Expression of soybean plant hemoglobin gene family under abiotic stresses

Masato Araragi1, Airi Ikeura2, Toshiki Uchiumi1,*

1Graduate School of Science and Engineering, Kagoshima University, 1-21-35 Korimoto, Kagoshima 890-0065, Japan;
2Faculty of Science, Kagoshima University, 1-21-35 Korimoto, Kagoshima 890-0065, Japan
*E-mail: uttan@sci.kagoshima-u.ac.jp  Tel: +81-99-285-8164  Fax: +81-99-285-8163

Received June 19, 2020; accepted September 7, 2020 (Edited by K. Suzuki)

Abstract  Many abiotic stresses induce the generation of nitric oxide (NO) in plant tissues, where it functions as a signal molecule in stress responses. Plants modulate NO by oxidizing it to NO3− with plant hemoglobin (GLB), because excess NO is toxic to cells. At least eight genes encoding GLB have been identified in soybean, in three clades: GLB1, GLB2, and GLB3. However, it is still unclear which GLB genes are responsible for NO regulation under abiotic stress in soybean. We exposed soybean roots to flooding, salt, and two NO donors—sodium pentacyanonitrosylferrate (III) dihydrate (SNP) and S-nitroso-N-acetyl-d,l-penicillamine (SNAP)—and analyzed expression of GLB genes. GmGLB1, one of two GLB1 genes of soybean, significantly responded to both SNP and SNAP, and its induction was almost completely repressed by a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. GmGLB1 responded to flooding but not to salt, suggesting that it is responsible for NO regulation under NO-inducing abiotic stresses such as flooding. GmGLB3, one of two GLB3 genes of soybean, did not respond to NO donors at all but did respond to flooding, at a lower level than GmGLB1. These results suggest that flooding induces not only NO but also unknown factor(s) that induce GmGLB3 gene in soybean.

Key words: flooding, nitric oxide, plant hemoglobin, salt, soybean.

Introduction

Soybean is indispensable as a source of protein and oil with isoflavones and phenolic compounds (Kim et al. 2012; Sugiyama et al. 2017). As populations continue to increase (Angel et al. 2011; Seto et al. 2011), the demand for soybean will increase too. Constraints on soybean production imposed by abiotic stresses such as flooding and salt are of concern all over the world, especially in island countries because of rising sea levels (Muis et al. 2015).

Stresses applied to soybean root significantly inhibit root growth (Liu et al. 2017; Valliyodan et al. 2014). In tomato, sodium pentacyanonitrosylferrate (III) dihydrate (SNP), a NO donor, inhibits elongation of the main root and induces lateral root formation [as does 1-naphthylacetic acid (NAA), a synthetic auxin], and higher concentrations (>200 µM) decrease total root length in a dose-dependent manner (Correa-Aragunde et al. 2004). The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), reduces the effect of NAA on the number of lateral roots, suggesting that NO is involved in the regulation of root development by auxins. In addition, NO reacts with O2− to generate peroxynitrate, a powerful oxidant that can mediate the tyrosine nitration of proteins, which damages plant cells (Del Río 2015). Therefore, excess NO produced by abiotic stresses (Simontacchi et al. 2015) inhibits plant growth.

Flooding causes hypoxic conditions in soil. As NO is generated in plants under hypoxic conditions (Benamar et al. 2008; Hebelstrup et al. 2012; Shimoda et al. 2005), excess NO might be involved in inhibition of root growth by flooding. So if NO could be controlled, plants might grow better under flooding. Some plant hemoglobins (GLBs) scavenge NO. GLBs are divided into three clades—GLB1, GLB2, and GLB3 (Supplementary Figure S1)—on the basis of their amino acid sequences (Hunt et al. 2001; Watts et al. 2001). GLB1 is involved in NO control in various plants. In Arabidopsis, AtGlb1 is induced by flooding. AtGlb1-overexpressors had very low NO emission, and AtGlb1-silenced lines had very

Abbreviations: cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAR-4M, diaminorhodamine-4M; GLB, plant hemoglobin; NAA, 1-naphthylacetic acid; NO, nitric oxide; PF, potassium ferrocyanide; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SNAP, S-nitroso-N-acetyl-d,l-penicillamine; SNP, sodium pentacyanonitrosylferrate (III) dihydrate; SS, soybean-culture solution.

