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Cloning and Expression Analysis of 2-on-2 Hemoglobin from Soybean

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Hemoglobins (Hbs) are heme proteins found in all five kingdoms of living organisms. In plants, three different classes of Hbs have been identified-nonsymbiotic Hbs from diverse species, symbiotic Hbs from nitrogen-fixing plants, and so-called 2-on-2 Hbs. Here, we report the cloning and expression analysis of the 2-on-2 Hb gene, *GmGLB3*, from soybean. The *GmGLB3* cDNA clone encodes a protein for 172 amino acid residues. Its deduced amino acid sequence shows the highest identity (74%) with 2-on-2 Hb from *Medicago truncatula*. Multiple sequence alignment confirms the conserved and signature amino acid residues previously reported with plant 2-on-2 Hbs. Genomic Southern hybridization demonstrates that *GmGLB3* has two copies in the soybean genome. Based on our northern hybridization, the *GmGLB3* gene is specifically expressed in root nodules, with levels increasing in the late stage during nodule development. Its transcript level is also increased under flooding and kinetin treatments in the roots, or under flooding and 2-iP treatments in the stems. However, no transcript is detected in the leaves regardless of treatment. Therefore, we propose that the *GmGLB3* gene is specifically expressed in root nodules and that its expression in other plant organs is regulated by cytokinin and/or flooding.

Keywords: 2-on-2 hemoglobin, cytokinin, flooding, root nodule, soybean

Hemoglobins (Hbs), found in all five kingdoms of living organisms, are primarily involved in the binding and transport of oxygen and other gaseous ligands (Weber and Vinogradov, 2001). They function via reversible binding during the storage and transport, as well as by facilitating the diffusion of oxygen. In plants, three different types of hemoglobins have been reported -- symbiotic, nonsymbiotic, and truncated 2-on-2 Hb.

Symbiotic Hbs (GLB2S) are found in leguminous plants and in actinorrhizal plants that form a symbiosis with N_2-fixing bacteria. They function as oxygen buffers and carriers, and provide O_2 to the micro-symbionts actively fixing N_2 in root nodules. These Hbs also prevent O_2 inhibition of the nitrogenase complex by facilitating its diffusion to N_2-fixing symbionts (Appleby, 1992).

Nonsymbiotic Hbs (GLB1) are ubiquitous in plants, and have been found in virtually all examined species, from liverworts (*Marchantia polymorpha*, AY026341) to dicots, such as soybean (*Glycine max*; Andersson et al., 1996), *Arabidopsis thaliana* (Trevaskis et al., 1997), and small radish (*Raphanus sativus*; Kim and An, 2003). Their expression patterns differ from those of the symbiotic Hbs, generally being detected in a variety of tissues, including the roots, stems, and leaves of both monocots and dicots (Hill, 1998). These patterns also vary significantly among different species, with the highest levels of expression often being found in metabolically active or stressed organs/tissues.

Recently a third group of Hbs (GLB3), originally identified in prokaryotes, protozoa, and algae, have been reported in plants. A so-called truncated Hb was first isolated from *Arabidopsis* (Watts et al., 2001). This AtGLB3 protein has a three-dimensional structure similar to the characteristic 2-on-2 arrangement of α-helices, but which is distinct from the 3-on-3 arrangement of the standard globin fold (Pesce et al., 2000). *AtGLB3* is expressed throughout the plant; its transcript level is reduced by hypoxia but increased slightly by treatment with 2,4-D or 2-iP. Therefore, expression of *AtGLB3* is regulated differently from the two other *Arabidopsis* Hbs (Watts et al., 2001). Expressed sequence tags with high similarity to *AtGLB3* have been identified from barley (AF376063) and *Medicago truncatula* (TC76399), and clones have been reported from wheat (*Triticum aestivum*; Larsen, 2003) and *Datisca glomerata* (CAD33536).

