Three distinct *Arabidopsis* hemoglobins exhibit peroxidase-like activity and differentially mediate nitrite-dependent protein nitration

Atsushi Sakamoto[a,b,c,*], Sho-hei Sakurao[b], Keiko Fukunaga[b], Toshiyuki Matsubara[c], Manami Ueda-Hashimoto[a], Shigefumi Tsukamoto[a], Misa Takahashi[a,b,c], Hiromichi Morikawa[a,b,c]

[a] *Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan*
[b] *Department of Biological Science, Faculty of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan*
[c] *Core Research for Evolutional Science and Technology (CREST), Project of Japan Science and Technology Agency, Kawaguchi 332-0012, Japan*

Received 29 April 2004; revised 21 June 2004; accepted 7 July 2004

Available online 14 July 2004

Edited by Peter Brzezinski

Abstract All plants examined to date possess non-symbiotic hemoglobin whose physiological role remains unclear. The present study explored the catalytic function of three representative classes of the plant hemoglobin from *Arabidopsis thaliana*: AtGLB1, AtGLB2, and AtGLB3. Purified recombinant proteins of these hemoglobins displayed hydrogen peroxide-dependent oxidation of several peroxidase substrates that was sensitive to cyanide, revealing intrinsic peroxidase-like activity. In the presence of nitrite and hydrogen peroxide, AtGLB1 was the most efficient at mediating tyrosine nitration of its own and other proteins via the formation of reactive nitrogen species as a result of nitrite oxidation. AtGLB1 mRNA significantly accumulated in *Arabidopsis* seedlings exposed to nitrite, supporting the physiological relevance of its function to nitrite and nitrite-derived reactive nitrogen species.

Keywords: Non-symbiotic hemoglobin; Nitrite; Nitrogen dioxide; Protein tyrosine nitration; Reactive nitrogen species; Peroxidase

1. Introduction

Hemoglobins have been found in living organisms from virtually all kingdoms and they exhibit diverse biological functions [1,2]. Higher plants contain two classes of this hemoprotein [3]. Symbiotic hemoglobins, better known as leghemoglobins, play an important role for controlling oxygen tension in legume-rhizobium symbiosis, which limits the distribution of this class to the root nodules of leguminous plants [4]. In contrast, both leguminous and non-leguminous species contain non-symbiotic hemoglobin that is genetically and structurally distinct from its symbiotic counterpart [3,4]. Plant non-symbiotic hemoglobin is often encoded by a small multigene family. For example, *Arabidopsis thaliana* contains all three known molecular species termed class 1, class 2 and truncated hemoglobins (hereafter referred to as AtGLB1, AtGLB2, and AtGLB3, respectively), which differ from each other in primary structure and physicochemical properties, and probably also in function [5,6]. In addition, their expression is differentially regulated and hence differentially distributed in organs, and they exhibit unique expression patterns during development and in response to certain stress conditions [7]. Whereas recent transgenic studies suggest the importance of this ubiquitous hemoprotein family in survival under hypoxia [8,9], its exact physiological roles remain obscure and several functions are conceivable. Being present at low concentrations [7], this hemoprotein may carry out as yet undocumented enzymatic activities because hemoglobin is long known to be able to mimic the activities of various heme-containing enzymes [10]. As has been demonstrated for other organisms of non-plant origin [2,11], it has recently been suggested that the plant hemoglobin is involved in the modulation of nitric oxide ($NO^{\bullet}$) levels, thereby controlling its bioactivity and toxicity [7,12,13].

The present study examined the possible catalytic function of hemoglobins, particularly in relation to recently explored novel aspects of plant metabolism of inorganic nitrogen and reactive nitrogen species (RNS) [14–16]. Focusing on three representative non-symbiotic hemoglobins, AtGLB1, AtGLB2, and AtGLB3, we provide in vitro evidence that all of the recombinant proteins intrinsically possess hydrogen peroxide (H_2O_2)-dependent activities that oxidize several compounds known as peroxidase substrates. Among the three types of hemoglobin, AtGLB1 was the most efficient at utilizing nitrite (NO_2^-) – a metabolic intermediate of the primary nitrogen assimilation – to generate RNS that nitrate protein

* Corresponding author. Fax: +81-82-424-0749.
E-mail address: ahkkao@hiroshima-u.ac.jp (A. Sakamoto).

Abbreviations: AtGLB, *Arabidopsis thaliana* hemoglobin; AtGLB1, *A. thaliana* class 1 hemoglobin; AtGLB2, *A. thaliana* class 2 hemoglobin; AtGLB3, *A. thaliana* truncated hemoglobin; BSA, bovine serum albumin; DHR123, dihydrorhodamine 123; metAtGLB, methohemoglobin form of AtGLB; oxyAtGLB, oxyhemoglobin form of AtGLB; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RNS, reactive nitrogen species; RT, reverse transcription; SOD, superoxide dismutase; Succ, succinate

0014-5793/$22.00
doi:10.1016/j.febslet.2004.07.005

tyrosine residues. The relevance of these findings is discussed with respect to plant metabolism for nitrogen oxides and RNS.

