Glutaredoxins Play an Important Role in the Redox Homeostasis and Symbiotic Capacity of *Azorhizobium caulinodans* ORS571

Yajun Cao,¹ Gaofei Jiang,² Mingxu Li,¹ Xingxing Fang,¹ Dan Zhu,¹ Wei Qiu,¹ Juanjuan Zhu,¹ Daogeng Yu,³ Yangchun Xu,² Zengtao Zhong,^{1,*} and Jun Zhu¹

¹ Department of Microbiology, College of Life Sciences, Nanjing Agricultural University, 210095 Nanjing, PR China

² Jiangsu Provincial Key Lab for Organic Solid Waste Utilization, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, National Engineering Research Center for Organic-based Fertilizers, Postdoctoral Station of Agricultural Resources and Environment, Nanjing Agricultural University, 210095 Nanjing, PR China

³ Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Science, 571737 Danzhou, Hainan, PR China

Accepted 23 September 2020.

Glutaredoxin (GRX) plays an essential role in the control of the cellular redox state and related pathways in many organisms. There is limited information on GRXs from the model nitrogen (N₂)-fixing bacterium *Azorhizobium caulinodans*. In the present work, we identified and performed functional analyses of monothiol and dithiol GRXs in *A. caulinodans* in the free-living state and during symbiosis with *Sesbania rostrata*. Our data show that monothiol GRXs may be very important for bacterial growth under normal conditions and in response to oxidative stress due to imbalance of the redox state in *grx* mutants of *A. caulinodans*. Functional redundancies were also observed within monothiol and dithiol GRXs in terms of different physiological functions. The changes in catalase activity and iron content in *grx* mutants were assumed to favor the maintenance of bacterial resistance against oxidants, nodulation, and N₂ fixation efficiency in this bacterium. Furthermore, the monothiol GRX12 and dithiol GRX34 play a collective role in symbiotic associations between *A. caulinodans* and *Sesbania rostrata*. Our study provided systematic evidence that further investigations are required to understand the importance of glutaredoxins in *A. caulinodans* and other rhizobia.

Keywords: *Azorhizobium caulinodans*; glutaredoxin; ROS; symbiosis

Azorhizobium caulinodans exists in a nitrogen (N₂) fixation-based symbiosis with the semiaquatic and water-tolerant tropical legume *Sesbania rostrata*, an important green manure for rice (Dreyfus and Dommergues 1981). *A. caulinodans* is recognized as a remarkable rhizobium model for the study of symbiosis because of the unique ability of this organism to initiate the formation of N₂-fixing nodules on both roots and stems of *Sesbania rostrata* (Den Herder et al. 2006). Root nodule formation is initiated either by lateral root base invasion via epidermal fissures in waterlogged soils or by root hair curling invasion of root tips in aerated and nonaquatic soils (Goormachtig et al. 2004). Stem nodules are developed by lateral root base invasion on adventitious roots (Tsien et al. 1983). While nodulation occurs via a complex and close association between *A. caulinodans* and *Sesbania rostrata*, bacteria are initially recognized as invaders and trigger an oxidative burst at the early stage of plant infection (D'Haeze et al. 2003). If this association is to be successful, the *A. caulinodans* population must be maintained in the soil and must come into contact with the host plants.

Increased production of reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), superoxides, and organic hydroperoxides, is a common plant defense mechanism against rhizobial infection (Becana et al. 2000; Gourion et al. 2015; Pauly et al. 2006). That Nod factor may induce endogenous ROS synthesis has been observed in *Sesbania rostrata* and a few other legumes (D'Haeze et al. 2003). ROS signals lead to a hypersensitive reaction that is probably involved in the auto-regulation of nodulation (Fernandez-Göbel et al. 2019). Indeed, rhizobia have many antioxidant systems, such as thioredoxin (TRX)- and glutathione (GSH)-dependent systems, superoxide dismutases (SODs), and catalases (Kats), to cope with ROS and maintain efficient N₂ fixation (El Msehli et al. 2011; Pauly et al. 2006). *A. caulinodans* produces anti-ROS enzymes such as Fe/Mn/Zn SODs (Puppo et al. 1986; Rubio et al. 2009), Kat (Zhao et al. 2016), alkylhydroperoxide reductase (AhpCD) (Jiang et al. 2019), and bacterioferritin comigratory protein (BCP) (Liu et al. 2019) to enable symbiosis. However, accumulation of H₂O₂ is required for *A. caulinodans* invasion and nodulation on *Sesbania rostrata* (Den Herder et al. 2006; D'Haeze et al. 2003). In addition to plant-derived ROS, endogenous ROS generated by metabolic processes are also harmful for normal cellular development and adaptation to environmental stress in rhizobia (Becana et al. 2000; Nanda

Yajun Cao, Gaofei Jiang, and Mingxu Li contributed equally to this work.

*Corresponding author: Z. Zhong; ztzhong@njau.edu.cn

Funding: This study was supported by National Key R&D Program of China (2019YFA09004700), National Natural Science Foundation of China (31770096, 31970266), China Postdoctoral Science Foundation (2019M651848) and Natural Science Foundation of Jiangsu Province (SBK2019040382).

*The e-Xtra logo stands for “electronic extra” and indicates a supplementary materials file is published online.

The author(s) declare no conflict of interest.

© 2020 The American Phytopathological Society

et al. 2010). The central role of GSH is that of a redox buffer within cells and the function of the GSH-dependent system is involved in iron metabolism and symbiosis in other rhizobia (Benyamina et al. 2013; Harrison et al. 2005; Taté et al. 2012). But how the GSH-dependent system affects ROS resistance and stem nodule formation is still limited in *A. caulinodans*.

The GSH-dependent antioxidant system, referred to as the glutaredoxin (GRX) system, is composed of GRX, NADPH (nicotinamide adenine dinucleotide phosphate), GSH, and GSH reductase. The GRX system controls the cellular redox state together with the TRX system (Fernandes and Holmgren 2004; Lu and Holmgren 2014). GRXs are small redox enzymes of approximately 100 amino acid residues that belong to the TRX family and are widely present in prokaryotes and eukaryotes (Holmgren 1989; Lillig et al. 2008; Meyer et al. 2012; Michelet et al. 2006). Similar to TRX, GRX contains an active disulfide bond (Foloppe and Nilsson 2004) that is either reduced by GSH or oxidized by proteinic cysteine residues (Holmgren 1988; Lillig et al. 2008). GRXs act as electron donors or regulators against oxidative stress (Berndt et al. 2008) in sulfur assimilation and delivery (Lillig et al. 1999; Wang et al. 2012b), transcription (Bandyopadhyay et al. 1998; Fan et al. 2016), iron homeostasis (Rouhier et al. 2010), and cellular differentiation (Benyamina et al. 2013; Takashima et al. 1999). Interestingly, a LysR-NodD family transcription regulator (OxyR) regulates the production of many antioxidant enzymes, such as AhpCD, Kat, TRX, and GRX (Aslund et al. 1999; Zheng et al. 1998) but can be deactivated by GRXs in *Escherichia coli* (Aslund et al. 1999).

Based on the active site motifs, GRXs are divided into two classes, dithiol GRXs (with the CxxC motif) and monothiol GRXs (with the CxxS motif) (Lillig et al. 2008). Distinct roles have also been described for different GRXs in some rhizobia, such as *Sinorhizobium meliloti* (Benyamina et al. 2013), *Bradyrhizobium japonicum* (Pessi et al. 2007), and *Rhizobium etli* (Taté et al. 2012). In *S. meliloti*, the expression of two dithiol *grx* genes was induced during symbiosis compared with the free-living state, but the monothiol *grx* was expressed similarly under both conditions (Capela et al. 2006). Deficiency of dithiol or monothiol GRXs can impair rhizobial growth and H₂O₂ resistance as well as symbiosis with *Medicago truncatula* (Benyamina et al. 2013). To study the role of GRX in *A. caulinodans*, we performed an *in silico* analysis of the entire set of *grx* genes in *A. caulinodans* ORS571 and conducted functional characterization both *in vitro* and *in vivo*. Our data suggested that monothiol GRXs play a more important role than dithiol GRXs in the resistance of *A. caulinodans* to ROS in the free-living state and during symbiosis with plants.

RESULTS

Four *A. caulinodans* GRXs belonging to two classes.

A BLAST search of the *A. caulinodans* ORS571 genome for homologs of *S. meliloti* GRXs revealed four paralogues encoding proteins that shared high sequence similarities with the GRXs of *S. meliloti* 1021 (Benyamina et al. 2013), namely, *grx1* (locus AZC_2234, accession BAF88232.1), *grx2* (locus AZC_3438, accession BAF89436.1), *grx3* (locus AZC_3593, accession BAF89591.1), and *grx4* (locus AZC_4296, accession BAF90294.1). The nucleotide lengths of the *A. caulinodans* *grx* genes ranged from 252 to 339 bp, while the amino acid (aa) lengths of GRXs ranged from 83 to 136 aa. An analysis of these four GRX sequences revealed a conserved N-terminal CxxC/S active site and the residues of GSH-binding sites (Fig. 1A). GRX1 (136 aa, 14.97 kDa) and GRX2 (109 aa, 11.88 kDa) contain the CGFS active site that is typical of classical

monothiol GRXs, while GRX3 (83 aa, 9.85 kDa) and GRX4 (85 aa, 9.33 kDa) possess a dithiol C(PR/SY)C active site.