The expression patterns of five cloned Hb genes, i.e., *GmGLB1*, *GmGLB2S; a*, *GmGLB2S; c1*, *GmGLB2S; c2*, and *GmGLB2S; c3*, have now been investigated in

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soybean (Ellfolk and Sievers, 1973; Hurrell and Leach, 1977; Hyldig-Nielsen et al., 1982; Wiborg et al., 1982; Andersson et al., 1996). Among them, four are known to be symbiotic. The *GmGLB2S* group is expressed at high concentrations only in root nodules (Appleby, 1992), whereas mRNAs of the non-symbiotic Hb gene (*GmGLB1*) are detected in the stem, seedling, leaf, and root as well as at increased levels in the nodule (Andersson et al., 1996). However, no report has been made on the cloning and expression analysis of 2-on-2 Hb, except that its homologous sequence can be found from the public soybean EST database (TC200098).

In the present study, we cloned *GmGLB3*, a 2-on-2 Hb gene from soybean, and analyzed its expression pattern in different organs and in response to environmental stresses and plant hormones. This is the first report on the presence of three different types of Hb genes in a single genome, and on their expression in the root nodules of a legume.

MATERIALS AND METHODS

Bacterial Strain and Plant Material

To nodulate soybean (*Glycine max* L. cv. Backtae) seeds were placed for imbibition on paper towels moistened with distilled water. After 3 d, the sprouted seeds were inoculated with 100 mL of approximately 5×10^7 colony-forming units mL^{-1} (cfu mL^{-1}) of *Bradyrhizobium japonicum* strain USDA 110, which had been cultured in YEM (Vincent, 1970) and suspended in a buffered nodulation medium (BNM, Ehrhardt et al., 1992). The inoculated seedlings were planted in pots filled with vermiculite, reared in a growth chamber at 27°C under a 16-h photoperiod, and watered with 0.5X BNM during the test period. To isolate the root nodules at various stages of their development, the plants were removed from the vermiculite and washed in tap water. The nodules were removed with forceps. As a control, non-inoculated plants were used for isolating total RNA from their roots and other tissues. All harvested tissues were frozen in liquid nitrogen and stored at -80°C.

Stress and Hormone Treatments

Six, 15-d-old seedlings per treatment were used in each experiment. For the cold treatment, seedlings were transferred from 27°C to 4°C and incubated for 24 h. To simulate flooding conditions, seedlings were completely immersed in H_2O for 24 h. For the hormone treatments, seedlings were carefully pulled out of the soil and immersed, with their roots, for 24 h in one of the following solutions: 100 μM indoleacetic acid (IAA), 90 μM kinetin, or 90 μM 6-(γ-γ-dimethyl-allylamino) purine (2-iP) (He et al., 2002). Afterward, all samples were frozen in liquid nitrogen and stored at -80°C.

Isolation of Nucleic Acids

Total RNA was isolated as previously described (Uhde-Stone et al., 2003) and genomic DNA was extracted according to Doyle and Doyle (1990). RNase-free DNase (Promega, USA) was used to remove genomic DNA contamination in the RNA samples during RNA purification.

Cloning and Sequence Analysis

The cDNA clone containing the 2-on-2 Hb gene was selected from our unpublished soybean root nodule EST database, and was sequenced using ABI PRISM 3700™ (Applied Biosystems, USA). Sequences were analyzed with the BLAST (Altschul et al., 1990), while the amino acid alignments were carried out via the CLUSTAL W program.

DNA and RNA Gel Blot Analysis

For the DNA analysis, genomic DNA (10 μg) purified from soybean leaves was digested with restriction enzymes, separated on a 0.8% agarose gel, and transferred to a nylon membrane (Amersham, UK) by the capillary blotting method (Sambrook et al., 1989). For RNA analysis, total RNA (10 μg) was separated on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane. These blots were hybridized overnight with a ^{32}P-labeled DNA probe, under the following conditions: 6X SSC [0.9 M NaCl and 0.09 M sodium citrate (pH 7.0)], 5X Denhardt's solution, and 0.1% SDS, at 63°C. The hybridized blots were washed at 63°C, with the salt concentration gradually decreasing to 1X SSC, and were exposed to X-ray film (Fuji, Japan).

