Acta Biochim Biophys Sin 2013, 45: 882–888 | © The Author 2013. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmt083.
Advance Access Publication 24 July 2013

Short Communication

The *Sinorhizobium meliloti* LysR family transcriptional factor LsrB is involved in regulation of glutathione biosynthesis

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Glutathione, a key antioxidant in *Sinorhizobium meliloti*, is required for the development of alfalfa (*Medicago sativa*) nitrogen-fixing nodules. This tripeptide can be synthesized by both γ-glutamyl cysteine synthetase (GshA) and glutathione synthetase (GshB) in *Escherichia coli* and *S. meliloti*. Genetic evidence has indicated that the null mutant of *S. meliloti gshA* or *gshB1* does not establish efficient symbiosis on alfalfa. However, the transcriptional regulation of *gshA* and *gshB* has not been well understood. Here, *S. meliloti* LsrB, a member of LysR family transcriptional factors, was found to positively regulate glutathione biosynthesis by activating the transcription of *gshA* and *gshB*1 under both free-living and symbiotic conditions. The decrease in glutathione production in the *lsrB* in-frame deletion mutant (*lsrB1-2*) was determined by using quadrupole time-of-flight liquid chromatography-mass spectrometry. The expression of *gshA* and *gshB1* was correspondingly reduced in the mutant under free-living and symbiotic conditions by analyses of real-time quantitative reverse transcription-polymerase chain reaction and promoter-*GUS* fusions. Interestingly, LsrB positively regulated the transcription of *oxyR*, which encodes another member of LysR family regulators and responds to oxidative stresses in *S. meliloti*. The *oxyR* null mutant produced less glutathione, in which the transcription of *gshA* was consistently down-regulated. These findings demonstrate that glutathione biosynthesis is positively regulated by both LsrB and OxyR in *S. meliloti*.

Keywords *Sinorhizobium meliloti*; lsrB; glutathione; regulation; oxyR

Received: March 21, 2013 Accepted: May 14, 2013

Introduction

Rhizobium, a Gram-negative soil bacterium, infects specific legumes and induces the formation of root nodules to fix atmospheric dinitrogen as ammonia for exchange of plant photosynthetic products, which is very important in agriculture and ecology. This process is modulated by reactive oxygen species (ROS) [1,2]. At the early stage, rhizobium colonized tips of root hairs enter host cell via an infection channel (thread). ROS regulates the formation of infection threads. For example, *Sinorhizobium meliloti* strains carrying the mutation of *sodC* (encoding a superoxide dismutase) or the over-expression of *katB* (a catalase gene) induce the formation of irregular infection threads on alfalfa seedlings [3–5]. *Rhizobium* releases into the cells of nodule primodia and differentiates as bacteroids to fix atmospheric dinitrogen. The nitrogenase activity and respiration of bacteroids lead to accumulation of ROS, which will destroy the integrity of cell membranes and oxidize proteins and DNA [1]. Therefore, Rhizobia have to fight against ROS during symbiotic interactions with their hosts.

To protect rhizobia against ROS, scavenging systems have been perfectly evolved, including detoxifying enzymes such as superoxide dismutases, peroxidases, catalases, and antioxidants of glutaredoxin, thioredoxin, and glutathione (GSH) [3,4,6]. GSH, a tripeptide (γ-glutamyl- L-cysteinylglycine) directly scavenges free radicals and works as a cofactor of detoxifying enzymes. This peptide is produced through two different pathways in cells. Firstly, the γ-glutamyl cysteine synthetase (γECS, GshA) catalyzes glutamate and cysteine to form γ-glutamyl cysteine (γEC), and then glycine is added by glutathione synthetase (GSHS, GshB) to generate GSH [6]. Secondly, the oxidized GSH (GSSG) is reduced by the glutathione reductase (Gor) as GSH [7].