Phylogenetic analysis of bacterial GRXs showed that these four proteins of *A. caulinodans* ORS571 belong to two GRX classes (Fig. 1B). Based on the taxonomy of reported GRXs (Couturier et al. 2009), GRX1 and GRX2 belong to class II GRXs, and GRX3 and GRX4 belong to class I GRXs. GRX1 and GRX2, containing a CGFS active site, share 77 and 58% identity, respectively, with SmGRX2 (Benyamina et al. 2013), 78 and 62% identity, respectively, with GRX BMEI1129 of *Brucella melitensis* (Uzureau et al. 2010), and high identities (>58%) with CC2505 of *Caulobacter crescentus* (Nierman et al. 2001). GRX1 and GRX2 share 61% sequence identity with each other. GRX3 shares only 32% identity with GRX4 of *A. caulinodans* and shares 32% identity with GrxC of *Synecocystis* sp. strain PCC 6803 (Sánchez-Riego et al. 2013). GRX4 shares 55% identity with SmGRX1 (Benyamina et al. 2013), 61% identity with *Methylobacterium oryzae* GrxC (Kwak et al. 2014), and 59% identity with the GRX (PA5129) of *Pseudomonas aeruginosa* (Reen et al. 2013).

We further compared the compositions of the GRX family from different model strains from four rhizobial genera (Supplementary Fig. S1). The GRX family of *S. meliloti* strain 2011 comprised three members, the dithiol SmGrx1, the monothiol SmGrx2, and the elongated monothiol SmGrx3 containing C-terminal transmembrane domains (Benyamina et al. 2013). The same composition of the GRX family was observed in other model strains of *Sinorhizobium* spp., such as *S. fredii* NGR234 and *S. meliloti* 1021. We also found that *A. caulinodans* ORS571 harbors more GRX-encoding genes than *Azorhizobium doebereineriae* UFLA1-100 and six other reference strains of *Bradyrhizobium* spp., *Mesorhizobium* spp., and *Rhizobium* spp. Interestingly, both *R. etli* CFN 42 and *R. leguminosarum* WSM1689 have three GRXs, one of which is a short dithiol GRX (73 aa) that contains the CVQC active site. The unrooted tree of rhizobial GRXs exhibits a similar phylogeny in which GRXs are divided into two classes (Supplementary Fig. S1). Phylogeny and alignment suggested that *grx* genes in each class could be largely redundant in the growth rate of *A. caulinodans* under normal conditions and in response to ROS.

Bacterial growth rate of *grx* mutants under ordinary conditions.

To assess the contribution of different GRXs to the growth rate of *A. caulinodans* in the free-living state, we constructed four single mutants with in-frame deletion of *grx1*, *grx2*, *grx3*, or *grx4*. As shown in Figure 2A, no differences were observed between $\Delta grx1$, $\Delta grx2$, $\Delta grx3$, $\Delta grx4$, and the wild-type strain *A. caulinodans* ORS571 (WT) after incubation for 34 h. We further generated the double mutant $\Delta grx12$, lacking monothiol GRX12, the double mutant $\Delta grx34$, lacking dithiol GRX34, and the quadruple mutant $\Delta grx1234$, to examine the redundancy of *grx* genes in bacterial growth rates under normal growth conditions (Fig. 2B). The $\Delta grx12$ and $\Delta grx1234$ mutant strains grew significantly slower than WT after incubation for 34 h, while the $\Delta grx1234$ mutant strain showed the strongest effects on *A. caulinodans* growth rate. In contrast, the double mutant $\Delta grx34$ grew at a similar rate compared with WT under ordinary conditions. Our data suggested that *grx* genes belonging to the same class were functionally redundant in the growth rate of *A. caulinodans*, while the monothiol GRX12 seemed to be more essential than the dithiol GRX34 for bacterial growth rate under normal growth conditions. The decreased growth rate of the $\Delta grx12$ and $\Delta grx1234$ mutant strains demonstrated a critical function of monothiol GRXs in ordinary growth of *A. caulinodans*. Similarly, SmGrx2 (with a CGFS

active motif) plays an important role in both the growth rate of free-living *S. meliloti* and the facilitation of bacterial resistance to peroxides (Benyamina et al. 2013).

Inhibition by oxidants on *grx* mutants.

We performed disc diffusion assays to determine the resistance of the above *grx*-deleted mutant strains to H₂O₂ and cumene hydroperoxide (CuOOH) (Fig. 3A; Supplementary Fig. S2A) to investigate the importance of the entire set of *grx* genes in the protection of *A. caulinodans* against both inorganic and organic oxidative stress. As shown in Figure 3A, compared with WT, the growth rates of the single mutants ($\Delta grx1$, $\Delta grx2$, $\Delta grx3$, $\Delta grx4$) and the double mutant $\Delta grx34$ were not affected by the addition of H₂O₂. However, addition of H₂O₂ significantly inhibited the growth of $\Delta grx12$ and $\Delta grx1234$, as

compared with WT on Petri dishes. The growth rate of $\Delta grx12$ was significantly lower than that of the quadruple mutant $\Delta grx1234$ on Petri dishes in the presence of H₂O₂. Upon treatment with organic CuOOH (Supplementary Fig. S2A), the single *grx* mutants $\Delta grx1$, $\Delta grx2$, $\Delta grx3$, $\Delta grx4$, and the double mutant $\Delta grx34$ also exhibited growth rates similar to that of WT, while the double mutant $\Delta grx12$ and the quadruple mutant $\Delta grx1234$ exhibited significantly reduced growth rate on agar plates. The results show there exists the functional complementation between *grx1* and *grx2*. The $\Delta grx34$ strain exhibited a better growth rate than $\Delta grx12$ and $\Delta grx1234$ on Petri dishes upon the addition of CuOOH, while growth rates of the latter two strains were similar to each other. Thus, *grx* genes encoding monothiol or dithiol GRXs exhibited functional redundancy in response to various types of oxidative stress.

Fig. 1. Phylogenetic analysis of glutaredoxin (GRX) proteins. **A**, Domain architecture of GRX homologs in *Azorhizobium caulinodans* ORS571. The conserved GRX domain is depicted as a black box in which the active sites are indicated. The numbers refer to the positions of the amino acid residues in the sequences. **B**, Phylogenetic analysis of GRX sequences from some bacteria. The neighbor-joining method (Howe et al. 2002) was used to infer the unrooted tree for GRXs. Branch lengths are proportional to the evolutionary distances. The unrooted tree is further classified into dithiol and monothiol classes based on the active motifs CGFS and CxxC. The sequence logos of the active site and GSH-binding site motifs were generated in Weblogo (Crooks et al. 2004).

Furthermore, monothiol GRXs contributed significantly to the protection of *A. caulinodans* against peroxides in comparison with dithiol GRXs on agar plates. To further confirm these results, we constructed different complementary strains and performed disc diffusion assays to determine the resistance of the complementary strains to H₂O₂ and CuOOH (Fig. 3B; Supplementary Fig. S2B). After complementation of monothiol GRX genes (*grx1* or *grx2*) into both double mutant Δ *grx12* and quadruple mutant Δ *grx1234*, peroxide-resistance ability of the strains reverted similarly to that of WT strain. Thus, dithiol GRX-gene (*grx3* or *grx4*) complementary strains reverted peroxide resistance ability partially, less than those of monothiol GRX-gene complementary strains.

Bacterial survival during oxidant killing assays.

According to the above data, gene redundancy of *grx* genes that encode either monothiol or dithiol GRXs was observed in the bacterial growth rate under normal conditions and in response to ROS stress. Thus, we investigated the bacterial sensitivities of the monothiol GRX-deficient mutant Δ *grx12*, the dithiol GRX-deficient mutant Δ *grx34*, and the quadruple mutant Δ *grx1234* to different levels of H₂O₂, tert-butyl hydroperoxide (tBOOH), and CuOOH in liquid tryptone-yeast extract (TY) medium via killing assays. Our data revealed that all the mutants were as sensitive as WT to a relatively low H₂O₂ level, but monothiol double mutant Δ *grx12* and Δ *grx1234* exhibited significantly increased bacterial sensitivity in response to a relatively high H₂O₂ level (10 mM) (Fig. 3C). In addition, the survival of Δ *grx34* was not affected by high concentrations of H₂O₂ compared with the survival of WT, while the survival of Δ *grx12* was not distinguishable from that of the Δ *grx1234* strain. In killing assays with inorganic peroxides, the sensitivities of the tested strains were reduced by increased concentrations of tBOOH and CuOOH. The sensitivities to both low and high levels of CuOOH were ranked as WT > Δ *grx34* > Δ *grx12* > Δ *grx1234* (Fig. 3E). The ranking of the bacterial sensitivity to various concentrations of tBOOH was similar to that observed for the CuOOH treatment (Fig. 3D). With only one exception, the survival of Δ *grx12* exhibited increased sensitivity to high levels of tBOOH (2.2 mM) and

was not distinguishable from the survival of Δ *grx34*. The killing assay results indicated that monothiol GRXs play an important role in bacterial resistance against both inorganic and organic peroxides, which was also confirmed by observing the bacterial growth rate in liquid TY medium with the addition of the above peroxides (Supplementary Fig. S3).

Cellular redox state in *grx* mutants.

The difference between GSH and glutathione disulfide (GSSG) is an important indicator of the cellular redox state, which can be affected by GRXs in yeast, rhizobia, and fungi (Benabdellah et al. 2009; Benyamina et al. 2013; Luo et al. 2010). We quantified the differences in GSH redox ratios in cells of the Δ *grx12*, Δ *grx34*, Δ *grx1234*, and WT strains to investigate the roles of two types of GRXs in the cellular redox state of *A. caulinodans* under free-living conditions (Fig. 4A), by ultra-performance liquid chromatography (UPLC) assay. Strong reduction of the GSH/GSSG ratio was observed in the monothiol double mutant Δ *grx12* and quadruple mutant Δ *grx1234*, whereas dithiol double mutation of *grx34* genes had a weaker impact on the GSH redox ratios compared with that of WT (Fig. 4A). No difference in the GSH/GSSG ratio was observed between the Δ *grx12* and Δ *grx1234* strains. Our data suggested that monothiol GRXs were strongly correlated with cellular redox homeostasis compared with the dithiol GRX in *A. caulinodans*. We assumed that the hypersensitivity of *A. caulinodans* to peroxides may be caused by alteration of the cellular redox state due to mutation of *grx* genes because GRXs regulate redox homeostasis via deglutathionylation in many organisms (Lillig et al. 2008). We found that loss of monothiol GRXs but not of the dithiol GRXs lowers the GSH/GSSG ratio in *A. caulinodans* (Fig. 4A). This result is similar to the finding that the GSSG content was enriched in a dithiol *SmGrx1* mutant in comparison with two mutants lacking a monothiol GRX in *S. meliloti* (Benyamina et al. 2013). Indeed, dithiol GRXs control the cellular redox state via the dual capacity to reduce disulfide bonds via the CxxC motif and to deglutathionylate target proteins using only the N-terminal cysteine residue (Fernandes and Holmgren 2004; Lillig et al. 2008). Our finding is consistent with a monothiol-based mechanism involving reduction of mixed disulfides by the monothiol Grx5 in *Saccharomyces cerevisiae* (Shenton et al. 2002). Thus, monothiol GRXs are required for the reduction of mixed disulfides to control the cellular redox state in *A. caulinodans*, perhaps via a monothiol-based mechanism, which could be interesting to study further.

