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A novel intergenic *dSH3-irr* insertion mutation in *Bradyrhizobium diazoefficiens* causes its symbiotic incompatibility with soybean

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Running title: Novel symbiotic mismatching bacteroid

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Abstract

Legumes and rhizobia collaborate to fix nitrogen in the host plant nodules, but some inadvertent mutation may generate unexpected symbiosis. We reported an altered host range symbiosis of *Bradyrhizobium diazoefficiens* USDA110 mutant dSH3_IP when associating with *Sophora flavescens*. Recently, we found SH3_IP cannot symbiose with original host soybean (*Glycine max*), and mutant influenced iron response regulator, which forms convergent gene pair with *dSH3* in strain USDA110. The reason of symbiotic mismatching between soybean and dSH3_IP was explored through dual RNA-seq transcriptome and symbiotic characterization. We discovered dSH3_IP bacteroid assimilated more nutrition, triggered stronger immune responses and perturbed the symbiotic components in soybean nodules. Anyway, the incompatible symbiosis between dSH3_IP and soybean originated from intergenic insertion of *dSH3-irr*, which importance was not uncovered previously and may be used as a potential regulatory element in the future to regulate the SNF efficiency by artificial genetic design.

Key words:

Dual RNA-seq transcriptome, dSH3, Irr, soybean, *Bradyrhizobium diazoefficiens*, Symbiosis

Introduction

Biological nitrogen fixation (BNF) is a complex process not only by genomic structure¹ and gene expression², but also by environmental factors, especially by the ammonia³ or nitrate contents⁴. Symbiotic nitrogen fixation (SNF) is more intricate than BNF since process of nodulation is essential first, and coadaptation between host and endosymbionts must be harmonious when they get together. SNF needs complicated molecular communications between rhizobia and legumes⁵, the infection and immunity inhibition^{6,7}, the differentiation and metabolic alternation from saprophytes or free-living rhizobia to nitrogen-fixing bacteroids⁸⁻¹⁰, the control of oxygen pressure by leghemoglobin (LHb)¹¹, the availability and homeostasis of metal ions¹², and the inhibition from high level of nitrate^{13,14}.

Dual RNA-seq determination captures the RNA transcripts from the intracellular pathogens or symbiotic bacteria and host cells simultaneously¹⁵, and it can provide insights into metabolic alternations and responses from both the included parties^{16,17}, and was ever applied to establish bacterial infection model^{15,18}. However, dual RNA-seq has not been used to explore the transcriptional variation of symbiotic nitrogen fixation between legumes and rhizobia. Currently, gene *dSH3* insertion mutant dSH3_IP of strain USDA110 was found to have an undesirable impact on SNF, causing non-N₂-fixing phenotype on legume. Dual RNA-seq and symbiotic characterization were cooperated to explain the cause between legume and dSH3_IP mutant. We also firstly demonstrated the importance of iron response regulator (Irr), which was most studied in free-living cell in responding to iron homeostasis^{19,20}, and we discovered its functions on determining the SNF efficiency partly. Further, *dSH3*

and *irr* are a pair of convergent genes, and the interaction of them especially in symbiosis has not been covered. Our results showed that the importance of symbiotic match genetically between legume and rhizobia as evidenced in previous study²¹, and will highlight the essential of the mutated gene in symbiosis in regulating the SNF efficiency.

Results

Rhizobial mutant used in this study and its lower symbiosis efficiency

Bradyrhizobium diazoefficiens USDA110 Tn5-18 (BDT18) was a mutant isolated from the strain USDA110 Tn5 transposon insertion library²². The Tn5 insertion broke the terminator of *blr0767* gene and increased the length of non-coding region between *blr0767* and *bll0768* (Fig. 1A). Besides, *blr0767* and *bll0768* are a pair of convergent genes and their terminators are linked side by side (Fig. 1A). *blr0767* gene encodes a protein containing double SH3 domains, so it is assigned as *dSH3* hereinafter. Deletion of *blr0767* (*dSH3*) gene failed, meaning the essentiality of this gene to the cell viability of the strain *B. diazoefficiens* USDA110. Plasmid pVO155 derived from pUC119 was inserted at the end of *dSH3* gene²². Actually, insertion with plasmid pVO155 in *dSH3* (*dSH3_IP*) changed the last amino acid from N (Asn) to L (Leu) and 14 extraneous amino acids (EDLDHGPSCRNPNP*) were added as a hydrophilic tail (Fig. 1A). According to the structure prediction by *trRosetta* server at YangLab²³, *dSH3_IP* retained the complete double SH3 domains compared to *dSH3* in wild type (Fig. 1B and 1C). Distances from *dSH3* to terminator and to *bll0768* are lengthened in both insertion mutants (Fig. 1A). *bll0768* is on the opposite of *blr0767* and it encodes an iron response regulator (*Irr*), a global transcriptional repressor for iron homeostasis^{19,20}.

Comparing with wild type strain induced nodule section, both of mutants *dSH3_IP* and BDT18 induced small and white root nodules on soybean (Fig. 1D). The nitrogen-fixing efficiency represented by ARA (acetylene reduction assay) of the

root nodules induced by two mutants were much lower than those of the nodules induced by the wild type strain USDA110 (Fig. 1E). While nodule numbers induced by the two mutants increased greatly compared with that induced by the wild type strain (Fig. 1F).

To avoid uncertain effect of transposon Tn5, dSH3_IP was selected for research. And we will focus on the dual RNA-seq transcriptome and symbiotic characterization of the soybean root nodules and the bacteroids inside the nodules induced by dSH3_IP mutant and wild type strain USDA110, respectively.

General description of the transcriptional changes revealed by dual RNA-Seq

The expression profiles from dual RNA-Seq transcriptomes were presented as heatmaps (Fig. 2A for the soybean nodules, and Fig. 2C for the bacteroids, with three repeats), which showed 8355 differential expressed genes (DEGs) in soybean nodules and 862 DEGs in USDA110 bacteroids ($|\log_2(FC)| > 1$, $FDR < 0.05$). Some DEGs from soybean nodules and bacteroids were picked up for qRT-PCR to verify the dual RNA-Seq data, and the results showed that they are excellent consistent respectively (Fig. 2B and D).

Gene Ontology (GO) enrichment analysis in dSH3_IP bacteroids revealed that there were many down-regulated genes compared with the wild type bacteroids in anaerobic respiration, fermentation, electron transport, redox and symbiosis in biological process (BP), while amounts of up-regulated genes were enriched in protein folding (Fig. S1). Comparing with the wild type strain induced nodule, most genes related to the nutrient transport, including carboxylic acids, amino acids, ions and organic acids, and related to responding to acid, stimulus, chemical, reactive oxygen species (ROS) and compounds were up-regulated in dSH3_IP induced nodules (Suppl. Fig. S2).

Metabolic changes in free-living bacteria and symbiotic bacteroids

DEG analysis of free-living dSH3_IP mutant vs. USDA110 wild type showed that 1437 genes were up-regulated and 314 genes were down-regulated ($|\log_2(\text{FC})| > 1$, $\text{FDR} < 0.05$, Fig. 3A). Verification of transcriptome by qRT-PCR was shown in Fig. 3B. Furthermore, qRT-PCR of BDT18 mutant showed the same gene expression trend as qRT-PCR and transcriptome of dSH3_IP (Fig. 3B). GO enrichment revealed that the DEGs enriched in oxidation–reduction process, oxidoreductase activity, chaperone complex, chaperonin ATPase complex, GroEL–GroES complex, and chaperone–mediated protein folding, metal ion transport, cation transport and ion transport, which were consistent with those enriched in bacteroids (Fig. 3C and Supp. Fig. S1).

Then, we analyzed DEGs between symbiosis and free living in USDA110 and dSH3_IP respectively. Apart from common genes, up-regulated genes are 2387 in USDA110 and 364 in dSH3_IP when they turn to symbiosis (Fig. 3Da), while down-regulated genes are 441 and 4552 respectively (Fig. 3Db).

KEGG pathway showed metabolic pathways and biosynthesis of secondary metabolites downregulated in root nodule induced by the dSH3_IP mutant in contrast to that induced by wild type (Fig. S3A). However, the most significantly downregulated genes in bacteroids of dSH3_IP compared with the wild type were involved in flagellar assembly, quorum sensing, nitrotoluene degradation and nitrogen metabolism. In addition, there were 12 pathways about different compound biosynthesis were downregulated among top 30 (Suppl. Fig. S3B).

Combat between dSH3_IP mutant and host lower the efficiency of root nodule

KEGG pathway enrichment analysis revealed plant–pathogen interaction, Toll–like receptor signaling pathway, Toll and Imd signaling pathway and MAPK signaling pathway were upregulated in root nodule induced by the dSH3_IP mutant in

contrast to that induced by the wild type strain (Fig. 4A). Specifically speaking, genes involved in immune responses, including *BAK1* (BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1), *RBOH*, *CDPK*, *MAPKKK* in PTI and *GmNNL1*, *GmRj1*, *GmRj2* in ETI, were upregulated in root nodule (Fig. S4 A and B). And DEGs about plant stress response transcriptional factors revealed majority of them were upregulated, like WRKY, MYB and BZIP (Suppl. Fig. S4 A and B). Moreover, gene expression level responsible for ethylene response factor (AP2/ERF) also showed a substantial increase (Fig. S5). All of them showed that the host plant had more intense immune responses to bacteroids inside the root nodule induced by dSH3_IP mutant than that of the nodule induced by the wild type strain.