Although the expression of *gor* is induced by hydrogen peroxide in OxyR-dependent manner from *Escherichia coli*, the expressions of *gshA* and *gshB* are independent of OxyR, the conserved member of LysR family regulators in bacteria. This regulator senses oxygen stresses and positively regulates the transcription of catalase genes [8]. In *S. meliloti*, *gshA* or

gsh1 (SMc00825) encodes γECS; GSHS is encoded by two genes, *gshB1* and *gshB2*. Genetic evidence has suggested that this biosynthetic pathway plays a fundamental role in GSH production and development of alfalfa root nodules [6]. The *gshA* null mutant grows slow and is not able to induce the formation of root nodules on alfalfa plants. Moreover, *gshB1* null mutant elicits deficient alfalfa root nodules of premature senescence. However, we did not know how the expressions of *gshA* and *gshB* are regulated in this bacterium.

In our previous work, a new LysR family regulator gene (*lsrB*) from *S. meliloti* has been identified to be essential for alfalfa nodulation [9]. The *lsrB* null mutant induces alfalfa plants to form root nodules of premature senescence, similar to the *gsh* minus mutants [6]. In addition, *oxyR*, another LysR family gene, has been found to be required for *S. meliloti* cells responding to oxidative stresses [10]. In the present study, the level of GSH, the expressions of *gshA* and *gshB* under free-living and symbiotic conditions, and the relationship between LsrB and OxyR were analyzed by using *lsrB* in-frame deletion (*lsrB1-2*) and *oxyR* null mutants in order to explore whether GSH biosynthesis is regulated by *S. meliloti* LsrB or OxyR.

Materials and Methods

Bacterial strains and growth conditions

Sinorhizobium meliloti 1021, *lsrB1-2* [11] and *oxyRsm* [10] were grown in Luria-Bertani (LB) medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LB/MC) at 28°C [12]. The following antibiotics were used at the concentrations indicated: chloramphenicol, 10 μg/ml; neomycin, 200 μg/ml; spectinomycin, 100 μg/ml; tetracycline, 10 μg/ml; and streptomycin, 500 μg/ml.

Assay of the level of GSH in *S. meliloti*

Extraction and quantification protocol for GSH was performed as described by Pan *et al*. [13] with some modifications. Briefly, *S. meliloti* cells in 50 ml of LB/MC cultures were prepared by centrifugation at 13,000 *g* for 1 min, frozen in liquid nitrogen, crushed using an electric drill (J1Z-GL-10, 220 V, 50 Hz, 400 W; Modong Company, Shanghai, China). The cell lysate was suspended in 500 μl of distilled water. After centrifugation at 13,000 *g* for 10 min, supernatants were used for analyses of 6520 Accurate-Mass quadrupole time-of-flight liquid chromatography-mass spectrometry (Q-TOF LC/MS) (Agilent, San Jose, USA). Purification and analysis conditions were as follows: column, Zorbax extend-C_{18} 4.6 × 50 mm, 1.8 μm; volume, 5 μl injection; flow, 0.2 ml/min; flow phase, $A = 0.1\%$ trifluoroacetic acid (TFA) in H_2O, $B = 0.1\%$ TFA in methanol; detection wavelength, diode array detector, 210, 254, 280, 320, 360, and 226 nm; mass range 50–400; nebulizer pressure, 40 psig; drying gas N_2 350°C, 9 l/min; electrospray ionization voltage, cap 3500 V; capillary, fragmentor 160 V, skimmer 65 V, Oct RFV pp750 V; scanning mode, negative MS scan mode 2 GHz Ext Dyn (1700).

RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction

Total RNA for reverse transcription was isolated from cells grown to log phase at an OD_{600} of ~0.8 in LB/MC medium by TransZol plant kit (Transgen, Beijing, China). RNA quality was analyzed by 1% agarose gel electrophoresis, and then treated with RNase-free DNase (TaKaRa, Dalian, China) for 30 min at 37°C to digest any remaining genomic DNA, which was analyzed by polymerase chain reaction (PCR) using a pair of primers (P15–P16). The qualified RNA products were used to synthesize cDNAs by Primerscript RT Master mix perfect Real-Time kit (TaKaRa), and the first-strand cDNA was used as the template for PCR amplification. Real-time quantitative PCR (qRT-PCR) was carried out using SYBR green Supermix (Toyobo, Osaka, Japan). The qPCR reaction system (20 μl) was composed of the following components: 10 μl of SYBR Green Real-time PCR Master mix, 0.5 μl of each primer, 1 μl of diluted cDNA sample, and 9 μl of sterile water. All analyses were performed in triplicate on real-time PCR system (Bio-Rad, Hercules, USA) using a program consisting of a denaturing cycle at 95°C for 3 min; 40 cycles comprising 95°C for 10 s, 62°C for 30 s, and 72°C for 30 s; and a final step in which the temperature was elevated on a gradient from 65 to 95°C to dissociate double-stranded DNA products. The primers P1–P16 of *gshA*, *gshB1*, *gshB2*, *gor*, *oxyR*, *katA*, and *rpsF* were used in qRT-PCR (**Table 1**).

Plant nodulation tests

Assays of alfalfa nodulation on Jenson's medium plates were performed as described previously [9], or in a vermiculite-perlite (3 : 1) mixture as demonstrated by Wang *et al*. [14].

Construction of promoter-*GUS* fusion and GUS activity assay

The 300 bp DNA fragments containing *gshA* and *gshB1* promoter regions were amplified with primer pairs P17–P18 and P19–P20, digested by *Bam*HI, *Sma*I, or *Pst*I, and cloned into pRG960 [15] to harvest recombinant plasmids, pPgshA and pPgshB1. To examine β-glucuronidase activity, the *S. meliloti* strain carrying an empty or a recombinant plasmid was grown in LB/MC medium to reach an OD_{600} of ~0.8, and then bacterial cells were collected by centrifugation at 6000 *g* for 2 min at 4°C. The cells were crushed using the electric drill at 4°C. The cell lysate was kept on ice to analyze β-glucuronidase activity with the substrate of *p*-nitrophenyl-β-D-glucuronide (Sigma, St Louis, USA) as described by Jefferson *et al*. [16]. The 3-week-old alfalfa nodules were

Table 1 Primers used in this study

Name	Gene	Sequence	Use
P1	*gshA*	5′-GCTGAACTGACCGCCTATCTG-3′	For qRT-PCR
P2	*gshA*	5′-CGTTCCTTTCGGCCATACC-3′	For qRT-PCR
P3	*gshB1*	5′-ACTTCACTCTCGGCGAAGAC-3′	For qRT-PCR
P4	*gshB1*	5′-TCGAGCAGATGCGTCGAGGT-3′	For qRT-PCR
P5	*gshB2*	5′-AACGACCCTTCCGAAGACGC-3′	For qRT-PCR
P6	*gshB2*	5′-AGTTGATCTTGAAGACGTTC-3′	For qRT-PCR
P7	*gor*	5′-TGGCGGGACGTGCGTCATT-3′	For qRT-PCR
P8	*gor*	5′-GCCGGGCGATTTCCTGTT-3′	For qRT-PCR
P9	*lsrB*	5′-TCCGGAAATCCAGGTACAGC-3′	For qRT-PCR
P10	*lsrB*	5′-ATGCGATATGCGCGGAT-3′	For qRT-PCR
P11	*katA*	5′-TCGCACACCTATTCCTTC-3′	For qRT-PCR
P12	*katA*	5′-GCGTAGAACAGTGACTCC-3′	For qRT-PCR
P13	*oxyR*	5′-ACGGCAGCTCCTGAGTTTCG-3′	For qRT-PCR
P14	*oxyR*	5′-TGCGGCTTCGCTCGGTAT-3′	For qRT-PCR
P15	*rpsF*	5′-GATCGTCATGTAGCGCAGGA-3′	For qRT-PCR
P16	*rpsF*	5′-CCTCGCTCGGCAGGACAT-3′	For qRT-PCR
P17	*gshA*	5′-TGCACTGCAGTCGTGACGGCGATCCCTCT-3′	For construction of promoter-*GUS* fusion
P18	*gshA*	5′-CGGGATCCTGTTCGTCTTCTTTGCCTGGG-3′	For construction of promoter-*GUS* fusion
P19	*gshB1*	5′-TGCACTGCAGCGTAACGCCGTGGCGCT-3′	For construction of promoter-*GUS* fusion
P20	*gshB1*	5′-TCCCCCGGGTTTTCCCGTTCCCGATCTGG-3′	For construction of promoter-*GUS* fusion

