Hydrogen Sulfide Promotes Nodulation and Nitrogen Fixation in Soybean–Rhizobia Symbiotic System

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The rhizobium–legume symbiotic system is crucial for nitrogen cycle balance in agriculture. Hydrogen sulfide (H₂S), a gaseous signaling molecule, may regulate various physiological processes in plants. However, whether H₂S has regulatory effect in this symbiotic system remains unknown. Herein, we investigated the possible role of H₂S in the symbiosis between soybean (*Glycine max*) and rhizobium (*Sinorhizobium fredii*). Our results demonstrated that an exogenous H₂S donor (sodium hydrosulfide [NaHS]) treatment promoted soybean growth, nodulation, and nitrogenase (Nase) activity. Western blotting analysis revealed that the abundance of Nase component nifH was increased by NaHS treatment in nodules. Quantitative real-time polymerase chain reaction data showed that NaHS treatment upregulated the expressions of symbiosis-related genes *nodA*, *nodC*, and *nodD* of *S. fredii*. In addition, expression of soybean nodulation marker genes, including early nodulin 40 (*GmENOD40*), ERF required for nodulation (*GmERN*), nodulation signaling pathway 2b (*GmNSP2b*), and nodulation inception genes (*GmNIN1a*, *GmNIN2a*, and *GmNIN2b*), were upregulated. Moreover, the expressions of glutamate synthase (*GmGOGAT*), asparagine synthase (*GmAS*), nitrite reductase (*GmNiR*), ammonia transporter (*GmSAT1*), leghemoglobin (*GmLb*), and *nifH* involved in nitrogen metabolism were upregulated in NaHS-treated soybean roots and nodules. Together, our results suggested that H₂S may act as a positive signaling molecule in the soybean–rhizobia symbiotic system and enhance the system's nitrogen fixation ability.

Keywords: gene expression, hydrogen sulfide, molecular signaling, nitrogen fixation, nitrogenase activity, nodulation, rhizobium–legume symbiosis, symbiosis

Symbiosis is ubiquitous in terrestrial, freshwater, and marine communities. It has played a key role in the emergence of major life forms on Earth, and in the generation of biological diversity (Moran 2006). Among all plant–microorganism symbioses, mutualism between legumes and bacteria known as rhizobia is the most well studied (Caballero-Mellado and Martinez-Romero 1999). Symbiotic infection by rhizobia into legume roots leads to the formation of a specialized organ known as the root nodule, in which rhizobia differentiate into nitrogen-fixing bacteroids (Mergaert et al. 2006). Root nodules not only create an optimized environment for nitrogen fixation but also provide a site where substance exchange can take place in plants (Becana and Sprent 1987). In exchange for atmospheric nitrogen fixed by bacteroids, legumes provide energy and carbon sources to the bacterial partner (Werner et al. 2015).

In recent years, overuse of nitrogen fertilizers aimed at increasing soybean (*Glycine max*) and other crop yields has led to widespread environmental problems (Tian et al. 2012). Counterproductively, nitrogen fertilizer overuse does not promote soybean production because the redundancy of inorganic nitrogen in the soil layer surrounding roots inhibits nodulation and biological nitrogen fixation (Tilman et al. 2002). This reduced the utilization efficiency of nitrogen fertilizers and caused acidification and hardening of soil, along with various environmental problems. Thus, enhancing the nitrogen-fixing ability of the soybean–rhizobia symbiotic system is a practical solution for reducing the overuse of nitrogen fertilizers and preventing subsequent environmental problems (Masclaux-Daubresse et al. 2010).

The establishment of symbiosis between plants and microorganisms involves a complex regulatory network that includes nitric oxide (NO) and reactive oxygen species (ROS) (Fukuto et al. 2012; Li et al. 2011; Oldroyd 2013). NO is produced in functional root nodules and is crucial for the establishment of symbiosis between *Medicago truncatula* and *Sinorhizobium meliloti* (Baudouin et al. 2006; del Giudice et al. 2011). The production of NO during the infection process indicates a role for this gaseous molecule in recognition between plants and their bacterial partners (Hichri et al. 2016). Similar findings reported by Pii et al. (2007) suggested that NO and auxins may control the formation of indeterminate nodules in *M. truncatula*. Meanwhile, NO is also crucial for the development of functional nodules in soybean, as demonstrated by Leach et al. (2010) using a NO synthase-specific inhibitor. Furthermore, ROS and NO may synergistically control the early stages of the formation of

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legume–rhizobia symbiosis (Damiani et al. 2016). Additionally, NO have a negative effect on nitrogenase (Nase) activity in plants (Cam et al. 2012; Meilhoc et al. 2010).

Recently, the role of hydrogen sulfide (H_2S) as a signaling molecule in regulating physiological processes in plants has become a hot topic. In addition to participating in adventitious root and lateral root formation and seed germination (Lin et al. 2012; Zhang et al. 2009; Zhang et al. 2008), H_2S is reportedly involved in alleviating oxidative damage caused by heavy metals such as aluminum, copper, boron, cadmium, and chromium (Chen et al. 2013; Fang et al. 2016; Li et al. 2012b; Sun et al. 2013; B. L. Wang et al. 2010; Zhang et al. 2010). Moreover, H_2S also acts as an antioxidant signaling molecule to modulate ROS and antioxidant levels, thereby improving drought tolerance in soybean (Zhang et al. 2010). Alternatively, H_2S enhances the drought tolerance of plants by affecting the biosynthesis and expression levels of genes associated with polyamines and soluble sugars (Chen et al. 2016). H_2S also appears to alleviate saline-induced stress responses in bermudagrass (*Cynodon dactylon*) (Shi et al. 2013). Shen et al. (2013) found that H_2S conferred drought tolerance in *Arabidopsis thaliana* through affecting microRNA expression. Moreover, H_2S regulates stomatal movements together with NO, abscisic acid, and inward-rectifying K^+ channels (García-Mata and Lamattina 2010; Papanatsiou et al. 2015; Scuffi et al. 2014). Chen et al. (2011) reported that H_2S promotes photosynthesis by increasing ribulose-1,5-bisphosphate carboxylase activity, and through thiol redox modification in *Spinacia oleracea*. Other studies indicate a role for H_2S in autophagy (Álvarez et al. 2012; Laureano-Marín et al. 2016; Romero et al. 2014). In both animals and plants, most H_2S responses are related to or mediated by NO or ROS (Laureano-Marín et al. 2016; Li et al. 2013; Scuffi et al. 2014; Zhang et al. 2009). However, whether H_2S exerts synergistic or similar functions in the formation of legume–rhizobia symbiosis remains unclear.