Correspondingly, T3SS (Type 3 secretory system) and T4SS (Type 4 secretory system) secretion systems, which could secrete Nop proteins to nodule to weaken the resistance of leguminous immunity, were upregulated in dSH3_IP bacteroids. The expression of toxin-antitoxin (TA) systems in dSH3_IP bacteroids that influences the persisted cell formation and the general stress response differed from their original expression model (Suppl. Fig. S4C). KEGG pathways of mutant bacteroids were also found to be enriched in plant–pathogen interaction, bacterial secretion system, apoptosis and microbial metabolism in diverse environments.

The numbers of infected cells in the dSH3_IP mutant induced nodule were less than the numbers of that in nodule induced by the wild type strain (Fig. 4C, 4D). In summary, the combat between soybean and dSH3_IP mutant escalated significantly in the root nodule.

Disorder of metabolism in dSH3_IP mutant bacteroids and soybean symbiosis pathway

The genes expression of transporters and regulators participating in assimilation of magnesium, sulfate and potassium increased but decreased in molybdenum (Mo), and expression pattern of genes participating in phosphorus and iron transports

showed completely different between the dSH3_IP mutant bacteroids and wild type bacteroids (Fig. 5A). The genes of *pstSCAB* and *phnEEDC* were down-regulated while *pit-orf* genes were up-regulated in phosphorus transport, and *feoAB* was upregulated while heme uptake-related genes were downregulated in iron transport (Fig. 5A). Nodules induced by dSH3_IP mutant contained more nutritive elements, especially elements of manganese (Mn), nickel (Ni), calcium (Ca), potassium (K) and iron (Fe), but were opposite for molybdenum (Mo) and magnesium (Mg) elements (Fig. 5B).

The dSH3_IP bacteroids displayed the increased accumulation of heterochromatin (red arrows) but the decreased content of poly- β -hydroxybutyrate (PHB) (yellow arrows) (Fig. 5Ca) compared with that in the wild type bacteroids (Fig. 5Cb). Genes involved in amino acids and C₄-dicarboxylate transporter (*dctA*) decreased, but genes of malonate transporter increased in dSH3_IP bacteroids (Fig. 2D). And the genes encoding pyruvate dehydrogenase (acetyl-transferring) E1 component subunit alpha (PdhA) and pyruvate dehydrogenase (PdhB) were upregulated in dSH3_IP bacteroids (Suppl. Fig. S8D).

The *fixL-fixJ-fixK2* genes were expressed higher in dSH3_IP bacteroids, but the expression of *regS-regR-nifA* genes were expressed lower. And all of them were involved in regulation at low oxygen concentration (Fig. 5D). B-O₂ redox was downregulated in dSH3_IP bacteroids, while C-O₂ redox (*fixNOQP* and *fixGHIS*) and A-O₂ redox were up-regulated (Fig. 5D). These results showed that the great changes of oxygen concentration in dSH3_IP bacteroids.

In nodulation signal pathway of the host nodule infected by dSH3_IP mutant, *CNGC15*, *NUP133*, *DMI3*, *NSP1* and *NSP2* were upregulated, however, *CYCLOPS* was not differentially expressed and *DMI1* was downregulated (Fig. 5E). In addition, *NLP4* (GLYMA_04G017400 and GLYMA_06G017800) were up-regulated, while *NIN* (GLYMA_04G000600 and GLYMA_06G000400), *NLP2* (GLYMA_11G125500 and GLYMA_12G050100), *GmRIC1*, *GmRIC2* and *GmNNC* were downregulated (Fig. 5E). Early nodulin (ENOD) were upregulated but *ENOD40* and late nodulin

including *LBA*, *LBC1*, *LBC2* and *LBC3* were downregulated in the nodule induced by dSH3_IP mutant (Fig. 5F).

Symbiosis efficiency influenced by convergent gene pair of *blr0767* and *bll0768*

It was reported that convergent genes may exist collision of RNA polymerase when they share a common terminator and gene pair may have unknown function. Terminator 1 of *dSH3* and terminator 2 of *irr* are so close in strain USDA110, so we try to knockout the intergenic region (after TAG of *blr0767* and before TAC of *bll0768*) and *bll0768* (*irr*) to explore their function in symbiosis (Fig. 6A). The role of *irr* in symbiosis has not been mentioned in literature. The deletion of *irr* led to the reduction of nitrogen fixation efficiency represented by the decreased chlorophyll content and nitrogenase activity (Fig. 6B, 6C and 6D). However, the deletion of intergenic region (Δ int.reg.) had no significant effects on symbiosis (Fig. 6B, 6C, 6D).

Based on our results, two models representing the transcriptional and symbiotic changes in soybean nodule cell (Fig. 7A) and dSH3_IP mutant bacteroid (Fig. 7B) were proposed. The changes of symbiotic pathway, immune response, biosynthesis of leghemoglobin and key transcriptional factors in soybean inoculated by dSH3_IP mutant were emphasized in Fig. 7A. Correspondingly, the changes involved in the transporters, nitrogen fixation pathway and regulation, variation of Irr, heme, Fe^{2+} and PO_4^{3+} , uncoupling of carbon and nitrogen due to crucial enzymes linked the TCA and GAC cycles, key secretion systems, regulation of oxygen level were highlighted in Fig. 7B.

Discussion

General discussion about the dSH3_IP mutant and SH3 domain-containing proteins/genes

Wild type strain USDA110 of *B. diazoefficiens* is one of highly efficient N₂-fixing bacteria when symbiosis with soybean²⁴. This strain USDA110 is widely used not only in scientific researches as a model bacterium²⁴, but also in agriculture as a commercial inoculant in some countries²⁵. Although many genes required for symbiosis have been identified in different rhizobial strain, still the functions of many others in different rhizobia, especially in specific rhizobial strain, have not yet been explored²². The function of *dSH3* gene (*blr0767*) was firstly described during the screening of the Tn5 transposon insertion library and the insertion mutant strain (BDT18) extended its host range from soybean to *S. flavescentis*²². Another insertion mutant dSH3_IP leads to the similar nodulation and non-N₂-fixing phenotype in both original host soybean and the extended host *S. flavescentis*. Nitrogen fixation of both mutants (pVO155/dSH3_IP and BDT18) on soybean could not be detected. Failure of deletion of *dSH3* gene further confirmed the indispensability of this gene in symbiosis, especially in the influence of SNF efficiency. Maybe it is the distinct structure of SH3 domain as a molecular ‘velcro’²⁶ specially mediating the protein-protein interaction (PPI), making it a crucial role in various metabolism and physiology.

Zhang *et al.* ever detected a protein in strain USDA110, which was one of the candidate proteins participating in the symbiotic interactome associating with soybean²⁷. This protein numbered Q89WC4 is dSH3 protein in strain USDA110. That is to say, dSH3 protein may be important in the molecular interaction between strain USDA110 and legume including soybean and *S. flavescentis*, though the function has not been uncovered. Another protein homologous to dSH3 was found to participate in the morphological differentiation and its expression was strictly regulated by cell cycle transcriptional regulator CtrA in *Sinorhizobium meliloti* (Smc02848)²⁸. Vriezen

et al. reported a protein containing one SH3 domain (numbered Smc00195) functions in resisting the heat and desiccation stress in *S. meliloti*. They speculated this protein (Smc00195) might be involved in signal transduction directly or indirectly and involved in sensing and maintaining membrane stability²⁹. A conserved DipI protein (CC3721), homologue to dSH3 protein in strain USDA110, is essential for the cell constriction in *Caulobacter crescentus*³⁰. The cells of *C. crescentus* devoid of DipI cannot constrict and cause cell filamentation³⁰. In addition, DipI interacts with the FtsQLB protein complex to involve in the activity of bacterial divisome³⁰. Based on these previous and current studies, we infer that the SH3 or dSH3 domain-containing proteins may participate in various physiological process and they are essential either in free-living status or in symbiotic bacteroid in root nodule. Even the destruction of dSH3 terminator in strain USDA110 leads to significant reduction of SNF in soybean root nodules.

Symbiotic incompatibility between dSH3_IP mutant bacteroid and soybean

Nodule symbiosis is a metabolic trading of reduced carbon (C₄-dicarboxylate) from legumes for reduced nitrogen (ammonia) from rhizobial bacteroids to survive the legume hosts in nitrogen-limited environment³¹. The transition to SNF is a complicated process that requires the participation of many components from two partners⁵.

Iron (Fe) is a main metal component for ferroprotein, Mo-Fe nitrogenase, iron-molybdenum cofactor (FeMo-co), Bfd (bacterioferritin-associated ferredoxin) and many other Fe-dependent enzymes. Therefore, genes involved in Fe homeostasis are tightly regulated. The Irr was ever identified as a transcriptional repressor of gene *hemB* in *B. diazoefficiens*, which encodes δ -aminolaevulinic acid dehydratase, an enzyme in the heme biosynthetic pathway³². Upregulated expression of *irr* gene in dSH3_IP mutant bacteroid suggest limited iron content in it induced root nodule³³.

