A GMC Oxidoreductase GmcA Is Required for Symbiotic Nitrogen Fixation in *Rhizobium leguminosarum* bv. *viciae*

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GmcA is a FAD-containing enzyme belonging to the GMC (glucose-methanol-choline oxidase) family of oxidoreductases. A mutation in the *Rhizobium leguminosarum* gmcA gene was generated by homologous recombination. The mutation in gmcA did not affect the growth of *R. leguminosarum*, but it displayed decreased antioxidative capacity at $\text{H}_2\text{O}_2$ conditions higher than 5 mM. The gmcA mutant strain displayed no difference of glutathione reductase activity, but significantly lower level of the glutathione peroxidase activity than the wild type. Although the gmcA mutant was able to induce the formation of nodules, the symbiotic ability was severely impaired, which led to an abnormal nodulation phenotype coupled to a 30% reduction in the nitrogen fixation capacity. The observation on ultrastructure of 4-week pea nodules showed that the mutant bacteroids tended to start senescence earlier and accumulate poly-$\beta$-hydroxybutyrate (PHB) granules. In addition, the gmcA mutant was severely impaired in rhizosphere colonization. Real-time quantitative PCR showed that the gmcA gene expression was significantly up-regulated in all the detected stages of nodule development, and statistically significant decreases in the expression of the redoxin genes *katG*, *katE*, and *ohrB* were found in gmcA mutant bacteroids. LC-MS/MS analysis quantitative proteomics techniques were employed to compare differential gmcA mutant root bacteroids in response to the wild type infection. Sixty differentially expressed proteins were identified including 33 up-regulated and 27 down-regulated proteins. By sorting the identified proteins according to metabolic function, 15 proteins were transporter protein, 12 proteins were related to stress response and virulence, and 9 proteins were related to transcription factor activity. Moreover, nine proteins related to amino acid metabolism were over-expressed.

**Keywords:** *Rhizobium leguminosarum*, the glucose-methanol-choline oxidoreductase GmcA, symbiotic nitrogen fixation, antioxidant and symbiotic gene expression, quantitative proteomics

**INTRODUCTION**

*Rhizobium leguminosarum* bv. *viciae* is an aerobic, Gram-negative, nitrogen-fixing bacterium that can live under the conditions of microaerobe, aerobe and form symbiotic relationships with *Pisum sativum* (pea) and *Vicia cracca* (vetch) under the condition of nitrogen limitation (Karunakaran et al., 2009). Organisms of this genus play a critical role in soil fertility, inducing the formation
of symbiotic nodules on the roots of leguminous plants, where bacteroids reduce atmospheric nitrogen to ammonia available for plant uptake (Bhat et al., 2015). The symbiosis between rhizobia and legumes can be characterized by a mutual exchange of signal molecules between the two partners (Janczarek et al., 2015; López-Baena et al., 2016). After attachment of the bacteria to the plant root, the plant supports bacterial infection via host-derived infection threads (Haney and Long, 2010). Successful nodulation requires the activation of cell division in the cortex to form the nodule primordium (Blanco et al., 2009). In nodules, the nitrogenase enzyme, which is extremely sensitive to oxygen, has a low turnover number and a large requirement of chemical energy in the form of ATP and reducing potential (Clarke et al., 2011; Okazaki et al., 2015). In addition to reducing N₂ and protons, nitrogenase can also reduce several small, non-physiological substrates, including a wide array of carbon-containing compounds (Seefeldt et al., 2013). It was found that uptake hydrogenases allow rhizobia to recycle the hydrogen generated in the nitrogen fixation process within the legume nodule (Baginsky et al., 2002).

Oxidoreductases catalyze a large variety of specific reduction, oxidation, and oxyfunctionalization reactions, which are important in redox processes, transferring electrons from a reductant to an oxidant (Hollmann and Schmid, 2004; Jeelani et al., 2010). Oxidoreductases included laccases, GMC (glucose-methanol-choline) oxidoreductases, copper radical oxidases and catalases (Beckett et al., 2015). The family of GMC flavoprotein oxidoreductases, which includes glucose/alcohol oxidase and glucose/choline dehydrogenase from prokaryotic and eukaryotic organisms, was first outlined by Cavener (1992). Members of the GMC oxidoreductase family share a common structural backbone of an adenine-dinucleotide-phosphate-binding βαβ-fold close to their amino terminus (Iida et al., 2007). The group of GMC flavoprotein oxidoreductases encompasses glucose oxidase from the mold Aspergillus niger, the glucose dehydrogenase from Thermoplasma acidophilum and Drosophila melanogaster, methanol oxidase from yeast Hansenula polymorpha, and choline dehydrogenase from Escherichia coli (Ahmad et al., 2010; Liu et al., 2013). In the leaf beetle subtribe Chrysomelina sensu stricto, GMC oxidoreductases enabled chemical defenses and were important for adaptive processes in plant–insect interactions (Rahfeld et al., 2014). In E. coli, choline dehydrogenase catalyzes the flavin-dependent, two-step oxidation of choline to glycerine betaine, which acts as an osmoprotectant compatible solute that accumulates when the cells are exposed to drastic environmental changes in osmolarity (Yilmaz and Bülow, 2010). However, little is known about the functional diversity of the rhizobium GMC family.

Rhizobium leguminosarum bv. viciae, which has been widely used as a model to study nodule biochemistry, is able to nodulate and fix nitrogen in symbiosis with several legumes (Karunakaran et al., 2009). Here, we investigated the roles of a GMC oxidoreductase GmcA in free-living bacteria and during nitrogen-fixing symbiosis on pea by analyzing the phenotypes of a mutant strain. Proteome analysis provides clues to explain the differences between the gmcA mutant and wild-type nodules.