In the present study, we found that treatment with an H_2S donor (sodium hydrosulfide [NaHS]) promotes infection of *Sinorhizobium fredii* into soybean roots, and enhances the biological nitrogen-fixation ability of the symbiotic system by upregulating symbiosis and nitrogen-fixation-related genes and proteins. Stimulation of nitrogen fixation and metabolism led to enhanced nitrogen assimilation and photosynthesis in soybean plants, which eventually promoted plant growth. This is the first study to investigate the regulatory role of H_2S in the legume–rhizobia symbiotic system, and our findings suggest that H_2S is a positive regulator in the establishment of symbiosis and symbiotic nitrogen fixation between *G. max* and *S. fredii*. This research will likely inspire future study of the role of H_2S in plant physiology, and might offer a possible solution to increasing soybean production.

RESULTS

Exogenous addition of H_2S donor promotes soybean plant growth.

To find the optimal concentration of NaHS for the growth of soybean, different concentrations (0, 10, 25, 50, 100, 250, and 500 μM) were used to treat soybean plants and *S. fredii* in liquid culture medium in our preliminary experiments. For *S. fredii*, 100 μM NaHS was shown to be the maximal concentration that did not significantly impact rhizobial growth (Supplementary Fig. S1). For soybean plants, 100 μM NaHS was the optimal concentration for root length, shoot length, and biomass yield (Supplementary Fig. S2). These preliminary results were in agreement with those of previous studies by Chen et al. (2011), Christou et al. (2014), and Tamizhselvi et al. (2007) using other plant materials. Then, to explore the regulatory effects of H_2S on soybean plant growth and symbiosis with *S. fredii*, 10 ml of

100 μM NaHS solution was used to treat each soybean roots, and the growth of roots and shoots was measured after inoculation. In both inoculated and noninoculated groups, NaHS treatment promoted root elongation (Fig. 1A). In the inoculated plants, average root length was promoted by NaHS treatment at most of the checkpoints, and this increase in root length was turned out to be statistically significant ($F = 7.318$, $P = 0.042$) by repeated measure (RM) analysis of variance (ANOVA) of the general linear model (GLM). In the noninoculated plants, NaHS treatment also slightly extended the average root length but the extension of root length by NaHS treatment was not significant (RM GLM, $F = 1.393$, $P = 0.197$). Similar results were observed in soybean shoots. NaHS treatment did not have a significant effect on shoot elongation in noninoculated soybean plants (RM GLM, $F = 1.618$, $P = 0.227$) (Fig. 1B). However, in the inoculated soybean plants, the average shoot length was significantly increased by NaHS treatment (RM GLM, $F = 8.774$, $P = 0.037$) (Fig. 1B). The biomass of dry matter in inoculated soybean roots treated with NaHS was significantly higher compared with control plants (RM GLM, $F = 6.475$, $P = 0.043$). However, there was no significant difference observed among the non-inoculated groups (RM GLM, $F = 3.017$, $P = 0.061$) (Fig. 1C). NaHS also significantly increased the biomass of dry matter in soybean shoots (RM GLM, $F = 6.911$, $P = 0.041$). No difference in shoot dry weight was observed in the noninoculated plants (Fig. 1D). Taken together, these results demonstrated that NaHS treatment promoted the growth of soybean seedlings, especially the growth of soybean roots under symbiotic conditions.

Exogenous addition of the H_2S donor promotes rhizobial infection, root nodulation, and Nase activity.

We counted the number of nodules on each harvested soybean root at 7, 14, 21, 28, 35, and 42 days postinoculation (DPI) to determine the effects of NaHS on nodulation in soybean plant. The result showed that H_2S promoted soybean nodulation (RM GLM, $F = 7.733$, $P = 0.037$) (Fig. 2A). Meanwhile, the addition of NaHS promoted not only soybean nodulation but also the nitrogen fixation ability of these nodules. The Nase activity assay, which was measured by the acetylene reduction (AR) analysis, showed that NaHS treatment significantly increased Nase activity in root nodules (RM GLM, $F = 17.094$, $P = 0.015$) (Fig. 2B). The AR ability showed significant increase compared with the control; these results suggested that H_2S promoted soybean nodulation and enhanced the nitrogen-fixing potential of the soybean–rhizobia symbiotic system.

Additionally, another phenotypic analysis of infection events was conducted to further delineate the effect of NaHS treatment on soybean nodulation. Root hair curling, infection threads, and nodule primordia formed during the nodulation process were observed and counted (Fig. 2C). At 5 DPI, more root hair curling and developing infection threads were found in NaHS-treated soybean roots (Fig. 2C). However, no significant difference in number of developing infection threads and nodule primordia were found between NaHS-treated and untreated soybean roots. At 7 DPI, cortex infection threads in NaHS-treated soybean roots were more abundant than those in untreated controls (Fig. 2D). These results suggested that H_2S may act as a positive regulator of soybean nodulation.

Exogenous addition of an H_2S donor increases endogenous H_2S concentration in soybean roots and nodules.

To confirm that the observed changes were caused by the H_2S released from NaHS, we used an H_2S -specific fluorescence probe to measure the amount of endogenous H_2S in soybean plant tissues, including lateral root, main root, and nodule. Endogenous H_2S was measured in different soybean tissues after loading with the sulfidefluor-7-acetoxymethyl ester

(SF7-AM) probe (Fig. 3; Supplementary Fig. S3). Before NaHS treatment, the observed low fluorescence intensity indicated a low concentration of endogenous H_2S in these tissues (Fig. 3G, H, and I). After treatment with 100 μM NaHS, higher fluorescence intensity was observed, indicating that NaHS increases the endogenous H_2S concentration in these tissues (Fig. 3J, K, and L).

Due to the drastic increase of fluorescence intensity by the NaHS treatment, we may rule out the possibility that this strong fluorescence was caused by autofluorescence. The effect of NaHS treatment on endogenous H_2S concentration in plant tissues was also verified by quantification of the fluorescence intensity in these tissues.

Fig. 1. Hydrogen sulfide's effect on the growth of soybean seedlings. Time course of **A** and **B**, root and shoot length and **C** and **D**, dry weight for soybean plants. **E**, Soybean plants following different treatments at 14 days postinoculation (DPI). Soybean plants were harvested every 7 days since inoculation. CK = controls, H = 100 μM sodium hydrosulfide (NaHS), Q = soybean seedlings inoculated with *Sinorhizobium fredii* Q8 strain, and QH = soybean seedlings inoculated with *S. fredii* Q8 strain and treated with 100 μM NaHS. Three independent experiments were conducted. Values are means $\pm$ standard error ($n = 60$).

To further evaluate the effect of NaHS treatment, we measured the H_2S content in plant tissues using methylene blue method, as described by Zhang et al. (2008). As the results of the methylene blue assay suggested, the H_2S contents in lateral roots, main roots, and root nodules were significantly increased by NaHS treatment (Fig. 4). Together, these results indicated that NaHS treatment could significantly increase the H_2S concentration in soybean tissues.