This article can be found at http://www.jspcmb.jp/
Published online January 30, 2021

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high NO emission (Hebelstrup et al. 2012). One of the GLB1 genes of *Lotus japonicus*, *LjGLb1-1*, is a NO- and hypoxia-inducible gene encoding NO-scavenging hemoglobin (Fukudome et al. 2016; Shimoda et al. 2005, 2009). In soybean, *GmGLB1* is expressed in response to flooding (Vallyyanan et al. 2014). Although the function of GmGLB1 is unclear, it might scavenge NO induced by flooding in the same way as LjGLb1-1 does (Fukudome et al. 2019b). *GmGLB3* expression is also induced by flooding (Lee et al. 2004), but its function is still unknown. *GmGLB2* genes encode leghemoglobins that are specific to nodules and show varieties of expression profile through nodule development (Fuchsan and Appleby 1979). Because of their high oxygen affinity (Kundu and Hargrove 2003), GmGLB2s might contribute to nitrogenase activity of rhizobia inside nodules by controlling oxygen partial pressure as same as leghemoglobins of other legumes (Appleby 1992).

NO is increased by salt in many plants. In *Hylotelephium erythroictum*, salt-induced NO promotes Ca\(^{2+}\) influx, which modifies the ion balance by promoting Na\(^{+}\) efflux and suppressing K\(^{+}\) efflux (Chen et al. 2019). In *Arabidopsis*, salt-induced NO decreases root meristem size (Liu et al. 2015). In soybean, salt increases root concentrations of Na\(^{+}\) and Cl\(^{-}\) (Umezawa et al. 2000) without an increase of NO (Kataria et al. 2020). Thus, soybean may respond to salt differently from other plants. It is important to know whether *GmGLB1* contributes to the maintenance of NO concentrations under salt stress in soybean.

At least eight genes encoding GLB have been identified in soybean. Two genes, *GmGLB1* and *GmGLB1-like*, are classified as GLB1 (Anderson et al. 1996; Schmutz et al. 2010). Four genes are classified as GLB2: genes for leghemoglobin A, C1, C2, and C3, which are specific to the root nodules (Hyldig-Nielsen et al. 1982; Wiborg et al. 1982). One gene, *GmGLB3*, is classified as GLB3 (Lee et al. 2004). The NCBI database lists one uncharacterized gene (https://www.ncbi.nlm.nih.gov/nuccore/NM_001249232.2) with high similarity to *GmGLB3* (79% amino acid identity), which we refer to here as *GmGLB3-like*. We could find no comprehensive report on the relationship between these GLB genes and NO in soybean. Here, we focused on the expression of all eight GLB genes of soybean under flooding and salt, and identified one that is induced by NO and may be responsible for NO modulation under these abiotic stress conditions.

**Materials and methods**

**Plants and bacteria**

*Glycine max* ‘Fukuyutaka’ was used in all experiments. *Bradyrhizobium diazoefficiens* USDA110, the genome of which is sequenced (Kaneko et al. 2002), was used as a microsymbiont. For preparation of rhizobial inoculant, USDA110 was cultured in a conical flask in 50 ml of yeast-mannitol medium (Kelee et al. 1969) for 7 days and diluted with distilled water to the desired concentration.

**Seeds sterilization and thinning**

Seeds were sterilized in 0.5% sodium hypochlorite for 1 min and washed 10 times with distilled water, then stirred in distilled water for 10 min. Four seeds were sown at a depth of 2.5 cm in an autoclaved Leonard jar filled with autoclaved vermiculite 300 ml of soybean-culture solution (SS, Supplementary Table S1). After 4 days (16 h light/8 h dark at 28°C), the germinated seeds were thinned to one per jar; we define this day as 0 days post-germination (dpg).

**Long-term stress treatment of plants**

Sterilized seeds were sown without rhizobial inoculation and maintained as above. After thinning, plants were treated with flooding or salt at 0 dpg. For flooding, Leonard jars were submerged in SS for 10 days. For salt treatment, jars received 300 ml of NaCl solution (5 or 50 mM in SS). 300 ml of SS was used for the control. Plants were grown in an incubator (16 h light/8 h dark at 28°C) for 10 days, and fresh weights of roots and leaves were measured.

**Detection of NO released from roots after salt treatment**

Two hundred non-sterilized seeds were sown in a tray (37 cm width × 26 cm length × 5 cm depth) filled with vermiculite and 21 of distilled water. After 5 or 6 days, six seedlings were moved into each of 6 Leonard jars filled with vermiculite moistened with 300 ml of SS. The plants were grown in an incubator (16 h light/8 h dark at 28°C) for 13 or 14 days and then treated with salt: 100 ml of SS containing NaCl (50 mM) was added per jar. 100 ml of SS was used for the control. 3, 6 or 24 h after each treatment, 100 ml of distilled water was passed through the jar, and the leachate was collected. We added 10 µl of diamorphodamine-4 M solution (Dar-4M: Goryo Chemical, Inc., Chuo-ku, Sapporo, Japan; diluted 1:500 with distilled water) to 10 µl of the leachate, and measured the intensity of fluorescence on a microfluorescence spectrophotometer ES-2 (Malcom Co., Ltd., Honmachi, Shibuya, Tokyo), with excitation at 560 nm and emission at 575 nm. The released NO was expressed as relative fluorescence intensity per dry weight of roots.