But nodule cytosol was not deficient in iron³⁴. So, contradiction existed in iron content. FeoABI is a ferrous iron transporter system regulated by Fur (ferric uptake regulator) protein and up-regulated under iron-deficient environment³⁵. Similarly, *feoAB* may be regulated by Irr in strain USDA110. However, *feoAB* was upregulated in dSH3_IP bacteroids and heme synthesis genes *hemABN* (repressed by Irr³⁶) were also up-regulated, which means iron is scarce. But ferric siderophore receptor genes (activated by Irr³⁷) were downregulated in the dSH3_IP mutant bacteroids and the expression of genes *bfr-BFD* (repressed by Irr) increased, which shows iron overload. Therefore, we speculated the disequilibrium of iron homeostasis in dSH3_IP induced nodules, which is a key factor for the failure of SNF.

As for wild type bacteroids of USDA110, the cascade genes for FixL-FixJ-FixK2-NnrR/RpoN1/FixK1 regulation system are activated when oxygen concentration is less than 5%³⁸⁻⁴², the expression of *fix/nif* genes controlled by RegS-RegR-NifA systems are significantly induced when oxygen level is lower than 0.5%⁴²⁻⁴⁵. Both of cascades are required for micro-oxygen adaptation of bacteroids in nodules, but the threshold of the oxygen level for effective SNF must be lower than 0.5%. The expression of *nifA* gene decreased significantly in the dSH3_IP mutant bacteroids leading to the few expressions of N₂-fixing genes directly. These changes meant that the oxygen concentration for nitrogenase activity in wild type bacteroids was rigidly controlled, but it was out of control in the mutant bacteroids.

For another, heme synthesis genes were also regulated by oxygen and FixK2⁴⁶⁻⁴⁸. Cascade *regS-regR-nifA* downregulated and *fixL-fixJ-fixK2* upregulated revealed concentration of oxygen is higher than 0.5%, and not reach to the level that protects the nitrogenase activity. And, the variation of A-O₂ redox, B-O₂ redox and C-O₂ redox (*fixNOQP* and *fixGHIS* genes) in nodule induced by dSH3_IP mutant suggested oxygen level is different from that in nodule induced by wild type. Besides, degradation of Irr protein needed oxygen, ferrochelatase, and heme^{49,50}. When oxygen decreased to a certain level, Irr protein would keep its activity for a longer time and

the FixK2 protein would decrease, and this will promote the Irr protein to suppress *hemABN* expression and the *fixK2* gene would be expressed weaker, causing reduced synthesis of heme in free-living bacteria finally. While the situation has been reversed in symbiotic bacteroids, there were more oxygen but expressions of heme synthesis related genes were higher. Thus, expressions of *irr* gene and its regulated genes were clearly aberrant. Comparatively, the wild type bacteroids might assimilate some hemes from root nodule of soybean.

The changes on oxidoreductase activity and electron transfer activity might be the consequence of unbalanced expression of iron- and heme-related metabolic genes because iron and heme are involved in various metabolites and enzyme activities as cofactors⁵¹. This may also result from that the dSH3_IP mutant bacteroids had changed greatly in its respiratory mode and direction of substance metabolism.

Phosphorous (P) and iron (Fe) are two essential nutrients for SNF, plant growth and development, but they have an antagonistic interaction⁵². Their contents in the nodules induced by dSH3_IP mutant were higher than that induced by the wild strain. We guess that there might be some regulators for the balance of P and Fe ratio in cells. Further, dSH3 was predicted to correlate with phosphorus metabolism. So, it was possible that the imbalance of P and Fe was due to the insertion in the dSH3_IP mutant. The dysfunction of Irr may be like polar effect on account of change dSH3 and their intergenic region, which may influence the efficiency of SNF. The imbalance of P and Fe would lead to the severe consequence of deficiency of the SNF. Therefore, it is critical to stabilize the P and Fe homeostasis in the root nodule for higher efficient SNF.

The organic α -ketoglutaric acid (α -KG) is an important intermediate connecting carbon and nitrogen metabolism⁵³, and higher C/N ratio (40:1-400:1) would promote the accumulation of α -KG in cells of legume *Medicago truncatula*⁵⁴. The expressions of α -ketoglutarate dehydrogenase complex (KGDHC) were down-regulated in tricarboxylic acid (TCA) cycle in dSH3_IP mutant bacteroids. The consequence of

down-regulated expressions of KGDHC is higher level of α -KG. In addition, the expression of gene for isocitrate lyase (*icl/aceA*), one of the most important enzymes involved in glyoxylate cycle (GAC), increased significantly and this may reduce over accumulation of α -KG. The changes of KGDHC and Icl/AceA suggested dSH3_IP mutant is avoided to produce excessive α -KG since no enough nitrogen for synthesis of amino acid.

We also discovered the higher pH level in cultures of dSH3_IP mutant than that the cultures of wild strain in free-living status (data not shown). And in dual RNA-seq, we found expressions of *nhaA* (Na^+/H^+ antiporter) genes were upregulated, while the expressions of genes related to cation/citrate: proton symporter and cation: proton antiporter were down-regulated. Changes of the above genes could affect intracellular and extracellular pH. Since peribacteroid space (PBS) acidification can be regarded as a marker of bacteroid nitrogen fixation⁵⁵, we inferred that dSH3_IP bacteroids changed the pH of PBS, disrupting the PBS acidification and failing to initiate nitrogen fixation.

The toxin-antitoxin (TA) systems were required to adapt to the environmental stresses rapidly⁵⁶, stabilize the integron⁵⁷, or form the persisted cell⁵⁸. Toxin gene BJA_RS08285 involved in TA system was significantly up-regulated in dSH3_IP mutant bacteroid in symbiosis. The change of the gene BJA_RS08285 may be related to the alternation of the environment from the inner and external of the bacteroid.

Immunity from legumes will be suppressed by rhizobia for establishment and maintenance of symbiosis. However, immune response, plant stress response transcriptional factors, ethylene response factor, the PTI and ETI were all upregulated in the aberrant nodules. To repress the immunity from the host, the mutant may secrete more Nops protein to the host plant through increased T3SS and T4SS⁵⁹⁻⁶³. In brief, the antagonistic relationship between dSH3_IP mutant and the host soybean increased and damage the effective SNF.

An appropriate low-oxygen environment was created inside the root nodule through leghemoglobin that is necessary for nitrogenase activity in the bacteroid. However, expressions of leghemoglobin genes including *LBA*, *LBC1*, *LBC2* and *LBC3* were all downregulated in the dSH3_IP mutant induced nodule, leading to improper oxygen concentration in the nodule and inactivation of nitrogenase in the bacteroids. Correspondingly, the expression of *regS-regR-nifA* genes down-regulated in the dSH3_IP bacteroid, so *nifA* may be not enough to activate the expression of nitrogenase gene. Besides, It was ever reported that the NLP2 (NIN-like protein 2) and NIN proteins can directly activate the expression of leghemoglobin gene¹¹ and the nodule inception (NIN) is proteolytic processed by a signal peptidase complex (SPC) to release a fragment that regulates transition to nitrogen fixation⁶⁴. RNA-seq showed that the expression of the homologous genes *NIN* (GLYMA_04G000600 and GLYMA_06G000400) and *NLP2* (GLYMA_11G125500 and GLYMA_12G050100) decreased significantly in the dSH3_IP mutant induced soybean root nodules, which made the SNF even worse.

NLPs were involved in the primary nitrate signaling pathway⁶⁵ and were essential for nodulin and AON^{11,64}. The expressions of *NLP2* and *NIN* genes were down-regulated in soybean nodules induced by the dSH3_IP mutant. All the *NLP6* (Glyma_09G137000, Glyma_10G234100 and Glyma_20G160200) and *NLP7* (Glyma_16G182400) genes from soybean are homologous to nitrate sensor *NLP7* from *Arabidopsis thaliana*⁶⁶, while the expression of them showed no difference in the nodules induced by the mutant and wild type strain. The expression of the *NLP4* genes (GLYMA_04G017400 and GLYMA_06G017800) were up-regulated in the mutant induced soybean nodules. *OsNLP4* in rice is homologous to GmNLP4 in soybean and OsNLP4 protein involve in the nitrogen uptake, assimilation and signal transduction in rice⁶⁷. Overexpression of *OsNLP4* gene significantly increased the nitrogen use efficiency and yield in rice⁶⁷. In order to alleviate pressure of low nitrogen, *GmNLP4* may be up-regulated in root nodules. Thus, it was essential to activate expression of *NLP2* and *NIN* genes to start nitrogen fixation inside the

nodules to obtain more nitrogen

When we focused on nodulation signal pathway, *DMI3/CCaMK*, which involved in decoding of symbiotic Ca^{2+} oscillations, and its downstream components *NSP1* and *NSP2* were upregulated in the nodule induced by dSH3_IP mutant. It seemed that the dSH3_IP mutant recruited more *DMI3*, *NSP1* and *NSP2* to participate in the nodulation pathway. However, *GmNIN* and *ENOD40* genes, regulated by *NSP1* and *NSP2*^{68,69}, were down-regulated significantly in the dSH3_IP mutant induced root nodule. *GmNIN* and *ENOD40* are critical in nodule development and transition to nitrogen fixation status. Besides, the *GmNIN* and *ENOD40* genes were regulated by cytokinin, but the expressions of the genes related to cytokinin synthesis were up-regulated. It implied that cytokinin may be not a hindrance and the problem might lie in the regulation of *GmNIN* and *ENOD40* by complex consisting of *NSP1*, *NSP2*, *DELLAs* and phosphorylated *IPD3*. Furthermore, the expressions of *GmRIC1*, *GmRIC2* and *GmNNC* genes were all down-regulated, which all function in AON (autoregulation of nodulation). Their reduced expression may produce more nodules in soybean infected by dSH3_IP. In brief, the nodulation signaling pathway was active but the SNF in the root nodule induced by dSH3_IP mutant could not be initiated.

