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Wheat truncated hemoglobin interacts with photosystem I PSK-I subunit and photosystem II subunit PsbS1

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Abstract

Recently, the truncated hemoglobin gene (*trHb*) was discovered in plant species, however, its role has not yet been determined. In this study, the gene expression of wheat *trHb* (*TatrHb*) was analyzed under various biotic and abiotic stresses. *TatrHb* transcript levels increased in NaCl-treated leaves and gibberellic acid (GA_3)-treated roots. In addition, sodium nitroprusside (SNP), a nitric oxide donor, induced an increase in *TatrHb* transcript levels in roots and leaves. A yeast two-hybrid assay (YIIH) was used to screen a hypoxia-treated wheat seedling library with the goal of determining the putative function of TatrHb. In this YIIH assay, photosynthesis-related genes that showed high homology to the *Hordeum vulgare* chloroplast photosystem I PSK-I subunit and *Zea mays* photosystem II subunit PsbS1 were detected and their interactions with TatrHb were confirmed. Subcellular localization of a TatrHb-green fluorescent protein (GFP) fusion protein and bimolecular fluorescence complementation (BiFC) assay suggested that TatrHb is involved in photosynthesis. The TatrHb-GFP fusion protein was localized in the plastids and the yellow fluorescent protein signal indicated that the TatrHb protein interacted with PSK-I and PsbS1 in the chloroplast.

Additional key words: chloroplast, gibberellic acid, NaCl, *Triticum aestivum*, nitric oxide, photosynthesis, YIIH assay.

Introduction

Hemoglobins (Hbs) are ubiquitous proteins found in many organisms including plants and animals (Bolognesi *et al*. 1997, Arredondo *et al*. 1998, Trent *et al*. 2002) and are categorized as symbiotic, nonsymbiotic, or truncated Hbs (trHbs). Leghemoglobin (legHb) is an oxygen carrier and an Hb-like protein that is found in the nitrogen-fixing root nodules of leguminous plants (Appleby 1992). This protein is produced by *Leguminosae* in response to infection of the roots by symbiotic rhizobia. Therefore, legHb can be classified as symbiotic Hb. Nonsymbiotic Hbs are believed to be present in all monocotyledons and dicotyledons (Bogusz *et al*. 1988, Jacobsen-Lyon *et al*. 1995). These proteins are unrelated to the symbiotic Hbs and have not been implicated in nitrogen fixation. Nonsymbiotic Hbs are expressed in diverse plant tissues in response to various stresses.

Andersson *et al*. (1996) found that nonsymbiotic Hb was expressed in soybean cotyledons, stems, roots, young leaves, and also in the nodules whereas legHb only in the infected cells of the nodules. Trevaskis *et al*. (1997) cloned 2 Hbs, *AHB1* and *AHB2*, in *Arabidopsis*. The nonsymbiotic *AHB1* was induced in both roots and rosette leaves by low oxygen levels whereas the symbiotic-like *AHB2* gene was expressed at low levels in rosette leaves and was induced by low temperature. In addition, *AHB1* expression increased under hypoxic conditions suggesting that AHB1 is a stress-related protein (Trevaskis *et al*. 1997). In addition, an *Hb* from a monocot was cloned by Taylor *et al*. (1994) using an aleurone cDNA library from barley and the analysis of rice nonsymbiotic *Hb* genes under normal conditions showed that both *Hb1* and *Hb2* were expressed in leaves but only *Hb1* was expressed in roots (Arredondo-Peter *et al.* 1997).

Another Hb family has been identified from prokaryotes, protozoa, eukaryotic algae, and plants. The

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Abbreviations: ABA - abscisic acid; BiFC - bimolecular fluorescence complementation; c-PTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxidase; GA_3 - gibberellic acid; GFP - green fluorescent protein; Hb - hemoglobin; MeJA - methyl jasmonate; NOS - NO synthase; ONPG - *O*-nitrophenyl β-D-galactopyranoside; PEG - polyethylene glycol; RT-PCR - reverse transcriptase-polymerase chain reaction; SA - salicylic acid; SNP - sodium nitroprusside; TatrHb - *Triticum aestivum* L. truncated hemoglobin; YIIH - yeast 2-hybrid; YFP - yellow fluorescent protein.

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amino acid sequence of this group of Hbs is shorter (by 20 - 40 amino acids) than that of nonsymbiotic Hb and this group of Hb is referred to truncated Hb (trHb). The tertiary structure of trHbs accommodates the deletion of the N-terminal α-helix and replacement of the crucial heme-binding F-helix with an extended polypeptide loop. TrHbs have a typical structure based on a "2-on-2" α-helical sandwich globin fold (helices B/E and G/H) whereas most globins have a "3-on-3" α-helical sandwich fold (helices A/E/F and B/G/H) (Pesce *et al.* 2000, Hoy *et al*. 2008).

TrHb genes can be divided phylogenetically into 3 groups: group I (*trHbN*), group II (*trHbO*), and group III (*trHbP*) (Wittenberg *et al.* 2002). Vuletich *et al*. (2006) reported that the Group II *trHb* gene was the original gene, whereas the group I and group III *trHb* genes were obtained *via* duplication and transfer events. The trHb of the unicellular green alga *Chlamydomonas eugametos*, which belongs to group I, is expressed in response to active photosynthesis and is localized along the chloroplast thylakoid membranes (Couture *et al.* 1994). The soluble trHb protein of the cyanobacterium *Nostoc commune*, which belongs to group I, is localized on the cytoplasmic face of the cell membrane and is expressed only under anaerobic conditions (Hill *et al.* 1996).

In higher plants, *GLB3* (a *2-on-2 Hb*-like gene) of *Arabidopsis* is expressed throughout the plant; however, it does not respond to hypoxia or cold stresses that are known to induce plant 3-on-3 Hbs. *AtGLB3* expression is regulated differently from the 2 other *Arabidopsis* Hbs (Watts *et al.* 2001). Peroxidase activity of recombinant AtGLB3 proteins was weaker than the signals developed by AtGLB1 and AtGLB2, indicating that the catalytic activity of AtGLB3 is lower than the other AtGLBs. Moreover, reaction of AtGLBs proteins with NO_2^- in the presence of H_2O_2 demonstrated that AtGLB3 is inactive for nitrite-dependent protein nitration (Sakamoto *et al.* 2004).