**Detection of NO released from roots after flooding**

In a large Petri dish (16 cm diameter × 4 cm depth), hundred seeds in autoclaved vermiculite with 200 ml of distilled water were incubated for 24 h. Germinated seeds were sterilized with 0.5% sodium hypochlorite, and three were placed in each of 18 watertight pots (7 cm width × 7 cm length × 9 cm depth) with 5 ml of 1% agarose. After 7 days’ incubation at 28°C (16 h light/8 h dark), pots were flooded with 100 ml of distilled water. As a control, pots were flooded with 100 ml of distilled water.
and immediately drained. After incubation for 3, 6, and 24 h, each pot was washed out with 100 ml of distilled water, and then 5 ml of distilled water was added into the pot, which was then incubated for 30 min at room temperature. We mixed 10 μl of the incubated water with 10 μl of DAR-4M solution (diluted 1:100 with distilled water) and measured NO released from roots as above.

**Short-term stress and SNP treatment of plants**

Sterilized seeds without rhizobial inoculation were used. After thinning, plants were grown for 9 days (16 h light/8 h dark at 28°C). They were then treated for 24 h with flooding or salt and SNP (Nacalai Tesque Inc., Nakagyo-ku, Kyoto, Japan) as a NO donor. As an analog of SNP, potassium ferrocyanide (PF; MP Biomedicals, LLC, Irvine, CA, USA) was used. For flooding, Leonard jars were submerged in SS. For salt treatment, jars received 100 ml of SS containing NaCl (50 mM). For SNP treatment, each jar also received 100 ml of SS containing 500 μM SNP. 100 ml of SS was used for the control. After 24 h, roots were quickly frozen in liquid N₂ for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis.

**SNAP and cPTIO treatment of plants**

Sterilized seeds without rhizobial inoculation were used. S-nitroso-N-acetyl-d,l-penicillamine (SNAP; Cayman Chemical Company, Ann Arbor, MI, USA) was used as a NO donor, and cPTIO (Dojindo Laboratories, Kamimashiki, Kumamoto, Japan) was used as a NO scavenger. After thinning, plants were grown for 7 days (16 h light/8 h dark at 28°C). Then roots cut from plants were put between filter papers moistened with 5 ml of distilled water containing 1 mM SNAP or 1 mM SNAP + 1 mM cPTIO at room temperature for 12 h in the dark. The roots were quickly frozen in liquid N₂ for qRT-PCR analysis.

**Soybean plants for analysis of tissue-specific expression of GLB genes**

Just after sowing, 10 ml of rhizobial inoculant (1×10⁷ cells ml⁻¹ in distilled water) was added. After rhizobial inoculant was added into the Leonard jars, seedlings were grown for 4 weeks (16 h light/8 h dark at 28°C). Then nodules, roots, and leaves were detached from the plants and quickly frozen in liquid N₂ for isolation of total RNA.

**Isolation of total RNA**

Total RNA was prepared with a Plant Total RNA Extraction Miniprep System (Viogene BioTek Corp., Sijihh, New Taipei City, Taiwan). It was purified by DNase treatment with RT-grade DNase (Fujifilm Wako Pure Chemical Industries, Ltd. Corp., Osaka, Japan) and recombinant RNase inhibitor (TaKaRa Bio Inc., Kusatsu City, Shiga, Japan). It was then precipitated by isopropanol in the presence of 3 M NaOAc and dissolved in RNase-free water.

**Analysis of GLB expression by qRT-PCR**

qRT-PCR was conducted with RNA samples from roots, nodules, and leaves. Reactions were performed in 96-well plates on an Applied Biosystems 7300 Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) with a One Step TB Green PrimeScript Plus RT-PCR Kit (TaKaRa Bio Inc., Kusatsu City, Shiga, Japan). TB Green mix was used in a final volume of 15 μl per well containing 18.75 ng cDNA. The thermal profile of the qRT-PCR was 42°C for 5 min, 95°C for 10 s, and 40–50 cycles of 95°C for 5 s, 61°C for 30 s, and 72°C for 1 min. The raw data were analyzed in 7300 Systems SDS v. 1.2.2 software (Thermo Fisher Scientific). The F-BOX gene was used as an endogenous control. qRT-PCR primers designed with Primer3 Web v. 4.1.0 software (http://primer3.sourceforge.net/) are listed in Supplementary Table S2. Six primer sets were designed for GLB family genes and one for the F-BOX gene of soybean. The primer set for GLB2 genes was based on the conserved region of the four GLB2 genes. Other primers were based on each GLB gene. Raw data (ΔCt) of each experiment are available (Supplementary Figures S2–S6).