In addition, Ca^{2+} oscillation is the upstream signal of the above enzyme complex (*NSP1*, *NSP2*, *DELLAs* and phosphorylated *IPD3*). Ca^{2+} oscillation is encoded by *DMI1* and *CNGC15*, and decoded by *LjCCaMK/MtDMI3-LjCYCLOPS/MtIPD3*⁷⁰⁻⁷². The abnormality of the Ca^{2+} oscillation could lead to the aberrant symbiosis⁷². *DMI1* and *CNGC15* displayed slight difference, while *DMI3* increased significantly. And *NUP133* also upregulated, which is required for Ca^{2+} oscillation. Therefore, we guess there was something wrong about Ca^{2+} oscillation and disorder of Ca^{2+} oscillation caused the interruption of symbiotic signal delivery between dSH3_IP mutant bacteroid and soybean root nodule. The importance of ion channel and signal transduction was ever certificated by the soybean-*B. diazoefficiens* interactome proteomics²⁷. The root nodules induced by dSH3_IP mutant contained more Ca^{2+}

content, and its efficient concentration might be altered due to change of other elements like phosphorus and the alteration of metabolism.

In conclusion, we first pay attention to the *dSH3* gene and its convergent gene *irr* (*bll0768*) in *B. diazoefficiens*. This gene pair may influence the efficiency of SNF between *B. diazoefficiens* and soybean. The changed expression of the ion transporter genes result in the severe consequence not only influencing the physiology of free-living bacteria, but also affecting the bacteroids in SNF, and the imbalance of ions in soybean root nodule. All the changes come from the insertion mutant of intergenic region between *dSH3* and *irr* convergent genes in *B. diazoefficiens* USDA110. The mechanism behind them deserves to survey and the region might be used to engineer a novel strain to improve the SNF.

Material and method

Bacterial strain, plasmids and plant

Soybean (*Glycine max*) variety C08 was used as host plant, and grown in greenhouse (24 °C with 12 h light) for 28 days after inoculating with the *B. diazoefficiens* USDA110 wild type or the mutant dSH3_IP. Strain or the mutant was cultured in 100 mL TY broth medium⁷³ at 28°C with rotation at 180 r/min to density of OD₆₀₀ 0.7-0.8. Cells were cleaned and resuspended with sterile saline solution if inoculating, or directly centrifuged with 4°C, 6,000 g for RNA isolation. Mutant was constructed with pVO155 based on conjunction and homologous recombination with detail in Liu *et al.*^{22,74}. Deletion mutants was also constructed by conjunction and homologous recombination. Acetylene reduction assay (ARA) was tested for nitrogenase activity by gas chromatography system (7820A, Agilent Technology) according to methods described previously⁷⁵.

RNA isolation, cDNA library and sequencing

Total RNA was extracted using TRIzol® Reagent according the manufacturer's instructions (Invitrogen) and genomic DNA was removed using DNase I (TaKara). RNA samples tested quality using 2100 Bioanalyser (Agilent) and were quantified using the ND-2000 (NanoDrop Technologies) ($OD_{260/280} = 1.8\sim 2.2$, $OD_{260/230} \geq 2.0$, $RIN \geq 6.5$, $28S:18S \geq 1.0$, total RNA $>10 \mu\text{g}$). Total RNA was prepared for RNA-seq strand-specific libraries following TruSeq RNA sample preparation Kit from Illumina (San Diego, CA), and took $5 \mu\text{g}$ of total RNA for following experiments. The rRNA of bacteroids was removed by RiboZero rRNA removal kit (Epicenter), fragmented using fragmentation buffer. Then cDNA synthesis, end repair, A-base addition and ligation of the Illumina-indexed adaptors were performed followed the basis of Illumina's protocol. Finally, a 200-300bp cDNA target fragment library was selected on 2% Low Range Ultra Agarose, and 15 PCR cycles were amplified by Phusion DNA polymerase (NEB). Paired-end libraries were sequenced by Illumina NovaSeq 6000 sequencing after quantified by TBS380 ($150 \text{ bp} \times 2$, Shanghai Biozeron).

RNA isolation, cDNA synthesis and RT-qPCR

Total RNA of root nodules and rhizobia was extracted separately using TRIzol reagent. cDNA was synthesized by FasKing RT Kit containing gDNase (TIANGEN, China). Some DEGs from bacteroids and soybean nodules were selected to verify the RNA-seq data by RT-qPCR. Primers were designed by online (<http://www.oligoarchitect.com/OligoArchitect/LoginServlet>) and listed in Suppl. Table S1. RT-qPCR used 2×RealStar Power SYBR qPCR Mix (Low ROX) (GenStar, China) and was performed using QuantStudio 6 Flex using $\Delta\Delta C_t$ method. Three technical replicates were included for three biology replicates, the constitutive 6 gene of soybean and 16S gene of strain USDA110 served to normalize the expression levels⁷⁶. Data represented the mean $\pm$ standard deviation (SD) of five replicates at least.

Differential expression analysis and Functional enrichment

To identify DEGs between the two different samples, the expression level for each transcript was calculated using the fragments per kilobase of read per million mapped reads (RPKM) method. The R package of *edgeR* was used for differential expression analysis. The DEGs between two samples were selected using the following criteria: i) the $|\log_2(\text{Fold Change})|$ was > 1 and the false discovery rate (FDR) should be < 0.05 . GO functional enrichment analysis was carried out by Goatools (<https://github.com/tanghaibao/Goatools>) to classify the functions of the DEG. DEGs were significantly enriched in GO terms and metabolic pathways if their Bonferroni-corrected P value was less than 0.05.

Determination of elements using ICP-MS

Root nodules were collected 28 days after inoculation with mutant and wild type rhizobial strain USDA110. Nodules were washed twice using TSE buffer (0.1mol/L Tris, 0.1 mol/L succinate, 10mM EDTA). Drying them completely and grind them into power with pestle on white paper. Total 0.25 g root nodules were firstly digested by using 6 ml nitric acid for 1 hour and then digested using 2 ml hydrogen peroxide for in CEM Mars digestion oven (MARS5). Finally, volume is fixed to 100 g and detected on the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (7700X, Agilent). Technical parameters: RF power: 1280 W, atomization chamber temperature: 2 °C, sampling depth: 8 mm, cooler flow rate: $1.47 \text{ L} \cdot \text{min}^{-1}$, carrier gas flow rate: $1 \text{ L} \cdot \text{min}^{-1}$, auxiliary flow rate: $1 \text{ L} \cdot \text{min}^{-1}$. Two repetitions were carried out for the determination of the element content using ICP-MS.

Data analysis

Protein structure prediction was performed online using *trRosetta*²³. Venn plots, bubble diagram and volcano plot were generated on the platform of Biozeron (<http://www.biozeron.com>). Heatmaps were plotted using R package *pheatmap*. Histograms were graphed by GraphPad Prism software (Ver. 8).

Data availability

The transcriptome data have been submitted to NCBI database with the accession numbers of (under certification).