Additionally, the *GLB3* of soybeans, which is a *2-on-2 Hb*-like gene, was specifically expressed in root nodules with levels increasing in the late stage during nodule development (Lee *et al*. 2004). In wheat, the expression of the *TaHb1* gene, which shows a very high similarity to nonsymbiotic *Hbs* from *Hordeum vulgare*, is induced in roots exposed to anaerobic conditions. *TaHb2* exhibits strong similarity to *trHbs* and has been demonstrated to be constitutively expressed in different tissues, such as roots, stems, leaves, and flowers (Larsen 2003).

The aim of this study was to elucidate the role of trHb in wheat. The wheat *trHb* gene and its promoter region were isolated from cDNA and genomic DNA, respectively, and were used to predict the putative function of *TatrHb*. In addition, the expression profile of *TatrHb* was evaluated to elucidate the response of the *TatrHb* gene to various stimulations or stresses. Furthermore, yeast two-hybrid (YIIH) assays were conducted to assess the putative role of TatrHb. Finally, determination of the subcellular localization of the TatrHb-green fluorescent protein (GFP) fusion protein and BiFC assay demonstrated that TatrHb may be involved in photosynthesis.

Materials and methods

Plants: The common wheat (*Triticum aestivum* L.) cv. Geumgangmill (developed by the National Institute of Crop Science, RDA, Suwon, Republic of Korea) seeds were germinated for 36 h at room temperature and transferred to *Magenta* boxes (6.6 × 6.5 × 20 cm, Greenpia Technology, Yeoyu, Korea) containing a polypropylene mesh and filled with 180 cm^3 of half-strength Hoagland's nutrient solution No. 2 (*Sigma*, St. Louis, MO, USA). Seedlings were grown in growth chambers (*VS-91G09M*, *Vision Scientific*, Bucheon, Korea) at day/night temperature of 23/20 °C, 14-h photoperiod, irradiance of 350 μmol m^{-2} s^{-1}, and relative humidity of 60 %. The Hoagland's nutrient solution was exchanged daily (for detail see Hong *et al*. 2010).

Isolation of the TatrHb gene and promoter region: We collected 74 expressed sequence tag (EST) sequences to isolate *TatrHb* using the term "2-on-2 Hb *Triticum aestivum*" in the National Center for Biotechnology database (http://www.ncbi.nlm.nih.gov/). To perform multiple alignments of the EST sequences, we used *ClustalW* through the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/clustalw/). Two pairs of forward and reverse primers were designed from the sequences of the putative 5′ and 3′ untranslated regions (UTRs) by comparison with the EST sequences. Total RNA was extracted from whole 2-week-old wheat seedlings to isolate cDNA for the *TatrHb* gene. The cDNA from wheat seedlings was synthesized from 1 μg of total RNA using *SuperScript* and *oligo-dT* primers (*Invitrogen*, Grand Island, NY, USA). *TatrHb* F1 and *TatrHb* R1 primers were used for the initial PCR amplification of cDNA (Table 1). The PCR protocol consisted of 30 cycles of 94 °C for 60 s, 56 °C for 60 s, and 72 °C for 90 s. Primary PCR products were purified, and 100-fold-diluted primary PCR products were used for nested PCR with *TatrHb* F2 and *TatrHb* R2 under the same conditions as the primary PCR (Table 1). Amplification products were cloned into the T&A cloning vector (*Real Biotech Cooperation*, Taipei, Taiwan) and sequenced.

The universal *Genome Walker* kit (*Clontech*, Palo Alto, CA, USA) was used to investigate the upstream region of the *TatrHb* gene. Two gene-specific primers (TatrHb UP1 and UP2) were used with 2 adapter primers that were included in the universal *Genome Walker* kit (Table 1). Amplification products were cloned into the *T&A* cloning vector (*Real Biotech Cooperation*) and sequenced.

RNA extraction and gene expression analysis: Total RNA was isolated from wheat seedlings using *TRIzol* reagent (*Invitrogen*) according to the manufacturer's instructions. The first strand of cDNA was synthesized from approximately 1 μg of total RNA using the *DyNamo* cDNA synthesis kit (*Finnzymes*, Vanda, Finland). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in 96-well plates using an *iCycler iQ* real-time PCR (*BioRad*, Hercules, USA) and *SYBR Green I Master* mix (*Finnzymes*) in a 0.05 cm^3 reaction volume with TatrHb qRT5' and TatrHb qRT3' primers (sequences are shown in Table 1). Each target gene in the PCR experiment was normalized against constitutively expressed *Taactin*.

Table 1. List of primer sequences and their purposes.

Name	Sequence	Purpose
TatrHb F1	5′-AGCAGCAGGAACGATGCAGT-3′	isolation of *TatrHb* cDNA
TatrHb R1	5′-AGGAATATGGGAGCTGAAAC-3′	isolation of *TatrHb* cDNA
TatrHb F2	5′-ATGCAGTCGCTGCAGGACAAG-3′	isolation of *TatrHb* cDNA
TatrHb R2	5′-CCGTTATTCAACTGGTTAAGCTTG-3′	isolation of *TatrHb* cDNA
TatrHb UP1	5′-GTTAAGCTTGAGCGGTTACTCGGCTGGTT-3′	isolation of the *TatrHb* promoter
TatrHb UP2	5′-TTACTCGGCTGGTTTGCTCGTTGCATGTT-3′	isolation of the *TatrHb* promoter
TatrHb qRT5′	5′-CGCGATCCAGAACCAGTACGAGTTCTT-3′	analysis of *TatrHb* gene expression
TatrHb qRT3′	5′-TTGGGTTTGCCTTGTCATCTCATTACCG-3′	analysis of *TatrHb* gene expression
Taactin RT5′	5′-GCCACACTGTTCCAATCTATGA-3′	analysis of *TatrHb* gene expression
Taactin RT3′	5′-CTTCGTGTATACCAGGAACTTC-3′	analysis of *TatrHb* gene expression
YIIH-TatrHb-F	5′-GAATTCATGCAGTCGCTGCAGGACAA-3′	yeast II hybridization
YIIH-TatrHb-R	5′-GGATCCTTACTCGGCTGGTTTGCTCGT-3′	yeast II hybridization
GFP-TatrHb-F	5′-TCTAGAATGCAGTCGCTGCAGGACAA-3′	subcellular localization of *TatrHb*
GFP-TatrHb-R	5′-GGATCCTTACTCGGCTGGTTTGCTCGT-3′	subcellular localization of *TatrHb*
PSK-I-F	5′-ATGGCTTCCCAGCTCTCCGCCATGA-3′	isolation of *PSK-I* cDNA/BiFC assay
PSK-I-R	5′-CTAGCCGATGATCTGGTCGAGGA-3′	isolation of *PSK-I* cDNA/BiFC assay
PsbS1-F	5′-ATGTCCGGCGTCAACGGCGTGG-3′	isolation of *PsbS1* cDNA/BiFC assay
PsbS1-R	5′-CTAAGTACACCCCAAGGTCTCACG-3′	isolation of *PsbS1* cDNA/BiFC assay