Reverse Transcription-PCR

Semi-quantitative RT-PCR was performed to analyze how expression patterns responded to various stresses and plant hormones. PCR primers were designed for gene-specific amplification on the UTR

of *GmGLB1* and *GmGLB3*; their sequences were: for GmGLB1F, 5′-TGGGAAGAGCAAGAAGC-3′; for GmGLB1R, 5′-AAGAGGAGGGTGGTTTCA-3′; for GmGLB3F, 5′-TTTACATGGGTGCTGCTTCA-3′; and for GmGLB3R, 5′-TGCTTTTGCCATAGACGAGG-3′. Primers for amplifying actin GmActinF, 5′-CCCCT-CAACCCAAAGGTCAACAG-3′; and GmActinR, 5′-GGAATCTCTCTGCCCCAATTGTG-3′, were used as a quantitative control. Total RNA (1 μg) was used as template for reverse transcription after treatment with RNase-free DNase (Promega, USA). The PCR cycles included 95°C for 5 min of initial denaturation, followed by 94°C for 15 sec, 52°C for 50 sec, and 72°C for 50 sec (for a total of 25 cycles), followed by a 5-min final extension at 72°C. The amplified PCR products were separated on a 1% agarose gel.

RESULTS AND DISCUSSION

Isolation and Characterization of the cDNA Clone Encoding 2-on-2 Hemoglobin

We have isolated a cDNA clone showing high sequence homology with previously reported plant 2-on-2 Hbs from our unpublished soybean root nodule EST database. The full-length sequence of this cDNA clone, named *GmGLB3*, has been determined and analyzed (GenBank Accession No. AY547292). This *GmGLB3* clone encodes a protein for 172 amino acid residues, with a molecular weight of 19.8 kDa and a calculated pI value of 7.16. The deduced amino acid sequence shows high identity values, ranging from 65.7% with *T. aestivum* to 74% with *M. truncatula*. However, identities with previously reported soybean Hb genes are only 8.6% with *GmGLB1* and 12.1% with *GmGLB2S;c3*.

We performed multiple sequence alignment of GmGLB3 and other previously reported plant 2-on-2 Hbs (Fig. 1). The alignment revealed a remarkably high homology among them. In particular, amino acids, such as Phe_{43}, Tyr_{44}, and His_{78}, which are conserved between 2-on-2 Hbs from plants and some microorganisms (Watts et al., 2001), are also found in GmGLB3. Moreover, GmGLB3 possesses Ala_{68}, which is characteristic of plant 2-on-2 Hbs (Fig. 1). Based on these results, we have now demonstrated that *GmGLB3* encodes a typical plant 2-on-2 Hb gene.