**MATERIALS AND METHODS**

**Bacterial Growth and Media**

The strains, plasmids and primers used in this study are listed in Table 1. Rhizobium strains were grown at 28°C in either Tryptone Yeast extract (TY) (Beringer and Hopwood, 1976) or Acid Minimal Salts medium (AMS) (Poole et al., 1994) with D-glucose (10 mM) as a carbon source and NH₄Cl (10 mM) as a N source (referred to as AMS Glc/NH₄⁺). For growth and qRT-PCR experiments, cells were grown in AMS Glc/NH₄⁺. Antibiotics were used at the following concentrations (µg/mL): ampicillin (Amp), 50; gentamicin (Gm), 20; kanamycin (Km), 20; neomycin (Neo), 80; spectinomycin (Spe), 100; streptomycin (Str), 500; tetracycline (Tc), 5. Strains were grown at 28°C with shaking (200 rpm) for liquid media. To monitor culture growth, optical density at 600 nm (OD₆₀₀) was measured on three independent cultures.

**Construction and Complementation of the gmcA Gene Mutant of R. leguminosarum 3841**

Primers gmcAF and gmcAR were used to PCR amplify an internal region of the gmcA gene from R. leguminosarum bv. viciae 3841 genomic DNA (Johnston and Beringer, 1975). The 650-bp gmcA PCR product was cloned into the PsI and XbaI sites of pK19mob, resulting in plasmid pKgmCA. The plasmid pKgmCA was conjugated with R. leguminosarum bv. viciae 3841 using pRK2013 as a helper plasmid, as previously described (Figurski and Helinski, 1979; Karunakaran et al., 2010). Insertions into the gmcA gene of strain RL3841 were selected by neomycin resistant AMS medium with 30mM pyruvate as a sole carbon source and confirmed by PCR using MgmCA and a pK19mob-specific primer (either pK19A or pK19B) (Karunakaran et al., 2010).

To complement the gmcA mutant, primers cgmcAF and cgmcAR were used to amplify the complete gmcA gene from strain RL3841. The PCR product was digested with KpnI and XbaI and cloned into pBR1MCS-5, resulting in plasmid pBBRgmcA. Plasmid pBBRgmcA was conjugated into the mutant strain RLgmcA using pRK2013 as a helper plasmid to provide the transfer genes, as previously described (Karunakaran et al., 2010).

**Hydrogen Peroxide Resistance Activity**

Logarithmic phase cultures of mutant strain RLgmcA and wild-type RL3841 were collected and washed twice in sterile phosphate-buffered saline (PBS) (1x; 136 mM NaCl, 2.6 mM KCl, 8.0 mM Na₂HPO₄, and 1.5 mM KH₂PO₄). Cells with an optical density (OD₆₀₀) 1 were treated with H₂O₂ at different concentrations (0, 1, 5, and 10 mmol/L) for 1 h. Strains were thoroughly washed with distilled water to remove any remaining oxidant, and the diluted TY plate method was used to evaluate the bacterial survival rate. The experiment consisted of three independent experiments, each of which had three repeats, and statistical differences were analyzed with one-way ANOVA (P < 0.05).
### TABLE 1 | Strains, plasmids, and primers.

| Strains       | Description                                           |
|---------------|-------------------------------------------------------|
| RL3841        | *Rhizobium leguminosarum* bv. *viciae*, Str<sup>f</sup> |
| RLgmcA        | RL3841 gmcA-pk19mob, Str<sup>f</sup> Neo<sup>f</sup> |
| RLgmcA (pBBRgmcA) | RLgmcA carrying gmcA gene, Str<sup>f</sup> Neo<sup>f</sup> Gm<sup>f</sup> |

| Plasmids      | Description                                           |
|---------------|-------------------------------------------------------|
| pK19mob       | pk19mob pUC19 derivative lacZ mob; Km<sup>f</sup>  |
| pRK2013       | Helper plasmid for mobilizing plasmids; Km<sup>f</sup> |
| pKgmcA        | gmcAF/gmcAR PCR product in pk19mob, Km<sup>f</sup>  |
| pBBRgmcA      | cgmcAF/cgmcAR PCR product in pBBR1MCS-5, Gm<sup>f</sup> |

| Primer        | Description                                           |
|---------------|-------------------------------------------------------|
| gmcAF         | Sense primer for pRL100444 (gmcA) mutation            |
| gmcAR         | Antisense primer for pRL100444 (gmcA) mutation        |
| MgmCA         | Mapping PCR primer for gmcA gene                      |
| pk19A         | pk19mob mapping primer                                |
| pk19B         | pk19mob mapping primer                                |
| gyrB1-F       | Sense primer for qRT-PCR of GyrB1                     |
| gyrB1-R       | Antisense primer for qRT-PCR of GyrB1                 |
| cgmcAF        | Sense primer for gmcA complementation                 |
| cgmcAR        | Antisense primer for gmcA complementation             |
| QgmcA-forward | Sense primer for qRT-PCR of gmcA                     |
| QgmcA-reverse | Antisense primer for qRT-PCR of gmcA                  |
| gyrb1-F       | gyrB1 primers for qRT-PCR                             |
| gyrb1-R       | gyrB1 primers for qRT-PCR                             |
| QkatG-F       | katG primers for qRT-PCR                              |
| QkatG-R       | katG primers for qRT-PCR                              |
| QkatE-F       | katE primers for qRT-PCR                              |
| QkatE-R       | katE primers for qRT-PCR                              |
| QcorB-F       | Sense primer for qRT-PCR of orhB                      |
| QcorB-R       | Antisense primer for qRT-PCR of orhB                  |
| QhmuS-F       | hmuS primers for qRT-PCR                              |
| QhmuS-R       | hmuS primers for qRT-PCR                              |
| QnfdX-F       | Sense primer for qRT-PCR of nfdX                      |
| QnfdX-R       | Antisense primer for qRT-PCR of nfdX                  |
| Qfdx2-F       | Sense primer for qRT-PCR of fdx2                      |
| Qfdx2-R       | Antisense primer for qRT-PCR of fdx2                  |