Application of phytohormones and stress treatments: In order to examine the responses of the *TatrHb* gene to phytohormones, 14-d-old seedlings were transferred to 180 cm^3 of half-strength Hoagland's nutrient solutions containing 100 mM salicylic acid (SA), 100 μM methyl jasmonate (MeJA), 100 μM gibberellic acid (GA_3), or 100 μM abscisic acid (ABA) (An *et al*. 2008). Ethylene treatment was performed by exposing young seedlings to 100 cm^3 m^{-3} ethylene gas in an air-tight, transparent plastic bag (Jung *et al*. 2008). The roots of wheat seedlings (fully expanded second-leaf stage) were immersed in 180 cm^3 of half-strength Hoagland's solution containing 25 % (m/v) polyethylene glycol (PEG, Mr 10 000) or 250 mM NaCl to assess the response of *TatrHb* to drought and salinity stress. Seedlings were also subjected to hydrogen peroxide treatment by dissolving 100 mM H_2O_2 in half-strength Hoagland's solution (An *et al*. 2008). Leaf and root samples were harvested 24 h after treatment. Fourteen days old seedlings were pretreated with 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1-oxyl-3 oxidase (c-PTIO, a nitric oxide [NO] scavenger) for 2 h in the light to suppress NO accumulation. Wheat seedlings were treated with four combinations of c-PTIO and sodium nitroprusside (SNP, a NO donor): 1 mg dm^{-3} c-PTIO, 0.1 mg dm^{-3} SNP, 1 mg dm^{-3} SNP, and 1 mg dm^{-3} c-PTIO + 1 mg dm^{-3} SNP, for an additional 3 h. The solution was continuously flushed for 72 h with N_2 gas (99.99 % N_2) for hypoxic treatment. The oxygen concentration in the vessels was monitored using an oxygen meter (*DO-24-P*, *DKK-TOA Corporation*, Tokyo, Japan). Concentration of dissolved oxygen was less than 1 % (v/v) during the treatment. Following the treatments, seedlings were dissected into roots and leaves and were immediately frozen in liquid N_2 and stored at -80 °C until further use.

YIIH cDNA library construction, screening, and co-transformation: To construct a cDNA library for YIIH screening, total RNA was extracted from hypoxia-treated leaves and roots with *TRIzol* (*Invitrogen*). Total RNA (2 μg) that was prepared by a mixture of 1 μg of RNA from leaves and 1 μg of RNA from roots was used for this library. The cDNA library was prepared according to the manuals supplied by the manufacturer (*Make Your Own Mate & Plate Library System*, *Clontech*). The cDNA library was washed out from 100 plates with 500 cm^3 of yeast peptone dextrose adenine media (YPDA, including 25 % (v/v) glycerol and 25 μg cm^{-3} kanamycin) and incubated at 30 °C for 30 min with rotation (220 rpm). Aliquots (1 mm^3) of the cDNA were stored at -80 °C until use. The bait vector was constructed by cloning full-length *TatrHb* coding regions which were amplified by PCR using specific primer pairs with *Eco*RI/*Bam*HI restriction sites (Table 1). PCR products were digested with the corresponding restriction enzymes and ligated into pGBKT7 (*Clontech*) using T4 ligase (*Roche*, Indianapolis, USA). The pGBKT7-trHb construct was transformed into the yeast strain YIIHGold and tested for

autoactivation of *ADE2*, *HIS3*, and *AUR1-C* reporter genes. A GAL4-based YIIH library was constructed in the pGADT7-Rec vector using the *Matchmaker* library construction and screening kit (*Clontech*) according to the manufacturer's protocol. YIIH screening was conducted according to the manufacturer's protocol. Mating products were plated onto a synthetic dropout (SD) medium without leucine (Leu) and tryptophan (Trp; DDO) until colonies appeared after about 5 - 7 d. The resulting diploid yeasts were selected on SD medium without adenine (Ade), histidine (His), Leu, and Trp (QDO). All SD mediums contained X-α-Gal and Aureobasidin A. *Matchmaker* insert check PCR mix 2 (*Clontech*) was used to amplify positive clones.

To further confirm the screening results from the paired YIIH assays, full-length photosystem I PSK-I subunit and photosystem II subunit PsbS1 cDNAs were amplified using the primer pairs listed in Table 1. The PCR products were cloned into pGADT7 vectors in frame. Interaction of the proteins was tested using co-transformed AH109 yeast cells with pGBKT7:*TatrHb* as bait and its putative interacting proteins (pGADT7:PSK-I and pGADT7:PsbS1) as prey. The transformants were plated onto SD medium lacking Leu and Trp and a single colony was plated onto SD medium containing X-α-gal and lacking Ade, His, Leu, and Trp. Selected colonies of each transformants were used for β-galactosidase activity assay.

β-Galactosidase activity was measured in liquid yeast cultures using *O*-nitro-phenyl β-D-galactopyranoside (ONPG) as a substrate. Cultures of each yeast cotransformant were grown overnight at 30 °C in 5 cm^3 of SD selective medium diluted with YPD medium and growth continued at 30 °C for 3 - 5 h until the cultures reached an absorbance (A_{600}) of 0.5 - 0.8. Next, 1.5 cm^3 of each culture was transferred to Eppendorf tubes, centrifuged, and resuspended in approximately one-fifth of the original volume with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$). Cells were frozen in liquid nitrogen and thawed at 37 °C in water bath for 1 min. This step was repeated 3 times to lyse the yeast cells. Then, 0.7 cm^3 of Z buffer with 0.27 % (v/v) β-mercaptoethanol was added to the tube, followed by 0.16 cm^{-3} of 4 mg cm^{-3} ONPG in Z buffer (*Sigma*). The samples were incubated at 30 °C until the reactions became yellow, and reactions were then terminated by the addition of 0.4 cm^3 of 1 M Na_2CO_3. The absorbance (A_{420}) of each supernatant was determined, and activity was calculated according to the following equation:

β-galactosidase units = $1000 \times A_{420}/(T \times V \times A_{600})$, where T was the incubation time in min, V was 0.1 cm^3. The yeast strain AH109 (*Clontech*), cotransformed with pGADT7-T and pGBKT7-53, was used as the positive control for protein-protein interactions, while cells containing pGADT7 and pGBKT7-Lam, whereas do not interact, were used as negative controls for these experiments.