GmGLB3 Genes in the Soybean Genome

Since a truncated 2-on-2 Hb was first reported from

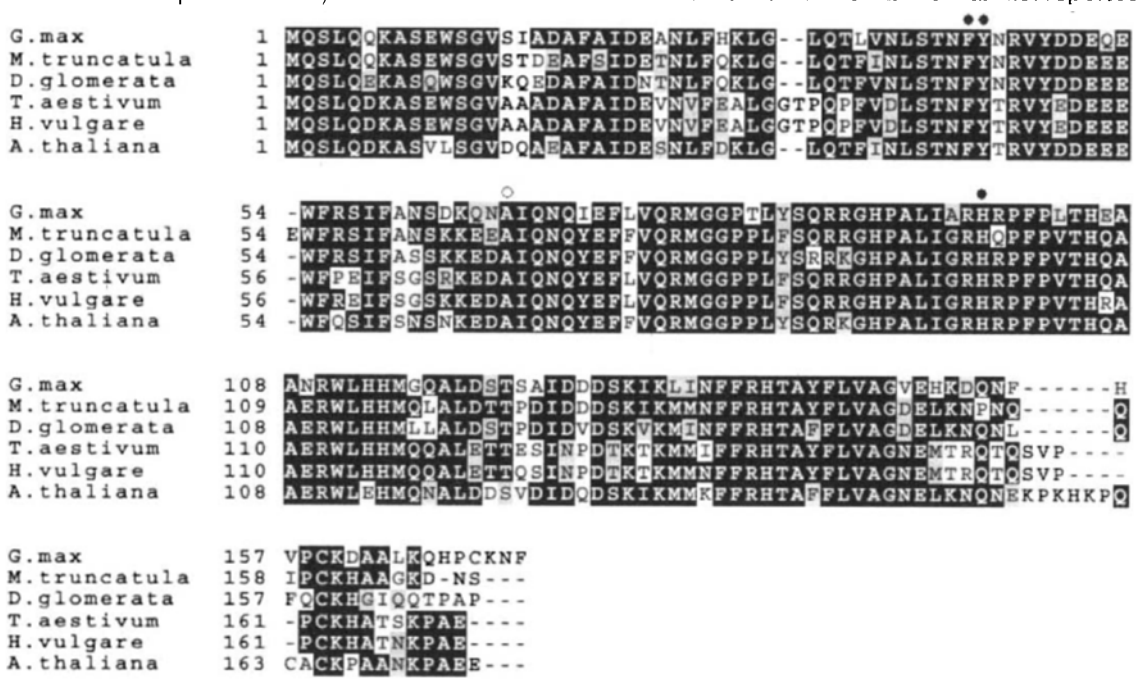

Figure 1. Multiple sequence alignment of GmGLB3 and previously reported plant 2-on-2 hemoglobins using CLUSTAL W. Amino-acid residues conserved among plant 2-on-2 hemoglobins are box-shaded, residues conserved between 2-on-2 Hbs from plants and some microorganisms are indicated with filled circles, and a residue conserved specifically in plant 2-on-2 Hbs is indicated with an open circle. Sources and GenBank accession numbers of sequences used in alignment are G. max, *Glycine max* (in this study); M. truncatula, *Medicago truncatula* (TC76399); D. glomerata, *Datisca glomerata* (CAD33536); T. aestivum, *Triticum aestivum* (AY151391); H. vulgare, *Hordeum vulgare* (AF376063); A. thaliana, *Arabidopsis thaliana* (AF376062).

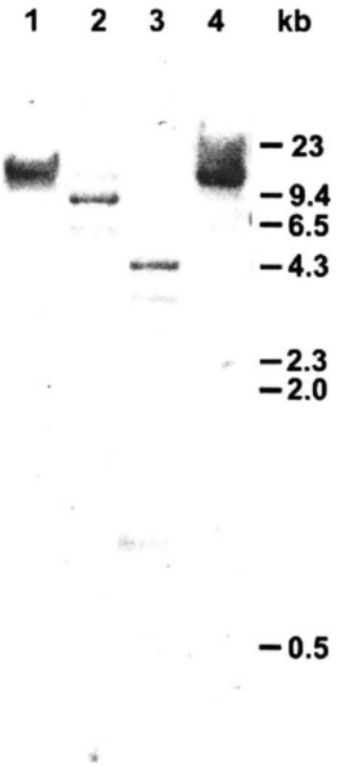

Figure 2. Genomic Southern analysis for *GmGLB3* gene. DNA blot was probed with ^{32}P-labeled full-length *GmGLB3* clone and washed under low-stringency conditions. Lane 1, *Bam*HI; Lane 2, *Eco*RI; Lane 3, *Hin*dIII; Lane 4, *Pst*I.

Arabidopsis, four more such genes have been cloned from plants. However, their copy number had not yet been determined in those genomes. Therefore, we performed Southern blot analysis, using the full-length insert of the *GmGLB3* cDNA clone as a probe (Fig. 2). More than two hybridizing bands were detected in all lanes containing restricted soybean genomic DNA, and each lane had hybridizing bands with two different levels of intensity. Because the *GmGLB3* clone comprised a *Hin*dIII site, and hybridizing bands were not matched with those of *GmGLB1* and *GmGLB2* (data not shown), we conclude that the soybean genome contains two copies of the 2-on-2 Hb gene.