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Legends

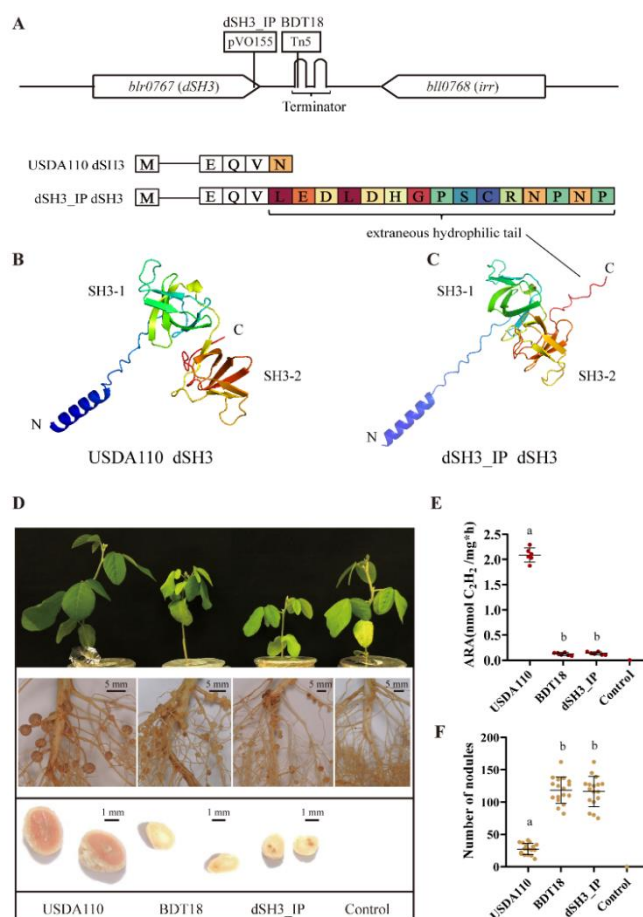
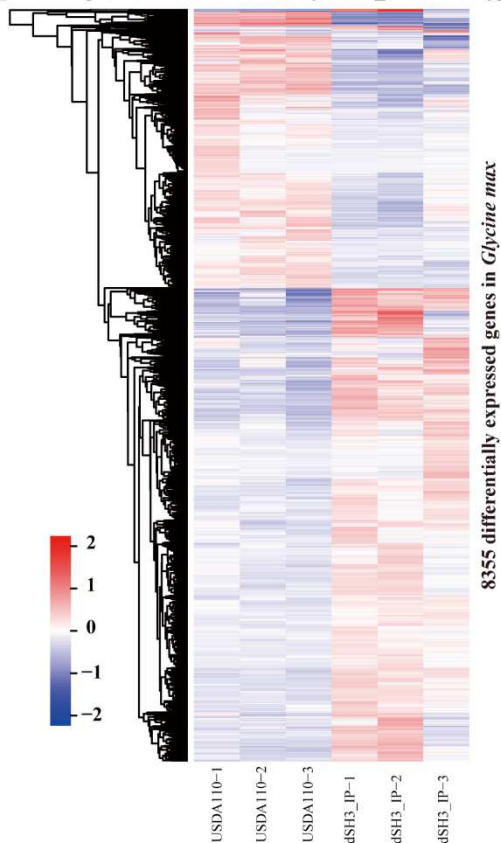


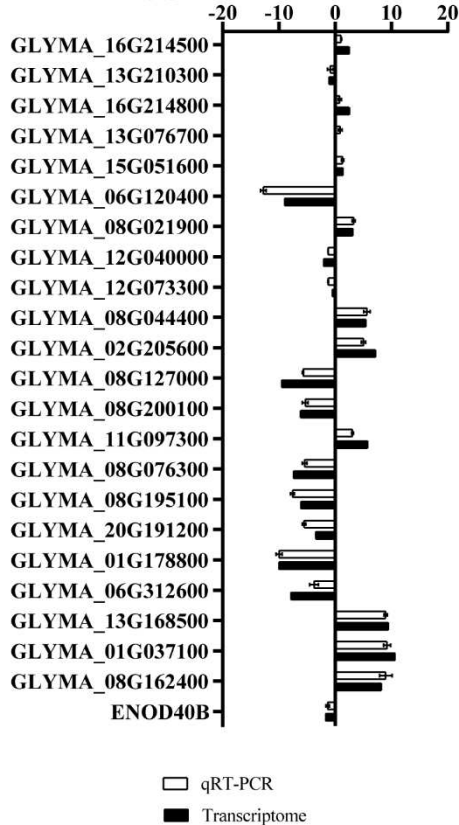
Fig. 1. dSH3_IP mutant and phenotype of symbiosis with soybean. (A) Genome structure changes of *dSH3_IP* mutant or BDT18 and amino acid sequence change of *dSH3* in *dSH3_IP* mutant. (B) 3D structure of *dSH3* in wild type. (C) 3D structure of *dSH3* in *dSH3_IP* mutant. (D, E and F) Phenotype of soybean 28 days post inoculation by wild type, BDT18 and *dSH3_IP* mutant. Data are mean $\pm$ SD (E: n=6, F: n=17, n represents the number of independent biological samples). Letters denote statistically significant groupings analyzed by Scheffe's method ($P < 0.01$).

Experiments were repeated three times.

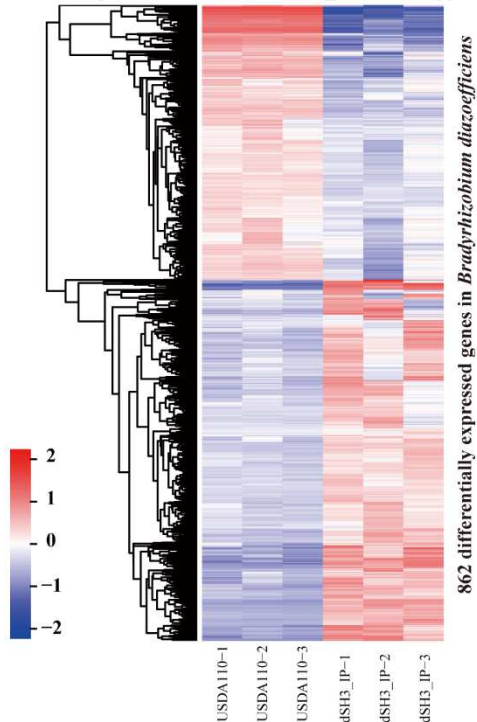
A Heatmap of root nodule induced by dSH3_IP vs. wild type



B Validation of root nodule RNA-Seq results by qRT-PCR



C Heatmap of bacteroids from dSH3_IP vs. wild type



D Validation of bacteroids' RNA-Seq results by qRT-PCR

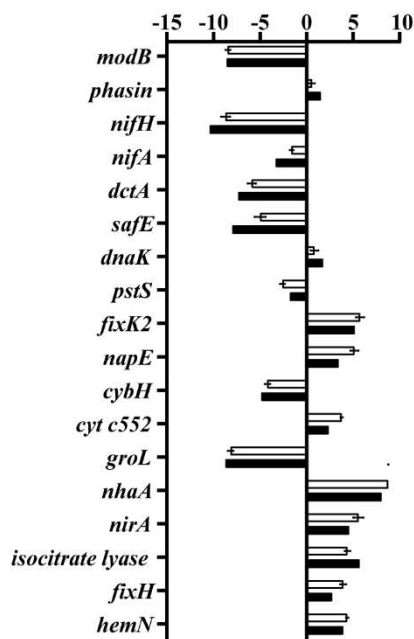


Fig. 2. Transcriptional profiles of *G. max* cv. C08 and *B. diazoefficiens* in root nodule. (A) Hierarchical clustering of differentially expressed genes ($|\log_2(\text{FC})| > 1$, $\text{FDR} < 0.05$) from three biological replicates in *G. max* cv. C08 transcriptomes. (B) Up-regulated and down-regulated genes in root nodule using RNA-Seq and qRT-PCR data. qRT-PCR data were represented as mean $\pm$ SD and repeated three times. (C) Hierarchical clustering of differentially expressed genes ($|\log_2(\text{FC})| > 1$, $\text{FDR} < 0.05$) from three biological replicates in *B. diazoefficiens* transcriptomes. (D) Up-regulated and down-regulated genes in root nodule bacteroids using RNA-Seq and qRT-PCR data. qRT-PCR data were represented as mean $\pm$ SD and repeated three times.