Effect of *grx* genes on iron homeostasis.

In many organisms, GRXs play an important role in iron (Fe) regulation via distinct mechanisms (Rouhier et al. 2010). We assessed the intracellular Fe content in the Δ *grx12*, Δ *grx34*, Δ *grx1234*, and WT strains to determine the importance of *grx* genes in the regulation of Fe metabolism in *A. caulinodans* (Fig. 4B). Deletions of the monothiol *grx12* and *grx1234* genes resulted in a dramatic reduction of total Fe content within the cells of *A. caulinodans* (Fig. 4B). No significant difference in Fe content was observed between the WT strain and the dithiol GRX-deficient mutant strain Δ *grx34* or between the Δ *grx12* mutant strain and the quadruple mutant strain Δ *grx1234*. This result provided strong evidence that monothiol GRXs but not dithiol GRXs are required for the control of Fe homeostasis in *A. caulinodans*.

Influence of *grx* mutations on Kat activity.

Kat plays an essential role in cellular redox homeostasis via the elimination of H₂O₂ in bacteria (Mishra and Imlay 2012). A Kat-encoding gene was significantly overexpressed due to

Fig. 2. Growth of *Azorhizobium caulinodans* mutants. **A**, Single, **B**, double, and quadruple mutant strains of *grx* genes and *A. caulinodans* ORS571 (WT) were used to study the contribution of *grx* genes to bacterial growth under normal conditions. Δ *grx1*, Δ *grx2*, Δ *grx3*, and Δ *grx4* are the single mutants; Δ *grx12* and Δ *grx34* are double mutants lacking monothiol and dithiol glutaredoxin (GRX), while Δ *grx1234* is the quadruple mutant. Bacterial cultures were grown in tryptone-yeast extract medium at 28°C, and the optical density at 600 nm value was determined to monitor normal growth. The letter on the right side of each curve indicates significant differences in the mean rank of different strains at the last sampling time (34 h) by one-way analysis of variance. The same letter indicates *P* value > 0.05; ns = no significant difference. Experiments were repeated at least three times.

mutation of the *grx* gene encoding the monothiol SmGrx2 in *S. meliloti* (Benyamina et al. 2013). This observation prompted us to determine the influence of *grx* mutations on the Kat activity of *A. caulinodans*, using the $\Delta grx12$, $\Delta grx34$, $\Delta grx1234$, and WT strains. In the absence of H_2O_2 stress (Fig. 4C), no difference in Kat activity was observed between WT and $\Delta grx34$. The Kat activity increased significantly in the double mutant strain $\Delta grx12$ and the quadruple mutant strain $\Delta grx1234$

compared with the WT strain. The $\Delta grx1234$ mutant strain exhibited the highest Kat activity both in the absence and presence of H_2O_2 among the four tested strains. Under H_2O_2 stress, the Kat activities of the $\Delta grx2$, $\Delta grx12$, and $\Delta grx1234$ strains were significantly higher than those of the single mutants, $\Delta grx34$, and WT strains (Fig. 4C; Supplementary Fig. S4A). Based on the response to H_2O_2 , mutations of functional *grx* genes may enhance Kat activity to regulate the cellular

Fig. 3. Sensitivity of *Azorhizobium caulinodans* mutants to peroxides. **A**, Inhibition of 10 M H_2O_2 for the single mutant ($\Delta grx1$, $\Delta grx2$, $\Delta grx3$ and $\Delta grx4$), double mutant ($\Delta grx12$ and $\Delta grx34$), quadruple mutant ($\Delta grx1234$), and wild type (WT) strains of *A. caulinodans* and **B**, complementary strains of quadruple mutant ($\Delta grx1234^{cpSRK}$, $\Delta grx1234^{c1}$, $\Delta grx1234^{c2}$, $\Delta grx1234^{c3}$, $\Delta grx1234^{c4}$) and WT strains with plasmid of *A. caulinodans* (WT^{cpSRK}) were tested by disk diffusion assays. Approximately 10^7 bacterial cells of *A. caulinodans* were mixed and spread on tryptone-yeast extract (TY) agar plates. A disc was placed in the center of each plate and was saturated with the above oxidants. Then, the plates were incubated at 28°C for 24 h until measurement of inhibition zones. Survival of the double mutants ($\Delta grx12$ and $\Delta grx34$), quadruple mutant ($\Delta grx1234$), and WT *A. caulinodans* was tested by killing assays with **C**, H_2O_2 (0, 8, and 10 mM), **D**, tert-butyl hydroperoxide (tBOOH) (0, 1.8, 2.0, and 2.2 mM), and **E**, cumene hydroperoxide (CuOOH) (0, 0.8, and 1.0 mM). Bacterial cultures were grown in liquid TY medium for 5 h (optical density at 600 nm of 0.2) at 28°C until the early log phase. Each strain was treated with the indicated oxidants and was incubated for 4 h. The letters on the right side indicate significant differences in mean rank between tested strains at the highest concentration, by one-way analysis of variance. The same letter indicates P value > 0.05. Experiments were repeated at least three times.

redox state under normal conditions and in response to H_2O_2 stress. After complementation of GRX genes into both double mutant $\Delta grx12$ and quadruple mutant $\Delta grx1234$, Kat activity of the strains reverted similarly to that of WT strain (Supplementary Fig. S4B). The rise of Kat activity was enhanced by the addition of exogenous H_2O_2 to *A. caulinodans*, which was consistent with our previous work (Zhao et al. 2016) and with the observed results for yeast (Mühlenhoff et al. 2010), *E. coli* (Mancini and Imlay 2015), *S. meliloti* (Jamet et al. 2003), and other bacteria (Mishra and Imlay 2012). In *S. meliloti*, *katC*, encoding Kat, is overexpressed in the monothiol GRX-deficient mutant (Benyamina et al. 2013), which supports our finding at the transcriptional level. This overexpression was restricted in only the monothiol *SmGrx2*

mutant under normal conditions but not in the mutant lacking the dithiol *SmGrx1* (Benyamina et al. 2013). We observed stronger Kat activity of *A. caulinodans* in the double mutants of both *grx12* and *grx34* in the presence of oxidative stress. Taken together with the strongest phenotype of the *grx1234* mutant, our data suggested a synergistic effect of the two GRX classes on the Kat activity of *A. caulinodans* for the manipulation of the redox state in this bacterium.

Bacterial colonization in soils.

Bacteria are subjected to various forms of stress, leading to the accumulation of ROS within bacterial cells in the environment, such as those in free-living states in soil (Mishra and Imlay 2012; Nanda et al. 2010). We investigated the impacts of *grx* genes on the colonization ability of *A. caulinodans*, using the $\Delta grx12$, $\Delta grx34$, $\Delta grx1234$, and WT strains in both natural and sterile soils. All streptomycin (Sm) spontaneous strains were compared with original strains by growth rate and there were no significant differences (data not shown). Figure 5 exhibits fluctuations in the output/input ratios for monocultures of each strain during soil incubation. Over a 10-day period, the bacterial loads of most strains exhibited reduction compared with the initial input into the natural soil but showed weak fluctuations at 3 days postinoculation (dpi). Monothiol double mutant $\Delta grx12$ showed the strongest decrease in cell count compared with other strains 10 days after soil inoculation, except $\Delta grx1234$ (Fig. 5A). The bacterial load of the WT strain increased in sterilized soil, whereas the two double mutant strains $\Delta grx12$ and $\Delta grx34$ exhibited reduced bacterial loads compared with the WT strain (Fig. 5B). The bacterial loads of all strains increased in sterilized soil.

Impact of *grx* genes on symbiotic capacity.

GRXs have been reported to be essential for the symbiotic performance of *S. meliloti* with the host legume *Medicago truncatula* (Benyamina et al. 2013). To understand the role of *grx* genes in *A. caulinodans* during symbiosis with host plants, we quantified the number and N_2 fixation efficiency of stem nodules and the plant biomass on *Sesbania rostrata*. The $\Delta grx12$ and $\Delta grx1234$ mutant strains initiated a slight increase in the number of stem nodules, but statistical analysis was unable to distinguish a significant difference between the stem nodule number of the $\Delta grx1234$ strain and that of the WT strain (Fig. 6A). There was also no difference within WT, $\Delta grx12$, $\Delta grx34$, and $\Delta grx1234$ strains in the formation of root nodules (Supplementary Fig. S5). All the stem nodules of each plant were harvested for the acetylene reduction assay (ARA) to determine the capacity of N_2 fixation. As shown in Figure 6B, all the mutant strains exhibited significant reduction in N_2 fixation efficiency to different extents. No significant difference in ethylene (C_2H_2) production was observed between plants inoculated with the $\Delta grx12$ and $\Delta grx34$ mutant strains. Plants inoculated with the quadruple mutant strain $\Delta grx1234$ exhibit the lowest N_2 fixation ability compared with plants inoculated with the other mutants and the WT strain. The total dry biomass of aerial plants reduced to different extents due to inoculation of *grx* mutant strains compared with that of plants inoculated with the WT strain (Fig. 6C). There were significant differences in biomass between the plants inoculated with the two double mutants ($\Delta grx12$ and $\Delta grx34$) and with WT, while plants inoculated with the $\Delta grx1234$ mutant strain were the lightest. The photographs of the *Sesbania rostrata* plant in Figure 6D show the symbiotic capacities of each mutant strain on the host plants. Our data suggested that *A. caulinodans grx* genes collectively contribute to symbiotic associations with *Sesbania rostrata* plants.