| Primers       | Description                                           |
|---------------|-------------------------------------------------------|
| gyrB1-F       | gyrB1 primers for qRT-PCR                             |
| gyrB1-R       | gyrB1 primers for qRT-PCR                             |
| QkatG-F       | katG primers for qRT-PCR                              |
| QkatG-R       | katG primers for qRT-PCR                              |
| QkatE-F       | katE primers for qRT-PCR                              |
| QkatE-R       | katE primers for qRT-PCR                              |
| QcorB-F       | sense primer for qRT-PCR of orhB                      |
| QcorB-R       | Antisense primer for qRT-PCR of orhB                  |
| QhmuS-F       | hmuS primers for qRT-PCR                              |
| QhmuS-R       | hmuS primers for qRT-PCR                              |
| QnfdX-F       | Sense primer for qRT-PCR of nfdX                      |
| QnfdX-R       | Antisense primer for qRT-PCR of nfdX                  |
| Qfdx2-F       | Sense primer for qRT-PCR of fdx2                      |
| Qfdx2-R       | Antisense primer for qRT-PCR of fdx2                  |

<sup>1</sup> Restriction sites in primer sequences are underlined.

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**Enzyme Activity Experiments**

For analysis of glutathione reductase and glutathione peroxidase activities, logarithmic phase cultures of mutant strain RLgmcA and wild-type RL3841 with an optical density (OD<sub>600</sub>) 1 were collected, and treated with 5 mM H<sub>2</sub>O<sub>2</sub> for 1 h. H<sub>2</sub>O<sub>2</sub>-treated PBS cells were collected by centrifugation at 5,000 rpm for 5 min at 4°C. The cells were held in an ice-water bath and sonicated for 15 min. The sonicate was centrifuged at 12,000 rpm for 10 min at 4°C. Glutathione reductase and glutathione peroxidase activities were determined using a peroxidase assay kit (Beyotime, China). The experiment consisted of three independent experiments, each of which had three repeats, and statistical differences were analyzed with one-way ANOVA (P < 0.05).

**Plant Growth and Microscope Study of Nodules**

Pea seeds were surface sterilized in 95% ethanol for 30 s and then immersed in a solution of 2% sodium hypochlorite for 10 min. *R. leguminosarum* bv. *viciae* strains were inoculated with 10<sup>7</sup> CFU per seed at the time of sowing. Plants were incubated in a controlled-environment chamber with an 18-h photoperiod (day/night temperature, 22 and 20°C). For dry weight determination, plants were grown in a 2-L beaker filled with sterile vermiculite, watered with nitrogen-free nutrient solution and harvested at 7 weeks (Cheng et al., 2017). The shoot was removed from the root and dried at 70°C in a dry-heat incubator for 3 days before being weighed. Acetylene reduction was determined at flowering (4 weeks) in peas, as previously described (Allaway et al., 2000). The experiment consisted of two independent experiments, each of which had five repeats, and statistical differences were analyzed with one-way ANOVA (P < 0.05).

Nodules at 4 weeks post infection were fixed in 2.5% glutaraldehyde and postfixed in 1.5% osmium tetroxide. Root nodules were sectioned and were then stained with toluidine blue. Ultra-thin sections stained with uranyl acetate and lead citrate were observed using a Hitachi H-7100 transmission electron microscope (Yan et al., 2004). For light microscopy, thick sections were cut on a microtome and stained.
Rhizosphere Colonization

Rhizosphere colonization assays were performed as previously described (Cheng et al., 2017). Pea seedlings were grown for 7 days, as described above, for acetylene reduction, and inoculated with RLgmcA and RL3841 in the cfu ratios 1000:0, 1000:1000, and 10000:1000. After 7 days (14 days after sowing), shoots were cut-off and 20 mL of sterile phosphate-buffered saline (PBS) was added to the roots and vortexed for 15 min at speed 10 (Karunakaran et al., 2006). After vortexing, the samples were serially diluted and plate counted on TY medium plates containing either streptomycin (for wild-type RL3841 and mutant RLgmcA together) or streptomycin and neomycin (for RLgmcA), giving the total number of viable rhizosphere- and root-associated bacteria (Barr et al., 2008). Each treatment consisted of one-way ANOVA (P < 0.05).

RNA Isolation and Quantitative Reverse Transcription–PCR (RT-PCR)

Quantitative Real-Time RT-PCR was used to determine differences in the expression of genes. Cell samples were collected from free-living R. leguminosarum cultivated in AMS liquid medium, or free-living cells treated with 5 mM H2O2 for 1 h or root nodules, which were harvested from pea that had been inoculated with R. leguminosarum strains at 2, 4, and 6 weeks. The nodules of plants were harvested and grinded into a regular fine powder with liquid nitrogen. Total RNA of each sample was extracted using TRIzol Reagent (Invitrogen) and quantified by NanoDrop (Thermo Fisher Scientific) (Smith et al., 1985). cDNA was prepared using SuperScript™ II reverse transcriptase and random hexamers. Quantitative real-time PCR was performed using the SYBR Premix ExTaq (Takara, Dalian, China) on the BIO-RAD CFX96 Real-Time PCR Detection System. Primers for katG, katE, hmuS, ohrB, rhtA, and nifD are detailed in Table 1. Gyrb1 was used as a reference housekeeping gene and the obtained data were analyzed as previously described (Prell et al., 2009). Statistical analysis of data sets was performed using REST (Pfaffl et al., 2002).

Protein Extraction and LC-MS/MS Analysis

The 4-week-nodule samples were grinded into cell powder in liquid nitrogen. The cell powder was transferred to a 5-mL centrifuge tube. Four volumes of lysis buffer (8 M urea, 1% protease inhibitor cocktail) was then added to the cell powder, and the slurry was sonicated three times on ice using a high intensity ultrasonic processor. Cellular debris was removed by centrifugation at 12,000 g for 10 min at 4°C, the supernatant was collected, and the protein content was determined using the BCA protein assay kit (Pierce, Rockland, IL, United States). The resulting proteins were reduced by 5 mM dithiothreitol at 56°C for 30 min, and then alkylated in 11 mM iodoacetamide for 15 min at room temperature in the dark. Each protein sample was then diluted by 100 mM tetraethyl ammonium bromide (TEAB) to obtain a urea concentration of less than 2 and 1:100 trypsin-to-protein mass ratio for a second digestion of 4 h. Following trypsin digestion, the peptides then were desalted by Strata X C18 SPE column and vacuum-dried. The peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's instructions of the tandem mass tag (TMT) kit (Thermo Fisher Scientific, Bremen, United States). Concisely, one unit of TMT reagent was dissolved and reconstituted in acetonitrile. The peptide mixtures were then incubated at room temperature for 2 h and pooled, desalted and dried by vacuum centrifugation.