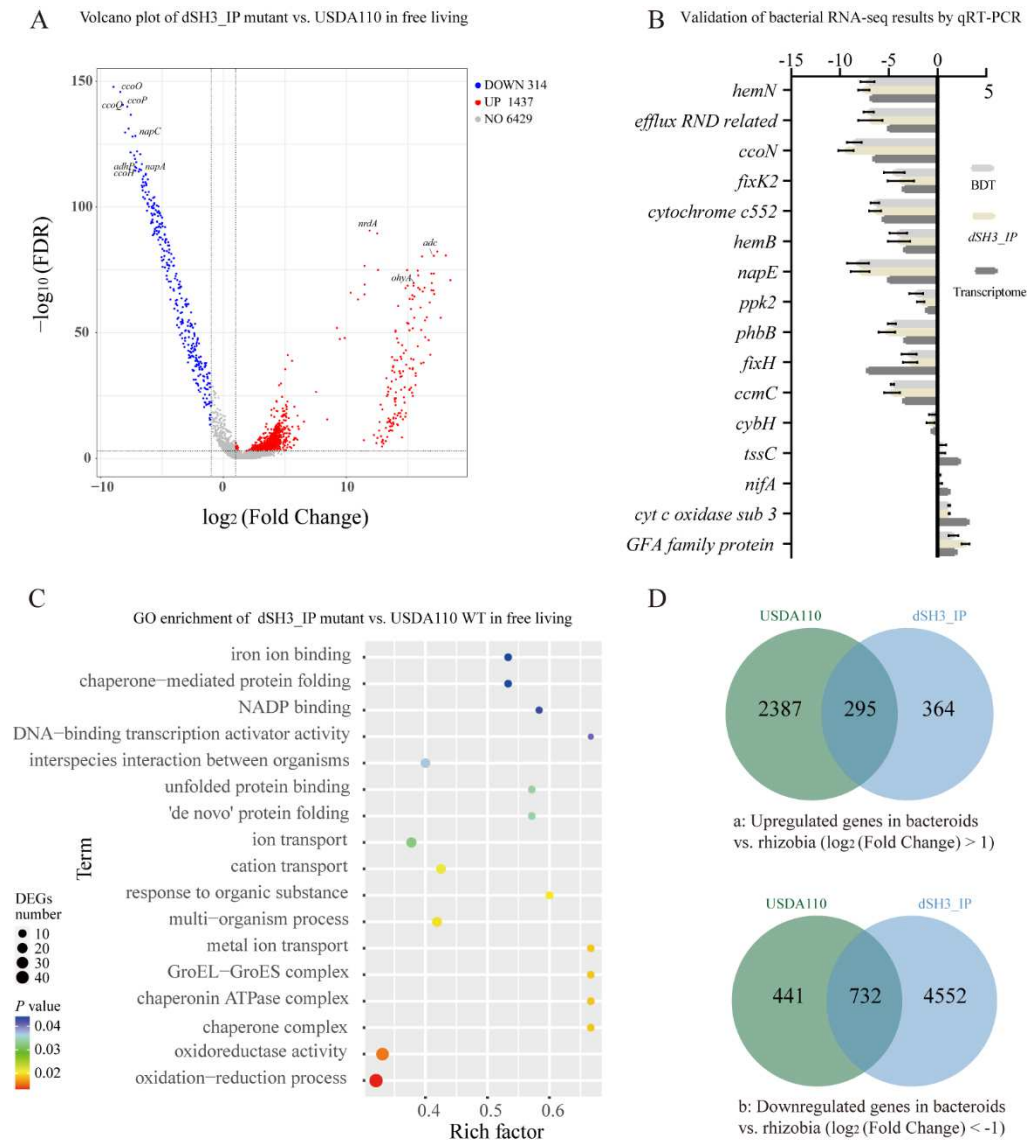


Fig. 3. Transcriptional profiles of *B. diazoefficiens* in free living and comparison with bacteroids in root nodules. (A) Volcano plots exhibit differentially expressed genes ($|\log_2(\text{FC})| > 1$, $\text{FDR} < 0.05$). Red and blue dots represent up-regulated and down-regulated genes respectively. (B) Up-regulated and down-regulated genes in free-living *B. diazoefficiens* using RNA-Seq and qRT-PCR data. qRT-PCR data were represented as mean $\pm$ SD and repeated three times. (C) Top seventeen GO terms for transcriptional profile of *B. diazoefficiens*. Size of circle represents number of enriched genes, colors represent significant. (D) a: Venn diagram illustrates up-regulated genes ($\log_2(\text{FC}) > 1$) in wild type and dSH3_IP mutant and their overlap using bacteroids vs. bacteria. b: Venn

diagram illustrates down-regulated genes ($\log_2(\text{FC}) < -1$) in wild type and *dSH3_IP* mutant and their overlap using bacteroids vs. bacteria.

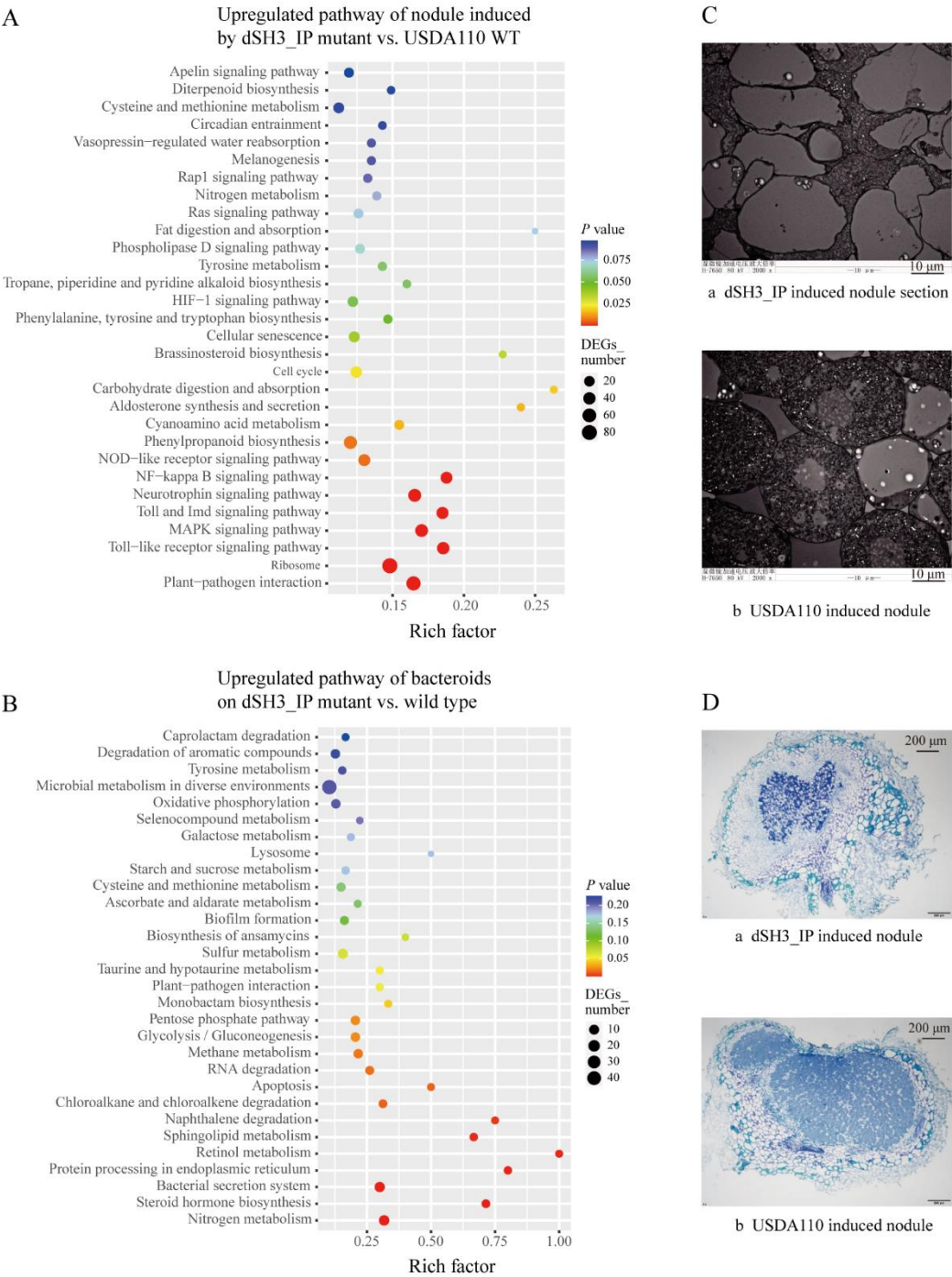
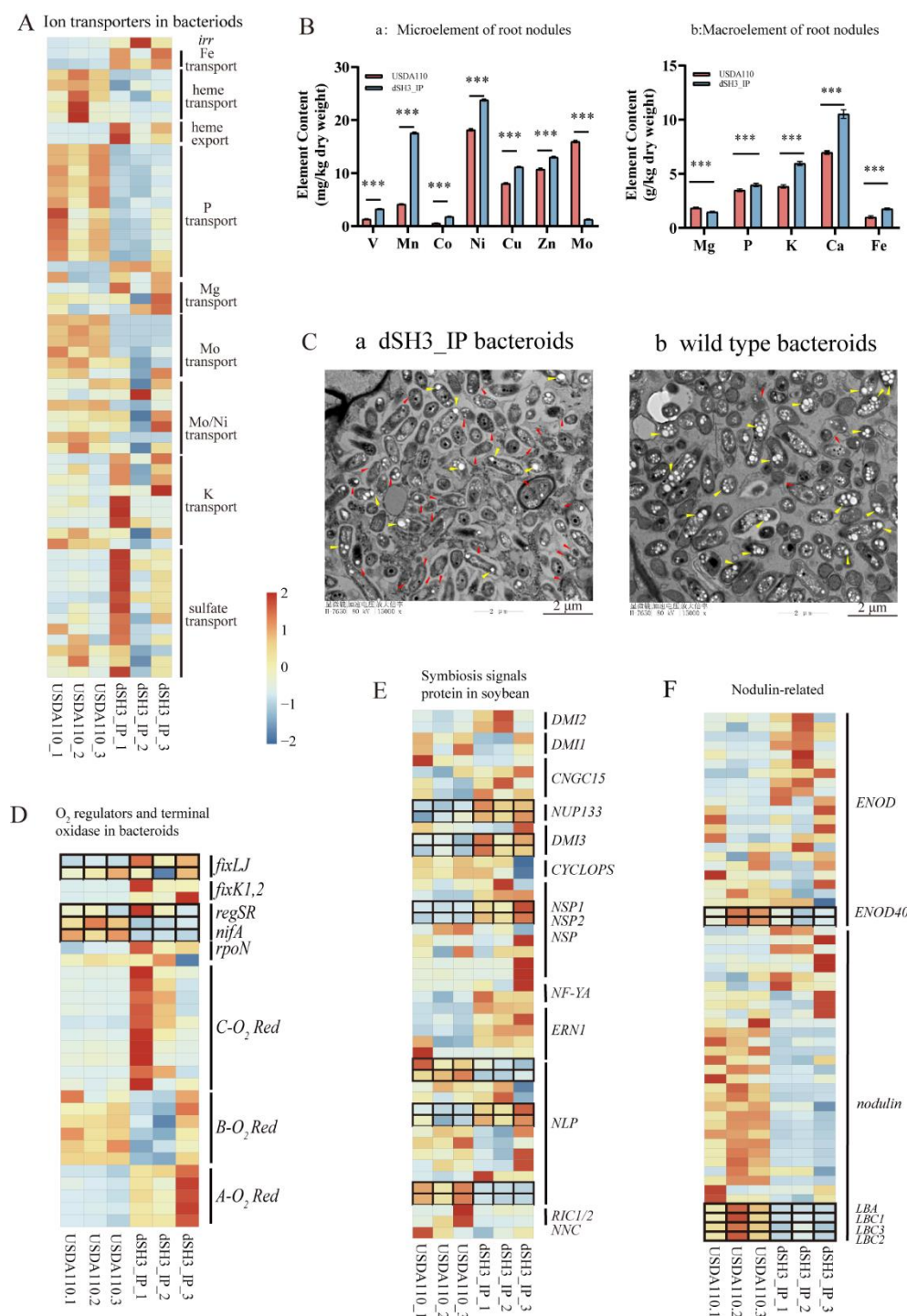