H_2S did not influence nodule structure.

We used a light microscope to determine whether H_2S could lead to structural change in nodules. According to the observation, the paraffin sections of nodules obtained at different times did not exhibit any significant difference between NaHS-treated nodules and control nodules (Supplementary Fig. S4).

Similarly, transmission electron microscopy (TEM) was then conducted to observe the microscopic structure of infected cells and bacteroids in nodules. Bacteroids in nodules harvested at 14, 21, and 28 DPI exhibited typical morphological characteristics, and no obvious differences were found between NaHS-treated nodules and controls. Taken together, H_2S did not exhibit any significant effect on rhizobia colonization and bacteroid differentiation in root nodules.

Chlorophyll content, photosynthesis intensity, and photosystem II activity are elevated by H_2S .

NaHS treatment increased the chlorophyll content in both inoculated and noninoculated plants from 7 to 42 DPI. In the

inoculated soybean plants, the chlorophyll content in soybean leaves was significantly increased by NaHS treatment (RM GLM, $F = 15.991$, $P = 0.020$) whereas, in the noninoculated plants, NaHS did not make any significant difference (RM GLM, $F = 3.512$, $P = 0.094$) (Fig. 5A). A net photosynthetic rate (Pn) curve showed that NaHS treatment enhanced the photosynthesis in soybean leaves in the inoculated plants (RM GLM, $F = 19.243$, $P = 0.013$). Again, NaHS treatment did not influence Pn in noninoculated plants (RM GLM, $F = 3.249$, $P = 0.081$) (Fig. 5B).

In terms of chlorophyll fluorescence parameters, the quantum yield of photosystem II photochemistry (PSII), the ratio of variable fluorescence (Fv) to maximum fluorescence (Fm) (Fv/Fm), and photochemical efficiency of PSII in the light (Fv'/Fm') followed a similar trend after treatment with 100 μM NaHS (Fig. 6). In the noninoculated group, there was not a significant difference in PSII between NaHS-treated soybean plants and control plants (RM GLM, $F = 3.119$, $P = 0.068$). However, in the inoculated group, PSII in NaHS-treated plants was higher than in controls (RM GLM, $F = 8.739$, $P = 0.031$) (Fig. 6A). Fv/Fm in inoculated plants was higher than in noninoculated plants. NaHS treatment significantly increased Fv/Fm in the inoculated soybean leaves (RM GLM, $F = 9.287$, $P = 0.034$). However, in the noninoculated group, there was no significant difference between NaHS-treated and control plants during the entire testing period (RM GLM, $F = 2.668$, $P = 0.148$) (Fig. 6B). In noninoculated plants, NaHS treatment did not affect Fv'/Fm' (RM GLM, $F = 1.577$, $P = 0.203$). However, in inoculated plants, Fv'/Fm' in

Fig. 2. Hydrogen sulfide's effect on soybean nodulation and nitrogenase (Nase) activities. **A**, Root nodule number and **B**, nitrogenase activity assay. Infection events assay at **C**, 5 and **D**, 7 days postinoculation (DPI). An asterisk (*) indicates significant difference ($P < 0.05$). Q = soybean seedlings inoculated with *Sinorhizobium fredii* Q8 strain and QH = soybean seedlings inoculated with *S. fredii* Q8 strain and treated with 100 μM sodium hydrosulfide. Three independent experiments were conducted. For nodule number, values are means $\pm$ standard error (SE) ($n = 60$). For Nase activity, values are means $\pm$ SE ($n = 45$).

NaHS-treated plants was higher than in controls (RM GLM, $F = 8.442$, $P = 0.038$) (Fig. 6C).

H₂S affects symbiosis and nitrogen fixation-related protein expression.

Western blotting analysis of chalcone synthase (CHS) and the Nase iron-containing protein (nifH) were conducted to investigate the regulatory effects of H₂S on the expression of key proteins involved in symbiotic establishment and nitrogen fixation. The result of Western blotting indicated that NaHS treatment significantly increased the protein expression level of nifH in root nodules. At 14, 21, 28, and 35 DPI, the abundances of nifH protein were significantly higher than that in the control nodules (Fig. 7A and B). However, H₂S did not trigger any significant changes in the protein expression abundance of CHS in NaHS-treated soybean root nodules compared with the nontreated controls (Fig. 7A and C).

Quantitative real-time polymerase chain reaction analysis of the expression of symbiosis-related gene.

To further elucidate the mechanism with which H₂S promoted nodulation in soybean, we examined the expression profile of several nodulation marker genes in soybean roots, including *GmENOD40*, *GmERN*, *GmNSP2b*, *GmNIN1a*, *GmNIN2a*, and *GmNIN2b*. We selected 12 h postinoculation and 1, 3, 5, and 7 DPI of *S. fredii* as checkpoints. The quantitative real-time polymerase chain reaction (qRT-PCR) results

demonstrated that H₂S stimulated the expression levels of these genes in soybean roots. For instance, RM ANOVA of the GLM suggested that NaHS treatment affected the expression level of these genes (Fig. 8). In the inoculated roots, the expression level of *GmENOD40* gene was higher in NaHS-treated roots (RM GLM, $F = 10.253$, $P = 0.021$). NaHS did not make any significant difference in expression of *GmENOD40* of the non-inoculated soybean roots (RM GLM, $F = 2.032$, $P = 0.169$) (Fig. 8A). Similarly, expression of *GmERN* (RM GLM, $F = 9.369$, $P = 0.034$) and *GmNSP2b* (RM GLM, $F = 7.318$,

Fig. 4. Quantification of hydrogen sulfide (H₂S) in soybean tissues with methylene blue method. NaHS = sodium hydrosulfide. An asterisk (*) indicates significant difference ($P < 0.05$). Three independent experiments were conducted. Values are means $\pm$ standard error ($n = 30$).

Fig. 3. Fluorescence probe assay of endogenous hydrogen sulfide (H₂S) in different soybean plant tissues. **A to C**, Bright field images of untreated plant tissues. **D to F**, Fluorescent field images of untreated plant tissues. **G to I**, Fluorescent field images of tissues loaded with H₂S-specific fluorescence sulfidefluor-7-acetoxymethyl ester (SF7-AM) probe. **J to L**, Fluorescent field images of tissues loaded with SF7-AM and treated with 100 μ M sodium hydrosulfide (NaHS). Bar = 200 μ m and $\lambda_{ex} = 488$ nm.