2. Materials and methods

2.1. Plasmid construction and recombinant production of AtGLB proteins

The plasmids for the production of recombinant *A. thaliana* hemoglobin (AtGLB) in *Escherichia coli* were constructed using amino-terminal fusion of a native protein to a histidine (His) tag in the vector pET16b (Novagen, Madison, WI, USA). A cDNA fragment encoding each AtGLB protein was amplified by polymerase chain reaction (PCR) using previously prepared *Arabidopsis* cDNAs [15] and a pair of gene-specific primers that generate PCR products with *Nde*I and *Bam*HI sites at the translation initiation codon and immediately downstream of the termination codon, respectively. The following primers were designed for PCR according to the published sequences [5,6] (restriction enzyme sites are underlined): 5′-ttcatatggagagtgaaggaaagattgtgttc-3′ (forward) and 5′-ttggatccttagttggaaagattcatttcagctt-3′ (reverse) for AtGLB1, 5′-ttcatatgggagagattgggtttacag-3′ (forward) and 5′-ttggatccttatgactcttcttgtttcatctcgg-3′ (reverse) for AtGLB2, and 5′-ttcatatgcaatcgctgcaagataag-3′ (forward) and 5′-ttggatccttattcttctgctggtttattggct-3′ (reverse) for AtGLB3. Each amplified product was initially cloned into pGEM-T Easy (Promega, Madison, WI, USA) for sequence verification and then subcloned between *Nde*I and *Bam*HI sites of pET16b.

For the production of recombinant protein, *E. coli* BL21(DE)pLysS cells were transformed with these pET16b-based vectors and grown at 37 °C for 12 h in Luria–Bertani medium in the presence of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. The recombinant proteins were extracted and purified by nickel-affinity chromatography as described previously [16]. Purified proteins were desalted on a gel-filtration column (PD-10; Amersham–Pharmacia Biotech, Uppsala, Sweden) and the concentration was determined using the dye-binding assay (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA; Sigma Chemicals, St. Louis, MO, USA) as the standard. Proteins were used in further experiments immediately after purification from bacteria. Methemoglobin and oxyhemoglobin were obtained by incubation of purified recombinant proteins with potassium ferricyanide and sodium dithionite, respectively, with subsequent purification on the PD-10 column. Absorption spectra of recombinant proteins were recorded at 25 °C in 20 mM Na-PO_4 buffer (pH 7.4) using a UV/Vis light spectrophotometer (BioSpec-1600; Shimadzu, Kyoto, Japan).

2.2. Peroxidase assay

Peroxidase activity was visualized in situ after proteins were separated by polyacrylamide gel electrophoresis (PAGE; 10% (w/v) gel) under non-denaturing conditions. For activity staining, the gels were immersed in 20 mM Na-PO_4 buffer (pH 7.4) containing 1 mM H_2O_2 and 10 μM *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red; Molecular Probes, Eugene, OR, USA) as the chromogenic reagent. Peroxidase activity was also examined using four substrates as the electron donor [guaiacol ($\varepsilon_{470} = 26.6\ mM^{-1}\ cm^{-1}$), Amplex Red ($\varepsilon_{563} = 54\ mM^{-1}\ cm^{-1}$), dihydrorhodamine 123 (DHR123; Molecular Probes) and NO_2^-] and potassium cyanide (KCN) as the inhibitor. A standard assay was performed using spectrophotometry at 25 °C in a 100-μl volume that contained 20 mM Na-PO_4 (pH 7.4), 0.1 mM EDTA, 0.5 mM H_2O_2, 1 mM (guaiacol and DHR123) or 0.1 mM (Amplex Red) of reducing substrate, and various concentrations of purified recombinant proteins in native or heat-denatured form. DHR123 oxidation was monitored at 500 nm and calibrated with its oxidized product rhodamine 123 (Sigma Chemicals). These peroxidase assays were initiated by the addition of H_2O_2. For the reaction with NO_2^-, a typical assay was conducted at 25 °C for 30 min in a 40-μl volume that included 20 mM Na-PO_4 (pH 7.4), 0.1 mM EDTA, 1 mM H_2O_2, 1 mM KNO_2 and 10 μM AtGLB protein. Optionally, BSA (10 μM) or superoxide dismutase (SOD; Sigma Chemicals) was additionally included in the reaction. The oxidation of NO_2^- was manifested as the appearance of tyrosine-nitrated proteins that were detected by immunoblotting analysis [17] (see Section 2.3).

2.3. Immunoblotting

A 20-μl aliquot of the reaction with NO_2^- was separated by SDS–PAGE on a 15% (w/v) polyacrylamide gel and electroblotted onto a polyvinylidene fluoride membrane (Immobilon™-P; Millipore, Bedford, MA, USA). To confirm equal loading of the protein, an identical gel for the remaining aliquot was stained in parallel with Coomassie brilliant blue (CBB). Protein tyrosine nitration was detected using an anti-nitrotyrosine rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA) and the secondary goat antibody conjugated with horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA), both at a dilution of 1:5000 (v/v) in phosphate-buffered saline (pH 7.4) plus 0.1% (v/v) Tween 20. A Western Lightning Chemiluminescence Reagent kit (Perkin–Elmer Life Sciences, Boston, MA, USA) was employed to visualize the signals derived from the antigen-antibody complex, which were then digitized by a VersaDoc 5000 imaging system and Quantity One software (Bio-Rad Laboratories).