collected, soaked in staining solution containing 25 mg/ml chromogenic substrate X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) as described by Jefferson *et al.* [17], and then visualized to analyze the β-glucuronidase activity of root nodules under a Danish micro engineering microscope (Leica, Solms, Germany).

Catalase activity assay

The native catalase activity assay was performed as described by Luo *et al.* [10] with some modifications [18]. *Sinorhizobium meliloti* strains were grown to an OD_{600} of ~0.8. Rhizobial cells were collected by centrifuging at 6000 *g* for 10 min and washed twice with phosphate-buffered saline (PBS) (pH = 7.4). Cells were frozen in liquid nitrogen, and then crushed by using the electric drill for 30 s up to three times at 4°C. The cell lysate was suspended in 200 μl of PBS (pH = 7.4) containing 1 mM phenylmethanesulfonylfluoride, and then sonicated with a TY92-II sonifier (Scientz, Ningbo, China) at a power of 300 W for 20 min in 5-s pulses. The supernatant was collected, after centrifugation of 13,000 *g* for 10 min at 4°C. Total protein was quantified using a spectrophotometer (Jenway, Staffordshire, UK). Crude protein (0.5 μg) was loaded onto a native polyacrylamide gel electrophoresis (PAGE) gel. After running for 5 h at 4°C, the native PAGE gel was thoroughly soaked in 0.06% H_2O_2 solution and slowly shaken for 20 min, washed twice with distilled water, and stained by mixture of 2% $FeCl_3$ and $KFe(CN)_4$ (1 : 2, v/v). When the gel became black green, the solution was discarded, washed with water, and pictures were taken using a digital camera (Nikon, Tokyo, Japan).

Results and Discussion

S. meliloti lsrB1-2 and *oxyRsm* mutants produces less GSH

Both *S. meliloti lsrB 1-2* (*lsrB* in-frame deletion, described in [11]) and *gshB1* minus mutants elicited similar, deficient root nodules on alfalfa plants [6], which prompted us to examine the GSH level in the *lsrB* mutant. Q-TOF LC/MS analysis showed that the level of GSH decreased by 14.28%–18.58% in the *lsrB1-2* mutant compared with the wild-type (**Fig. 1**). After introduction of an expressible *lsrB* gene on the pSRK-Tc plasmid into the mutant, this defect was rescued (**Fig. 1**). These data indicated that LsrB is associated with positive regulation of GSH biosynthesis in *S. meliloti*.

In *E. coli*, the LysR family regulator, OxyR, modulates the redox state of GSH [8]. Therefore, it is possible that *S. meliloti* OxyR regulates GSH production under free-living conditions. To test this possibility, the GSH level in the *S. meliloti oxyR* null mutant (*oxyRsm*) was determined. It was decreased by 26.13%–37.95% in the mutant in comparison with the wild-type (**Fig. 1**), suggesting that OxyR is also a positive regulator of GSH production in *S. meliloti*.