$P = 0.038$) were also induced by NaHS treatment in inoculated roots. In the noninoculated soybean roots, the expression of both *GmERN* (RM GLM, $F = 2.798$, $P = 0.137$) and *GmNSP2b* ($F = 2.301$, $P = 0.148$) were not affected by NaHS treatment (Fig. 8B and C). Expression level of *GmNIN1a* was also stimulated by H_2S in the inoculated soybean roots (RM GLM, $F = 14.837$, $P = 0.015$). In noninoculated roots, *GmNIN1a* was not influenced by H_2S . On the other hand, *GmNIN2a* ($F = 11.311$, $P = 0.020$) and *GmNIN2b* ($F = 7.079$, $P = 0.041$) expression in inoculated roots were stimulated by NaHS treatment only from 3 to 7 DPI (Fig. 8E and F). Again, NaHS treatment did not provoke any significant difference in the noninoculated soybean roots.

We also used exogenous flavonoids to treat *S. fredii*. In this circumstance, the expression of *nodA*, *nodC*, and *nodD* genes of *S. fredii* was examined to verify whether NaHS treatment could also induce the symbiotic reaction in rhizobia. In this quantitative assay, both NaHS-treated and nontreated *S. fredii* cells in tryptone-yeast (TY) liquid medium were collected at 0, 0.5, 2, 5, 12, and 24 h after the addition of the mixture of genistein and glycine. The qRT-PCR result suggested that exogenous flavonoids successfully simulated the symbiotic signaling by soybean roots which induced the expression of rhizobial *nodA*, *nodC*, and *nodD* genes, and the expression levels of *nodA*, *nodC*, and *nodD* genes were upregulated by NaHS treatment (RM GLM, $F = 9.183$, $P = 0.021$; $F = 12.619$, $P = 0.018$; and $F = 21.773$, $P = 0.008$, respectively) (Fig. 8).

qRT-PCR analysis of the relative expression of key enzymes related to nitrogen metabolism.

Expression of nitrogen-metabolism-related genes was investigated to determine the possible influence of H_2S on the

molecular mechanisms of symbiotic nitrogen fixation and nitrogen metabolism in soybean plants. Glutamate synthase (*GmGOGAT*), asparagine synthase (*GmAS*), nitrite reductase (*GmNiR*), ammonia transporter *SAT1* (*GmSAT1*), leghemoglobin (*GmLb*) in soybean plant, and *nifH* in *S. fredii* were selected for qRT-PCR analysis.

The transcript abundances of *GmGOGAT* was increased in the inoculated roots by NaHS treatment from 14 to 35 DPI (RM GLM, $F = 9.335$, $P = 0.034$) (Fig. 9A). Similarly, the expression of *GmAS* and *GmSAT1* was also enhanced in the NaHS-treated roots from 14 to 35 DPI (RM GLM, $F = 8.821$, $P = 0.036$; and $F = 8.013$, $P = 0.039$, respectively) (Fig. 9B and D). However, NaHS treatment significantly upregulated the expression of *GmNiR* in the whole experimental period from 5 to 35 DPI (RM GLM, $F = 13.337$, $P = 0.026$) (Fig. 9C). Moreover, NaHS treatment also significantly upregulated the expression of nitrogen-fixation-related *GmLb* and rhizobial *nifH* genes (RM GLM, $F = 8.973$, $P = 0.032$; and $F = 6.991$, $P = 0.047$, respectively) (Fig. 9E and F).

DISCUSSION

H_2S promotes soybean plant growth in the soybean–rhizobia symbiotic system.

NaHS has been used as an exogenous H_2S donor to study the physiological effects of H_2S in many studies (Z. G. Li et al. 2012; Shi et al. 2013; Tamizhselvi et al. 2007; M. J. Wang et al. 2010). Numerous experiments have shown that H_2S can cause various biological effects in plants. In addition to alleviation of abiotic stresses such as heavy metals, heat, drought, and mineral salts (Chen et al. 2016; L. Li et al. 2012; Z. G. Li et al. 2012; Shi et al. 2013; Zhang et al. 2008), H_2S was also found to be

Fig. 5. Hydrogen sulfide's effect on photosynthetic parameters. **A**, Chlorophyll content and **B**, net photosynthetic rate (Pn). CK = controls, H = 100 μ M sodium hydrosulfide (NaHS), Q = soybean seedlings inoculated with *Sinorhizobium fredii* Q8 strain, and QH = soybean seedlings inoculated with *S. fredii* Q8 strain and treated with 100 μ M NaHS. DPI = days postinoculation. Three independent experiments were conducted. Values are means $\pm$ standard error ($n = 60$).

Fig. 6. Hydrogen sulfide's effect on chlorophyll fluorescent parameters. **A**, Quantum yield of photosystem II photochemistry (PSII). **B**, Ratio of variable fluorescence to maximum fluorescence (F_v/F_m). **C**, Photochemical efficiency of PSII in the light (F_v'/F_m'). CK = controls, H = 100 μ M sodium hydrosulfide (NaHS), Q = soybean seedlings inoculated with *Sinorhizobium fredii* Q8 strain, and QH = soybean seedlings inoculated with *S. fredii* Q8 strain and treated with 100 μ M NaHS. DPI = days postinoculation. Three independent experiments were conducted. Values are means $\pm$ standard error ($n = 60$).

involved in regulating plant growth via different mechanisms (Chen et al. 2011; Dooley et al. 2013; Zhang et al. 2011). In the present study, we found that exogenous H_2S promoted the growth of soybean plants under symbiotic conditions with *S. fredii* (Fig. 1). In the inoculated soybean plants, NaHS treatment increased the root length and dry weight (Fig. 1A and C). This indicated that H_2S promoted the formation and development of roots, which is consistent with the findings of Zhang et al. (2009) in sweet potato. Moreover, the shoot length and dry weight were also promoted by NaHS treatment (Fig. 1B and D). Although sulfur (S) is an essential nutrient element for plants, complex regulatory networks have evolved to tightly balance S, uptake, transport and metabolism during changes of supply and demand. Although, in the present study, the nitrogen-free nutrient solution supplied sufficient S for plant growth (64.33 mg/liter) according to the description by Chandra and Pandey (2016), soybean plants were unlikely to suffer from S starvation during the treatment. In addition, the S content in the NaHS treatment was much lower than that in the nitrogen-free nutrient solution (approximately 1:25); thus, we may rule out the possibility that the dry weight accumulation and growth increase were due to the NaHS-treated soybean receiving more S as nutrient. Together, these results indicated a potential role for H_2S in regulating the symbiosis between *G. max* and *S. fredii*, which could promote symbiotic nitrogen fixation and, consequently, plant growth of soybean.

H_2S promotes the formation of root nodules and Nase activity.