Fig. 4. Physiological properties of *Azorhizobium caulinodans* mutants. Double mutants ($\Delta grx12$ and $\Delta grx34$), a quadruple mutant ($\Delta grx1234$), and wild type (WT) strains of *A. caulinodans* were used in this study. **A**, Glutathione (GSH) redox ratio of *A. caulinodans* strains. The ratios of reduced GSH/GSSG (glutathione disulfide) is an indicator of the redox balance, comparing the area of GSH with that of GSSG by ultra-performance liquid chromatography. **B**, The Fe content of *A. caulinodans* strains. **C**, Catalase activities of *A. caulinodans* strains with or without the addition of 8 mM H_2O_2 . The order of letters at the top of each panel in A and B and each condition in C indicates significant differences in mean rank of different strains by one-way analysis of variance and, in C, by *t* test. Identical letters indicate P value > 0.05 , ns = no significant difference, two asterisks (**) indicate P value < 0.01 . Experiments were repeated at least three times.

DISCUSSION

The main function of the GSH-dependent antioxidant system is to keep the balance of reduction-oxidation potential intracellular. This work focused on four GRX genes in *A. caulinodans* and how they contribute to peroxides scavenged. We showed that the essential role of monothiol GRXs in *A. caulinodans* is resistance to exogenous inorganic and organic peroxides, since the monothiol double mutant $\Delta grx12$ and quadruple mutant $\Delta grx1234$ were extremely sensitive to those peroxides (Fig. 3; Supplementary Fig. S2). Additionally, these two mutants, $\Delta grx12$ and $\Delta grx1234$, showed high intracellular oxidative level and low Fe content (Fig. 4A and B). The data showed that high oxidative stress exists in these mutant strains. As a result, Kat activity of the mutant strains was increased in order to confront the pressure (Fig. 4C). GRXs are known to be important for the control of Fe metabolism in both eukaryotic and prokaryotic organisms, either via incorporation of Fe-S clusters directly or via indirect control of iron-responsive transcription factors (Rouhier et al. 2010). It is not unexpected that mutation of the monothiol *SmGrx2* leads to increased accumulation of intracellular Fe content compared with that in the *smGrx1* mutant and WT strains of *S. meliloti* (Benyamina et al. 2013). Indeed, monothiol GRXs have a conserved CGFS active motif, which can bind the Fe-S cluster and transfer the cluster to client proteins, whereas dithiol GRXs often do not harbor such a motif (Li and Outten 2012). However, our data (Fig. 4B) are paradoxical to some extent, because $\Delta grx12$ mutant cells were expected to amass Fe in *A. caulinodans* due to the lack of monothiol GRXs that were expected to strongly affect Fe uptake systems, such as in *E. coli* (Fernandes et al. 2005), yeast (Mühlenhoff et al. 2010; Ojeda et al. 2006), and *S. meliloti* (Benyamina et al. 2013). Interestingly, we found a slight but not

significant increase in Fe content within cells of the mutant lacking dithiol GRXs compared with the WT strain. This effect was observed for Grx2 (CSYC motif), which can impair Fe-S cluster assembly and an iron-regulatory protein in *Rattus norvegicus* (Lee et al. 2009). In this study, we did not completely characterize the mechanism of GRX-dependent iron regulation, and it would be interesting to further study the regulatory effects of GRX on iron metabolism in *A. caulinodans*.

Having determined that GRX is responsible for ROS resistance in free-living conditions, we investigated the phenotype of GRXs in soil conditions. No difference in output/input ratios were observed between mutant strains beyond 3 dpi in sterile soil. Interestingly, the soil colonization experiment demonstrated the importance of monothiol GRXs for bacterial survival in natural soils, whereas no effect was observed for the quadruple *grx* mutant (Fig. 5A). As reported previously, there was a slight reduction in the bacterial population of *A. caulinodans* during the two months after inoculation in natural soils (Ladha et al. 1989). This bacterial density within soils was distinctly reduced after two cycles of the *Sesbania rostrata*–rice rotation (Ladha et al. 1989), which suggested reduced competitiveness of *A. caulinodans* compared with other soil microbes. In *Streptococcus sanguinis*, competition with related species was reported to be severely compromised due to the dependency on GSH synthetase (Zheng et al. 2013). Our data supported this hypothesis to some extent, as the colonization abilities of the *grx* mutant strains were inhibited in natural soil but slightly increased in sterilized soil.

To test whether GRXs affect symbiosis efficiency, we measured nitrogenase activity, the number of stem-root nodules, and plant biomass among WT, double mutants, and the quadruple mutant (Fig. 6; Supplementary Fig. S5). Interestingly, both the *grx12*- and *grx34*-deleted mutants were significantly

Fig. 5. Colonization of *Azorhizobium caulinodans* mutants in soil. Overnight cultures of double mutants ($\Delta grx12$ and $\Delta grx34$), a quadruple mutant ($\Delta grx1234$), and wild type (WT) strains of *A. caulinodans* were inoculated in **A**, natural soil and **B**, sterilized soil at 10^6 CFU per gram of soil as input. The survival of *A. caulinodans* were enumerated for output by a series of 10-time dilutions on a Petri dish at 3, 7, and 10 days postinoculation. The order of letters at the top of each panel indicates significant differences in mean rank of different strains, by *t* test. ns = no significant difference, one asterisk (*) indicates *P* value < 0.05, three (***) indicate *P* value < 0.001. Experiments were repeated at least three times.

compromised in terms of nitrogenase activity and plant biomass, which is consistent with the phenotypes of the two *S. meliloti* mutants with different *grx* genes (Benyamina et al. 2013). Furthermore, the lowest nitrogenase activity and plant biomass, observed for the quadruple *grx* mutant, suggest collective contribution by the two GRX classes to the N_2 fixation efficiency of *A. caulinodans*. This reduced N_2 fixation phenotype of the *grx* mutants may be linked to the synthesis of nitrogenase, which is composed of many Fe-S clusters (Chan et al. 1993). Nitrogenases are known to be very sensitive to oxygen and comprise the dimeric Fe protein as an electron donor for N_2 reduction and the MoFe protein for substrate reduction. The decreased Fe content within cells of *A. caulinodans* may have led to the decrease in the N_2 fixation efficiency. Indeed, other disruptions of the GRX system also profoundly impair the symbiotic interaction of rhizobia with their plant hosts, such as *gshA* and *gshB*, which are responsible for GSH biosynthesis, as described

by Taté et al. (2012), Muglia et al. (2008), and Tang et al. (2018). Thus, we hypothesize that, together with other proteins, the GRX system contributes to N_2 fixation, given the importance of nitrogenase synthesis in *A. caulinodans*. Further in-depth research is needed to verify this hypothesis.

In this study, we identified two gene copies for each GRX family in the rhizobium *A. caulinodans*. These genes exhibit functional redundancies in bacterial growth under normal conditions and in response to oxidative stress. Monothiol GRXs play a very important role in bacterial growth under varying conditions, in bacterial resistance against peroxides, and in symbiosis due to the pluripotency of deglutathionylation, inducing Kat activity and iron regulation (Benyamina et al. 2013). Our data revealed some distinct functions of the various bacterial GRXs in *A. caulinodans*. We believe that it would be very interesting to further investigate the functional diversity of GRXs in other rhizobial strains.

MATERIALS AND METHODS

Strains, plasmids, and plants.

Bacterial strains and plasmids used in this work are listed in Table 1 and Supplementary Table S1. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C (Green and Sambrook 2012). *A. caulinodans* ORS571 (Lee et al. 2008) and its derivative strains were grown in TY medium at 28°C (Beringer 1974). Spontaneous Sm-resistant mutants of WT, $\Delta grx12$, $\Delta grx34$, and $\Delta grx1234$ were obtained from each original strain on TY agar plates containing 100 μ g of Sm per milliliter and were used in soil colonization. All solid media contained 1.2% agar. Indicated antibiotics were used for selection at final concentrations as follows: ampicillin (100 μ g ml⁻¹), gentamicin (20 μ g ml⁻¹), and Sm (100 μ g ml⁻¹). All culture conditions were the same as above unless specifically noted. Seeds of *Sesbania rostrata* were surface-sterilized (Fernández-López et al. 1998), and the seedlings were inoculated with *A. caulinodans* and grown in pots inside a chamber under controlled conditions (D'Haeze et al. 1998).

Identification of the GRX family.

The protein sequences of SmGRXs in *S. meliloti* (Benyamina et al. 2013) were used to identify similar sequences of GRXs of *A. caulinodans*, using the BLASTP program from the National Center for Biotechnology Information. Four GRX proteins, AZC_2234, AZC_3438, AZC_3593, and AZC_4296, were retrieved from the proteome of *A. caulinodans* ORS 571 (Lee et al. 2008). These sequences were used as queries for the identification of homologs from bacteria and model rhizobial strains (Supplementary Fig. S1), using the BLASTP program. The selected rhizobial strains were *A. doebereineriae* UFLA1-100 (Seshadri et al. 2015), *Bradyrhizobium diazoefficiens* USDA 110 (Kaneko et al. 2002), *B. japonicum* USDA 6 (Kaneko et al. 2011), *Mesorhizobium huakuii* 7653R (Wang et al. 2014), *M. loti* MAFF 303099 (Kaneko et al. 2000), *R. etli* CFN 42 (González et al. 2006), *R. leguminosarum* bv. *trifolii* WSM1689 (Terpolilli et al. 2014), *Sinorhizobium fredii* NGR234 (Schmeisser et al. 2009), and *S. meliloti* 1021 (Galibert et al. 2001 and 2011 (Sallet et al. 2013)). The alignment of amino acid sequences was performed by CLUSTALW in MEGA version 7.0 (Kumar et al. 2016). Phylogenetic analyses were conducted by the neighbor-joining method with the pair-wise deletion option for handling alignment gaps and the Poisson correction model for distance calculation (Howe et al. 2002). Bootstrap tests were conducted with 1,000 replicates. Branch lengths are proportional to phylogenetic distances. The sequence logos of active site and GSH binding site motifs of GRXs were generated in Weblogo (Crooks et al. 2004).