The trypptic peptides were dissolved in solvent A (0.1% formic acid in aqueous solution) and loaded directly onto a reversed phase analytical column (75 µm i.d. × 15 cm length). The loaded material was eluted from this column in a linear gradient of 6–22% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23–35% in 8 min, climbing to 80% in 3 min and holding at 80% for 3 min with a flow rate of 400 nL/min. The MS proteomics data were deposited to NSI source, followed by tandem mass spectrometry (MS/MS) by using a Q Exactive™ Plus (Thermo) coupled online to the ultra-performance liquid chromatography (UPLC). The electrospray voltage was set to 2.0 kV. For the full scan mode, the m/z scan range was from 350 to 1,800. The intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected to run MS/MS analysis using NCE setting as 28 and the fragments were measured using a resolution of 17,500 in the Orbitrap. The MS analysis alternated between MS and data-dependent tandem MS scans with 15.0 s dynamic exclusion. Automatic gain control (AGC) was set to accumulate 5 × 10^4 ions with the Fixed first mass of 100 m/z. Experiments were conducted in triplicate.

Data Analysis

The resulting MS/MS data were processed and prepared for a database search using the MaxQuant version 1.5.2.8 (Cox and Mann, 2008). The resulting tandem mass spectra were searched against the R. leguminosarum genome database concatenated with a reverse decoy database (Young et al., 2006). Trypsin/P was specified as the cleavage enzyme allowing up to four missing cleavages. The precursor mass tolerance was set to 20 ppm for the first search and 5 ppm for the main search, and the tolerance of the ions was set to 0.02 Da for fragment ion matches. Carbamidomethylation of cysteines was considered as a fixed modification, and oxidation of methionine was specified as variable modifications. A false discovery rate (FDR) of 1% was specified, and the minimal peptide score for modified peptides was set to 40. Protein expression was analyzed statistically using Student’s t-tests (p < 0.05). Up-regulated and down-regulated proteins were defined as having fold changes (FC) > 1.2 and <0.83, respectively.

RESULTS

Bioinformatic Analysis of the R. leguminosarum gmcA Gene

Rhizobium leguminosarum gmcA gene (pRL100444) is predicted to encode a 550-amino acid polypeptide with an expected molecular mass of 60.5 kDa and a pl value of 8.19 (Young et al., 2006). The amino acid sequence of GmcA contained...
was significantly lower for the mutant than for the wild type. When inoculated alone into the rhizosphere, the percentage of bacteria recovered after 7 days was significantly lower for the mutant RLgmcA compared to the wild type RL3841 for growth in the pea rhizosphere. The results showed that the mutant RLgmcA was not significantly affected by hydrogen peroxide treatments at low concentrations of 0.5 and 1 mmol/L compared with the wild-type RL3841 strain, whereas the antioxidative capacity of mutant RLgmcA was significantly decreased by these treatments with H$_2$O$_2$ at higher concentrations of 5 and 10 mmol/L (Table 2). The role of R. leguminosarum GmcA in controlling protein glutathionylation status was investigated by quantifying glutathione reductase activity of the nodules (Table 3). Thus, GmcA may play important roles in oxidative stress resistance and cellular detoxification in R. leguminosarum.

### Antioxidation Analysis of a R. leguminosarum gmcA Mutant

To confirm the function of the gmcA gene in growth performance, antioxidation and symbiotic nitrogen fixation ability, a mutant RLgmcA strain of this gene was constructed by single crossover homologous recombination. In liquid AMS minimal medium with glucose as a carbon source and NH$_4$Cl as a nitrogen source, there is no significant difference in growth between the mutant RLgmcA and wild-type RL3841 (data not shown). The importance of GmcA for protection against oxidative stress was investigated by carrying out survival assays of the mutant RLgmcA in the presence of hydrogen peroxide (H$_2$O$_2$). The survival rates of RLgmcA were not significantly affected by H$_2$O$_2$ treatments at low concentrations of 0.5 and 1 mmol/L compared with the wild-type RL3841 strain, whereas the antioxidative capacity of mutant RLgmcA was significantly decreased by these treatments with H$_2$O$_2$ at higher concentrations of 5 and 10 mmol/L (Table 2).

### Pea Rhizosphere Colonization by R. leguminosarum Strains

Competition between the gmcA mutant RLgmcA and the wild type RL3841 for growth in the pea rhizosphere was measured by inoculating a low number of bacteria into the pea rhizosphere (10$^3$ to 10$^5$ bacteria per seedling) and determining total bacteria after 7 days. When the mutant RLgmcA and the wild type RL3841 were inoculated alone into short-term colonization of sterile pea rhizosphere, the percentage of bacteria recovered after 7 days was significantly lower for the mutant than for the wild strain (Figure 1).

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**Table 2** | Tolerance of R. leguminosarum strains to different concentrations of H$_2$O$_2$.

| Strain            | H$_2$O$_2$ (mM) |
|-------------------|-----------------|
|                   | 0               | 0.5            | 1.0            | 5.0            | 10.0           |
| RL3841            | (6.72 ± 0.71) x 10$^7$ | (4.27 ± 0.27) x 10$^7$ | (2.85 ± 0.18) x 10$^7$ | (2.73 ± 0.14) x 10$^7$ | (1.01 ± 0.21) x 10$^7$ |
| RLgmcA            | (7.03 ± 1.00) x 10$^7$ | (3.80 ± 0.17) x 10$^7$ | (2.21 ± 0.29) x 10$^7$ | (1.59 ± 0.19) x 10$^7$ | (4.07 ± 0.90) x 10$^7$ |

All data are averages (±SEM) from three independent experiments. Different letters indicates the value is significantly different from that of the wild-type RL3841 control (one-way ANOVA, P < 0.05).