Nitrogen-fixing nodules formed by the symbiosis of rhizobia and legume plants are essential for fixation of nitrogen in the

environment (Lee et al. 2014). The nodulation process is regulated by two critical systemic signaling events. The first is the recognition of the two symbiotic partners and the initiation of nodulation, which occurs in the rhizosphere. The second occurs within the plant to regulate the number of nodules as a means of balancing the resource cost and nitrogen benefit associated with nodulation (Hayashi et al. 2013). Many small molecules such as NO , H_2O_2 , and phytohormones have been reported to be involved in the regulation of nodulation between legumes and rhizobia (Hérouart et al. 2002; Hirsch et al. 1997; Puppo et al. 2013). Here, we demonstrated that exogenous H_2S could promote soybean nodulation (Fig. 2A). On the other hand, Nase is the essential enzyme for symbiotic nitrogen fixation that catalyzes the conversion of dinitrogen into ammonia (Fishbeck et al. 1973). Our results suggested that Nase activity in NaHS-treated nodules was enhanced in the NaHS-treated nodules (Fig. 2B). This enhanced Nase activity may promote the nitrogen fixation potential of the NaHS-treated soybean plants.

In the *M. truncatula*–*S. meliloti* system, del Giudice et al. (2011) confirmed the crucial position of NO in successful infection. Studies in *Arabidopsis* have shown that auxin accumulation is a prerequisite for organ formation in plants (Ferguson and Mathesius 2014). External treatment of roots with auxins and auxin action inhibitors suppressed nodule formation, indicating that auxins are required for nodulation within a certain concentration range in *M. truncatula* (van Noorden et al. 2006). Infection event assays showed that, during the early stages of the establishment of symbiosis, a larger number of infection events occurred in NaHS-treated soybean roots (Fig. 2C and D). Our results here suggested that H_2S may

Fig. 7. A, Western blotting analysis of the expression of the nitrogenase iron protein (nifH) and chalcone synthase (CHS) in soybean nodules at 14, 21, 28, and 35 days postinoculation (DPI). Expression levels of **B,** nifH and **C,** CHS are relative to actin. An asterisk (*) indicates significant difference ($P < 0.05$). Q = soybean seedlings inoculated with *Sinorhizobium fredii* Q8 strain and QH = soybean seedlings inoculated with *S. fredii* Q8 strain and treated with 100 μM sodium hydrosulfide. Values are means $\pm$ standard error ($n = 9$).

promote the bacterial infection in the nodulation process, and may enhance the colonization of *S. fredii* into soybean nodules.

Baudouin et al. (2006) demonstrated that NO is synthesized in *M. truncatula* nodules and is required for nodule functioning, and Leach et al. (2010) used NO synthase-specific inhibitors to show that NO is crucial for the development of functional nodules in soybean. In our current study, both paraffin section observation and TEM were conducted to explore whether H₂S could cause any structural change in soybean root nodules. According to the paraffin section observation, NaHS treatment did not cause any significant structural change in soybean nodules. As for TEM, the ultrastructure of soybean nodules was also not significantly affected by H₂S. The bacteroids in the infected cells exhibited typical characteristics in both NaHS-treated nodules and control nodules. Together, these results suggested that H₂S may not have significant effect on morphogenesis during the nodulation process, and it may promote Nase activity through other mechanism.

H₂S promotes photosynthetic and photochemical activity in soybean plants.

Herein, we demonstrated the regulatory role of H₂S in symbiosis, nitrogen fixation, and metabolism in the soybean–rhizobia symbiotic system. In plants, adequate nitrogen content can positively affect many physiological processes such as flowering, carbon assimilation in tissues, and ion uptake (Fernandes and Rossiello 1995; Xu et al. 2001). In plants, nitrogen content is closely related to chlorophyll content and photosynthesis in leaves (Gulmon and Chu 1981; Lapointe 1987), because nitrogen is a component of chlorophyll and photosynthesis-related proteins. Therefore, nitrogen content in soybean plants may influence the formation of chloroplasts and accumulation of chlorophyll in them. In this work, both chlorophyll content and Pn were elevated in leaves of NaHS-treated plants (Fig. 5). The soybean plants were cultured in perlite-vermiculite substrate and watered with nitrogen-free nutrient solution in our study, which caused nitrogen starvation during

Fig. 8. Gene expression level of symbiotic related genes. Relative expression levels of genes A, *GmENOD40*; B, *GmERN*; C, *GmNSP2b*; D, *GmNIN1a*; E, *GmNIN2a*; F, *GmNIN2b*; G, *nodA*; H, *nodC*; and I, *nodD* are displayed in multiple line charts with symbols. CK = controls, H = soybean plants treated with 100 μ M sodium hydrosulfide (NaHS), Q = soybean plants inoculated with the *Sinorhizobium fredii* Q8 strain, QH = Soybean plants inoculated with the *S. fredii* Q8 strain and treated with 100 μ M NaHS, F = *S. fredii* treated with exogenous flavonoids, and F + H = *S. fredii* treated with exogenous flavonoids and 100 μ M NaHS. Expression levels of genes *GmENOD40*, *GmERN*, *GmNSP2b*, and *GmNIN* were relative to the expression level of respective genes in CK roots at 0.5 days postinoculation (DPI). Expression levels of *nodA*, *nodC*, and *nodD* were relative to the expression level of respective genes in F rhizobia at 0 h. The *16s ribosomal RNA* gene and *Gmactin* were used as endogenous control to normalize the quantification of transcript abundances in *Sinorhizobium fredii* and *Glycine max*, respectively. Values are means $\pm$ standard error of three biological replicates and three technical replicates ($n = 9$).

plant growth. Because H_2S may promote soybean nodulation and nitrogen fixation ability, it may have increased the nitrogen supplement in the soybean plants, which eventually alleviated nitrogen starvation and increased chlorophyll content and Pn in the NaHS-treated soybean plants. Although H_2S significantly increased the chlorophyll content in the soybean leaves, it did not significantly affect the NH_4^+ and NO_3^- concentration in soybean roots and shoots (Supplementary Fig. S5). This result is understandable: that, under nitrogen deficiency conditions, limited nitrogen in plant tissues will be rapidly transformed into organic compounds.

H_2S also acted as a modulator of PSII activity in soybean. Specifically, PSII, Fv/Fm, and Fv'/Fm' parameters in inoculated plants were increased (Fig. 6), indicating that the photochemical efficiency in the NaHS-treated soybean leaves was increased. This is consistent with the increase in chlorophyll content caused by 100 μM NaHS treatment. Together with increased chlorophyll content and PSII activity, this enhanced photosynthesis led to higher carbon assimilation and, ultimately, promoted the soybean plant growth and symbiotic relationship between soybean and rhizobia.

H_2S regulates the expression of genes and proteins related to symbiosis and nitrogen metabolism.