2.4. Determination of NO_3^- and NO_2^- levels in the reaction of AtGLB1 with NO_2^- and H_2O_2

The levels of NO_3^- and NO_2^-, produced by the reaction of AtGLB1 with NO_2^- and H_2O_2, were measured by means of capillary electrophoresis using a capillary ion analyzer (Millipore/Waters, Milford, MA, USA) [18]. A total of 40 μl of reaction solution was heated at 95 °C for 2 min and then centrifuged to remove insoluble material before a 10-μl aliquot thereof was subjected to the analysis. Standard solutions for the calibration of NO_3^- and NO_2^- levels were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.5. Reverse transcription-PCR (RT-PCR)

Lots of 100 surface-sterilized seeds of *A. thaliana* (ecotype C24) were germinated in 4 ml of liquid medium [0.05% (w/v) 2-morpholinoethanesulfonate buffer (pH 5.5), 1% (w/v) sucrose, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 0.1 mM Fe–EDTA, 50 μM H_3BO_3, 12 μM $MnSO_4$, 1 μM $ZnCl_2$, 1 μM $CuSO_4$ and 0.2 μM Na_2MoO_4] that was supplemented with 2.5 mM ammonium succinate (NH_4^+-Succ) as the exclusive source of nitrogen [19]. Germinated seeds were cultured further at 22 °C under a cycle of 16-h light (0.07 mmol quanta $m^{-2}\ s^{-1}$)/8-h dark with gentle shaking. After 12 days of submerged growth, the seedlings were treated by adding either KCl, KNO_3 or KNO_2 at a final concentration of 5 mM, or an additional 2.5 mM NH_4^+-Succ. Seedlings were harvested following a 2-h incubation, rinsed, and immediately subjected to RNA extraction. A 1-μg aliquot from each RNA preparation was denatured at 65 °C for 15 min and then reverse-transcribed at 42 °C for 60 min in 20 μl of reaction solution containing ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and oligo$(dT)_{20}$ primers. Following heat denaturation at 95 °C for 5 min, a 1/100th fraction of this reaction was used as the matrix in 25 μl of reaction solution for PCR amplification with *TaKaRa Taq*™ (Takara Shuzo, Otsu, Japan) and the gene-specific primers described above. The following PCR protocol was used: 94 °C for 3 min; a variable number of cycles (15, 20, 25 or 30; to ensure that PCR assays were in a linear range) of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 90 s; and 72 °C for 7 min. As a control to check that an equal amount of the matrix was used for RT-PCR, a PCR was performed to amplify cDNAs for constitutively expressed actin transcripts using the following set of consensus primers, 5′-ggtaacattgtgctcagtggt-3′ (forward) and 5′-ctcggccttggagatccacat-3′ (reverse) [20]. PCR products were run on an 8% (w/v) polyacrylamide gel that was then stained with ethidium bromide.

3. Results and discussion

3.1. Peroxidase activity of recombinant AtGLB proteins

In an attempt to investigate possible catalytic activity of plant non-symbiotic hemoglobins, amino-terminally His-tagged proteins for each AtGLB were overexpressed in recombinant *E. coli* and purified from the crude extract by affinity column chromatography. The SDS–PAGE of the purified recombinant proteins showed bands around the predicted molecular masses, including the His tag and linker: 20.6 kDa for AtGLB1, 20.4 kDa for AtGLB2, and 22.7 kDa for AtGLB3 (Fig. 1A, left panel). The recombinant proteins resolved by native PAGE were colocalized with peroxidase activity stained in situ, although the signal generated by AtGLB3 was considerably weaker than the signals developed by the