Fig. 4. Upregulated KEGG pathway enrichment analysis and root nodule sections. (A) Upregulated pathway of root nodule. Size of circle represents number of enriched genes, colors represent significant. (B) Upregulated pathway of bacteroids in root nodule.

Size of circle represents number of enriched genes, colors represent significant. **(C)** Transmission electron microscope (TEM) photograph of (a) dSH3_IP induced nodule section and (b) Wild type strain USDA110 induced nodule section. **(D)** Toluidine blue stain (TBS) photograph of (a) dSH3_IP induced nodule section and (b) Wild type strain USDA110 induced nodule section.



repeated three times.

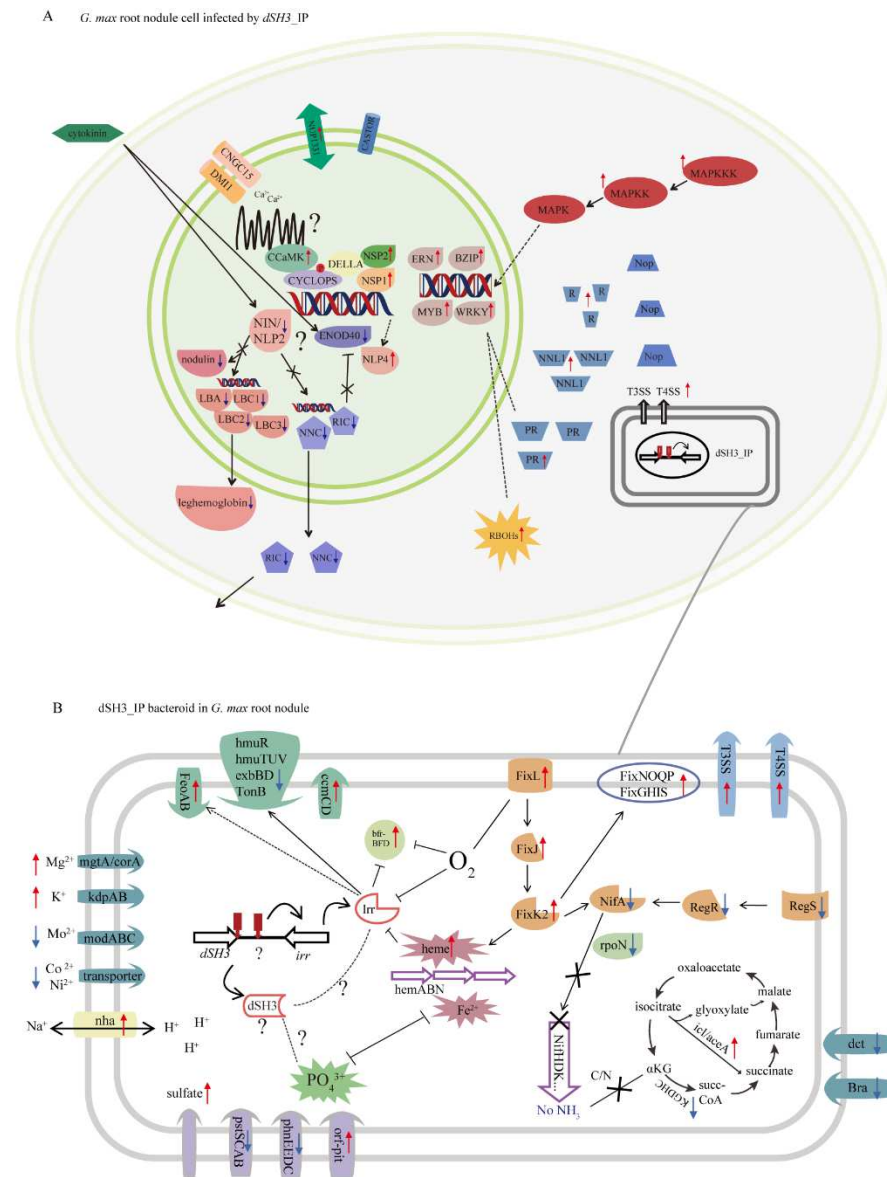


Fig. 7. Schematic overview for the dual RNA-seq transcriptome changes in root nodule (A) and bacteroid (B). (A) *G. max* root nodule cell infected by dSH3_IP. Immunity and stress response were upregulated, including PR, NNL, MAPKK, MAPKKK, ERN, MYB, BAIP and WRKY. Late nodulin, like LBA, LBC1, LBC2 and LBC3, and early nodulin NIN, ENOD40 were downregulated. But NSP1, NSP2

and CCaMK upregulated. GmRIC and GmNNC were downregulated. Diagram of root nodule shows greater immunity and symbiotic matter may be on calcium spiking and its downstream. (B) dSH3_IP bacteroid in *G. max* root nodule. Diagram exhibits changes of ion transport, oxygen regulation, TCA and secretion systems. Make the prediction that dSH3 may relate to phosphorus and propose potential relationship between *dSH3* and *irr*. Red arrow represents upregulation. blue arrow represents downregulation. Blank arrow represents activation or next step. Dotted line represents unknown relationship.

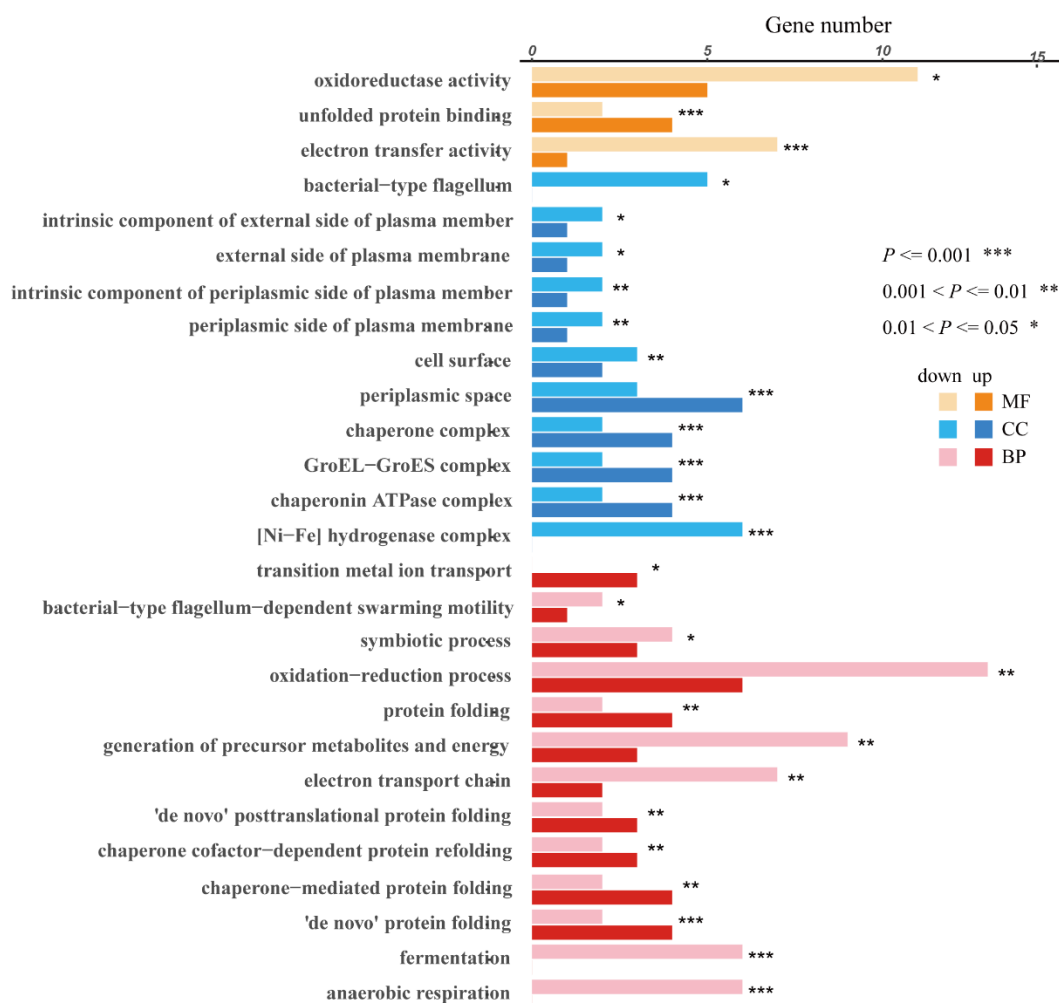


Fig. S1. GO enrichment analyses of dSH3_IP bacteroids.