Confocal microscopy: The pBIN35S-326-GFP vector, which contains the 35S promoter from PBI121 and the *GFP* gene from the 326-GFP vector, was used to determine the subcellular localization of the TatrHb protein. *Nicotiana tabacum* was grown in a greenhouse at 23 °C, relative humidity 60 %, 12-h photoperiod, and irradiance of 300 μmol m^{-2} s^{-1}. Full-length *TatrHb* coding regions were amplified by PCR using specific primer pairs that included *Xba*I/*Bam*HI restriction sites (Table 1). The PCR products were then digested with the corresponding restriction enzymes before ligation into pBIN-GFP.

For the BiFC assay, PCR fragments for TatrHb, photosystem I PSK-I subunit, and photosystem II subunit PsbS1 coding sequences (including stop codons) were generated using the primers listed in Table 1 and subsequently cloned into the pCR2.1 TOPO vector. *Gateway LR Clonase II* enzyme mix (*Invitrogen*) was used to clone *TatrHb* into the pE-SPYNE-GW destination vector and photosystem I PSK-I subunit and photosystem II subunit PsbS1 into the pE-SPYCE-GW vector according to the manufacturer's instructions. Briefly, the 0.01 cm^3 of LR reaction mixture contained: 0.05 cm^3 of entry vector, 0.001 cm^3 of destination vector, 0.002 cm^3 of *Gateway LR Clonase II* enzyme mix, and 0.002 cm^3 of TE buffer (pH 8.0). Following 1 h incubation at 25 °C, the LR reaction mixture was incubated with 0.001 cm^3 of proteinase K solution at 37 °C for 10 min and used for transformation of One Shot TOP10 competent cell. The *Gateway*-compatible *BiFC* binary vectors pE-SPYNE-GW and pE-SPYCE-GW were kindly provided by Dr. C. Carsjens and Dr. W. Dröge-Laser (University of Göttingen, Germany).

Each pE-SPYNE-GW:TatrHb and pE-SPYCE-GW:PSK-I or pE-SPYCE-GW:PsbS1 construct was introduced into the *Agrobacterium tumefaciens* strain GV3101 *via* the freeze-thaw method as described by Chen *et al*. (1994). Recombinant *A. tumefaciens* cultures were grown overnight at 28 °C in 100 cm^3 flasks containing 50 cm^3 of LB medium supplemented with 50 μg cm^{-3} kanamycin. A 0.1 cm^3 aliquot of *A. tumefaciens* cells were inoculated into 20 cm^3 of LB medium supplemented with 10 mM MES buffer, pH 5.7, 50 μg cm^{-3} kanamycin, and 0.15 mM aceto-syringone (3,5-dimethoxy-4′-hydroxyacetophenone). The *A. tumefaciens* cells were cultured at 28 °C with agitation until reaching the saturated growth phase and were then collected by centrifuging at 12 000 *g* for 10 min. After removing the supernatant, the pellet was washed twice with infiltration buffer (10 mM MES buffer, pH 5.7, 10 mM $MgCl_2$, and 0.15 mM acetosyringone) and then suspended in the same buffer (A_{600} = 0.8). After infiltration of *Agrobacterium* in 3-week-old soil-grown tobacco using a needless syringe, plants were placed in normal growing conditions for 72 h. Subcellular localization of the fusion proteins was assessed using a *LSM 5 Exciter* confocal laser scanning microscope (Carl Zeiss, Hamburg, Germany). The BiFC complexes were visualized using 488 nm excitation and 515 - 555 nm emission filters.

Results

Out of the 74 ESTs collected from the NCBI EST database, 21 ESTs were selected by cluster assignments using *ClustalW* to design specific primers for the isolation of the *TatrHb* gene. Two pairs of primers located in the 5′ and 3′ UTR regions of *TatrHb* were designed and used to amplify cDNA.

cDNAs corresponding to the *TatrHb* sequences were identified and contained a 516 bp open reading frame encoding 172 amino acids. The *Conserved Domain Database* (CDD) of the *NCBI* (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (Marchler-Bauer *et al*. 2009), a collection of multiple sequence alignments, was used for domain searching to predict the role of *TatrHb*. Three heme-binding sites were found within *TatrHb* using CDD (Fig. 1). Phe45, Tyr46, and His80, which are known conserved amino acids in 2-on-2 Hbs in plants and some microorganisms, were also found in *TatrHb* (Watts *et al*. 2001).

We isolated the 3′ upstream region of *TatrHb* in an attempt to elucidate the regulatory mechanisms of *TatrHb* using a genome walker strategy. The 1542-bp amplified fragment was analyzed for putative *cis*-acting elements of