Fig. 6. Symbiotic performance of *Azorhizobium caulinodans* mutants on *Sesbania rostrata*. Overnight cultures of double mutants ($\Delta grx12$ and $\Delta grx34$), a quadruple mutant ($\Delta grx1234$), and wild type (WT) strains of *A. caulinodans* were inoculated on the stem of *Sesbania rostrata*, to study the role of glutaredoxin (*grx*) genes in symbioses. **A**, Number of stem nodules on *Sesbania rostrata*. **B**, Nitrogen-fixation activities of stem nodules were measured by acetylene reduction assay (ARA). **C**, Dry weight of the aboveground plants inoculated with the indicated strain. **D**, Plants pictured at 5 weeks postinoculation. The order of letters at the top of each panel indicates significant differences in mean rank of different strains, by one-way analysis of variance. Identical letters indicate *P* value > 0.05. Experiments were repeated at least three times.

Construction of *A. caulinodans* mutants and complementary strains.

All constructs were verified by sequencing or PCR. PCR primers and restriction enzyme sites are listed in Table 2 and Supplementary Table S2. Molecular techniques were performed following standard protocols (Green and Sambrook 2012). *E. coli* DH5 α was used for general cloning, and *E. coli* SM10 λ pir was used for the bacterial conjugation. The *A. caulinodans* WT strain ORS571 was transformed by electroporation, as described previously (Zhao et al. 2016). To construct the in-frame deletion mutants, overlap extension PCR was used to create the deletions. In-frame deletion plasmids in *grx1* (AZC_2234), *grx2* (AZC_3438), *grx3* (AZC_3593), and *grx4* (AZC_4296) were constructed by overlapping PCR of the flanking regions (Table 2) of the target genes and cloning the resulting product into suicide vector pEX18Gm containing a *sacB* counter-selectable marker, by restriction enzyme digestion (Metcalf et al. 1996). The constructed plasmids, pEX18Gm-*grx1*, pEX18Gm-*grx2*, pEX18Gm-*grx3*, and pEX18Gm-*grx4*, were introduced into WT by conjugation, and deletion mutants were selected for double homologous recombination events. Double-crossover events were selected on

sucrose plates (10%) after the first “cross-in” homologous recombination. The positive clones were verified by PCR examination. The single mutant strains were named Δ *grx1*, Δ *grx2*, Δ *grx3*, and Δ *grx4* for in-frame deletions of *grx1*, *grx2*, *grx3*, and *grx4* genes. The double mutants Δ *grx12* and Δ *grx34* and the quadruple mutant Δ *grx1234* were derived from WT generated by the same method as above. Each plasmid that constitutively expressed *grx1*, *grx2*, *grx3*, and *grx4* was constructed by cloning these genes into the pSRK vector (Khan et al. 2008), and constructs were introduced into each *grx* mutant by electroporation (Supplementary Table S1). Primers used in this study are listed in Supplementary Table S2.

Monitoring bacterial growth.

A. caulinodans strains of this work were single mutants Δ *grx1*, Δ *grx2*, Δ *grx3*, and Δ *grx4*, double mutants Δ *grx12* and Δ *grx34*, and quadruple mutant Δ *grx1234*. *A. caulinodans* strains were grown overnight in liquid TY medium and were adjusted to an optical density at 600 nm (OD₆₀₀) of 1.0 as precultures. A total of 1% precultures were transferred into fresh TY medium and were re-incubated for the bacterial

Table 1. Strains and plasmids used in this study

Strains or plasmids	Relevant characteristics ^a	Source
<i>Escherichia coli</i> strains		
DH5 α λ pir	Host for cloning	Lab strain
SM10 λ pir	Host for conjugation	Lab strain
<i>Azorhizobium caulinodans</i> strains		
Wild type (WT)	<i>A. caulinodans</i> ORS571, AZC0 wild-type, Amp ^R	Dreyfus and Dommergues 1981
WT-Sm	Spontaneous Sm ^R mutant of WT, Sm ^R	
Δ <i>grx1</i>	Derivative of WT carrying a <i>grx1</i> in-frame deletion	This study
Δ <i>grx2</i>	Derivative of WT carrying a <i>grx2</i> in-frame deletion	This study
Δ <i>grx3</i>	Derivative of WT carrying a <i>grx3</i> in-frame deletion	This study
Δ <i>grx4</i>	Derivative of WT carrying a <i>grx4</i> in-frame deletion	This study
Δ <i>grx12</i>	Derivative of WT carrying a <i>grx12</i> double in-frame deletion	This study
Δ <i>grx34</i>	Derivative of WT carrying a <i>grx34</i> double in-frame deletion	This study
Δ <i>grx1234</i>	Derivative of WT carrying a <i>grx1234</i> quadruple in-frame deletion	This study
Δ <i>grx12</i> -Sm	Spontaneous Sm ^R mutant of <i>grx12</i> , Sm ^R	
Δ <i>grx34</i> -Sm	Spontaneous Sm ^R mutant of <i>grx34</i> , Sm ^R	
Δ <i>grx1234</i> -Sm	Spontaneous Sm ^R mutant of <i>grx1234</i> , Sm ^R	
Plasmids		
pEX18Gm	Suicide cloning vector, Gm ^R	Khan et al. 2008
pSRK	Expression vector, Tc ^R	Khan et al. 2008

^a Amp^R, Sm^R, Gm^R, and Tc^R indicate ampicillin-, streptomycin-, gentamycin-, and tetracycline-resistant.

Table 2. Primers in this study^a

Primer	Sequence (5' to 3') ^b	Description	Restriction sites	Size (bp)
<i>grx1</i> -1	CCCAAGCTTGTATGGTACCGCCCTCTGG	Upstream fragment of <i>grx1</i>	<i>Hind</i> III	758
<i>grx1</i> -2	GCGCTCAGCCGATAGGGGGCAAAGCGCAAGATG			
<i>grx1</i> -3	CGCTTTGCCCCCTATCGGCTGAGCGCGCGTCTTCG	Downstream fragment of <i>grx1</i>	<i>Xba</i> I	758
<i>grx1</i> -4	GCTCTAGAGGCCGCGCCTGAGATTCTGCTTTT			
<i>grx2</i> -1	CGGAATTCGTGTGGATCTTGTAAGGCAAAA	Upstream fragment of <i>grx2</i>	<i>Eco</i> RI	681
<i>grx2</i> -2	AGGCGGCCCTCTGCAGGGACATGTCTTTCGCTCTCCG			
<i>grx2</i> -3	AGACATGTCCCTGCAGAGGGCCGCCTGATACCGACGTG	Downstream fragment of <i>grx2</i>	<i>Xba</i> I	680
<i>grx2</i> -4	GCTCTAGAGGCTTTCGACAGGCCAATCTCT			
<i>grx3</i> -1	CCCAAGCTTTGGTTCTCAGGGTGAAAGTG	Upstream fragment of <i>grx3</i>	<i>Hind</i> III	762
<i>grx3</i> -2	GATGAATGAAGTGCAGCGCTTCTGATTGACGCGGAC			
<i>grx3</i> -3	ATCAGAAGCGCTGCAGTTTCATTCATCGGGGGTACCG	Downstream fragment of <i>grx3</i>	<i>Xba</i> I	768
<i>grx3</i> -4	GCTCTAGACTGTCCGACACCCATGACCC			
<i>grx4</i> -1	GCTCTAGACCACTGCATCGCCTGCAAG	Upstream fragment of <i>grx4</i>	<i>Xba</i> I	749
<i>grx4</i> -2	AGGCGGCCATCAAGGATTTCATGGGGTCTCTCA			
<i>grx4</i> -3	CCCCATGAAATCCTTGATGGCGGCGCTGACCCG	Downstream fragment of <i>grx4</i>	<i>Hind</i> III	756
<i>grx4</i> -4	CCAAGCTTTGTAGGCGCCGGTTTCTATG			

^a Primers were used for construction of in-frame deletion of Δ *grx1*, Δ *grx2*, Δ *grx3*, and Δ *grx4* genes in *Azorhizobium caulinodans* strains.

^b The underlined sequences are the restriction sites of indicated enzymes.

growth under normal conditions. Growth dynamics were monitored by measuring the OD₆₀₀ value every 2 h after inoculation in a UV-visible light spectrophotometer (Philes Ltd, Nanjing, China). When necessary, the method was simply adjusted to measure the bacterial growth rate under oxidative stress.

Disc diffusion assays of oxidants.

Inhibitions of inorganic oxidant H₂O₂ and organic oxidant CuOOH on *A. caulinodans* strains were determined by disc diffusion assays (Wang et al. 2012a). Single mutants of *Δgrx1*, *Δgrx3*, and *Δgrx4*, double mutants *Δgrx12* and *Δgrx34*, the quadruple mutant *Δgrx1234*, and complementary *A. caulinodans* strains were used in this work. Precultures were prepared as described above and were mixed with the semisolid TY medium (0.6% agar) at 1% rate at 42°C. The mixtures were poured onto solid TY agar plates (55 mm in diameter). The sterilized Whatman AA disc (6 mm) (Whatman International Ltd, Maidstone, U.K.) containing 4 μl of 10 M H₂O₂ or 6.1 M CuOOH was placed on the center of Petri plates for 24 h of incubation at 28°C. The inhibition zones were measured and adjusted by subtracting the diameter of the Whatman AA disc.

Peroxide killing assay.