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**Table 3** | Oxidase activity of R. leguminosarum gmcA mutant.

| Strains R. leguminosarum | Glutathione reductase (U/mg protein) | Glutathione peroxidase (U/mg protein) |
|--------------------------|--------------------------------------|-------------------------------------|
| RL3841                   | 0.399 ± 0.041$^a$                    | 0.406 ± 0.029$^a$                   |
| RLgmcA                   | 0.408 ± 0.031$^a$                    | 0.018 ± 0.006$^b$                   |
| RLgmcA(pBBRgmcA)         | 0.409 ± 0.030$^a$                    | 0.385 ± 0.031$^a$                   |

All data are averages (±SEM) from three independent experiments. Different letters indicates the value is significantly different from that of the wild-type RL3841 control (one-way ANOVA, P < 0.05).
by Rhizobia-infected cells (Figures 2B,C). The ultrastructural structure of the infected cells was observed by transmission electron microscopy. In the mutant infected nodule cells, bacteroids underwent premature senescence. Bacteroids in pea plants inoculated by R. leguminosarum bv. viciae usually did not produce visible PHB granules, but in the mutant bacteroids, the poly-b-hydroxybutyrate (PHB) was also distinctly observed (Figure 2).

Expression Level of the gmcA Gene in Nodules Induced by R. leguminosarum 3841

The expression of gmcA was significantly up-regulated in the early stage (14 days), maturation stage (28 days) and late stage (42 days) of nodule development and senescence in comparison to that in free-living cells (Figure 3). During symbiosis, gmcA gene has the highest expression level in nodules at 42 days after inoculation. Thus, these results showed that gmcA gene expression was induced during R. leguminosarum—pea symbiosis and suggest that this gene plays an important role in bacteroid persistence in old nodules.

Analysis of the Relative Expression of Genes Involved in Redoxin Production and Nitrogen Fixation in the gmcA Mutant

As shown in Figure 4A, under 5 mmol/L H₂O₂-induced oxidative stress condition, a significant decrease in katG, fdxB, and hmuS gene expression was observed in the gmcA mutant, suggesting that GmcA plays an important role in cellular redox balance. Since a large reduction in the nitrogen-fixing capacity of nodules inoculated with mutant strain was observed, qRT-PCR was used to assess whether the N-fixation system, e.g., nitrogenase genes, was affected in the transcription of ribosomal RNAs in the GmcA-deficient mutant. The expression of nifD and fdxB was analyzed in pea root nodules using qRT-PCR (Figure 4B). Unexpectedly, the expression level of nifD and fdxB was found to be significantly increased in 4-week-old nodules inoculated with gmcA mutant strain compared with control nodules. Thus, GmcA may function in redox balance and antioxidant defense system in the pea root nodules. hmuS gene expression was significantly down-regulated in gmcA mutant, both under H₂O₂-induced oxidative stress condition and in 4-week-old nodule, suggesting gmcA is involved in iron transport and regulation of iron homeostasis.

Protein Differential Expression Analysis

A quantitative proteomic approach using UPLC coupled with tandem mass spectrometry (LC/LC-MS/MS) was performed to compare differential gmcA mutant root bacteroids in response to wild type infection. Proteomics analysis identified peptides derived from a total of 2002 distinct protein groups in gmcA mutant bacteroids and 2000 in wild-type bacteroids, with molecular weights ranging from 7 to 317 kDa. A total of 60 differentially expressed proteins (P < 0.05) were identified. Among these proteins (Table 5), 33 proteins were up-regulated in gmcA mutant nodule bacteroids and 27 proteins were down-regulated. Cell surface protein (RL4381) was absent in the gmcA mutant bacteroids, while invasion associated protein (RL1020) and surface protein (RL4381) was absent in the wild-type bacteroids in response to wild type infection. Proteomics analysis identified peptides derived from a total of 2002 distinct protein groups in gmcA mutant bacteroids and 2000 in wild-type bacteroids, with molecular weights ranging from 7 to 317 kDa. A total of 60 differentially expressed proteins (P < 0.05) were identified. Among these proteins (Table 5), 33 proteins were up-regulated in gmcA mutant nodule bacteroids and 27 proteins were down-regulated. Cell surface protein (RL4381) was absent in the gmcA mutant bacteroids, while invasion associated protein (RL1020) and lipase-protein ligase B (RL2555) were not found in the wild-type bacteroids. Thirty-two differential protein-encoding genes were localized in plasmids pRL7, pRL8, pRL9, pRL10, pRL11, and pRL12. Cellular localization of the differentially expressed proteins showed that thirty-nine proteins localized to the cytoplasm, thirteen proteins localized to periplasmic

![Figure 1](image1.png)

**Figure 1** | Competition of the wild type (RL3841) (black bars) and the gmcA mutant (RLgmcA) (gray bars) in sterile rhizospheres. Inoculation ratios are given on the x-axis, with 1 corresponding to 1,000 CFU. Number of bacteria (per plant) recovered from 10 plants (mean ± SEM) from ten independent plants.

![Figure 3](image3.png)

**Figure 3** | Relative expression of gene gmcA in the RL3841 cells and mutant (RLgmcA) under oxidative stress conditions (5.0 mmol/L H2O2). Statistical analysis and analysis of variance (ANOVA) were performed to identify differentially expressed genes. * indicates P < 0.05.