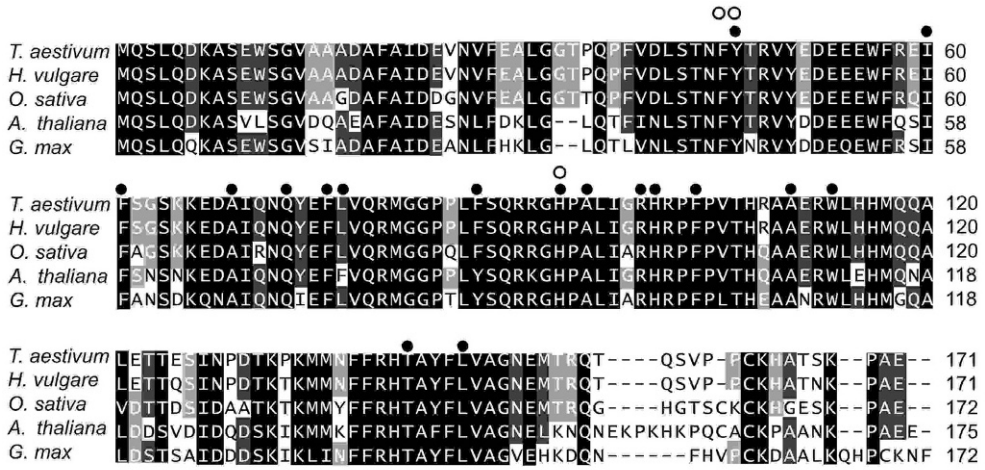

Fig. 1. Multiple sequence alignment of *Triticum aestivum* truncated hemoglobin (TatrHb) and previously reported plant 2-on-2 hemoglobin (Hbs) using *ClustalW*. Amino acid residues conserved among plant 2-on-2 Hbs are *boxed* and *shaded*, and residues conserved between 2-on-2 Hbs from plants and some microorganisms are indicated by *filled circles*. Residues related to heme-binding sites are indicated by *open circles*. Sources and *GenBank* acc. Nos. of sequences used in alignment are: *T. aestivum* (FJ006725), *H. vulgare* (AF376063), *O. sativa* (AK073898), *A. thaliana* (AF376062), and *G. max* (AY547292).

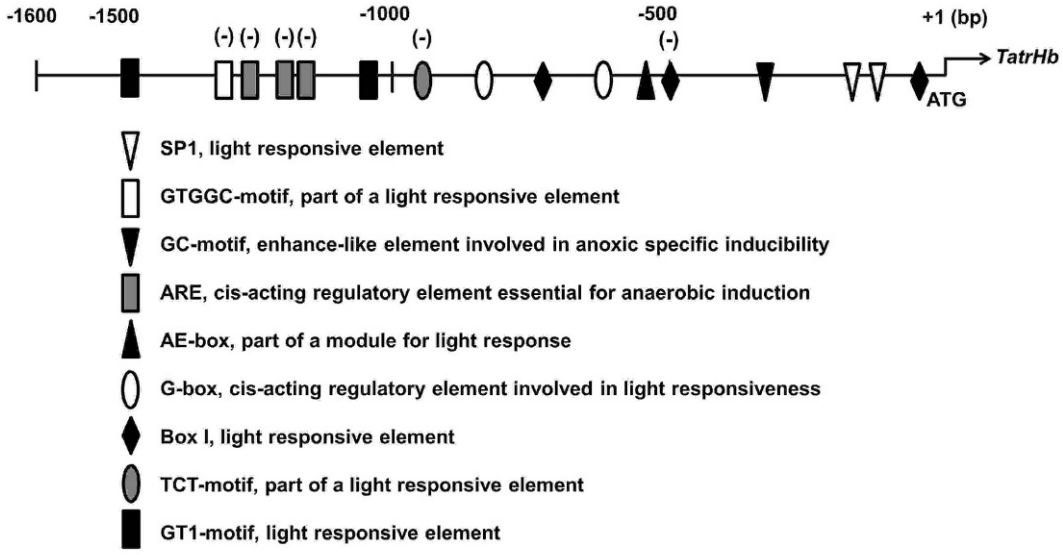

Fig. 2. Schematic map of promoter regions with putative *cis*-regulatory elements of the *TatrHb* gene. Seven *cis*-regulatory elements involved in the light response (SP1, GTGGC-motif, AE-box, G-box, Box I, TCT-motif, and GT1-motif) were found at 12 positions in the promoter region of *TatrHb*, according to the *PlantCARE* database using default parameters.

TatrHb using *PLACE* (http://www.dna.affrc.go.jp/PLACE) and *PlantCARE* (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Seven light response elements were identified at 12 positions (SP1, GTGGC-motif, AE-box, G-box, Box I, TCT-motif, and GT1-motif), and 1 regulatory element essential for anaerobic induction was identified at 4 positions (Fig. 2).

Quantitative PCR was used to measure the accumulation of *TatrHb* transcripts (Table 2). In general, the expression of *TatrHb* was reduced by the majority of treatments. In leaf tissue, the expression of *TatrHb* increased 2.5-fold after 24 h NaCl treatment; however, expression was reduced in response to treatment with ABA, ethylene, GA_3, H_2O_2, MeJA, PEG, and SA. *TatrHb* expression in response to ABA, ethylene, H_2O_2, MeJA, NaCl, PEG, and SA in roots was reduced, except in the case of GA_3 treatment. *TatrHb* expression increased 2.6-fold after 24 h GA_3 treatment in roots.

Since *trHb* is known to be involved in NO detoxification in bacteria (Ouellet *et al*. 2002, Pathania *et al*. 2002), SNP (an NO donor) and c-PTIO (an NO scavenger) were applied to young wheat seedlings in order to analyze the expression of *TatrHb* in response to exogenous NO treatment (Table 2). The expression of *TatrHb* was relatively low in both roots and leaves under c-PTIO treatment. However, we detected an approximate 2-fold increase in the expression of *TatrHb* in both leaves and roots following 0.1 mg dm^{-3} SNP treatment, a 9-fold increase in expression in leaves, and a 5.5-fold increase in roots following 1 mg dm^{-3} SNP treatment. In addition, when SNP + c-PTIO were applied to roots and leaves, the expression levels of *TatrHb* were not signifi-cantly increased as the transcript level under c-PTIO treatment.

Table 2. Transcript accumulation of *TatrHb* expressed in plants in response to phytohormones and abiotic stresses. The plant material used included 14-d-old leaves and roots of wheat treated for 24 h with 100 μM abscisic acid (ABA), 100 μM gibberellic acid (GA_3), 100 μM methyl jasmonic acid (MeJA), 100 mM H_2O_2, 200 mM NaCl, 20 % polyethylene glycol (PEG), 100 mM salicylic acid (SA), 100 cm^3 m^{-3} ethylene, 0.1 and 1 mg dm^{-3} NO donor (SNP), or 1 mg dm^{-3} NO scavenger (c-PTIO). Plants were also pretreated with c-PTIO for 2 h and then treated with SNP and c-PTIO for 3 h. Transcript levels were estimated by RT-PCR and were normalized to *Taactin* transcripts. Data are the means ± SEs of 3 plant replicates