After analysis at the phenotypic level, we sought to elucidate the effects of H_2S on the expression of genes and proteins related to symbiosis and nitrogen fixation in the soybean-rhizobia system. First, we focused on Nase, the enzyme which catalyzes the nitrogen-fixation process. The Nase complex consists of two metalloproteins that are highly conserved in sequence and structure throughout nitrogen-fixing bacteria (Halbleib and Ludden 2000). One protein component contains

the active site for substrate reduction with a molybdenum-iron (MoFe) cofactor, while the other iron (Fe)-containing protein acts as electron donor to the MoFe component (Roth et al. 2010). The Fe protein is a 64-kDa α_2 dimer of the *nifH* gene product (Halbleib and Ludden 2000). In this research, we detected the *nifH* protein as an indicator for Nase content using Western blotting analysis. The results showed that the abundance of the *nifH* protein was increased by NaHS treatment (Fig. 7A and B).

Then, we determined the content of CHS, an enzyme catalyzing the synthesis of chalcone, which is reported to be a key precursor of a series of flavonoids (Dao et al. 2011; Schijlen et al. 2007). Li et al. (1993) demonstrated that CHS-deficient *Arabidopsis* displayed lower production of flavonoids. In the present study, the results of Western blotting analysis showed that the abundances of CHS in root nodules were not affected by H_2S (Fig. 7A and C). This result suggested that H_2S may not regulate soybean nodulation via early signaling recognition between soybean and rhizobia.

Then, we investigated the expression of genes related to symbiosis and nitrogen metabolism in root nodules. *GmENOD40* was reported to be the downstream component of the perception of Nod factors (NFs) (Ferguson et al. 2010), which is expressed in pericycle cells of root vascular bundles, dividing cortical cells, the nodule primordium, and developing nodules (Ferguson and Mathesius 2014). Charon et al. (1999) reported that alteration in the expression of *ENOD40* could influence nodulation, suggesting that it plays a vital role in nodule organogenesis. In the present study, expression of gene *GmENOD40* in soybean was upregulated by NaHS treatment (Fig. 8A). *NIN* is essential for nodule organogenesis and is also required for the initiation of bacterial infection in the roots

Fig. 9. Gene expression level of nitrogen-metabolism related genes. Relative expression levels of **A**, *GmGOGAT*; **B**, *GmAS*; **C**, *GmNiR*; **D**, *GmSAT1*; **E**, *GmLb*; and **F**, *nifH* are displayed in multiple-line charts with symbols. CK = controls, H = soybean plants treated with 100 μM sodium hydrosulfide (NaHS), Q = soybean plants inoculated with the *Sinorhizobium fredii* Q8 strain, and QH = soybean plants inoculated with the *Sinorhizobium fredii* Q8 strain and treated with 100 μM NaHS. Expression levels of genes *GmGOGAT*, *GmAS*, *GmNiR*, and *GmSAT1* were relative to the expression levels of respective genes in CK roots at 5 days postinoculation (DPI). Expression levels of *GmLb* and *nifH* were relative to the expression levels of respective genes in Q nodules at 7 DPI. The *16s ribosomal RNA* gene and *Gmactin* were used as endogenous control genes to normalize the quantification of transcript abundances in *Sinorhizobium fredii* and *Glycine max*, respectively. Values are means $\pm$ standard error of three biological replicates and three technical replicates ($n = 9$).

(Madsen et al. 2010; Vernié et al. 2015). In this case, the expression levels of three *NIN* genes in soybean were upregulated by NaHS treatment (Fig. 8D, E, and F). In addition, the other two nodulation marker genes involved in the NF nodulation pathway (*GmERN* and *GmNSP2b*) were also activated in the NaHS-treated soybean roots (Fig. 8B and C). Together, the present results suggested that H₂S upregulated the expression of *GmENOD40*, *GmERN*, *GmNSP2b*, and *GmNIN* genes, which play a crucial role in the NF nodulation signaling pathway and are closely related to the organogenesis and development of root nodules. These results may have indicated that NaHS promoted soybean nodulation and nodule development through regulating the expression abundance of symbiotic-related genes.

On the bacterial side, *nod* genes in rhizobia are crucial for the early stages of recognition by legume hosts because they encode host-specific lipochito-oligosaccharidic NFs that can activate downstream symbiotic reactions by binding to plant kinase-like receptors (Giraud et al. 2007). Among the *nod* genes, *nodD* is a key player because, after sensing flavonoid signals secreted by legume roots into the soil, *nodD* initiates the expression of other *nod* genes (Machado and Krishnan 2003). The results of qRT-PCR demonstrated that transcript abundances of *nodA*, *nodC*, and *nodD* genes were increased in free-living *S. fredii*, suggesting that H₂S not only promotes symbiosis by affecting the legume host but also triggers stronger symbiotic responses in the rhizobia.

Uptake of nitrate by root cells followed by reduction and assimilation in plant tissues is the main route by which mineral nitrogen is converted into organic nitrogen by living organisms. Like photosynthesis, these are life-dependent processes (Imsande and Touraine 1994). In our study, the relative expression levels of six genes related to nitrogen metabolism, including soybean genes *GmGOGAT*, *GmAS*, *GmNiR*, *GmSAT1*, *GmLb*, and rhizobial gene *nifH*, were quantified by qRT-PCR (Fig. 9). These genes encode crucial players in amino acid metabolism and nitrogen assimilation. Expression of *GmGOGAT*, *GmAS*, *GmNiR*, and *GmSAT1* was strongly induced by H₂S compared with untreated controls (Fig. 9). Glutamic acid and aspartic acid are two essential amino acids that provide carbon skeletons for the synthesis of many amino acids by transamination. Indeed, δ -aminolevulinic acid, a precursor of chlorophyll, is synthesized from the intact carbon skeleton of glutamic acid (Beale 1990). Furthermore, asparagine is the common precursor of the essential amino acids lysine, threonine, methionine, and isoleucine in higher plants, and aspartate may also be converted to asparagine in a potentially competing reaction (Azevedo et al. 2006). The ammonium transporter encoded by *GmSAT1* was reported to be located in the peribacteroid membrane, and is believed to be responsible for the transportation of ammonium fixed by bacteroids in soybean nodules (Kaiser et al. 1998). Thus, upregulation of *GmGOGAT*, *GmAS*, and *GmSAT1* indicates that H₂S might enhance the transportation of fixed ammonium and its assimilation into amino acids (Fig. 9A, B, and D). In addition, nitrate assimilation can occur in either the roots or leaves via the nitrate reductase-nitrite reductase pathway (Evans 2001). The product of this assimilation event is NH₄⁺, and assimilation of NH₄⁺ occurs through the glutamine synthetase-glutamate synthase pathway. Assimilation occurs in the roots, near the site of uptake, to avoid toxic accumulation (Bloom 1988). Therefore, we can conclude that H₂S upregulated the expression of *GmGOGAT*, *GmAS*, *GmNiR* and *GmSAT1* genes, through which led to higher N supply to the soybean plants (Fig. 9).