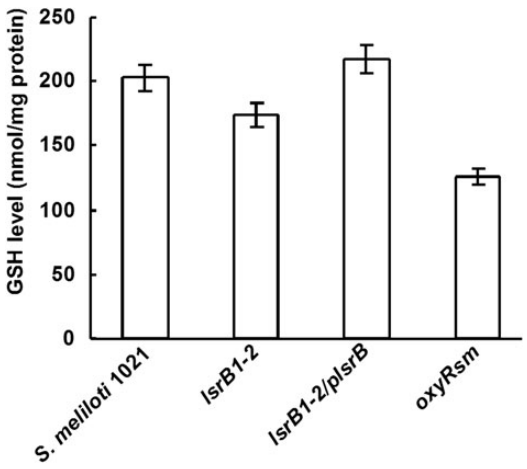

Figure 1 GSH production in *S. meliloti* strains *S. meliloti* 1021, the wild-type strain; *lsrB1-2*, *S. meliloti lsrB* in-frame deletion strain; *lsrB1-2*/p *lsrB*, the *lsrB1-2* mutant carrying the expressible *lsrB* gene on the pSRK-Tc plasmid. *oxyRsm*, *S. meliloti oxyR* null mutant (*oxyR*::pK19mob2ΩHMB). All *S. meliloti* strains were grown to an OD_{600} of ~0.8 in LB/MC medium. The experiment was repeated three times. $P = 0.058$, between the wild-type and the *lsrB1-2* (*oxyRsm*) mutant.

Sinorhizobium meliloti LsrB positively regulates the expression of GSHS genes

To determine how *S. meliloti* LsrB regulates GSH production, the expression of GSHS genes was analyzed first. qRT-PCR data showed that the transcript level of *gshA*, *gshB1*, and *gor* was differentially decreased in the *lsrB1-2* mutant compared with the wild-type, except *gshB2* under free-living conditions [**Fig. 2(A)**]. To confirm this result, the promoters of *gshA* and *gshB1* were successfully cloned and fused with the *GUS* reporter gene, respectively. The *lsrB1-2* mutant carrying each fusion showed much less β-glucuronidase activity than the wild-type [**Fig. 2(B)**]. These data demonstrated that *S. meliloti* LsrB acts as a positive regulator of GSH biosynthesis by up-regulating the expression of *gshA*, *gshB1*, and *gor* genes.

Sinorhizobium meliloti strains carrying each of *gshA* and *gshB1* promoter-*GUS* fusions were used for nodulation on alfalfa seedlings. GUS staining exhibited that *gshA* and *gshB1* were expressed in the infection zones and nitrogen-fixation zones of 3-week-old alfalfa root nodules hosting the wild-type strain [**Fig. 3(B,C)**]. Moreover, the β-glucuronidase activity was much higher in these root nodules than in those induced by the *lsrB1-2* mutant [**Fig. 3(E,F)**]. These data showed that *S. meliloti* LsrB positively regulates GSH biosynthesis in bacteroids (nitrogen-fixing *Rhizobium* cells) from alfalfa root nodules.

The expression of GSHS genes is regulated by *S. meliloti* OxyR

To explore why less GSH was produced in the *S. meliloti* *oxyRsm* mutant (**Fig. 1**), the expression of GSHS genes was analyzed by qRT-PCR. The transcript level of *gshA* in the *oxyRsm* mutant was decreased to nearly one-fifth of that in the wild-type, whereas the transcription level of *gshB2*, *gor*, *katA*, and *oxyR* was differentially increased in the mutant compared with the wild-type (**Fig. 4**). The transcriptional level of *gshB1* and *lsrB* was similar between the *oxyRsm* mutant and *S. meliloti* 1021 (**Fig. 4**). We concluded that *S. meliloti* OxyR positively regulates the expression of *gshA* to affect GSH production.