**Table 4** | Symbiotic behavior of R. leguminosarum gmcA mutant.

| Strain            | Nodules per plant | Acetylene reduction (µ moles acetylene per plant per h) | Dry weight per plant (g) |
|-------------------|-------------------|--------------------------------------------------------|--------------------------|
| RL3841            | 137.3 ± 13.2a     | 2.24 ± 0.14a                                            | 1.86 ± 0.20a             |
| RLgmcA            | 131.3 ± 11.3a     | 1.56 ± 0.05a                                            | 1.10 ± 0.18a             |
| RLgmcA(pBBRgmcA)  | 135.5 ± 11.3a     | 2.12 ± 0.16a                                            | 1.80 ± 0.16a             |
| WC                | 0                 | 0                                                      | 0.35 ± 0.09a             |

All data are averages (±SEM) from ten independent plants. a,b,c Different letters indicates the value is significantly different from that of the wild-type RL3841 control (one-way ANOVA, P < 0.05). WC, water control without inoculation.
space, five proteins located in the outer membrane, two were extracellular proteins, and one protein existed in the inner membrane (Table 5).

By sorting the identified proteins according to metabolic function, most of the differences in expression were found among transporter activity (15 proteins), followed by 12 proteins related to stress response and virulence, 9 proteins related to transcription factor activity, 7 proteins related to transcription factor activity, 6 proteins related to amino acid metabolism, 6 proteins related to carbohydrate metabolism, and 4 proteins related to nucleotide metabolism. This change in metabolism was mirrored by corresponding changes in proteins involved in the regulation of transcription, among which, a nitrogen-specific transcriptional activator NifA and a nitrogen regulatory protein PtsN were highly expressed in the mutant bacteroids. The main groups of differentially expressed proteins identified were transport proteins, of which 6 were ABC-type nitrate/nitrite transporters. The result showed gmcA mutant was affected in transport, especially in nitrate transport. Further analysis of the differentially expressed proteins identified a subset involved in stress response and virulence. The number of affected oxidoreductases, cytochrome oxidase, dehydrogenase, hydrolase, dehydrogenase, surface, and invasion associated proteins also...


### TABLE 5 | Differential expression proteins in 4-week nodule mutant bacteroids relative to wild-type bacteroids.

| Gene ID | Gene Name | Cellular localization | Protein description | MW [kDa] | pI | Ratio | P-value |
|--------|-----------|-----------------------|---------------------|----------|----|-------|---------|
| **Stress response and virulence** | | | | | | | |
| RL3853 | Cytoplasmic | FAD-dependent oxidoreductase | 47.38 | 5.71 | 6.15 | 0.0004 |
| RL4381 | Cytoplasmic | cell surface protein | 66.06 | 4.46 | 1.58 | NP1 |
| pRL90097 | Cytoplasmic | 4-hydroxytheonine-4-phosphate dehydrogenase | 34.66 | 6.39 | 1.23 | 0.0332 |
| pRL100245 | Cytoplasmic | LLM class flavin-dependent oxidoreductase | 38.97 | 5.20 | 1.22 | 0.0082 |
| pRL80022 | Cytoplasmic | alpha/beta hydrolase | 35.12 | 5.56 | -0.48 | 0.0011 |
| pRL80023 | Cytoplasmic | carbon monoxide dehydrogenase subunit M protein | 30.42 | 5.58 | -0.48 | 0.0008 |
| pRL80041 | Cytoplasmic | Histidinol dehydrogenase | 47.16 | 5.39 | -0.60 | 0.0115 |
| RL1020 | Cytoplasmic | invasion associated protein | 22.08 | 5.45 | -0.85 | NP2 |
| pRL120603 | Cytoplasmic | NAD-dependent succinate-semialdehyde dehydrogenase | 52.53 | 5.29 | -0.74 | 0.0344 |
| pRL90018 | Cytoplasmic | inner membrane Putative cytochrome oxidase transmembrane component FixN | 60.90 | 8.98 | -0.82 | 0.0074 |
| **Amino acid metabolism** | | | | | | | |
| pRL100242 | Cytoplasmic | amino acid synthesis family protein | 21.17 | 6.29 | 1.45 | 0.0159 |
| RL0044 | Cytoplasmic | glutamine amidotransferase | 31.99 | 5.21 | 1.42 | 0.0082 |
| RL0004 | Cytoplasmic | Phosphoribosyl-ATP pyrophosphatase | 11.51 | 5.19 | 1.30 | 0.0049 |
| pRL100099 | Cytoplasmic | Nif11 family protein | 14.45 | 8.86 | 1.23 | 0.0152 |
| RL2075 | Cytoplasmic | Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C | 10.19 | 4.73 | 1.22 | 0.0082 |
| pRL100192 | Cytoplasmic | glutamate N-acetyltransferase | 20.33 | 6.71 | 1.21 | 0.0024 |
| pRL90027 | Cytoplasmic | Putative glutamine amidotransferase protein | 28.31 | 5.18 | -0.72 | 0.0044 |
| **Carbohydrate metabolism** | | | | | | | |
| pRL110453 | Cytoplasmic | Concanavalin A-like lectin/glucanase domain | 22.05 | 5.00 | 1.25 | 0.0237 |
| RL0916 | Cytoplasmic | 2-dehydro-3-deoxygalactonokinase | 31.49 | 5.69 | -0.73 | 0.0008 |
| RL2475 | Cytoplasmic | L-fuconate dehydratase | 47.20 | 5.17 | -0.82 | 0.0137 |
| pRL110598 | Cytoplasmic | co-chaperone GroES | 26.70 | 5.22 | -0.99 | NP2 |
| RL2555 | Cytoplasmic | lipote-protein ligase B | 26.70 | 5.22 | -0.99 | NP2 |
| **Transporter activity** | | | | | | | |
| RL4326 | Periplasmic | Putative transmembrane protein | 98.15 | 5.59 | 1.39 | 0.0122 |
| RL3066 | Periplasmic | Putative transmembrane protein | 17.22 | 8.09 | 1.35 | 0.0090 |
| RL2491 | Periplasmic | Conserved hypothetical exported protein | 9.75 | 5.38 | 1.28 | 0.0001 |
| pRL103386 | Periplasmic | VWA domain-containing protein | 74.90 | 4.84 | 1.24 | 0.0122 |
| RL3065 | Periplasmic | Conserved hypothetical exported protein | 14.77 | 5.88 | 1.23 | 0.0151 |
| pRL70182 | Periplasmic | Conserved hypothetical exported protein | 36.84 | 5.32 | 1.21 | 0.0265 |
| pRL80026 | Periplasmic | ABC transporter substrate-binding protein | 45.36 | 5.81 | -0.33 | 0.0006 |
| pRL80060 | Periplasmic | ABC transporter substrate-binding protein | 29.75 | 5.35 | -0.53 | 0.0007 |
| pRL80085 | Cytoplasmic | Autoinducer 2 ABC transporter substrate-binding protein | 35.70 | 6.02 | -0.60 | 0.0448 |
| RL2775 | Periplasmic | ABC transporter substrate-binding protein | 36.80 | 3.92 | -0.71 | 0.0111 |
| RL4402 | Periplasmic | ABC transporter substrate-binding protein | 35.94 | 4.96 | -0.72 | 0.0115 |
| RL1499 | Periplasmic | ABC transporter substrate-binding protein | 36.71 | 4.01 | -0.77 | 0.0001 |
| pRL100415 | Periplasmic | ABC transporter substrate-binding protein | 37.92 | 5.17 | -0.79 | 0.0492 |
| pRL120671 | Periplasmic | nitrate ABC transporter substrate-binding protein | 36.00 | 5.50 | -0.83 | 0.0123 |
| pRL100325 | Outer membrane | outer membrane siderophore receptor | 78.19 | 4.64 | -0.83 | 0.0203 |
| **Nucleotide metabolism** | | | | | | | |
| RL0952 | Cytoplasmic | RNA-binding domain transcriptional regulator | 83.38 | 6.67 | 1.34 | 0.0278 |
| RL2475 | Cytoplasmic | Putative DNA polymerase III, delta subunit | 36.35 | 5.90 | 1.26 | 0.0409 |
| RL1785 | Cytoplasmic | 50S ribosomal protein L5 | 11.21 | 10.37 | 1.23 | 0.0038 |
| RL2183 | Cytoplasmic | nucleotidyltransferase | 33.59 | 8.80 | -0.82 | 0.017 |
| **Transcription factor activity** | | | | | | | |
| pRL100146 | Periplasmic | transcriptional regulator | 23.94 | 9.57 | 1.33 | 0.0243 |
| RL4412 | Cytoplasmic | prlA | 80.82 | 6.32 | 1.30 | 0.0382 |