Double mutants *Δgrx12* and *Δgrx34* and quadruple mutant *Δgrx1234* of *A. caulinodans* strains were used in this work. Various concentrations of H₂O₂, tBOOH, and CuOOH were used to test the sensitivity of *A. caulinodans* strains to organic and inorganic peroxides by killing assays (Jiang et al. 2019; Zhao et al. 2016). Overnight cultures were re-incubated to stationary phase (OD₆₀₀ = 1.0) and were treated with the final concentrations of H₂O₂ (8 and 10 mM), tBOOH (1.8, 2.0, and 2.2 mM), and CuOOH (0.8 and 1.0 mM). Then, bacterial cultures were incubated at 28°C for 4 h. A series of 10-times dilutions of treated cultures were placed on TY agar plates and were incubated for 3 days before the enumeration of the colony-forming units (CFU). Survival percent was assessed by the comparison of CFU with controls that were treated with TY medium.

Kat assay.

Double mutants *Δgrx12* and *Δgrx34* and the quadruple mutant *Δgrx1234* of *A. caulinodans* strains were used in this work. Approximately 1% of overnight precultures were reinoculated into the fresh TY medium containing appropriate antibiotics until the stationary phase (OD₆₀₀ = 1.0). H₂O₂ was added to 8 mM in cell cultures of *A. caulinodans* strains, and the mixtures were further incubated at 28°C for 1 h. Same quantities of rinsed cells of each strain were collected and lysed by sonication. The supernatant of lysates was used to test Kat activity by the Catalase Activity Assay Kit A007-1-1 (Nanjing Jiancheng Bioengineering institute, Nanjing, China) according to the manufacturer's instructions.

GSH and GSSG analysis by UPLC.

Glutathione and glutathione disulfide contents were measured and developed as the methods previously described (Queval and Noctor 2007; Cui et al. 2012). The monobromobimane (mBBR) derivatives were filtered through a 0.22 μm filter, 1 μL sample was injected into UPLC analysis (Agilent Technologies, infinity 1290). Thiol derivatives were separated on a ZORBAX Eclipse Plus C₁₈ column (2.1×50 mm, 1.8-Micron; Agilent) at a flow rate of 0.3 mL·min⁻¹. The linear gradient was from 0% to 10% (v/v) solution B (90% methanol, 0.25% acetic acid, pH4.3) within 10 min. Then the column was washed with 100% solution B for 4 min and re-equilibrated with 100% solution A (10% methanol, 0.25%

acetic acid, pH4.3) for 3 min. The fluorescent derivatives (mBBR) were quantified by fluorescence detection with excitation wavelength at 380 nm and emission wavelength at 480 nm. A standard solution of 0.1 mM GSH and hGSH were used for quantification, with retention times were 6 and 9 min, respectively. Peak area ratio of GSH/GSSG was calculated.

Determination of Fe content.

Double mutants of *Δgrx12*, *Δgrx34* and the quadruple mutant *Δgrx1234* of *A. caulinodans* strains were used in this work. *A. caulinodans* strains were grown in liquid TY medium overnight before the determination of the Fe content by the reported method (Fish 1988). Approximately 10⁹ cells of each strains were suspended with 1 mL sterile water and 0.5 mL freshly prepared reagent A (0.142 M KMnO₄ and 0.6 M HCl). The mixtures of each strains were incubated at 60°C for 2 h and added with 0.1 mL reagent B (13.1 mM neocuproin, 5 M ammonium acetate, 2 M ascorbic acid and 6.5 mM ferrozine). The subsequent cultures were centrifuged after a 30 min incubation at 25°C. The OD₅₆₂ values of each supernatant were measured with an UV-visible light spectrophotometry (Philes Ltd, Nanjing, China). The OD₅₆₂ values were converted to Fe content with a standard curve of ferrous sulfate (FeSO₄).

Soil colonization.

The Sm resistance strains of *Δgrx12*, *Δgrx34* and *Δgrx1234* of *A. caulinodans* were acquired by spontaneous mutation, then were used in this study. Soils (nature soils), half of which were autoclaved at 121°C for 30 min (sterile soils), were collected from the experimental base of Nanjing Agricultural University, Nanjing of China. Then 200 g soils were placed in a 500 mL square sterile glass bottle. Overnight cultures of each strain were inoculated into the bottle and well mixed at the final concentration of 10⁶ CFU·g⁻¹ soil in 28°C chamber. Colonization of nature and sterile soils was determined by the bacterial load of each strain in soil samples at 0, 3, 7, and 10 dpi. Bacterial enumeration was performed by plating on YEM Congo red medium through a series of 10-time dilutions of soil samples.

Plant assays.

Double mutants *Δgrx12* and *Δgrx34* and the quadruple mutant *Δgrx1234* of *A. caulinodans* strains were used in this work. Overnight cultures were adjusted to the OD₆₀₀ value of 0.1 (10⁸ cells per milliliter) and were sprayed onto the stem of *Sesbania rostrata*. The amount of stem nodule, N₂-fixing efficiency, and the biomass of aerial plants were determined at 45 dpi. The N₂-fixing efficiency was assessed by ARA, as reported previously (Ling et al. 2016). Briefly, all nodules were collected from each plant, were placed in 20-ml empty bottles with 2 ml of acetylene (10% vol/vol), and were incubated upside down at 28°C for 2 h. The ethylene production was detected by using a HP 7280A series gas chromatograph system. Gas chromatography was conducted to measure peak height of ethylene and acetylene with 100 μl of gas. The approximate nitrogenase activity was expressed as micromoles of C₂H₄ of acetylene production per gram of nodule dry weight.

ACKNOWLEDGMENTS

We thank H. Wang and Z. Huang for writing discussion.