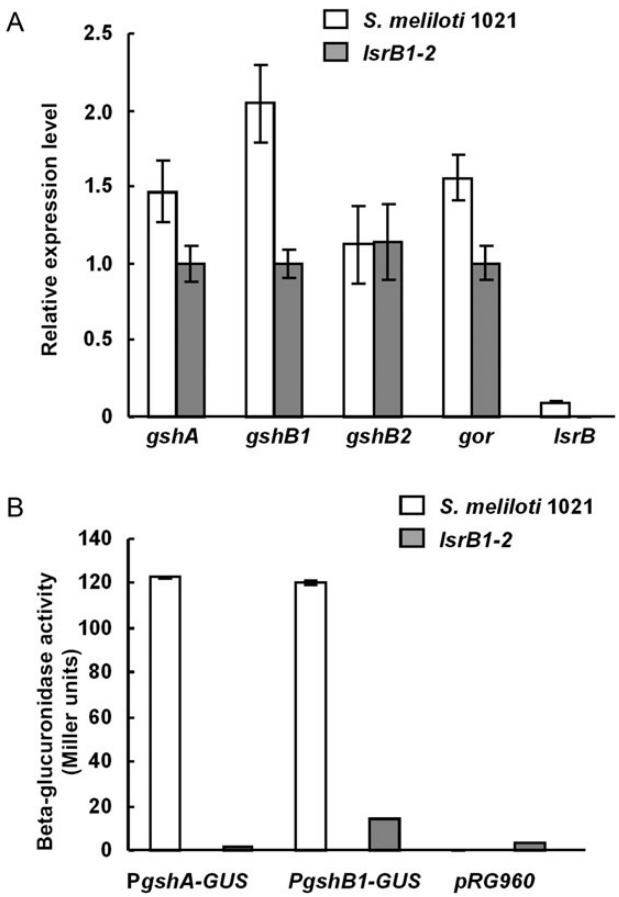

Figure 2 The expression of GSHS genes in the *S. meliloti lsrB1-2* mutant (A) The transcript levels of GSHS genes were determined by qRT-PCR. The ribosome protein gene *rpsF* was placed as the inner standard. The relative expression level = log2 (the expression level of the gene/the expression level of *rpsF*). (B) Glucuronidase activity of promoter-*GUS* fusions in *S. meliloti* strains. P*gshA-GUS*, the promoter of *S. meliloti gshA* fused with the *GUS* reporter gene on the plasmid of pRG960. All *S. meliloti* strains were grown to an OD_{600} of ~0.8 in LB/MC medium. Each experiment was performed in triplicate.

The expression of *oxyR* is positively regulated by *S. meliloti* LsrB

Both *S. meliloti* LsrB and OxyR positively regulate GSH production by activating the expression of *gshA*, suggesting that there is a connection between these two LysR family regulators. In order to test this hypothesis, the expression of *oxyR* was analyzed in the *lsrB1-2* mutant. qRT-PCR data

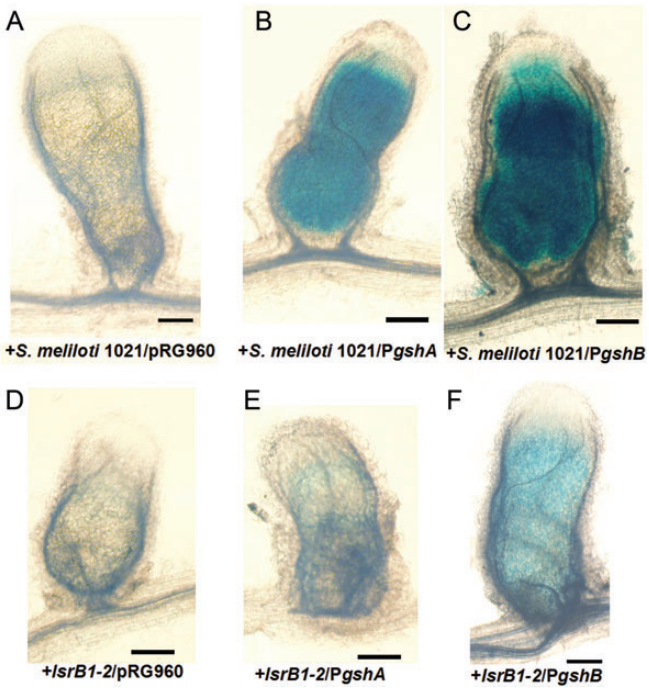

Figure 3 The expression of promoter-*GUS* fusions in alfalfa root nodules hosting the *lsrB1-2* mutant GUS activity of P_{gshA}-*GUS*, P_{gshB1}-*GUS* fusions, and the vector (pRG960) in alfalfa root nodules were induced by *S. meliloti* 1021 (A–C) or *lsrB1-2* mutant (D–F). More than 20 3-week-old root nodules were used in the experiments. Bar = 200 μm.