**Results**

**Plant growth after long-term stress treatment**

After 10 days of flooding or NaCl (5 or 50 mM) treatment, there was no significant difference from the non-flooded control group in the leaf fresh weight of plants treated with either stress (Figure 1A, B), but the root fresh weight was significantly decreased (Figure 1C, D).

**Amount of NO under abiotic stress conditions**

The fluorescence intensity in roots (measure of amount of NO released) increased significantly at 6 and 24 h...
GLB gene expression in soybean under abiotic stress

Expression of GLB genes responding to NO donor

We used SNP and SNAP as NO donors to identify NO-inducible GLB genes. SNP induced the expression of GmGLB1 with statistical significance compared with the control (Figure 3A). Although GmGLB1-like was also induced by SNP with statistical significance, the induction was not strong compared with that of GmGLB1 (Figure 3B). GmGLB3 and GmGLB3-like did not respond to SNP (Figure 3C, D).

SNP generates not only NO but also cyanide and/or an iron–cyanide complex (Bethke et al. 2006). Thus, we examined the expression of GLB genes with PF as an analog of SNP. PF generates cyanide and/or the complex but not NO. Although PF also promoted GmGLB1 expression with statistical significance compared with the control, no significant difference was detected between SNP and PF (Figure 3A). Therefore, we were not able to exclude the possibility that cyanide and/or the complex induced GmGLB1. GmGLB3 and GmGLB3-like did not respond to PF (Figure 3C, D).

Then, we employed another NO donor SNAP. Treatment of cut roots with 1 mM SNAP for 12 h induced the expression of GmGLB1 compared with the control (Figure 4). Because SNAP generates NO and disulfide (Singh et al. 1996), we also employed cPTIO as a NO scavenger to confirm that the expression of GmGLB1 was definitely due to NO (Figure 4). The addition of 1 mM cPTIO as a NO scavenger with SNAP induced lower expression of GmGLB1 than SNAP alone but still higher expression than the control (Figure 4), indicating that GmGLB1 responded to NO. GmGLB1-like was also induced by SNAP (Figure 5), suggesting that GmGLB1-like could be NO-inducible. GmGLB2 was not expressed in any roots (data not shown).

Figure 2. NO released from roots under stress conditions. (A) Relative amounts of NO released from roots at 3, 6, and 24 h after submergence in soybean-culture solution (SS). As a control, SS was added and immediately removed again. Values are means ± SE of three replicate experiments (n=3 in each experiment). (B) Relative amounts of NO released from roots at 3, 6, and 24 h after treatment with 50 mM NaCl. SS was used for control and dilution of NaCl. Values are means ± SE of three replicate experiments (n=1 in each experiment). *Significantly different from the control at the equivalent time point (p<0.05), Student's t-test.

Figure 3. Promotion of GLB gene expression by SNP and PF. Relative amounts of transcripts of (A) GmGLB1, (B) GmGLB1-like, (C) GmGLB3, and (D) GmGLB3-like in roots treated with 500 µM SNP and 500 µM PF for 24 h (estimated by qRT-PCR). Soybean-culture solution (SS) was used for the control. Expression levels were normalized using F-BOX gene as a reference gene. Fold changes in expression are shown relative to control. Values are means ± SE of five replicate experiments (n=3 in each experiment) for GmGLB1 and GmGLB1-like. Values are means ± SE of four replicate experiments (n=3 in each experiment) for GmGLB3 and GmGLB3-like. Means denoted by the same letter do not differ significantly by Student's t-test at p<0.05.
Expression of GLB genes under abiotic stress conditions
In the roots of 9-dpg-plants exposed to stress for 24 h, no GLB genes responded to 50 mM NaCl, but GmGLB1, GmGLB1-like, GmGLB3, and GmGLB3-like genes responded to flooding with statistical significance, notably GmGLB1 (Figure 6). GmGLB2 was not expressed in any roots (data not shown).