Leghemoglobin (Lb) is one of the main functional components that provides an optimal environment for the nitrogen-fixation process in root nodules. The main function of Lb is to supply oxygen to the bacteroids, to support the respiration during nitrogen fixation at a low concentration to avoid

disrupting Nase, which is highly sensitive to oxygen (Marino et al. 2013). Thus, the upregulated expression of gene *GmLb* may provoke a positive alteration in the redox environment in soybean nodules which will finally lead to higher nitrogen fixation (Fig. 9E). Moreover, NaHS treatment increased the transcription abundance of *nifH* during the treatment (Fig. 9F). Together with the results of Western blot analysis, these results indicated that the NaHS treatment promoted the Nase activity through upregulating the expression of the *nifH* gene and increasing Fe protein content in soybean root nodules.

According to our results, we hypothesized a model to explain the stimulatory effects of H₂S on symbiosis. H₂S may promote soybean nodulation through upregulating the expression of symbiosis-related genes in both plants and rhizobia (Fig. 10). Meanwhile, the nitrogen fixation was also enhanced by H₂S because it may upregulate the synthesis of *nifH* protein in root nodules and promote Nase activity. Moreover, H₂S also stimulated the expression of nitrogen-metabolism-related genes and led to higher nitrogen assimilation. This elevated nitrogen supply increased the chlorophyll synthesis and promoted photosynthesis in soybean leaves, which eventually led to soybean growth promotion..

Conclusion.

In this study, we demonstrated the potential role of H₂S in symbiosis and nitrogen fixation in the *G. max*–*S. fredii* symbiotic system. Our results suggested that H₂S enhances the symbiotic relationship of *G. max*–*S. fredii* because NaHS treatment promoted nodulation and Nase activity in soybean. In addition, H₂S promoted infection of rhizobia in both roots and nodules of soybean by upregulating the expression of symbiosis-related genes such as *GmENOD40*, *GmERN*, *GmNSP2b*, *GmNIN*, *nodA*, *nodC*, and *nodD*. Additionally, the abundance of *nifH* protein was increased in soybean nodules following NaHS treatment. Moreover, NaHS treatment stimulated the expression of nitrogen-metabolism-related genes such as *GmGOGAT*, *GmAS*, *GmNiR*, *GmSAT1*, *GmLb*, and *nifH*. The enhanced nitrogen fixation and assimilation increased the chlorophyll content, the Pn, and PSII activity in inoculated plants. Finally, this enhanced photosynthesis endowed soybean plants with promoted growth. Taken together, these findings suggested that H₂S

Fig. 10. Schematic model of the mechanisms underlying the positive effects of hydrogen sulfide (H₂S) on the *Glycine max*–*Sinorhizobium fredii* symbiotic system. The model, based on the results of the present study and the wider literature, explains how exogenous H₂S might influence the nodulation signaling pathway and the biological nitrogen fixation capacity in *G. max*. Pn = net photosynthetic rate. Arrows within smaller boxes indicate promotion effect. Arrows between and outside of the boxes indicate interactions.

promoted soybean growth under symbiotic conditions by enhancing the establishment of symbiosis and stimulating nitrogen fixation in the *G. max*–*S. fredii* symbiotic system. Further details of the underlying molecular and physiological mechanisms such as the targets of H₂S, and how H₂S fits into regulatory signaling pathways still need further study.

MATERIALS AND METHODS

Plant growth and H₂S treatment.

Soybean (*G. max* ‘Zhonghuang 13’) seed were surface sterilized with 75% ethyl alcohol and sodium hypochlorite, then placed on a 1% agar plate for 72 h at 28°C in the dark. Growth medium (800 ml) (vermiculite-perlite, 1:1 [vol/vol]) was watered with 400 ml of nitrogen-free nutrient solution (CaCl₂ at 100 mg/liter, KH₂PO₄ at 100 mg/liter, Ferric citrate at 5 mg/liter, NaH₂PO₄ at 150 mg/liter, MgSO₄ at 240 mg/liter, H₃BO₃ at 2.86 mg/liter, MnSO₄ · 4H₂O at 2.03 mg/liter, ZnSO₄ · 7H₂O at 0.22 mg/liter, Na₂MoO₄ · 2H₂O at 0.06 mg/liter, and CuSO₄ · 5H₂O at 0.08 mg/liter) and sterilized in a polypropylene planting bag. Germinated seed were transferred into growth medium (one seedling/bag). Seven-day-old soybean seedlings were divided into four groups: the first group served as controls, the second group was treated with 100 μM NaHS (H), the third group was inoculated with rhizobia (*S. fredii* Q8 strain) (Q), and fourth group inoculated with *S. fredii* and treated with NaHS (QH). All seedlings in the Q and QH groups were inoculated with 10 ml of a rhizobial suspension (optical density at 600 nm [OD₆₀₀] = 0.05) 10 days after sowing. NaHS was used as the H₂S donor (Christou et al. 2013). Seedlings in the H and QH groups were watered with 10 ml of NaHS solution (100 μM) every 3 days until harvest, and seedlings from the other two groups were watered with double-distilled H₂O instead. Sterile nitrogen-free nutrient solution (50 ml) was added to each bag every 7 days to maintain the steady humidity and ionic concentration. Seedlings from each treatment were harvested every 7 days since inoculation, and half of the samples were dried to a constant weight for dry matter determination while the other half was immediately frozen in liquid nitrogen and stored at –80°C. We grew a total of four sets of soybean seedlings at different times as four replicates.

Root length, shoot length, root weight, and shoot weight measurements.

The longest distance of root or shoot tip to the junction of root and hypocotyl were set as root length and shoot length. Five soybean seedlings of each treatment group were harvested every 7 days after inoculation for these measurements, and this experiment was repeated four times with four sets of soybean plants as biological replicates.

Nase activity determination.

Nase activity was quantified using the acetylene reduction method of Fishbeck et al. (1973), with slight modifications. Fresh soybean root nodules were transferred into a 10-ml rubber-capped airtight glass bottle filled with a mixture of acetylene and air (1:100 [vol/vol]). Bottles were incubated at 28°C for 3 h, and the content of ethylene was determined using a gas chromatography system (Agilent Technologies, La Jolla, CA, U.S.A.). Standard curves made by pure ethylene standard sample were used to calibrate the result of gas chromatography.

Infection event assay.