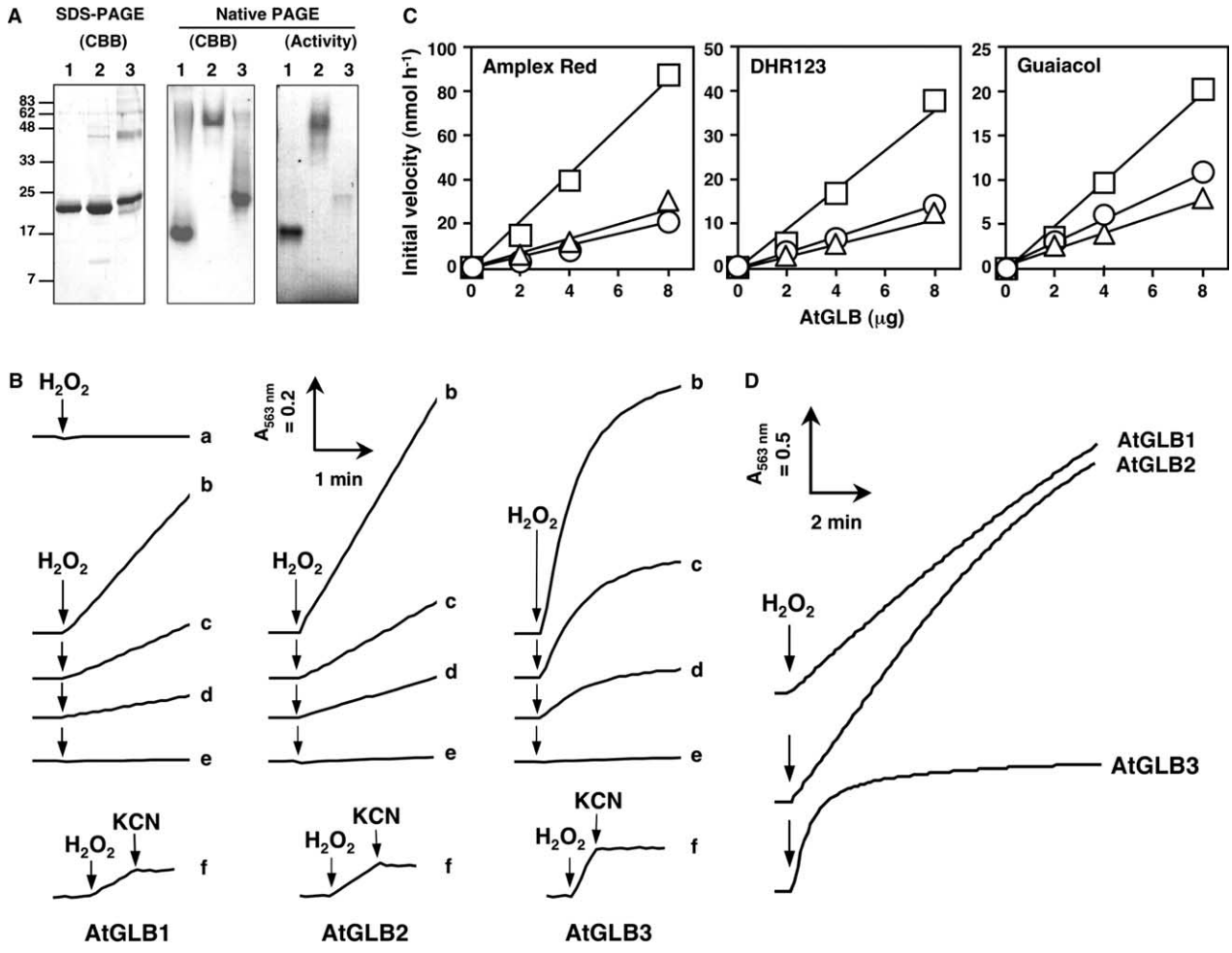

Fig. 1. (A) Gel electrophoretic analysis of recombinant AtGLB proteins. Left, CBB staining of purified proteins (5 μg) after SDS–PAGE (15% gel) with molecular masses given in kilodalton on the left; middle, CBB staining of purified proteins (20 μg) after native PAGE (10% gel); right, activity staining of a gel duplicate of the native PAGE. Lane 1, AtGLB1; lane 2, AtGLB2; lane 3, AtGLB3. (B) H_2O_2-dependent oxidation of Amplex Red by AtGLB proteins and inhibition by KCN. Protein concentrations were as follows (in micromolar): a, 0; b, 4; c and f, 2; d, 1; e, 2 (heat denatured). The addition of H_2O_2 (0.5 mM) and KCN (5 mM) is indicated by the downward arrows. (C) Linear relation of peroxidase activity of AtGLB proteins with various substrates as a function of protein quantity. Symbols: circle, AtGLB1; triangle, AtGLB2; square, AtGLB3. Means of two or three independent experiments are shown and S.D. falls within the symbol size. (D) The same results as trace b in panel B are shown on an extended time scale (up to 10 min).

other two proteins (Fig. 1A, middle and right panels). Fig. 1B shows the time course of peroxidase activity of recombinant AtGLB proteins as monitored spectrophotometrically. The oxidation of Amplex Red was absolutely dependent on the presence of both H_2O_2 and the recombinant protein, and it was inhibited by KCN. Neither H_2O_2 alone nor H_2O_2 with heat-denatured proteins oxidized Amplex Red. The initial rate of oxidation by recombinant proteins increased in a dose-dependent manner, with specific activities of 41 ± 4, 52 ± 7, and 175 ± 11 nmol Amplex Red oxidized $min^{-1}\,mg^{-1}$ protein (means $\pm$ S.D.; $n = 3$ for each value) for AtGLB1, AtGLB2 and AtGLB3, respectively. Essentially, the same profiles were obtained for the oxidation rate when DHR123 and guaiacol were used instead as reducing substrates, representing a linear relationship between the initial oxidation rate and protein quantity (Fig. 1C). Although the specific activity was greatest for AtGLB3, the oxidation rate of Amplex Red was linear only during the first 30 s, after which rapid inactivation occurred (Fig. 1D). This might explain the poor staining of the activity of AtGLB3 in native PAGE (Fig. 1A, right). Collectively, these results demonstrate that all of the recombinant AtGLB proteins intrinsically possess peroxidase-like activity. In terms of the mechanism underlying the reaction with H_2O_2, AtGLB proteins possibly form a derivative that is similar to compound I of peroxidases, which would then enable these hemoproteins to oxidize various reducing substrates [10].

3.2. Reaction of AtGLB proteins with NO_2^- in the presence of H_2O_2

Following the observation that the three AtGLB proteins could perform peroxidase-like reaction with the substrates tested, we next used NO_2^- as a source of electron. In the presence of H_2O_2, NO_2^- is oxidized by certain peroxidases and hemoproteins such as human hemoglobin to generate RNS that subsequently react with various biological targets including proteins, leading to nitration of protein tyrosyl residues [17,21,22]. Freshly prepared AtGLB proteins were incubated with NO_2^- and H_2O_2, and the self-nitration of protein tyrosine residues was examined by means of immunoblotting using anti-nitrotyrosine antibodies. As illustrated in