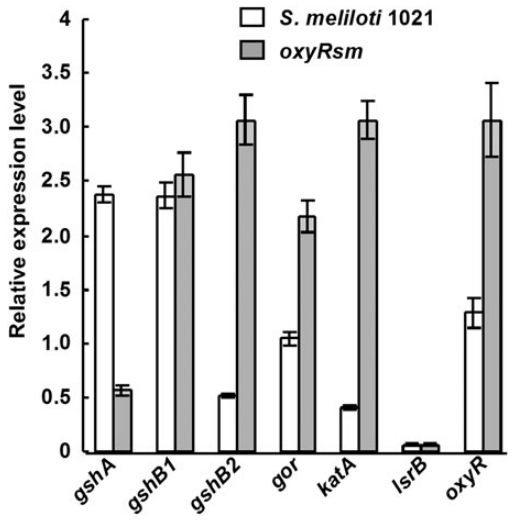

Figure 4 The expression of GSHS genes in the *S. meliloti oxyRsm* mutant The transcript levels of GSHS genes were determined by qRT-PCR. All *S. meliloti* strains were grown to an OD_{600} of ~0.8 in LB/MC medium. Experiments were repeated at least three times. The ribosome protein gene *rpsF* was placed as the inner standard. The relative expression level = log2 (the expression level of the gene/the expression level of *rpsF*).

showed that *S. meliloti oxyR* gene was down-regulated in the *lsrB1-2* mutant, whereas the expression of *katA* was doubled in the mutant [**Fig. 5(A)**]. The OxyR-dependent induction of *katA* in the *lsrB1-2* mutant was also confirmed by native PAGE gel staining [**Fig. 5(B)**]. These data further proved

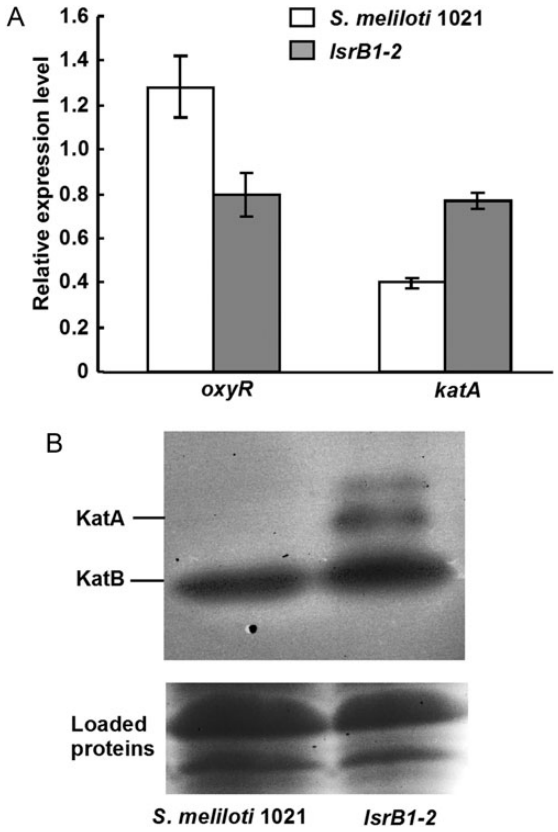

Figure 5 The expression of *oxyR* in *S. meliloti lsrB1-2* mutant (A) The transcription of *S. meliloti oxyR* and *katA* gene examined by qRT-PCR. The ribosome protein gene *rpsF* was placed as the inner standard. The relative expression level = log2 (the expression level of the gene/the expression level of *rpsF*). (B) The catalase activity was assayed in the native PAGE gel. All *S. meliloti* strains were grown to an OD_{600} of ~0.8 in LB/MC medium. The experiments were repeated three times.

that the expression of *oxyR* is positively regulated by LsrB, which is consistent with our previous result that *S. meliloti* OxyR suppresses the expression of *katA* without exogenous oxidative stresses [10].