(Continued)
suggests that GmcA function in antioxidant capacity in the root nodules and that the loss of these proteins could result in antioxidant defect. Finally, the loss of GmcA resulted in the differential expression of seven proteins with unknown function in the nodule bacteroids.

**DISCUSSION**

The family of GMC oxidoreductases includes glucose/alcohol oxidase and glucose/choline dehydrogenase. Members of this family catalyze a wide variety of redox reactions with respect to substrates and co-substrates (Sützl et al., 2018). An important issue is that *gmcA* expression is elevated in nitrogen-fixing bacteroids of the pea root nodules, but the function of GmcA in root nodule bacteria nitrogen fixing system is poorly understood. In this study, we took advantage of a *gmcA* mutant strain of *R. leguminosarum* to examine what role GmcA may play in symbiotic nitrogen fixation. Our data demonstrated that GmcA is required for the nodule senescence and cellular detoxification that is affected, regarding its nitrogen fixation capacity and oxidative stress response.

Mutation of *R. leguminosarum gmcA* did not affect the growth of free-living bacteria but led to decreased antioxidative capacity under the conditions of 5 and 10 mM hydrogen peroxide H$_2$O$_2$. The direct link between GmcA and H$_2$O$_2$-induced oxidative stress. It has been reported that GMC oxidoreductases are involved in extracellular iron transport protein HmuS are ubiquitous electron transfer proteins participating in the iron-sulfur cluster biosynthesis and a wide variety of redox reactions (Chao et al., 2005; Gu et al., 2008). In *Rhizobium*, the peroxidases and the catalases KatG (catalase HPI), KatE (catalase), and OhrB (organic hydroperoxide resistance) were known to participate in the antioxidant defense mechanism against H$_2$O$_2$-induced stress (Vargas Mdel et al., 2003), and the two electron transfer proteins FdxB and HmuS are also involved in a wide variety of redox reactions (Chao et al., 2005; Gu et al., 2008). This cell cytotoxicity was relieved by inducing transcription of antioxidant genes (Jung and Kim, 2003). Expression levels of *katG, fdxB*, and *hmuS* genes were significantly down-regulated in the *gmcA* mutant under H$_2$O$_2$-induced oxidative stress. It has been reported that decreased ferredoxin-NADP(H) oxidoreductase (FNR) results in a more oxidized glutathione pool, while increasing FNR content results in a more reduced glutathione pool (Goss et al., 2012). Glutathione reductase activity in mutant RLgmcA was not different from that wild-type strain, but the absence of GmcA was associated with a 96.5% decrease in cellular glutathione peroxidase activity. Cellular peroxide deficit damages cellular macromolecules by reactive oxygen species (ROS), and glutathione peroxidases are one of the important ROS scavengers in the cell (Islam et al., 2015). The decrease of glutathione peroxidase activity is related to an uncontrolled increase of ROS (Giergiel et al., 2012).