Fig. 2A, AtGLB1 showed overwhelmingly greater yields of self-nitration than AtGLB2 under the same condition, proceeding the reaction even in the absence of H_2O_2. In contrast, no such nitration was detected with AtGLB3, possibly due to its proclivity for inactivation (Fig. 1D). To investigate the effect of the redox state of heme iron on the self-nitrating activity, the same experiments were conducted for each AtGLB protein in oxyhemoglobin (oxyAtGLB) or methemoglobin (metAtGLB) form (Fig. 2B and C). The experiments with oxyAtGLB reproduced essentially the same results as those presented in Fig. 2A. In addition, when the reaction was carried out in the presence of BSA, this non-catalytic protein appeared to be a target of nitration (Fig. 2B and C). These results demonstrate that both AtGLB1 and AtGLB2 can mediate nitration of tyrosyl residues not only within the proteins, but also of coexisting proteins. The substantial difference in the effectiveness of nitration between AtGLB1 and AtGLB2 probably reflects different efficiencies when oxidizing NO_2^-, and hence when generating RNS. The oxidation to methemoglobin enhanced the nitrating capacity of AtGLB2 considerably (Fig. 2C), but not of AtGLB3 (data not shown). Although the proteins no longer proceeded the reaction upon the irreversible self-nitration, these results support that AtGLB proteins oxidize NO_2^- using a mechanism similar to heme-peroxidase catalysis because the ferric but not ferrous heme is the ground state of the prosthetic group in the peroxidases.

3.3. NO_2^--mediated protein nitration by AtGLB1 proteins

Protein tyrosine nitration by AtGLB1, the most efficient at nitration among the three proteins, was examined further. The degree of nitration increased with increasing concentrations of NO_2^- according to saturation kinetics, and the H_2O_2-concentration dependence gave a similarly shaped plot although the nitration already occurred in its absence as noted above (Fig. 3A). The reaction failed to occur when chemically related NO_3^- was used as the substrate, and was significantly inhibited by KCN (Fig. 3B), demonstrating that the nitration reaction was NO_2^--specific and heme-dependent. As mentioned above, NO_2^- alone (albeit to a lesser extent) was also able to induce the self-nitration of oxyAtGLB1 (Fig. 2A and B). During incubation with NO_2^-, the oxyAtGLB1 was converted to the methemoglobin as shown by the loss of the characteristic absorption at 538 and 576 nm of O_2-binding ferrous heme (Fig. 3C). Therefore, the self-nitration in the absence of H_2O_2 could be due to autocatalytic oxidation of the ferrous heme by NO_2^- that is known to produce the methemoglobin-peroxide complex and nitrogen dioxide ($NO_2^{\cdot}$), a strong nitrating agent [23,24]. The reaction was analyzed in the presence of SOD in order to examine the possibility of nitration mediated by $ONOO^-$, another potent nitrating species formed by spontaneous reaction between $NO^{\cdot}$ and superoxide anion radicals ($O_2^{\cdot-}$). The addition of SOD, which catalytically eliminates $O_2^{\cdot-}$, had little effect on the self-nitration, and indeed SOD experienced nitration of the tyrosine residues (Fig. 3D). These results suggest that the protein nitration is mediated by the formation of $NO_2^{\cdot}$ rather than $ONOO^-$. According to chemistry, $NO_2^{\cdot}$ dimerizes in aqueous solution to form N_2O_4 that spontaneously reacts with water to give rise to the equivalent molar formation of NO_3^- and NO_2^- [25]. Table 1 shows that there was a significant increase in NO_3^- formation, which is roughly equivalent to the AtGLB1 concentration when AtGLB1 and NO_2^- coexisted, and that negligible amounts of

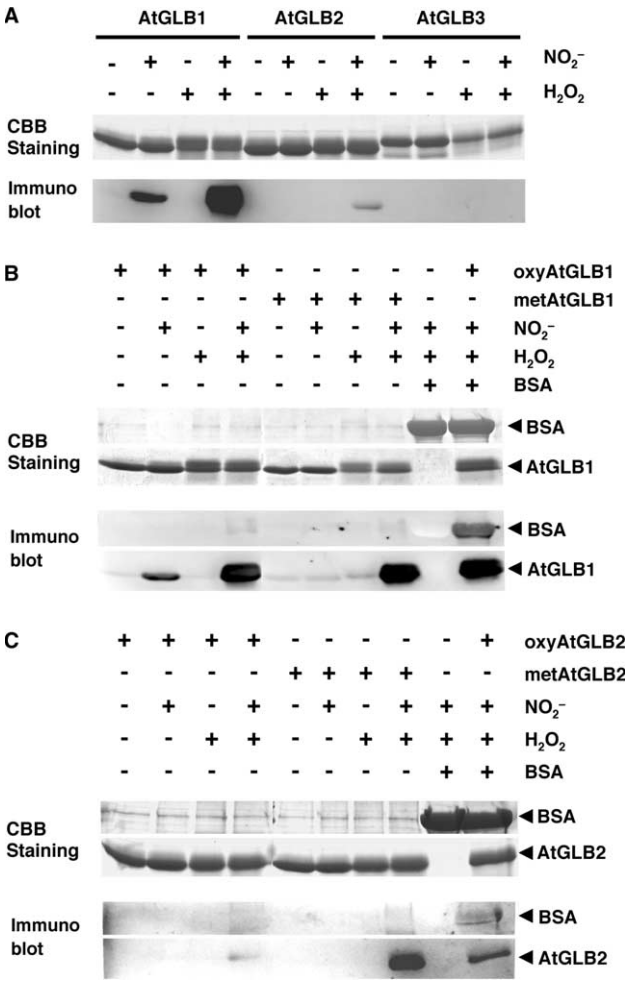

Fig. 2. (A) Self-nitration of tyrosyl residues of AtGLB proteins in the presence of NO_2^-. AtGLB proteins were incubated with 1 mM NO_2^- and/or 1 mM H_2O_2 at 25 °C for 30 min, subjected to SDS–PAGE and examined for protein nitrotyrosine formation by immunoblotting. (B and C) Protein tyrosine nitration by oxyhemoglobin and methemoglobin forms of AtGLB proteins (B, AtGLB1; C, AtGLB2). Protein tyrosine nitration was examined as in panel A in the presence of BSA or in its absence.