AUTHOR-RECOMMENDED INTERNET RESOURCE

Weblogo: <http://weblogo.berkeley.edu>

LITERATURE CITED

- Aslund, F., Zheng, M., Beckwith, J., and Storz, G. 1999. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc. Natl. Acad. Sci. U.S.A.* 96:6161-6165.
- Bandyopadhyay, S., Starke, D. W., Mieyal, J. J., and Gronostajski, R. M. 1998. Thioltransferase (glutaredoxin) reactivates the DNA-binding activity of oxidation-inactivated nuclear factor I. *J. Biol. Chem.* 273:392-397.
- Becana, M., Dalton, D. A., Moran, J. F., Iturbe-Ormaetxe, I., Matamoros, M. A., and Rubio, M. C. 2000. Reactive oxygen species and antioxidants in legume nodules. *Physiol. Plant.* 109:372-381.
- Benabdellah, K., Merlos, M.-A., Azcón-Aguilar, C., and Ferrol, N. 2009. GintGRX1, the first characterized glomeromycotan glutaredoxin, is a multifunctional enzyme that responds to oxidative stress. *Fungal Genet. Biol.* 46:94-103.
- Benyamina, S. M., Baldacci-Cresp, F., Couturier, J., Chibani, K., Hopkins, J., Bekki, A., de Lajudie, P., Rouhier, N., Jacquot, J.-P., Alloing, G., Puppo, A., and Frendo, P. 2013. Two *Sinorhizobium meliloti* glutaredoxins regulate iron metabolism and symbiotic bacteroid differentiation. *Environ. Microbiol.* 15:795-810.
- Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* 84:188-198.
- Berndt, C., Lillig, C. H., and Holmgren, A. 2008. Thioredoxins and glutaredoxins as facilitators of protein folding. *BBA-Mol. Cell Res.* 1783:641-650.
- Capela, D., Filipe, C., Bobik, C., Batut, J., and Bruand, C. 2006. *Sinorhizobium meliloti* differentiation during symbiosis with alfalfa: A transcriptomic dissection. *Mol. Plant-Microbe Interact.* 19:363-372.
- Chan, M. K., Kim, J., and Rees, D. C. 1993. The nitrogenase FeMo-cofactor and P-cluster pair: 2.2 Å resolution structures. *Science* 260:792-794.
- Couturier, J., Jacquot, J.-P., and Rouhier, N. 2009. Evolution and diversity of glutaredoxins in photosynthetic organisms. *Cell. Mol. Life Sci.* 66:2539-2557.
- Crooks, G. E., Hon, G., Chandonia, J.-M., and Brenner, S. E. 2004. WebLogo: A sequence logo generator. *Genome Res.* 14:1188-1190.
- Cui, W., Li, L., Gao, Z., Wu, H., Xie, Y., and Shen, W. 2012. Haem oxygenase-1 is involved in salicylic acid-induced alleviation of oxidative stress due to cadmium stress in *Medicago sativa*. *J. Exp. Bot.* 63:5521-5534.
- D'Haese, W., De Rycke, R., Mathis, R., Goormachtig, S., Pagnotta, S., Verplancke, C., Capoen, W., and Holsters, M. 2003. Reactive oxygen species and ethylene play a positive role in lateral root base nodulation of a semiaquatic legume. *Proc. Natl. Acad. Sci. U.S.A.* 100:11789-11794.
- D'Haese, W., Gao, M., De Rycke, R., Van Montagu, M., Engler, G., and Holsters, M. 1998. Roles for azorhizobial Nod factors and surface polysaccharides in intercellular invasion and nodule penetration, respectively. *Mol. Plant-Microbe Interact.* 11:999-1008.
- Den Herder, G., Schroyers, K., Holsters, M., and Goormachtig, S. 2006. Signaling and gene expression for water-tolerant legume nodulation. *Crit. Rev. Plant Sci.* 25:367-380.
- Dreyfus, B. L., and Dommergues, Y. R. 1981. Nitrogen-fixing nodules induced by *Rhizobium* on the stem of the tropical legume *Sesbania rostrata*. *FEMS Microbiol. Lett.* 10:313-317.
- El Msehli, S., Lambert, A., Baldacci-Cresp, F., Hopkins, J., Boncompagni, E., Smiti, S. A., Hérouart, D., and Frendo, P. 2011. Crucial role of (homo)glutathione in nitrogen fixation in *Medicago truncatula* nodules. *New Phytol.* 192:496-506.
- Fan, Q., Zhang, Y., Liu, Y., Yang, J., Li, D., Tang, Y., Cai, L., Zhao, Z., Zhou, P., and Lu, Y. 2016. Glutaredoxin desensitizes lens to oxidative stress by connecting and integrating specific signaling and transcriptional regulation for antioxidant response. *Cell. Physiol. Biochem.* 39:1813-1826.
- Fernandes, A. P., Fladvad, M., Berndt, C., Andréen, C., Lillig, C. H., Neubauer, P., Sunnerhagen, M., Holmgren, A., and Vlamis-Gardikas, A. 2005. A novel monothiol glutaredoxin (Grx4) from *Escherichia coli* can serve as a substrate for thioredoxin reductase. *J. Biol. Chem.* 280:24544-24552.
- Fernandes, A. P., and Holmgren, A. 2004. Glutaredoxins: Glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antioxid. Redox Signal.* 6:63-74.
- Fernandez-Göbel, T. F., Deanna, R., Muñoz, N. B., Robert, G., Asurmendi, S., and Lascano, R. 2019. Redox systemic signaling and induced tolerance responses during soybean-*Bradyrhizobium japonicum* interaction: Involvement of Nod factor receptor and autoregulation of nodulation. *Front. Plant Sci.* 10:141.
- Fernández-López, M., Goormachtig, S., Gao, M., D'Haese, W., Van Montagu, M., and Holsters, M. 1998. Ethylene-mediated phenotypic plasticity in root nodule development on *Sesbania rostrata*. *Proc. Natl. Acad. Sci. U.S.A.* 95:12724-12728.
- Fish, W. W. 1988. Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. *Methods Enzymol.* 158:357-364.
- Foloppe, N., and Nilsson, L. 2004. The glutaredoxin -C-P-Y-C- motif: Influence of peripheral residues. *Structure* 12:289-300.
- Galibert, F., Finan, T. M., Long, S. R., Puhler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M. J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R. W., Dreano, S., Federspiel, N. A., Fisher, R. F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R. W., Jones, T., Kahn, D., Kahn, M. L., Kalman, S., Keating, D. H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Peck, M. C., Pohl, T. M., Portetel, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorholter, F. J., Weidner, S., Wells, D. H., Wong, K., Yeh, K. C., and Batut, J. 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* 293:668-672.
- González, V., Santamaría, R. I., Bustos, P., Hernández-González, I., Medrano-Soto, A., Moreno-Hagelsieb, G., Janga, S. C., Ramírez, M. A., Jiménez-Jacinto, V., Collado-Vides, J., and Dávila, G. 2006. The partitioned *Rhizobium etli* genome: Genetic and metabolic redundancy in seven interacting replicons. *Proc. Natl. Acad. Sci. U.S.A.* 103:3834-3839.
- Goormachtig, S., Capoen, W., James, E. K., and Holsters, M. 2004. Switch from intracellular to intercellular invasion during water stress-tolerant legume nodulation. *Proc. Natl. Acad. Sci. U.S.A.* 101:6303-6308.
- Gourion, B., Berrabah, F., Ratet, P., and Stacey, G. 2015. *Rhizobium*-legume symbioses: The crucial role of plant immunity. *Trends Plant Sci.* 20:186-194.
- Green, M. R., and Sambrook, J., eds. 2012. *Molecular Cloning: A Laboratory Manual*, 4th Ed. Cold Spring Harbor Laboratory Press, New York.
- Harrison, J., Jamet, A., Muglia, C. I., Van de Sype, G., Aguilar, O. M., Puppo, A., and Frendo, P. 2005. Glutathione plays a fundamental role in growth and symbiotic capacity of *Sinorhizobium meliloti*. *J. Bacteriol.* 187:168-174.
- Holmgren, A. 1988. Thioredoxin and glutaredoxin: Small multi-functional redox proteins with active-site disulphide bonds. *Biochem. Soc. Trans.* 16:95-96.
- Holmgren, A. 1989. Thioredoxin and glutaredoxin systems. *J. Biol. Chem.* 264:13963-13966.
- Howe, K., Bateman, A., and Durbin, R. 2002. QuickTree: Building huge neighbour-joining trees of protein sequences. *Bioinformatics* 18:1546-1547.
- Jamet, A., Sigaud, S., Van de Sype, G., Puppo, A., and Hérouart, D. 2003. Expression of the bacterial catalase genes during *Sinorhizobium meliloti*-*Medicago sativa* symbiosis and their crucial role during the infection process. *Mol. Plant-Microbe Interact.* 16:217-225.
- Jiang, G., Yang, J., Li, X., Cao, Y., Liu, X., Ling, J., Wang, H., Zhong, Z., and Zhu, J. 2019. Alkyl hydroperoxide reductase is important for oxidative stress resistance and symbiosis in *Azorhizobium caulinodans*. *FEMS Microbiol. Lett.* 366:fzn2014.
- Kaneko, T., Maita, H., Hirakawa, H., Uchiike, N., Minamisawa, K., Watanabe, A., and Sato, S. 2011. Complete genome sequence of the soybean symbiont *Bradyrhizobium japonicum* strain USDA6T. *Genes (Basel)* 2:763-787.
- Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., Watanabe, A., Idesawa, K., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Mochizuki, Y., Nakayama, S., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., and Tabata, S. 2000. Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Res.* 7:331-338.
- Kaneko, T., Nakamura, Y., Sato, S., Minamisawa, K., Uchiike, T., Sasamoto, S., Watanabe, A., Idesawa, K., Iriguchi, M., Kawashima, K., Kohara, M., Matsumoto, M., Shimpo, S., Tsuruoka, H., Wada, T., Yamada, M., and Tabata, S. 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. *DNA Res.* 9:189-197.
- Khan, S. R., Gaines, J., Roop, R. M., 2nd, and Farrand, S. K. 2008. Broad-host-range expression vectors with tightly regulated promoters and their use to examine the influence of TraR and TraM expression on Ti plasmid quorum sensing. *Appl. Environ. Microbiol.* 74:5053-5062.
- Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33:1870-1874.