Expression Patterns of *GmGLB3* in Soybean Organs

Expression analysis of plant 2-on-2 Hbs has been very limited. The *AtGLB3* gene from *Arabidopsis* is expressed throughout various organs; its transcript is four times higher in the roots than in the shoots (Watts et al., 2001). *TaHb2*, a 2-on-2 Hb from wheat, is expressed at varying levels in all organs, being more

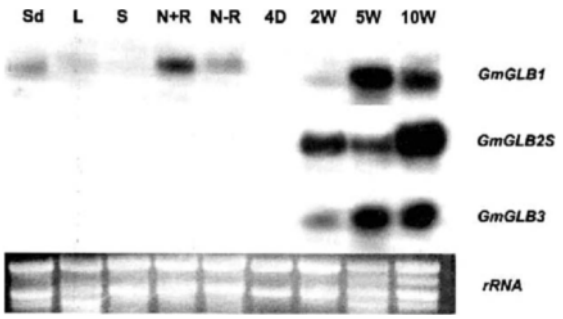

Figure 3. Expression patterns of three soybean hemoglobin genes for different organs and during root nodule development. *GmGLB1*, nonsymbiotic hemoglobin; *GmGLB2S*, symbiotic hemoglobin Lbc3; *Gm GLB3*, 2-on-2 hemoglobin; Sd, seedling; L, leaf; S, stem; N+R, root grown with nitrogen source; N-R, root grown without nitrogen source; 4D, root at 4 DAI (days after inoculation); 2W, nodule at 2 WAI (weeks after inoculation); 5 W, nodule at 5 WAI; 10 W, nodule at 10 WAI.

abundant in the flowers, stems, and leaves than in the roots (Larsen, 2003). In contrast, a 2-on-2 Hb gene from *Medicago truncatula* is expressed specifically in the root nodules but not in other organs (Fedorova et al., 2002).

Because it was isolated from a root nodule cDNA library, we hypothesized that the *GmGLB3* gene would show a nodule-specific or -enhanced expression pattern. Therefore, we purified total RNA from the seedling, leaves, stem, roots grown with and without a nitrogen source, and root nodules at various developmental stages. These samples were then subjected to northern hybridization using the full-length insert of the *GmGLB3* cDNA clone as a probe. Transcripts were specifically detected only in the root nodules, especially during their development (Fig. 3), with the highest expression level found at 5 to 10 weeks after inoculation (WAI). This pattern is similar to that of the symbiotic Hb gene *GmGLB2S; c3*.

Expression Patterns of *GmGLB1* and *GmGLB3* under Treatments with Various Plant Hormones and Abiotic Stresses

Expression and induction of nonsymbiotic Hb genes has been reported in several plant species. For example, accumulation has been detected under hypoxic conditions in barley (Taylor et al., 1994) and rice (Lira-Ruan et al., 2001). In *Arabidopsis*, *AHB1* is induced by hypoxia in the roots and rosettes, whereas *AHB2* expression is triggered by low temperature (Trevaskis

et al., 1997). *Mhb1*, the nonsymbiotic Hb gene of *Medicago sativa*, is also inducible by hypoxia but not by low temperature (Seregelyes et al., 2000). Finally, *RsHb*, the nonsymbiotic Hb gene of *Raphanus sativus*, is activated by flooding and mannitol treatments (Kim and An, 2003).

Despite those previous studies, expression of 2-on-2 Hb genes under stress conditions and hormone treatments has never been investigated in legumes. Therefore, to determine the expression patterns of *GmGLB3*, a 2-on-2 Hb from soybean, and to identify the relationship between expression of nonsymbiotic Hb and 2-on-2 Hb, we conducted semi-quantitative RT-PCR analysis of various organs under flooding, cold, auxin, and cytokinin treatments. Expression of *GmGLB1* was induced by cold stress in all tested organs, as well as by flooding, which causes hypoxia, in the roots and leaves but not in the stems (Fig. 4). In contrast, *GmGLB3* was not induced by cold treatment in any organs and was induced by flooding only in the roots and stems. In general, plant non-symbiotic Hb genes are highly expressed under hypoxic conditions as well as in metabolically activated tissues. This occurs to maintain the energy status of cells in low-oxygen environments (Sowa et al., 1998). *GmGLB3* might complement this role of *GmGLB1* in roots under low-oxygen conditions, and may be the primary gene functioning in stems under hypoxia.