NO_3^- were detected in reactions where each component was omitted individually (Table 1). These results indicate that AtGLB1 generated $NO_2^{\cdot}$ at the concentration more than that observed for NO_3^- because NO_3^- formation accounts only for a part of the fate of the produced $NO_2^{\cdot}$ that is also the source of nitrotyrosine residues and $NO_2^{\cdot}$-derived NO_2^- in the reaction. This is another indication of the catalytic action of AtGLB1.

3.4. Differential accumulation of AtGLB mRNAs in response to NO_2^-

Having found a substantial difference in NO_2^--mediated activities of protein tyrosine nitration catalyzed by the three AtGLB proteins, we examined the effect of NO_2^- on AtGLB gene expression. *Arabidopsis* seedlings that had been grown in the presence of NH_4^+-Succ were treated with KNO_3, KNO_2 or KCl, and total RNA was extracted and subjected to RT-PCR analysis. Fig. 4 shows the level of AtGLB mRNAs as well as that of the actin transcripts run as a constitutive control. Strong induction of AtGLB1 mRNA accumulation was observed when the seedlings were treated with either KNO_2 or KNO_3. KCl had

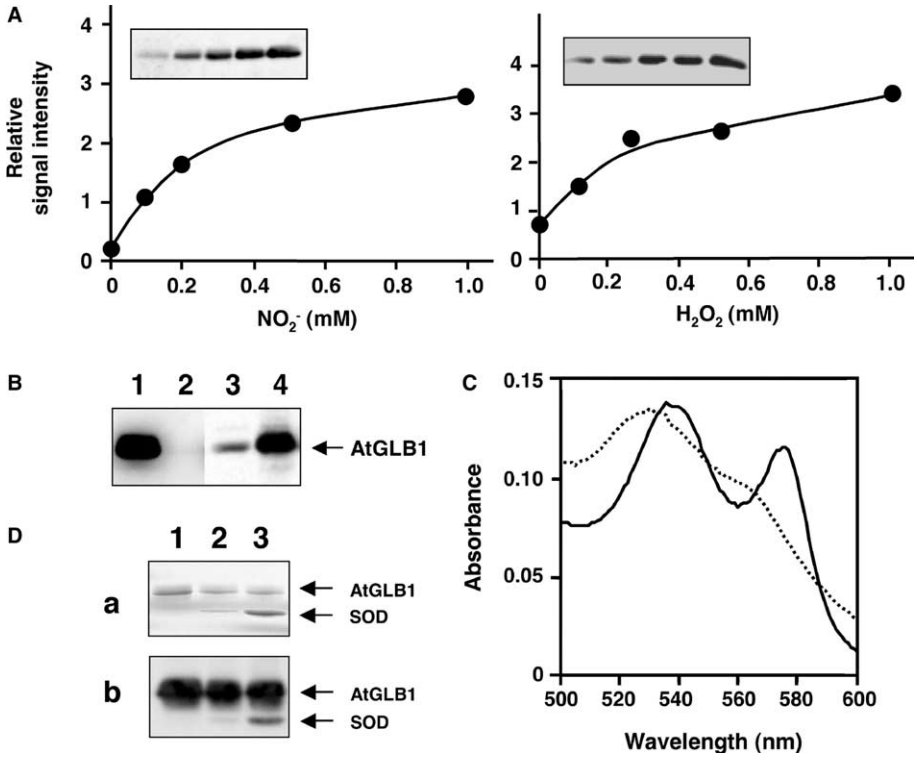

Fig. 3. (A) Self-nitration of AtGLB1 as a function of NO_2^- or H_2O_2 concentration. Left, AtGLB1 was incubated in the presence of 1 mM H_2O_2 and the indicated concentration of NO_2^- for 30 min, and the degree of protein nitration was calculated as a function of NO_2^- concentrations; right, similar reactions were carried out with various concentrations of H_2O_2 in the presence of 1 mM NO_2^-. Inset figures show typical results of immunoblotting. (B) Immunoblotting results after AtGLB1 was incubated with H_2O_2 in the presence of 1 mM KNO_2 (lanes 1 and 4), 1 mM KNO_3 (lane 2) or 1 mM KNO_2 plus 2 mM KCN (lane 3). (C) Spectral changes of oxyAtGLB1 during incubation with NO_2^-. Optical spectra between 500 and 600 nm were recorded for 10 μM oxyAtGLB1 in 20 mM Na-PO_4 before (solid line) and after (dashed line) incubation with 1 mM NO_2^- at 25 °C for 30 min. (D) Effects of SOD on the self-nitration of AtGLB1. a, CBB staining; b, immunoblots. Concentrations of SOD were as follows (in units per milliliter): lane 1, 0; lane 2, 10; lane 3, 100.

no effect on the steady-state AtGLB1 mRNA level, demonstrating that the response of the AtGLB1 gene is specific to NO_2^- and NO_3^-. AtGLB2 mRNA was also induced by the treatments, but the response was marginal compared with that seen for AtGLB1. In contrast, AtGLB3 mRNA was the least responsive to the treatments and the actin control showed little change. These results not only confirmed a previous report of the NO_3^--responsive expression of AtGLB1 gene [19], but also established NO_2^- as a novel and comparative inducer of the AtGLB1 gene expression. It is interesting to note that there is a positive correlation between the extent of NO_2^--induced accumulation of AtGLB mRNAs and the protein-nitrating capability of the corresponding AtGLB proteins (Fig. 2).