Expression of GLB genes in different tissues
At 4 weeks after inoculation, GmGLB1-like was expressed in all tissues (Figure 7A). GmGLB1-like was highly expressed in leaves with statistical significance (Figure 7B). GmGLB3 was expressed most in the nodules (Figure 7C). GmGLB3-like was mainly expressed in the nodules and the roots (Figure 7D). GmGLB2 was expressed only in the nodules (Figure 7E).

Discussion
LjGlb1-1, a GLB1 gene of L. japonicus, regulates NO, which induces its expression (Shimoda et al. 2005, 2009). Soybean has at least eight GLB genes (Anderson et al. 1996; Hyldig-Nielsen et al. 1982; Lee et al. 2004; Schmutz et al. 2010; Wiborg et al. 1982), but it is still unclear which GLB regulates NO in soybean. Here, to understand how soybean regulates NO, we identified which GLB genes respond to NO under abiotic stresses.

Flooding decreased root fresh weight (Figure 1C) and increased the release of NO from roots (Figure 2A). As SNP, a NO donor, also reduced root fresh weight (Supplementary Figure S7), it appears that increased NO decreased root fresh weight. Excess NO unbalances plant hormones. NO donors also inhibit root growth in A. thaliana (Wang et al. 2017). NO promotes the degradation of auxins (Terrile et al. 2012), which controls root growth and development by creating an auxin concentration gradient (Overvoorde et al. 2010). NO is also cytotoxic and reacts with O$_2^-$ to generate peroxynitrate, a powerful oxidant that can mediate tyrosine nitration of proteins (Del Rio 2015). Presumably, the cytotoxicity of NO and the phytohormonal
under flooding was not so high as that of GmGLB1. In the present study, we focused on the identification of GLB which is a candidate for NO regulation under stress condition. GmGLB1 showed the highest induction in the root under flooding (Figure 6). Therefore, we did not investigate the response of other GLB genes, including GmGLB1-like, against SNAP with cPTIO.

As NO induces GmGLB1, GmGLB1 would be induced by abiotic stresses that increase NO. Valliyodan et al. (2014) and Casarotto et al. (2019) have already reported that flooding induces expression of GmGLB1 and it may contribute to flooding tolerance. As NO was produced and the expression of GmGLB1 was strongly induced by flooding but not by salt (Figures 2, 6A). GmGLB1 might be responsible for NO regulation under flooding as well as LjGlb1-1, one of the GLB1s of Lotus japonicus (Fukudome et al. 2019b).

Interestingly, flooding also induced GmGLB1-like, GmGLB3, and GmGLB3-like (Figure 6). The expression of GmGLB3 was induced more than 40 times higher than that of control (Figure 6C), whereas GmGLB3 did not respond to both NO donors at all (Figures 3C, 5). These results suggest that factor(s) other than NO induces GmGLB3 under flooding. While GmGLB1 was expressed in roots, leaves, and nodules, the expression of other GLB genes showed a bias: GmGLB1-like mainly in leaves, GmGLB3 mainly in nodules and GmGLB3-like mainly in nodules and roots (Figure 7). GLB3 of legumes is expressed in the nodules of legumes (Bustos-Sanmamed et al. 2011; Lee et al. 2004). GmGLB3 is highly expressed during nodule development and responds to flooding and kinetin (Lee et al. 2004). GmGLB3-like, which slightly respond to NO donors (Figures 3D, 5) and flooding (Figure 6D), could be transcribed higher than other GLB genes throughout all experiments (Supplementary Figures S2–S6). However, we do not have enough knowledge about biochemical properties of GmGLB3 and GmGLB3-like to discuss their function. GmGLB2, expressed only in nodules (Figure 7E), encodes a leghemoglobin that creates low oxygen partial pressure for nitrogenase activity of rhizobia inside nodules (Appleby 1992). These results together show that GLB genes are functionally differentiated in soybean. For further understanding of their functions, the biochemical properties of each GLB, such as affinity to oxygen and NO, should be clarified.

In conclusion, among the eight GLB genes in soybean, GmGLB1 is induced by NO and might be responsible for NO regulation in soybean. The other GLB genes except nodule specific GmGLB2 were induced by flooding in the roots, so we cannot exclude the possibility that these GLBs are also functional under flooding in the roots. None of the genes was induced by salt. In Lotus japonicus–Mesorhizobium loti symbiosis, overexpression of LjGlb1-1 improves the efficiency of the symbiosis.
by increasing nodule activity and by delaying nodule senescence (Fukudome et al. 2019a), and confers flooding tolerance on the nodules (Fukudome et al. 2019b). Considering with these reports, GmGLB1 could be one of the candidates used to improve the symbiotic ability of soybean.

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