Treatments	Leaf	Root
Control	1.00 ± 0.226	1.00 ± 0.192
ABA	0.74 ± 0.168	0.81 ± 0.166
GA3	0.57 ± 0.166	2.64 ± 0.460
MeJA	0.39 ± 0.183	0.81 ± 0.172
H_2O_2	0.22 ± 0.064	0.02 ± 0.023
NaCl	2.46 ± 0.369	0.47 ± 0.075
PEG	0.18 ± 0.085	0.06 ± 0.078
SA	0.74 ± 0.216	0.21 ± 0.098
Ethylene	0.72 ± 0.096	0.60 ± 0.232
c-PTIO	0.08 ± 0.253	0.30 ± 0.215
SNP 0.1	2.83 ± 1.552	2.41 ± 1.064
SNP 1.0	8.98 ± 1.797	5.53 ± 1.851
SNP/c-PTIO	0.10 ± 0.540	0.20 ± 0.152

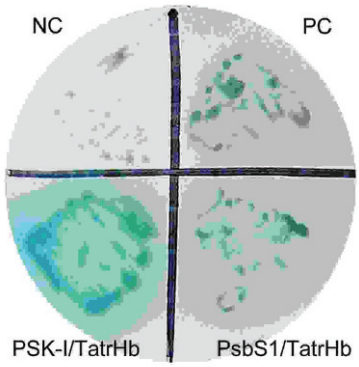

Fig. 3. Colony assay demonstrating the interactions of the PSK-I and PsbS1 subunits with TatrHb. The positive control (PC) for protein interaction was yeast cotransformed with pGADT7 and pGBKT7-53, whereas cells containing pGADT7 and pGBKT7-Lam were used as the negative control (NC). The intensity of staining indicates the qualitative binding strength of the interaction.

The YIIH system was used to identify proteins that interact with TatrHb. The TatrHb protein was fused to the GAL4 DNA-binding domain in pGBKT7. pGBKT7/TatrHb was transformed into the host strain (AH109) to generate transformants that interact with TatrHb. Thirty-nine colonies were identified by screening on double dropout (DDO; SD/-Leu/-Trp) medium and quadruple dropout (QDO; SD/-Ade/-His/-Leu/-Trp) medium with X-α-gal. Positive colonies contained the activation domain that led to vitality and blue color generation in the colonies. These were amplified by colony PCR and sequenced. Among the 39 clones, 10 clones were selected based on *BLASTX* alignment of the deduced amino acid sequences of each clone (Table 3). Seven of the 10 clones were identified as photosynthesis-related genes (YIIH 19, YIIH 23, YIIH 27, and YIIH 35) and showed high sequence similarity with the chloroplast DNA of *T. aestivum* (YIIH 19 and YIIH 27), photosystem I PSK-I subunit of *H. vulgare* (YIIH 23), and photosystem II subunit PsbS1 of *Z. mays* (YIIH 34). YIIH 18 showed high sequence homology to the small subunit precursor of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC/O) in *T. aestivum*. In addition, YIIH 31 and YIIH 35 showed high sequence homology to the large subunit of RuBPC/O (Table 3). To confirm the results of the YIIH assays, full-length cDNAs of PSK-I and PsbS1, which are involved in the photosynthetic light reactions, were cloned into pGADT7. Constructs were cotransformed with the pGBKT7-trHb bait plasmid into AH109 and protein interactions were tested by growth on selective media. The colony β-galactosidase assay demonstrated that TatrHb interacted to some degree with PSK-I and PsbS1 and the results of β-galactosidase

Table 3. List of proteins that potentially interact with *TatrHb* (RuBPC/O - ribulose-1,5-bisphosphate carboxylase/oxygenase).

Clone	Putative identification	Plant	E-value	Length
YIIH 8	expansin EXPB4	*Triticum aestivum*	8e-72	666
YIIH 13	mitochondrial transcription termination factor-like	*Oryza sativa*	4e-76	761
YIIH 17	mitochondrion rrn26 gene for rRNA large subunit	*T. aestivum*	0.0	570
YIIH 18	small subunit precursor of RuBPC/O	*T. aestivum*	2e-165	390
YIIH 19	chloroplast DNA	*T. aestivum*	0.0	457
YIIH 23	photosystem I PSK-I subunit	*Hordeum vulgare*	0.0	512
YIIH 27	chloroplast DNA	*T. aestivum*	0.0	485
YIIH 31	large subunit of RuBPC/O	*T. aestivum*	1e-117	260
YIIH 34	photosystem II subunit PsbS1	*Zea mays*	3e-111	519
YIIH 35	large subunit of RuBPC/O	*T. aestivum*	3e-142	286

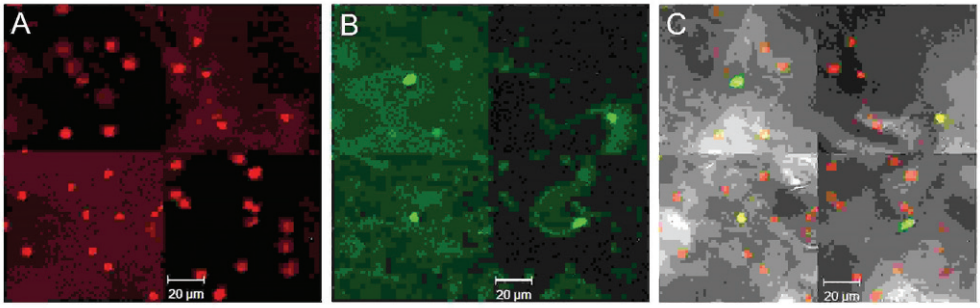

Fig. 4. Subcellular localization of TatrHb protein. Visualization of stroma-targeted TatrHb-green fluorescent protein (GFP) in living leaf epidermal cells of tobacco expressing the TatrHb-GFP fusion protein by laser scanning confocal microscopy. A fresh leaf excised from the plant was observed immediately. Confocal images of red autofluorescence from chlorophyll (*A*), green fluorescence from GFP (*B*), and the merged green and red signals (*C*).