Symbiotic Hb genes manifest a universal response to cytokinin. In legumes, for example, cytokinin activates legume nodulin genes such as *ENOD40* and *Sesbania ENOD2*, which normally are expressed in roots following *Rhizobium* infection (Dehio and de Bruijn, 1992; Fang and Hirsch, 1998). However, 2,4-D cannot activate *ENOD40* in white clover (Mathesius et al., 2000). Because cytokinins can induce a number of nodulin genes, it has been proposed that cytokinin production is an early event in *Rhizobium* infection (Jimenez-Zurdo et al., 2000; Mathesius et al., 2000), although the mRNA level of 2-on-2 Hb genes is not markedly changed by treatment with 2,4-D or 2-iP in *Arabidopsis* (Watts et al., 2001). In our study, *GmGLB1* was induced by auxin and cytokinin treatments in all tested organs, whereas *GmGLB3* was activated only by kinetin in the roots (Fig. 4). That pattern is similar to the one for nodulin genes expressed in cytokinin-treated legume roots. The variation in root response to kinetin and 2-iP seems to be a result of the difference in their chemical origins, that is, 2-iP is the natural form while kinetin is the synthetic version. Because specific expression was found in the root nodule, we suggest that the *GmGLB3* gene

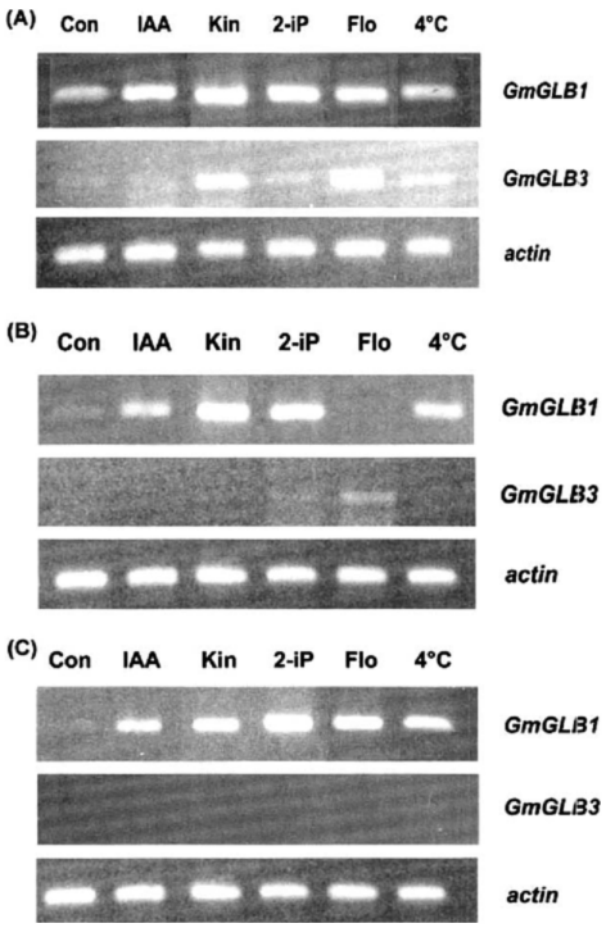

Figure 4. Semi-quantitative RT-PCR analysis of *GmGLB1* and *GmGLB3* genes under treatment with various plant hormones and stresses for root (**A**), stem (**B**), and leaf (**C**). Con, untreated control; IAA, 100 μM indoleacetic acid; Kin, 90 μM kinetin; 2-iP, 90 μM 6-(γ-γ-dimethylallylamino) purine; Flo, flooding; 4°C, cold treatment.

may be regulated by a mechanism similar to that for nodulin genes. In this respect, it would be useful to determine whether its promoter region contains the so-called nodulin motif, which lies in the promoter region of the symbiotic Hb gene (Uchiumi et al., 2002).

Our study has revealed the presence of three different kinds of Hb genes in the soybean genome. The truncated 2-on-2 type, *GmGLB3*, is specifically expressed in the root nodule, but its expression in non-nodular organs can also be induced by flooding and kinetin treatments. Distinct expression patterns for *GmGLB1* and *GmGLB3* in various organs suggest that the former is important for the development of normal tissues whereas, under hypoxia or cytokinin treatment, both genes might have redundant functions, depending on the type of organ.

Further examination, such as with promoter analysis of the *GmGLB3* genomic clone and *in situ* hybridization in the soybean nodule, will facilitate better understanding of the function and regulation of 2-on-2 Hb genes in plants.

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