- Kwak, M.-J., Jeong, H., Madhaiyan, M., Lee, Y., Sa, T.-M., Oh, T. K., and Kim, J. F. 2014. Genome information of *Methylobacterium oryzae*, a plant-probiotic methylophilic bacterium in the phyllosphere. *PLoS One* 9:e106704.
- Ladha, J. K., Garcia, M., Miyan, S., Padre, A. T., and Watanabe, I. 1989. Survival of *Azorhizobium caulinodans* in the soil and rhizosphere of wetland rice under *Sesbania rostrata*-rice rotation. *Appl. Environ. Microbiol.* 55:454-460.
- Lee, D. W., Kaur, D., Chinta, S. J., Rajagopalan, S., and Andersen, J. K. 2009. A disruption in iron-sulfur center biogenesis via inhibition of mitochondrial dithiol glutaredoxin 2 may contribute to mitochondrial and cellular iron dysregulation in mammalian glutathione-depleted dopaminergic cells: Implications for Parkinson's disease. *Antioxid. Redox Signal.* 11:2083-2094.
- Lee, K. B., De Backer, P., Aono, T., Liu, C.-T., Suzuki, S., Suzuki, T., Kaneko, T., Yamada, M., Tabata, S., Kupfer, D. M., Najjar, F. Z., Wiley, G. B., Roe, B., Binnewies, T. T., Ussery, D. W., D'Haese, W., Herder, J. D., Gevers, D., Vereecke, D., Holsters, M., and Oyaizu, H. 2008. The genome of the versatile nitrogen fixer *Azorhizobium caulinodans* ORS571. *BMC Genomics* 9:271.
- Li, H., and Outten, C. E. 2012. Monothiol CGFS glutaredoxins and BolA-like proteins: [2Fe-2S] binding partners in iron homeostasis. *Biochemistry* 51:4377-4389.
- Lillig, C. H., Berndt, C., and Holmgren, A. 2008. Glutaredoxin systems. *Biochim. Biophys. Acta* 1780:1304-1317.
- Lillig, C. H., Prior, A., Schwenn, J. D., Aslund, F., Ritz, D., Vlamis-Gardikas, A., and Holmgren, A. 1999. New thioredoxins and glutaredoxins as electron donors of 3'-phosphoadenylylsulfate reductase. *J. Biol. Chem.* 274:7695-7698.
- Ling, J., Wang, H., Wu, P., Li, T., Tang, Y., Naseer, N., Zheng, H., Masson-Boivin, C., Zhong, Z., and Zhu, J. 2016. Plant nodulation inducers enhance horizontal gene transfer of *Azorhizobium caulinodans* symbiosis island. *Proc. Natl. Acad. Sci. U.S.A.* 113:13875-13880.
- Liu, X., Qiu, W., Rao, B., Cao, Y., Fang, X., Yang, J., Jiang, G., Zhong, Z., and Zhu, J. 2019. Bacterioferritin comigratory protein is important in hydrogen peroxide resistance, nodulation, and nitrogen fixation in *Azorhizobium caulinodans*. *Arch. Microbiol.* 201:823-831.
- Lu, J., and Holmgren, A. 2014. The thioredoxin antioxidant system. *Free Radic. Biol. Med.* 66:75-87.
- Luo, M., Jiang, Y.-L., Ma, X.-X., Tang, Y.-J., He, Y.-X., Yu, J., Zhang, R.-G., Chen, Y., and Zhou, C.-Z. 2010. Structural and biochemical characterization of yeast monothiol glutaredoxin Grx6. *J. Mol. Biol.* 398: 614-622.
- Mancini, S., and Imlay, J. A. 2015. The induction of two biosynthetic enzymes helps *Escherichia coli* sustain heme synthesis and activate catalase during hydrogen peroxide stress. *Mol. Microbiol.* 96: 744-763.
- Metcalfe, W. W., Jiang, W., Daniels, L. L., Kim, S. K., Haldimann, A., and Wanner, B. L. 1996. Conditionally replicative and conjugative plasmids carrying *lacZ* alpha for cloning, mutagenesis, and allele replacement in bacteria. *Plasmid* 35:1-13.
- Meyer, Y., Belin, C., Delorme-Hinoux, V., Reichheld, J.-P., and Riondet, C. 2012. Thioredoxin and glutaredoxin systems in plants: Molecular mechanisms, crosstalks, and functional significance. *Antioxid. Redox Signal.* 17:1124-1160.
- Michelet, L., Zaffagnini, M., Massot, V., Keryer, E., Vanacker, H., Miginiac-Maslow, M., Issakidis-Bourguet, E., and Lemaire, S. D. 2006. Thioredoxins, glutaredoxins, and glutathionylation: New cross-talks to explore. *Photosynth. Res.* 89:225-245.
- Mishra, S., and Imlay, J. 2012. Why do bacteria use so many enzymes to scavenge hydrogen peroxide? *Arch. Biochem. Biophys.* 525:145-160.
- Muglia, C., Comai, G., Spegazzini, E., Riccillo, P. M., and Aguilar, O. M. 2008. Glutathione produced by *Rhizobium tropici* is important to prevent early senescence in common bean nodules. *FEMS Microbiol. Lett.* 286: 191-198.
- Mühlenhoff, U., Molik, S., Godoy, J. R., Uzarska, M. A., Richter, N., Seubert, A., Zhang, Y., Stubbe, J., Pierrrel, F., Herrero, E., Lillig, C. H., and Lill, R. 2010. Cytosolic monothiol glutaredoxins function in intracellular iron sensing and trafficking via their bound iron-sulfur cluster. *Cell Metab.* 12:373-385.
- Nanda, A. K., Andrio, E., Marino, D., Pauly, N., and Dunand, C. 2010. Reactive oxygen species during plant-microorganism early interactions. *J. Integr. Plant Biol.* 52:195-204.
- Nierman, W. C., Feldblyum, T. V., Laub, M. T., Paulsen, I. T., Nelson, K. E., Eisen, J. A., Heidelberg, J. F., Alley, M. R., Ohta, N., Maddock, J. R., Potocka, I., Nelson, W. C., Newton, A., Stephens, C., Phadke, N. D., Ely, B., DeBoy, R. T., Dodson, R. J., Durkin, A. S., Gwinn, M. L., Haft, D. H., Kolonay, J. F., Smit, J., Craven, M. B., Khouri, H., Shetty, J., Berry, K., Utterback, T., Tran, K., Wolf, A., Vamathevan, J., Ermolaeva, M., White, O., Salzberg, S. L., Venter, J. C., Shapiro, L., and Fraser, C. M. 2001. Complete genome sequence of *Caulobacter crescentus*. *Proc. Natl. Acad. Sci. U.S.A.* 98:4136-4141.
- Ojeda, L., Keller, G., Muhlenhoff, U., Rutherford, J. C., Lill, R., and Winge, D. R. 2006. Role of glutaredoxin-3 and glutaredoxin-4 in the iron regulation of the Aft1 transcriptional activator in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 281:17661-17669.
- Pauly, N., Pucciariello, C., Mandon, K., Innocenti, G., Jamet, A., Baudouin, E., Hérouart, D., Frendo, P., and Puppo, A. 2006. Reactive oxygen and nitrogen species and glutathione: Key players in the *legume-Rhizobium symbiosis*. *J. Exp. Bot.* 57:1769-1776.
- Pessi, G., Ahrens, C. H., Rehrauer, H., Lindemann, A., Hauser, F., Fischer, H.-M., and Hennecke, H. 2007. Genome-wide transcript analysis of *Bradyrhizobium japonicum* bacteroids in soybean root nodules. *Mol. Plant-Microbe Interact.* 20:1353-1363.
- Puppo, A., Dimitrijevic, L., Trinchant, J. C., and Rigaud, J. 1986. Acetylene reduction and superoxide dismutase activities in *Sesbania rostrata* root and stem nodules. *Physiol. Veg.* 24:689-686.
- Queval, G., and Noctor, G. 2007. A plate reader method for the measurement of NAD, NADP, glutathione, and ascorbate in tissue extracts: Application to redox profiling during *Arabidopsis* rosette development. *Anal. Biochem.* 363:58-69.
- Reen, F. J., Haynes, J. M., Mooij, M. J., and O'Gara, F. 2013. A non-classical LysR-type transcriptional regulator PA2206 is required for an effective oxidative stress response in *Pseudomonas aeruginosa*. *PLoS One* 8:e54479.
- Rouhier, N., Couturier, J., Johnson, M. K., and Jacquot, J.-P. 2010. Glutaredoxins: Roles in iron homeostasis. *Trends Biochem. Sci.* 35: 43-52.
- Rubio, M. C., Becana, M., Kanematsu, S., Ushimaru, T., and James, E. K. 2009. Immunolocalization of antioxidant enzymes in high-pressure frozen root and stem nodules of *Sesbania rostrata*. *New Phytol.* 183: 395-407.
- Sallet, E., Roux, B., Sauviac, L., Jardinaud, M.-F., Carrère, S., Faraut, T., de Carvalho-Niebel, F., Gouzy, J., Gamas, P., Capela, D., Bruand, C., and Schiex, T. 2013. Next-generation annotation of prokaryotic genomes with EuGene-P: Application to *Sinorhizobium meliloti* 2011. *DNA Res.* 20:339-354.
- Sánchez-Riego, A. M., López-Maury, L., and Florencio, F. J. 2013. Glutaredoxins are essential for stress adaptation in the *cyanobacterium Synechocystis* sp. PCC 6803. *Front. Plant Sci.* 4:428.
- Schmeisser, C., Liesegang, H., Kryciak, D., Bakkou, N., Le Quéré, A., Wollherr, A., Heinemeyer, I., Morgenstern, B., Pommerening-Röser, A., Flores, M., Palacios, R., Brenner, S., Gottschalk, G., Schmitz, R. A., Broughton, W. J., Perret, X., Strittmatter, A. W., and Streit, W. R. 2009. *Rhizobium* sp. strain NGR234 possesses a remarkable number of secretion systems. *Appl. Environ. Microbiol.* 75:4035-4045.
- Seshadri, R., Reeve, W. G., Ardley, J. K., Tennesen, K., Woyke, T., Kyrpides, N. C., and Ivanova, N. N. 2015. Discovery of novel plant interaction determinants from the genomes of 163 root nodule bacteria. *Sci. Rep.* 5:16825.
- Shenton, D., Perrone, G., Quinn, K. A., Dawes, I. W., and Grant, C. M. 2002. Regulation of protein S-thiolation by glutaredoxin 5 in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* 277:16853-16859.
- Takashima, Y., Hirota, K., Nakamura, H., Nakamura, T., Akiyama, K., Cheng, F. S., Maeda, M., and Yodoi, J. 1999. Differential expression of glutaredoxin and thioredoxin during monocytic differentiation. *Immunol. Lett.* 68:397-401.
- Tang, G., Li, N., Liu, Y., Yu, L., Yan, J., and Luo, L. 2018. *Sinorhizobium meliloti* glutathione reductase is required for both redox homeostasis and symbiosis. *Appl. Environ. Microbiol.* 84:e01937-e17.
- Taté, R., Cermola, M., Riccio, A., Diez-Roux, G., and Patriarca, E. J. 2012. Glutathione is required by *Rhizobium etli* for glutamine utilization and symbiotic effectiveness. *Mol. Plant-Microbe Interact.* 25:331-340.
- Terpolilli, J., Rui, T., Yates, R., Howieson, J., Poole, P., Munk, C., Tapia, R., Han, C., Markowitz, V., Tatiparthi, R., Mavrommatis, K., Ivanova, N., Pati, A., Goodwin, L., Woyke, T., Kyrpides, N., and Reeve, W. 2014. Genome sequence of *Rhizobium leguminosarum* bv *trifolii* strain WSM1689, the microsymbiont of the one flowered clover *Trifolium uniflorum*. *Stand. Genomic Sci.* 9:527-539.
- Tsien, H. C., Dreyfus, B. L., and Schmidt, E. L. 1983. Initial stages in the morphogenesis of nitrogen-fixing stem nodules of *Sesbania rostrata*. *J. Bacteriol.* 156:888-897.
- Uzureau, S., Lemaire, J., Delaive, E., Dieu, M., Gaigneaux, A., Raes, M., De Bolle, X., and Letesson, J.-J. 2010. Global analysis of quorum sensing targets in the intracellular pathogen *Brucella melitensis* 16 M. *J. Proteome Res.* 9:3200-3217.

- Wang, H., Chen, S., Zhang, J., Rothenbacher, F. P., Jiang, T., Kan, B., Zhong, Z., and Zhu, J. 2012a. Catalases promote resistance of oxidative stress in *Vibrio cholerae*. PLoS One 7:e53383.
- Wang, L., Ouyang, B., Li, Y., Feng, Y., Jacquot, J.-P., Rouhier, N., and Xia, B. 2012b. Glutathione regulates the transfer of iron-sulfur cluster from monothiol and dithiol glutaredoxins to apo ferredoxin. Protein Cell 3:714-721.
- Wang, S., Hao, B., Li, J., Gu, H., Peng, J., Xie, F., Zhao, X., Frech, C., Chen, N., Ma, B., and Li, Y. 2014. Whole-genome sequencing of *Mesorhizobium huakuii* 7653R provides molecular insights into host specificity and symbiosis island dynamics. BMC Genomics 15:440.
- Zhao, Y., Nickels, L. M., Wang, H., Ling, J., Zhong, Z., and Zhu, J. 2016. OxyR-regulated catalase activity is critical for oxidative stress resistance, nodulation and nitrogen fixation in *Azorhizobium caulinodans*. FEMS Microbiol. Lett. 363:fnw130.
- Zheng, M., Aslund, F., and Storz, G. 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. Science 279:1718-1722.
- Zheng, X., Zhang, K., Zhou, X., Liu, C., Li, M., Li, Y., Wang, R., Li, Y., Li, J., Shi, W., and Xu, X. 2013. Involvement of gshAB in the interspecies competition within oral biofilm. J. Dent. Res. 92:819-824.