Pea plants inoculated with the *gmcA* mutant exhibited a large decrease in the nitrogen-fixing activity of root nodules (reduced by more than 30%), although, the protein expression of NifA and PtsN was higher in the mutant bacteroids compared to that of wild type bacteroids. Two genes, *nifD* and *fdxB*, involved in metabolism related to nitrogen fixation and bacteroid maturation in pea root nodules (Capela et al., 2006) also had a higher level of expression in the mutant bacteroids. It has been reported that GMC oxidoreductases are involved in extracellular hydrogen peroxide and iron homeostasis (Rohr et al., 2013). Iron is required for symbiotic nitrogen fixation as a key component

### Table 5

| Gene ID | Gene Name | Cellular localization | Protein description | MW [kDa] | pl | Ratio | P-value |
|---------|-----------|-----------------------|---------------------|---------|----|-------|---------|
| RL3457  | Extracellular | SH3-like domain, bacterial-type; uncharacterized protein | 21.84 | 4.62 | 1.25 | 0.0082 |
| RL4025  | ptaN | Cytoplasmic | PTS IIA-like nitrogen regulatory protein PtsN | 16.65 | 5.70 | 1.23 | 0.0007 |
| RL1379  | rosR | Cytoplasmic | MucR family transcriptional regulator | 15.62 | 6.96 | 1.22 | 0.0055 |
| RL0133  | Cytoplasmic | YbaB/EbfC family nucleoid-associated protein | 11.42 | 5.18 | 1.22 | 0.0007 |
| pRL100196 | nIA | Cytoplasmic | nif-specific transcriptional activator | 56.46 | 9.05 | 1.21 | 0.0011 |
| pRL80079 | Cytoplasmic | Sugar-binding transcriptional regulator | 35.34 | 5.71 | −0.38 | 0.0072 |
| pRL80046 | Cytoplasmic | TetR/AcrR family transcriptional regulator | 25.32 | 8.01 | −0.46 | 0.0112 |
| pRL800106 | Periplasmic | Uncharacterized protein | 28.65 | 6.19 | 1.59 | 0.0363 |
| RL1874  | Cytoplasmic | Uncharacterized protein | 13.23 | 4.66 | 1.35 | 0.0185 |
| RL3516  | Extracellular | DUF2076 domain-containing protein | 26.14 | 5.91 | 1.23 | 0.0005 |
| RL4728  | Extracellular | DUF1013 domain-containing protein | 27.48 | 4.26 | 1.24 | 0.0017 |
| RL1874  | Cytoplasmic | Uncharacterized protein | 13.23 | 4.66 | 1.35 | 0.0185 |
| pRL800106 | Cytoplasmic | Uncharacterized protein | 9.58 | 9.51 | −0.21 | 0.0189 |
| pRL80006 | Cytoplasmic | Uncharacterized protein | 59.58 | 5.52 | −0.47 | 0.0028 |

Protein expression was analyzed statistically using Student’s t-tests (P < 0.05). Np1, no protein in mutant; Np2, no protein detected in wild type.
of multiple ferroproteins involved in this important biological process (Takanashi et al., 2013). hmsU was chosen based on previous studies, which showed that it was involved in iron transport (Chao et al., 2005). hmsU expression level in the mutant bacteroids, demonstrating the involvement of GMC in the regulation of iron homeostasis. Proteomic analysis of the mutant nodule bacteroids indicated that most of the differentially expressed proteins were involved in transporter activity, metabolism, and stress responses. These transporters may aid in regulation of ion and membrane potential homeostasis through their transport of nitrate, which is known to regulate the symbiosis (Vincill et al., 2005). These results indicated that GmcA is involved in a variety of metabolic processes, as has been described in A. niger and E. coli (Etchebeste et al., 2012; Liu et al., 2013).

The electron microscope investigation revealed that gmcA mutant altered the ultrastructure of pea nodules. GmcA can likely play a role in nodule senescence, since senescent parameters such as increased activities of enzymes of amino acid metabolism, PHB production, and an increase in the number of disintegrated bacteroids occurred. In addition, glutathione peroxidase activity dramatically decreased, and amino acid metabolism reflecting arginase activity was increased. R. leguminosarum bv. viciea forms determinate nodules on pea and usually does not produce PHB granules during symbiosis. PHB granules occurred in undergoing senescence bacteroids, which indicated that the energy and carbon metabolism has shifted (Xie et al., 2011). The PHB and tricarboxylic acid (TCA) cycles both start with acetyl-CoA. Under aerobic conditions, the TCA cycle is responsible for the complete oxidation of acetyl-CoA and formation of intermediates required for ATP production, but under oxygen limitation condition, when there is an inhibition of the TCA cycle by NADH or NADPH, the bacteroids favor PHB synthesis. During PHB synthesis, there is apparently a concomitant reduction in protein synthesis, a process coupled to ATP formation and utilization (Tal et al., 1990). In the symbiosis of the GmcA-deficiency mutant RLgmcA, the low expression of the catalase-peroxidase gene (katG), alpha/beta hydrolase (pRL80022), carbon monoxide dehydrogenase (pRL80023), succinate-semialdehyde dehydrogenase (pRL120603), and alcohol dehydrogenase (pRL90027) inhibited NAD(P)H oxidase activity. To allow continued operation of the TCA cycle by NADH or NADPH, the bacteroids favor PHB synthesis.

The gmcA gene expression is significantly up-regulated during the whole nodulation process, and its highest expression level occurred at 42 days after inoculation. Moreover, the R. leguminosarum gmcA mutant was unable to compete efficiently in the rhizosphere with its wild-type parent, which shows that bacterial GmcA is important for adaptation to the microenvironment of the plant host. Overall, considering the poor nitrogen-fixing ability of its nodules, the mutant in gmcA gene had a profound influence on the whole nodulation process.

DATA AVAILABILITY STATEMENT
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017485.

AUTHOR CONTRIBUTIONS
GC conceived and designed the study. QZ, SL, and HW performed the experiments. GC, QZ, DH, and XL analyzed the results. GC and QZ wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00394/full#supplementary-material

FIGURE S1 | Analysis of the protein domains of GmcA in Rhizobium leguminosarum 3841. BetA, choline dehydrogenase or related flavoprotein; GMC_oxred_C, GMC oxidoreductase; GMC_mycofac_2, GMC family mycofactocin-associated oxidoreductase.

FIGURE S2 | Plant growth test of the symbiotic ability of R. leguminosarum. (A) Control plant root inoculated with the wild type RL3841. (B) Plant root inoculated with RLgmcA.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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