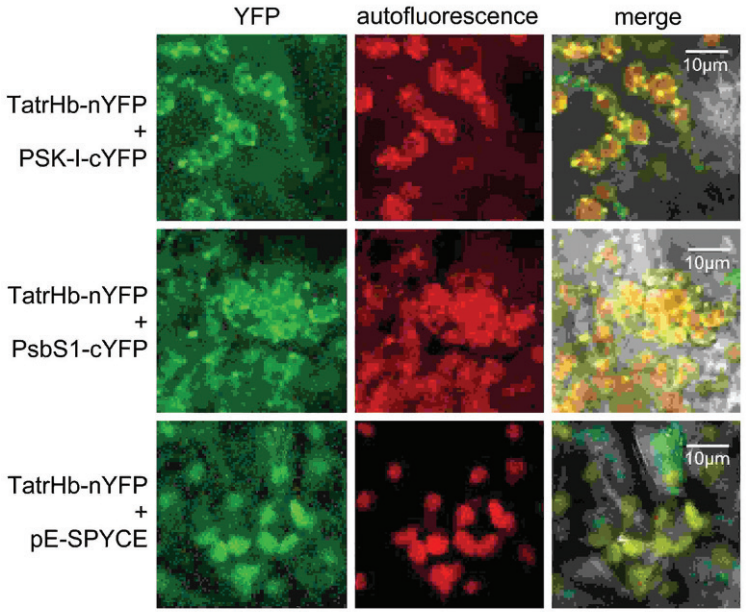

Fig. 5. Bimolecular fluorescence complementation detection of protein-protein interactions in tobacco leaf epidermal cells. The images demonstrate TatrHb and PSK-I interactions (*top row*), TatrHb and PsbS1 interactions (*middle row*), and TatrHb and empty pE-SPYCE-GW vector as a negative control (*bottom row*) in a split-yellow fluorescent protein (YFP) system. Images acquired using a confocal laser scanning microscope show YFP (505 - 530 nm) fluorescence in green (*left*), chlorophyll autofluorescence in red (*middle*), and the merged image (*right*).

Table 4. Liquid assay demonstrating the interaction of PSK-I and PsbS1 subunits with TatrHb. β-Galactosidase activities of cultures of PSK-I subunit/TatrHb and PsbS1 subunit/TatrHb cotransformants were determined as described in the Methods section. The positive control (PC) for protein interaction was yeast cotransformed with pGADT7 and pGBKT7-53 whereas cells containing pGADT7 and pGBKT7-Lam were used as the negative control (NC). Data are the means ± SE of 3 plant replicates.

Cotransformants	β-galactosidase
pGADT7:PSK-I / pGBKT7:TatrHb	2.269 ± 0.127
pGADT7:PsbS1 / pGBKT7:TatrHb	2.159 ± 0.192
pGADT7 / pGBKT7-53 (PC)	3.846 ± 0.229
pGADT7 / pGBKT7-Lam (NC)	0.185 ± 0.054

assays in liquid cultures of each yeast cotransformant were consistent with the colony assays (Fig. 3, Table 4).

A TatrHb-GFP fusion vector was constructed in order to determine the subcellular localization of the TatrHb protein in tobacco leaf cells *in vivo*. The fluorescence of the TatrHb-GFP protein was localized in the plastid where formation of saccharides occurs during photosynthesis (Fig. 4). The results further support the YIIH results and strongly suggest that TatrHb can interact with photosynthesis-related genes and photosystem subunits.

The protein-protein interactions of TatrHb with PSK-I and PsbS1 were also examined in living tobacco cells using a BiFC assay which produces reconstitution of yellow fluorescent protein (YFP). TatrHb was fused to the N-terminal of YFP using the pE-SPYNE-GW vector and 2 photosystem subunits, PSK-I and PsbS1, were fused to the C-terminal end of YFP using the pE-SPYCE-GW vector (Walter *et al*. 2004). The constructs were introduced into tobacco leaf epidermal cells by *Agrobacterium*-mediated infiltration for transient expression and association of TatrHb with TaPSK-I and TaPsbS1 was assessed in epidermal cells based on the reconstitution of YFP fluorescence (BiFC). The yellow fluorescence from YFP and red chlorophyll autofluorescence were monitored by laser scanning confocal microscopy.

The emission of YFP fluorescence was visualized at 505 - 530 nm and the autofluorescent signal was visualized at 560 nm. The merged images from YFP fluorescence and chlorophyll autofluorescence clearly indicated that TatrHb-nYFP specifically interacted with PSK-I-cYFP within the chloroplast. In addition, a strong YFP fluorescent signal was observed when TatrHb-nYFP was cotransformed with PsbS1-cYFP indicating that TatrHb and PsbS1 can associate *in vivo* (Fig. 5).

Discussion

Hbs are primarily involved in the binding and transport of oxygen and other gaseous ligands (Weber *et al.* 2001). In plants, 3 different types of Hbs have been reported: symbiotic, nonsymbiotic, and truncated 2-on-2 Hbs. In this study, we isolated trHb from wheat. Based on multiple sequence alignments of TatrHb with others trHbs, conserved amino acids between trHbs, such as Phe45, Tyr46, and HisT80, and a heme-binding site, which is a specific feature of Hb proteins, indicated that *TatrHb* encodes a typical plant 2-on-2 *Hb* gene (Fig. 1).

GLB3, a plant 2-on-2 *trHb* gene, was first isolated from *Arabidopsis* (Watts *et al*. 2001). The *Arabidopsis* GLB3 protein exhibits unusual concentration-independent binding of O_2 and CO. *GLB3* is expressed in both roots and aerial tissues and accumulation in roots was found to be 4 times higher than in shoots. Also, unlike for plant 3-on-3 *Hbs*, *Arabidopsis GLB3* mRNA levels in whole plants were reduced by hypoxia, but did not change significantly in the presence of exogenous phytohormones, such as 2,4-D, N6-(2-isopentenyl)-adenine (2iP) or ABA. In soybeans, the *trHb* gene (*GmGLB3*) was previously reported to have 2 genomic copies as demonstrated by Southern hybridization (Lee *et al*. 2004). Based on Northern hybridization, *GmGLB3* mRNA transcripts were expressed in root nodules and increased in the late stage during nodule development. *GmGLB3* transcript levels increased under flooding and kinetin treatments in soybean roots. Transcript accumulation was also observed after flooding and 2iP treatment in the stems. However, no transcripts were detected in the leaves regardless of treatment conditions (Lee *et al*. 2004).