3.5. Implications to plant physiology and the metabolism of nitrogen oxides and RNS

The results presented here revealed that plant non-symbiotic hemoglobins possess intrinsically peroxidase-like activity capable of mediating tyrosine nitration of proteins. Although rather high concentrations are required for the catalysis, we applied in our experiments the physiological range of the concentrations (1–20 μM; [7]) of non-symbiotic hemoglobins, and thus the present in vitro observation of the AtGLB actions likely reflects the reactions expected in vivo. In addition, the inducible nature of AtGLB genes expression would further increase the protein concentration, which is a compensating benefit for rather inefficient catalytic properties.

Table 1
Nitrate formation during the reaction of AtGLB1 with NO_2^- in the presence of H_2O_2

Reaction condition	NO_3^- (μM)[b]	NO_2^- (μM)[b]
Full composition[a]	27.8 ± 0.8	74.9 ± 2.7
Without KNO_2	1.8 ± 1.7	0.8 ± 1.4
Without AtGLB1	3.3 ± 0.9	100 ± 2.6

[a] Reaction was performed at 25 °C for 30 min in 20 mM Na-PO_4 buffer (pH 7.4) containing 30 μM AtGLB1, 0.1 mM KNO_2 and 0.1 mM H_2O_2.
[b] Values are means ± S.D. ($n = 3$).

The results are also strongly suggestive of a close relationship between NO_2^- and an as yet unknown function of plant non-symbiotic hemoglobins, notably AtGLB1. Although the physiological importance of a coupling between NO_2^--consumption and the generation of RNS is yet to be clarified, the marked transcriptional response of the AtGLB1 gene to NO_2^-, and its precursor NO_3^-, supports that the catalytic activity to NO_2^- is physiologically relevant to its function. Such activity might contribute to cellular detoxification of NO_2^- as a poisonous metabolite [26,27], or involve cellular signaling because the tyrosine nitration of proteins by RNS such as $NO_2^{\cdot}$ and $ONOO^-$ is emerging as a post-translational modification that critically regulates various cellular functions [28]. Meanwhile, there is growing evidence of physiological roles for hemoglobins in the context of NO·-related functions [2,11]. The NO_2^-/NO_3^--mediated activation of the AtGLB1 gene thus might be

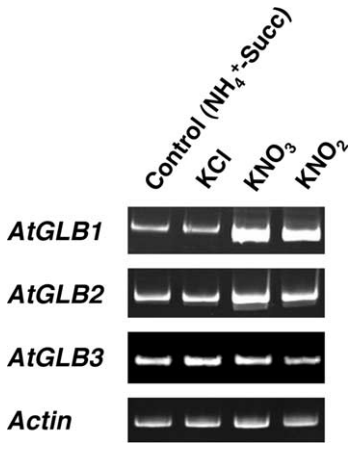

Fig. 4. Differential mRNA accumulation for the three AtGLB genes in *Arabidopsis* seedlings treated with various nitrogen sources. cDNA was generated from the seedlings that had been exposed to 5 mM KNO_2, KNO_3 or KCl for 2 h after a 12-day hydroponic culture in the presence of NH_4^+-Succ. The PCR was performed with 25 cycles for each AtGLB gene and with 20 cycles for the actin genes that served as the control to adjust the amount of PCR template. Shown are typical results of ethidium-bromide-stained gels from three independent experiments, which illustrate the consistency.

indicative of the response to NO· for modulation of its bioactivity and toxicity, because of the recent recognition of NO_2^- as a source of NO· biogenesis in plants [29,30]. Very recently, the interaction between NO· and barley class 1 hemoglobin has been reported [13].

Apart from the nitrate assimilation, many details of plant metabolism of nitrogen oxides and RNS remain to be elucidated [14]. In our previous studies, we hypothesized an enzymatic mechanism to describe the generation and decomposition of RNS that is linked to nitrate assimilation [15,16]. The present study, together with a recent report [26], poses the oxidation of NO_2^- by proteins with peroxidase-like activity as another potential route of deriving RNS from the primary nitrogen metabolism. In this context, it is noteworthy that nitrated proteins are formed in transgenic tobacco that accumulates excessive NO_2^- as a result of gene silence of nitrite reductase [31]. We recently found that a substantial proportion of nitrogen oxides such as $NO_2^{\cdot}$ and NO_3^- has an alternative metabolic fate in plant cells, being neither assimilated into organic compounds nor pooled as inorganic ions, but instead converted to previously unrecognized forms of nitrogen to which we referred as ''unidentified nitrogen'' [14]. The chemical signatures of such nitrogen species most probably include organic nitro/nitroso groups, suggesting the involvement of RNS in its formation [14]. These findings, supported by their occurrence in a broad spectrum of plant species surveyed, have led us to propose that a novel mechanism of nitrogen metabolism is ubiquitous in the plant kingdom [14]. Plant proteins with ambiguous function such as AtGLB1 might, by virtue of their ability to produce RNS from NO_2^-, contribute to the newly discovered but as yet unexplored mechanism of plant nitrogen metabolism.