Different from *oxyR*, the expression of *lsrB* was not changed in the *oxyRsm* mutant in comparison with the wild-type strain (**Fig. 4**), suggesting its expression is not affected by OxyR. In addition, *S. meliloti lsrB* is positively autoregulated [**Fig. 2(A)**]. However, *oxyR* is negatively autoregulated (**Fig. 4**), which is also consistent with our previous data [10]. Therefore, the expression of *oxyR* is dually regulated by LsrB and OxyR in *S. meliloti*.

GSH is produced by two different pathways. One pathway is composed of two enzymes, GshA and GshB. However, GshA is a rate-limiting enzyme and more important than GshB because its catalytic product, γEC, functions similar to GSH [19]. Although *S. meliloti gshA* and *gshB1* are required for alfalfa nodulation [6], transcriptional regulation of these genes was not yet clear in this bacterium. In this study, we found that both *S. meliloti* LsrB and OxyR were involved in the regulation of GSH biosynthesis. The single

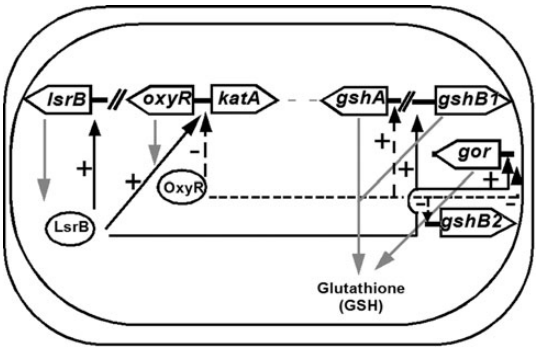

Figure 6 A possible model of LsrB regulating GSH biosynthesis LsrB positively regulated the expression of *oxyR*. OxyR up-regulated the transcription of GSHS genes, like *gshA*. These enzymes synthesized more GSH, which was required for redox balance of free-living and symbiotic *Rhizobium* cells. +, positive regulation; −, negative regulation. Dash line, regulatory pathways of OxyR; gray line, biosynthetic pathway.

null mutants of *S. meliloti lsrB* and *oxyR* produced less GSH, which matched well with the expression levels of *gshA* (**Figs. 2 and 4**). We are not clear why the expression of *gshB1* is not changed in the *oxyRsm* mutant under our tested conditions. Our unpublished data exhibited that *gshB2* is not required for alfalfa nodulation, suggesting that this gene is an alternative in GSH biosynthesis. Therefore, the elevated expression of *gshB1* and *gshB2* in the *oxyRsm* mutant could not dominate the GSH level ultimately.

We attempted to construct the double mutant of *oxyR* and *lsrB* in *S. meliloti*. However, no colonies were obtained without the expression of the *lsrB* or *oxyR* genes from the pSRK-Tc plasmids, suggesting that one OxyR and one LsrB protein are required for cellular redox regulation. Our unpublished chromatin immunoprecipitation assays showed that LsrB did not bind to the promoter region of *gshA in vivo*, which supported that LsrB affects GSH production mediated by other regulators, like OxyR. The binding of OxyR to the specific DNA region will be further tested to verify the regulation roles.

Different expression data of *gor* in the *lsrB1-2* and *oxyRsm* mutant [**Fig. 2(A)** and **Fig. 4**] implied that the transcription of this gene is dually regulated by both LsrB (positive) and OxyR (negative) in *S. meliloti*. In *E. coli*, *gor* is not essential for bacterial growth and survival [8]; however, the roles of *S. meliloti gor* in GSH biosynthesis and symbiosis need to be further studied.

In summary, a regulatory model of GSH production in *S. meliloti* was proposed (**Fig. 6**). LsrB positively regulated the expression of *oxyR*. OxyR up-regulates the transcription of GSHS genes like *gshA* in order to produce more GSH for modulation of bacterial physiology under free-living and symbiotic conditions. The biosynthesis of GSH is regulated by both LsrB and OxyR in *S. meliloti*. The regulation of LsrB is possibly in an OxyR-dependent manner.

Funding

This work was supported by grants from the National Key Program for Basic Research (2010CB126501 and 2011CB100702) and the National Natural Science Foundation of China (31070218).

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