In this study, we detected increased *trHb* transcripts in wheat in NaCl-treated leaves and GA_3-treated roots (Table 2). Similar to the levels of *GLB3* in *Arabidopsis* and *GmGLB3* in soybeans, accumulation of *TatrHb* transcripts was not affected by other treatments in leaves and roots, such as ABA, MeJA, ethylene, H_2O_2, PEG, and SA. This is consistent with previous results demonstrating that expression of *trHb* is not significantly involved in abiotic and biotic stress responses in *Arabidopsis* and soybeans.

NO is an important signaling molecule with diverse physiological functions in plants (Wendehenne *et al*. 2004, Crawford *et al*. 2006, Besson-Bard *et al*. 2008). Zhang *et al*. (2006) previously reported that SNP-derived NO enhanced maize tolerance to salt stress, and NaCl induced a transient increase in NO content in maize leaves by elevating the activities of the proton-pump and the Na^+/H^+ antiport of the tonoplast. *Arabidopsis* mutants (Atnoa1) with impaired *in vivo* NO synthase (NOS) activity displayed a higher ratio of Na^+ to K^+ in the shoots than wild-type plants due to enhanced accumulation of Na^+ and reduced accumulation of K^+ under salt stress. Germination of *Atnoa1* seeds was more sensitive to NaCl than that of wild-type seeds and wild-type plants exhibited higher survival rates than *Atnoa1* plants when grown under salt stress (Zhao *et al*. 2007). In addition, Qiao *et al*. (2008)

reported that *OsNOA1* transgenic expression rescued *Atnoa1* in seedling development under normal condition and enhanced the salt tolerance of *Atnoa1* during seed germination. When the *StNOA1* gene from *Solanum tuberosum* was inserted into the *AtNOA1* mutant plant genome using the *Agrobacterium*-mediated floral dip method, root elongation and survival rate in this transgenic plant were significantly higher than those in *Atnoa1* seedlings under salt stress. Moreover, *StNOA1* has been suggested to participate in *A. thaliana* salt-stress responses leading to increased salinity tolerance (Zhang *et al*. 2010). In tobacco leaf cells, increased salinity led to rapid changes in NO-induced fluorescence within cells of the palisade mesophyll and across all epidermal cell types including guard cells (Gould *et al*. 2003). *TatrHb* transcripts were highly expressed in leaves and roots treated with 1 mg dm^{-3} SNP (Table 2). Previous studies have shown that trHbN protein from *Mycobacterium tuberculosis* and *Mycobacterium bovis* contains an active site that transforms NO to nitrate anion (Ouellet *et al*. 2002, Pathania *et al*. 2002). Knockdown of trHbN in *M. bovis* resulted in a decrease in respiration activity upon exposure to NO. In our current study, increased exogenous NO induced by SNP treatment led to an increase in *TatrHb* transcript levels in leaves and roots which may enhance the catalysis of NO oxidation to nitrate. Roots and leaves were harvested 3 h after treatment and the accumulation of *TatrHb* transcripts was shown to be an immediate response to exogenous NO. Therefore, our results demonstrated that NO accumulation occurred following NaCl treatment in the leaves of wheat seedlings and increased NO led to a 2.5-fold upregulation of *TatrHb* transcripts (Table 2).

A YIIH assay was conducted in order to determine the function of trHb by identifying the proteins with which it interacts. Several candidate proteins were identified including 2 photosystem subunits, small and large RuBPC/O subunits and chloroplast DNA (Table 3). Ancestral Hbs may have displayed electron-transport functions. For instance, several bacterial and yeast Hbs are 2-domain proteins that contain globin and flavin domains which clearly function in transporting electrons (Bolognesi *et al*. 1997). The β-galactosidase and YIIH assays provided further evidence that TatrHb may have an electron-transfer function like ancestral Hbs based on the proteins with which TatrHb was shown to interact. Furthermore, 7 light-response elements, namely SP1, GTGGC-motif, AE-box, G-box, Box I, TCT-motif, and GT1-motif, were identified at 12 positions in the promoter region of *TatrHb* (Fig. 2). These *cis*-acting elements found in the promoter region of *TatrHb* support that TatrHb is potentially involved in photosynthesis, consistently with the implications of the results of the YIIH assay indicating candidate proteins that interacted with TatrHb.

The *trHb* gene of the unicellular green alga *Chlamydomonas eugametos* is expressed in response to active photosynthesis and is localized along the chloroplast thylakoid membranes (Couture *et al*. 1994). The *glbN* gene of *Nostoc commune* was shown to be expressed only under anaerobic conditions (Hill *et al*. 1996) and co-expressed with genes of the nitrogen fixation complex (Thorsteinsson *et al*. 1999). The various functions of trHbs indicate that the simplified 2-on-2 structure of the globin fold observed in trHbs may reflect biological functions distinct from O_2 storage or transport (Wittenberg *et al*. 2002).

Despite the above mentioned studies, the functional role of trHbs has not been well studied in plants. In our report, we examined *trHb* transcripts in wheat under phytohormone treatment and abiotic stresses and investigated trHb potential role in photosynthesis through YIIH and BiFC assays. Subcellular localization of the TatrHb-GFP fusion protein and BiFC assay conclusively demonstrated that TatrHb was expressed in plastids. The TatrHb-GFP fusion protein was detected in the plastids (Fig. 4) and the detection of the YFP signal demonstrated that TatrHb interacted with PSK-I and PsbS1 (Fig. 5). PSI-K has a role in organizing the peripheral LHCs on the core antenna of photosystem I. The PsbS subunit of PSII plays a crucial role in pH and xanthophyll-dependent nonphotochemical quenching (NPQ) of excess absorbed light energy, thus contributing to the photoinhibition defense mechanism (Bergantino *et al*. 2003). These results suggest that TatrHb has a putative role in photosynthesis and plays a similar role in active photosynthesis as the *trHb* gene of the unicellular green alga *C. eugameto*. However, the role of TatrHb in photosynthesis is not yet definitively established and many questions are still unanswered. Therefore, in further studies, it will be necessary to investigate how TatrHb acts with photosynthetic factors and to evaluate the physiological and chemical effects on transgenic plants in which TatrHb is constitutively expressed. Additionally, since *TatrHb* transcript levels in roots and leaves were increased following treatment with an NO donor, consistently with group I (trHbN) *trHbs* from bacteria that have roles in scavenging and detoxification of NO, TatrHb may still share some characteristics and functions with bacterial trHb as observed in its ancestors. Furthermore, it is necessary to investigate the putative role of TatrHb in the scavenging of NO.

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