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 15570038) to A.S. from the Japan Society for the Promotion of Science.

References

[1] Hardison, R. (1998) J. Exp. Biol. 201, 1099–1117.
[2] Weber, R.E. and Vinogradov, S.N. (2001) Physiol. Rev. 81, 569–628.
[3] Bogusz, D., Appleby, C.A., Landsmann, J., Dennis, E.S., Trinick, M.J. and Peacock, W.J. (1988) Nature 331, 178–180.
[4] Arredondo-Peter, R., Hargrove, M.S., Moran, J.F., Sarath, G. and Klucas, R.V. (1998) Plant Physiol. 118, 1121–1125.
[5] 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) Proc. Natl. Acad. Sci. USA 94, 12230–12234.
[6] Watts, R.A., Hunt, P.W., Hvitved, A.N., Hargrove, M.S., Peacock, W.J. and Dennis, E.S. (2001) Proc. Natl. Acad. Sci. USA 98, 10119–10124.
[7] Dordas, C., Rivoal, J. and Hill, R.D. (2003) Ann. Bot. 91, 173–178.
[8] Sowa, A.W., Duff, S.M.G., Guy, P.A. and Hill, R.D. (1998) Proc. Natl. Acad. Sci. USA 95, 10317–10321.
[9] Hunt, P.W., Klok, E.J., Trevaskis, B., Watts, R.A., Ellis, M.H., Peacock, W.J. and Dennis, E.S. (2002) Proc. Natl. Acad. Sci. USA 99, 17197–17202.
[10] Everse, J. and Hsia, N. (1997) Free Radic. Biol. Med. 22, 1075–1099.
[11] Patel, R.P. (2000) Free Radic. Biol. Med. 28, 1518–1525.
[12] Dordas, C., Hasinoff, B.B., Igamberdiev, A.U., Manac'h, N., Rivoal, J. and Hill, R.D. (2003) Plant J. 35, 763–770.
[13] Igamberdiev, A.U., Seregélyes, C., Manac'h, N. and Hill, R.D. (2004) Planta 219, 95–102.
[14] Morikawa, H., Takahashi, M., Sakamoto, A., Matsubara, T., Arimura, G.-I., Kawamura, Y., Fukunaga, K., Fujita, K., Sakurai, N., Hirata, T., Ide, H., Nonoyama, N. and Suzuki, H. (2004) Planta 219, 14–22.
[15] Sakamoto, A., Ueda, M. and Morikawa, H. (2002) FEBS Lett. 515, 20–24.
[16] Sakamoto, A., Tsukamoto, S., Yamamoto, H., Ueda-Hashimoto, M., Takahashi, M., Suzuki, H. and Morikawa, H. (2003) Plant J. 33, 841–851.
[17] Grzelak, A., Balcerczyk, A., Mateja, A. and Bartosz, G. (2001) Biochim. Biophys. Acta 1528, 97–100.
[18] Kawamura, Y., Takahashi, M., Arimura, G., Isayama, T., Irifune, K., Goshima, N. and Morikawa, H. (1996) Plant Cell Physiol. 37, 878–880.
[19] Wang, R., Guegler, K., LaBrie, S.T. and Crawford, N.M. (2000) Plant Cell 12, 1491–1509.
[20] Fotopoulos, V., Gilbert, M.J., Pittman, J.K., Marvier, A.C., Buchanan, A.J., Sauer, N., Hall, J.L. and Williams, L.E. (2003) Plant Physiol. 132, 821–829.
[21] van der Vliet, A., Eiserich, J.P., Halliwell, B. and Cross, C.E. (1997) J. Biol. Chem. 272, 7617–7625.
[22] Sampson, J.B., Ye, Y., Rosen, H. and Beckman, J.S. (1998) Arch. Biochem. Biophys. 356, 207–213.
[23] Kosaka, H., Imaizumi, K. and Tyuma, I. (1981) Biochim. Biophys. Acta 702, 237–241.
[24] Doyle, M.P., Herman, J.G. and Dykstra, R.L. (1985) J. Free Radic. Biol. Med. 1, 145–154.
[25] Bonner, F.T. and Stedman, G. (1996) in: Methods in Nitric Oxide Research (Feelisch, M. and Stamler, J.S., Eds.), pp. 3–18, Wiley, West Sussex.
[26] Klebanoff, S.J. (1993) Free Radic. Biol. Med. 14, 351–360.
[27] Sakihama, Y., Tamaki, R., Shimoji, H., Ichiba, T., Fukushi, Y., Tahara, S. and Yamasaki, H. (2003) FEBS Lett. 553, 377–380.
[28] Schopfer, F.J., Baker, P.R.S. and Freeman, B.A. (2003) Trends Biochem. Sci. 28, 646–654.
[29] Yamasaki, H., Sakihama, Y. and Takahashi, S. (1999) Trends Plant Sci. 4, 128–129.
[30] Rockel, P., Strube, F., Rockel, A., Wildt, J. and Kaiser, W.M. (2002) J. Exp. Bot. 53, 103–110.
[31] Morot-Gaudry-Talarmain, Y., Rockel, P., Moureaux, T., Quilleré, I., Leydecker, M.T., Kaiser, W.M. and Morot-Gaudry, J.F. (2002) Planta 215, 708–715.