The *S. fredii* Q8 strain harboring the enhanced green fluorescence protein encoded on the pMP2444 plasmid was employed. The plasmid was incorporated into the Q8 strain by triparent hybridization through the assistant plasmid pRK2013

(Nishikawa et al. 2008). We define the 2-cm segment below the root-hypocotyl junction as the infection zone as described by Wang et al. (2014), because most nodules appeared in this part of the root. All of the lateral roots from this segment were cut and observed, and typical infection events were determined at 5 and 7 DPI using a BX53 fluorescence microscope (Olympus, Tokyo, Japan). This experiment was repeated for four times with biological replicates.

Visualization of H₂S with fluorescent probe SF7-AM and H₂S content quantification.

H₂S fluorescent probe SF7-AM was purchased from Sigma-Aldrich (CAS: 1416872-50-8; Dallas, TX, U.S.A.). The experiment was performed on entire roots and fresh nodule slices (100 μm) obtained with a freezing microtome (Tissue-Tek, Sakura, Japan). As described by Lin et al. (2013), plant tissues were washed with phosphate buffer (pH = 7.4), then incubated in 5 mM SF7-AM for 1 h, washed with 20 mM HEPES, and visualized and photographed using a BX53 fluorescence microscope (Olympus). The fluorescence intensity was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A.).

The endogenous H₂S content was assayed using the methylene blue method described by Zhang et al. (2008). The calibration curve was made with NaHS solution concentrations. Plant tissues were collected at 14 DPI. The H₂S content was expressed on a fresh weight basis (micromoles per kilogram). This experiment was repeated three times with biological replicates.

Chlorophyll content determination.

Measurement of the chlorophyll content in soybean seedlings was carried out using a SPAD-502 Plus leaf chlorophyll meter (Konica Minolta, Kumamoto, Japan). In total, 10 leaves (third fully developed leaf) from 5 soybean seedlings per treatment were measured at 10:00 A.M. every 7 days until harvest. This experiment was repeated two times with biological replicates.

Determination of photosynthesis and chlorophyll fluorescence parameters.

Pn was measured using a portable photosynthesis system (Li-6400; Li-Cor, Lincoln, NE, U.S.A.) on the second fully expanded leaf of a soybean seedling. In all, 10 different leaves from five different soybean seedlings were tested, and the measurement was conducted twice. Air temperature, light intensity, CO₂ concentration, and air relative humidity were maintained at 25°C, 800 μmol m^{–2} s^{–1} photosynthetically active radiation, 380 μl l^{–1}, and 90%, respectively. Determination was conducted from 9:00 to 11:00 A.M. to avoid high temperature and air vapor pressure deficits. Light was supplemented using an LED light system. Vapor pressure deficit during measurement was approximately 1 kPa.

Chlorophyll fluorescence measurement was conducted on 10 leaves (third fully developed leaf) from five soybean seedlings using a Plant Efficiency Analyzer (Hansatech Instruments Ltd., Norfolk, England). Before measurement, leaves were pre-treated in the dark for 30 min. Minimal fluorescence (F_o), maximal fluorescence (F_m), F_v (= F_m – F_o), and F_v/F_m parameters were recorded for 15 s at a photon flux density of 4,000 μmol m^{–2} s^{–1}. Additionally, we measured the steady-state fluorescence level (F_s′) under continuous illumination, the maximal fluorescence level (F_m′) induced by a saturating light pulse at the steady state, and the minimum fluorescence level (F_o′) after exposure to far-red light for 3 s. Then, F_v′ was calculated using the formula F_v′ = F_m′ – F_o′. PSII was calculated using the formula PSII = 1 – (F_s′/F_m′). This experiment was repeated twice.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting assays.

Total protein of soybean samples was extracted following the protocol of Chen et al. (2011). After total protein isolation, protein concentrations were quantified using the Bradford (1976).

For Western blotting analysis, proteins (200 µg for each sample) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 12% acrylamide gels and transferred to a polyvinylidene difluoride membrane. The membrane was blocked overnight with 5% skim milk powder. Protein blots were probed with primary antibodies for nifH (1:2000, AS01 021A; Agrisera, Vännäs, Sweden) or CHS (1:1000, AS12 2615; Agrisera) at a dilution of 1:5000 or 1:3000, respectively, at 4°C overnight. Blots were washed three times in a solution of 50 mM TRIS-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween-20, followed by incubation with secondary antibody (antirabbit immunoglobulin G horse-radish peroxidase-conjugated, 1:5000 dilution; Sungene, Tianjin, China) overnight at 4°C. Actin (1:5000, AS13 2640; Agrisera) was used as an internal control. Blots were finally washed with phosphate-buffered saline-Tween three times, and imaged using a Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, U.S.A.). The OD value was determined using Image J software and used to estimate the protein abundance. This experiment was conducted with three biological replicates.

Exogenous flavonoids treatment.

TY liquid medium (30 ml of tryptone at 5 g/liter, yeast extract at 3 g/liter, and CaCl₂ at 0.5 g/liter) was inoculated with 20 µl of *S. fredii* preserved in 30% glycerin (OD₆₀₀ = 0.1). The medium was then shake cultured (200 rpm) for 24 h at 28°C. Glycitein and genistein were made into a 15-mg/ml stock solution and sterilized by filtration. Each stock solution (30 µl) was then added to the medium (final concentration = 15 µg/ml). After 0, 0.5, 2.5, 5, 12, and 24 h of shake culture (200 rpm) at 28°C, rhizobia samples were collected for RNA extraction.

Total RNA isolation, reverse transcription, and gene expression analysis.

Total RNA isolation was performed using the TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Beijing, China) according to the manufacturer's instructions. RNA integrity was examined by 1% agarose gel electrophoresis. RNA concentration was determined using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, U.S.A.). Reverse transcription was conducted using TaKaRa PrimerScript™ RT master Mix (TaKaRa) as suggested by the manufacturer. qRT-PCR was carried out with a Quantstudio 6 Flex real-time PCR system (Thermo Fisher, Carlsbad, CA, U.S.A.) and SYBR Premix Ex Taq II (TaKaRa). The qPCR programs are described in Supplementary Table S2. Primers used for expression assay were designed with Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, U.S.A.) according to the coding sequences (CDS) of the respective genes. The CDS of the genes were obtained from GenBank or EMBL. Target fragments amplified by the primers were sequenced and checked by agarose gel to ensure the accuracy of the qRT-PCR reaction. The sequences of the primers used in the present study are listed in Supplementary Table S1. The *16s ribosomal RNA* gene and *Gmactin* were used as endogenous control genes to normalize the quantification of transcript abundances in *S. fredii* and *G. max*, respectively. qRT-PCR was conducted with three biological replicates and three technical replicates.

Statistical analysis.

Statistical significance was tested by ANOVA using SPSS 19.0 (SPSS Inc., Chicago, IL, U.S.A.). All of the significances

in time course experiments were tested using the RM ANOVA of GLM; for these parametric tests, differences were considered statistically significant at $P < 0.05$.

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