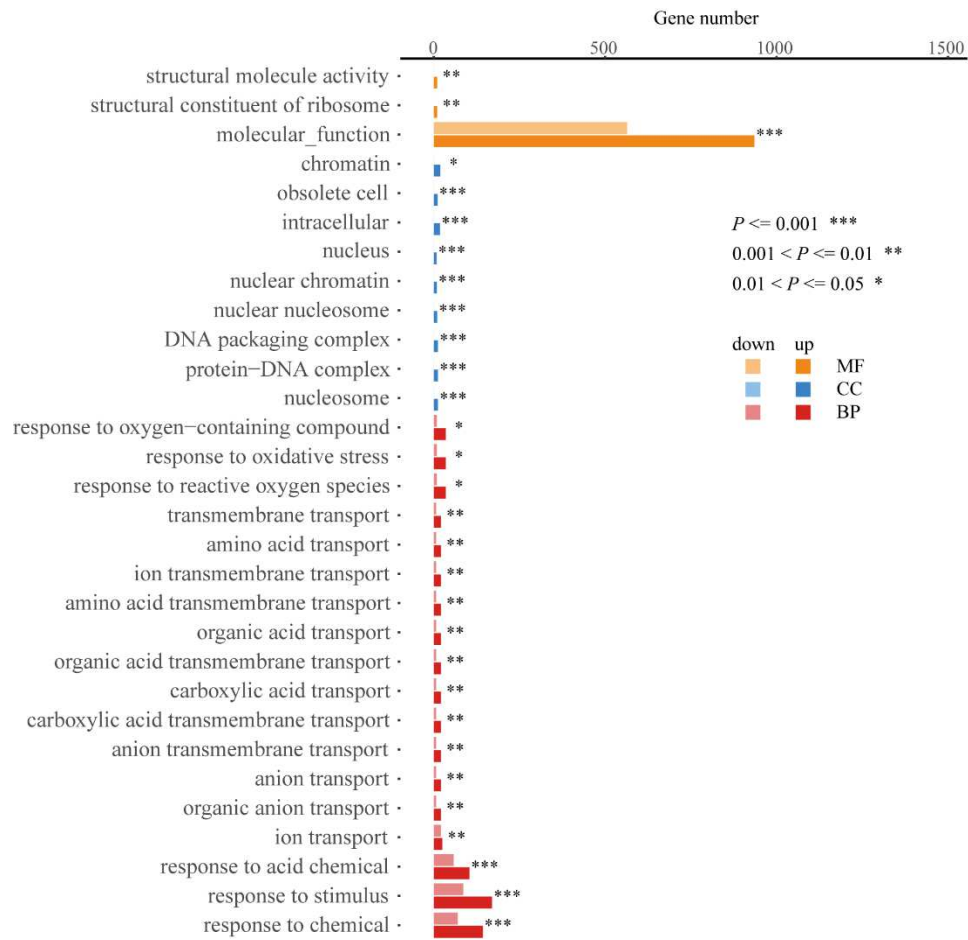
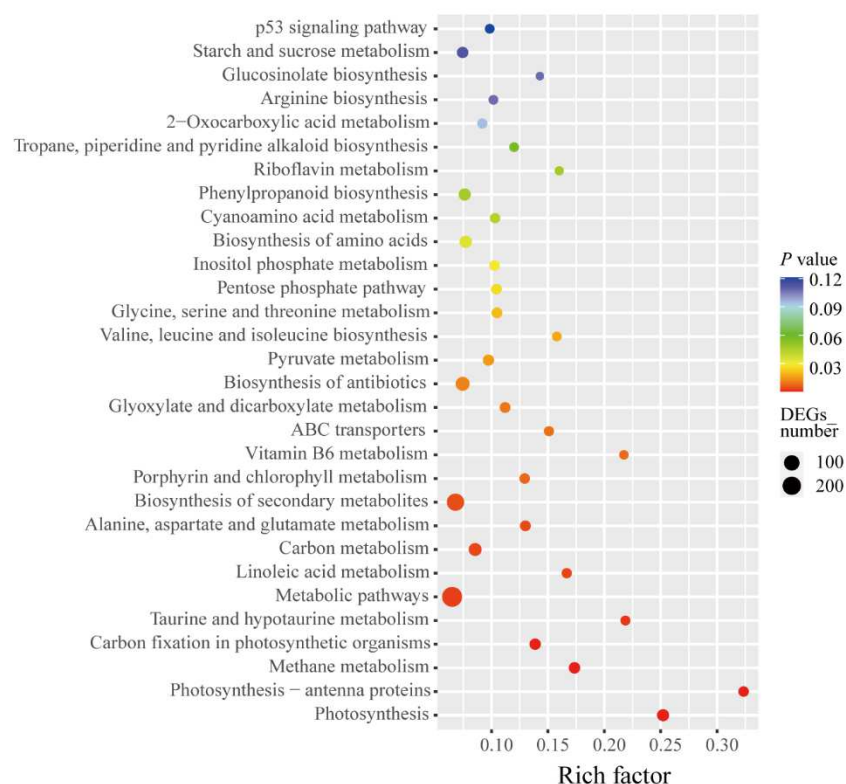


Fig. S2. GO enrichment analyses of dSH3_IP-induced root nodule.

A Downregulated pathway of root nodule induced by dSH3_IP mutant vs. wild type



B Downregulated pathway of bacteroids on dSH3_IP mutant vs. wild type

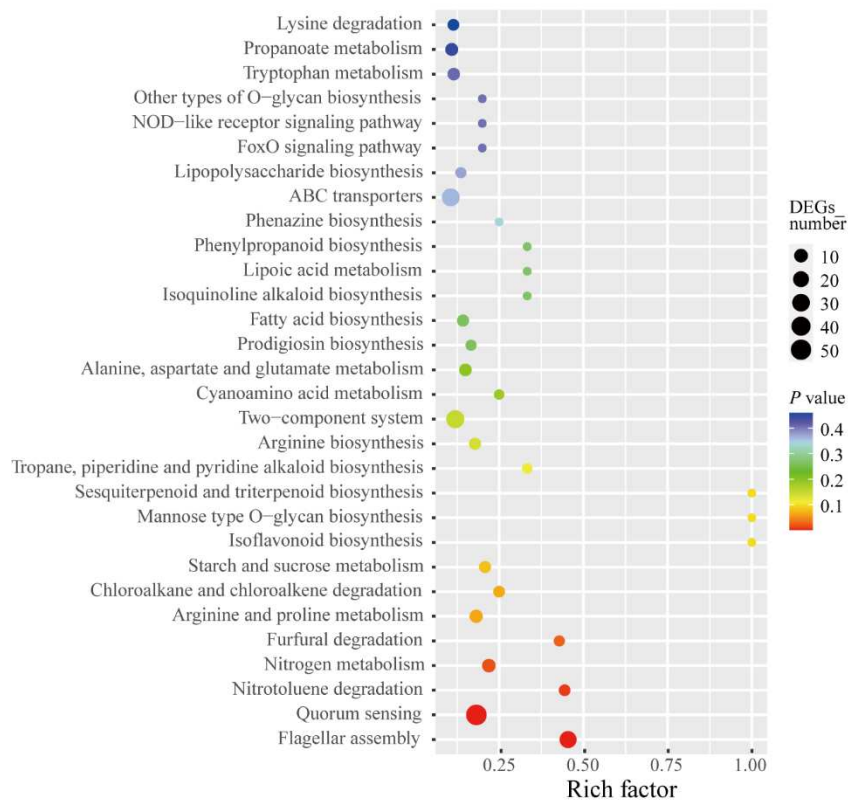


Fig. S3. Genes expression heatmaps of immunity and defense response. (A)

Differentially expressed genes ($|\log_2(\text{FC})| > 1$, $\text{FDR} < 0.001$) of stress response in root nodule from Fig 2A. **(B)** Genes expression distribution of significant or not in root nodule ($|\log_2(\text{FC})| > 1$, $\text{FDR} < 0.001$). **(C)** Genes expression heatmaps of T3SS, T4SS and TA system in bacteroids.

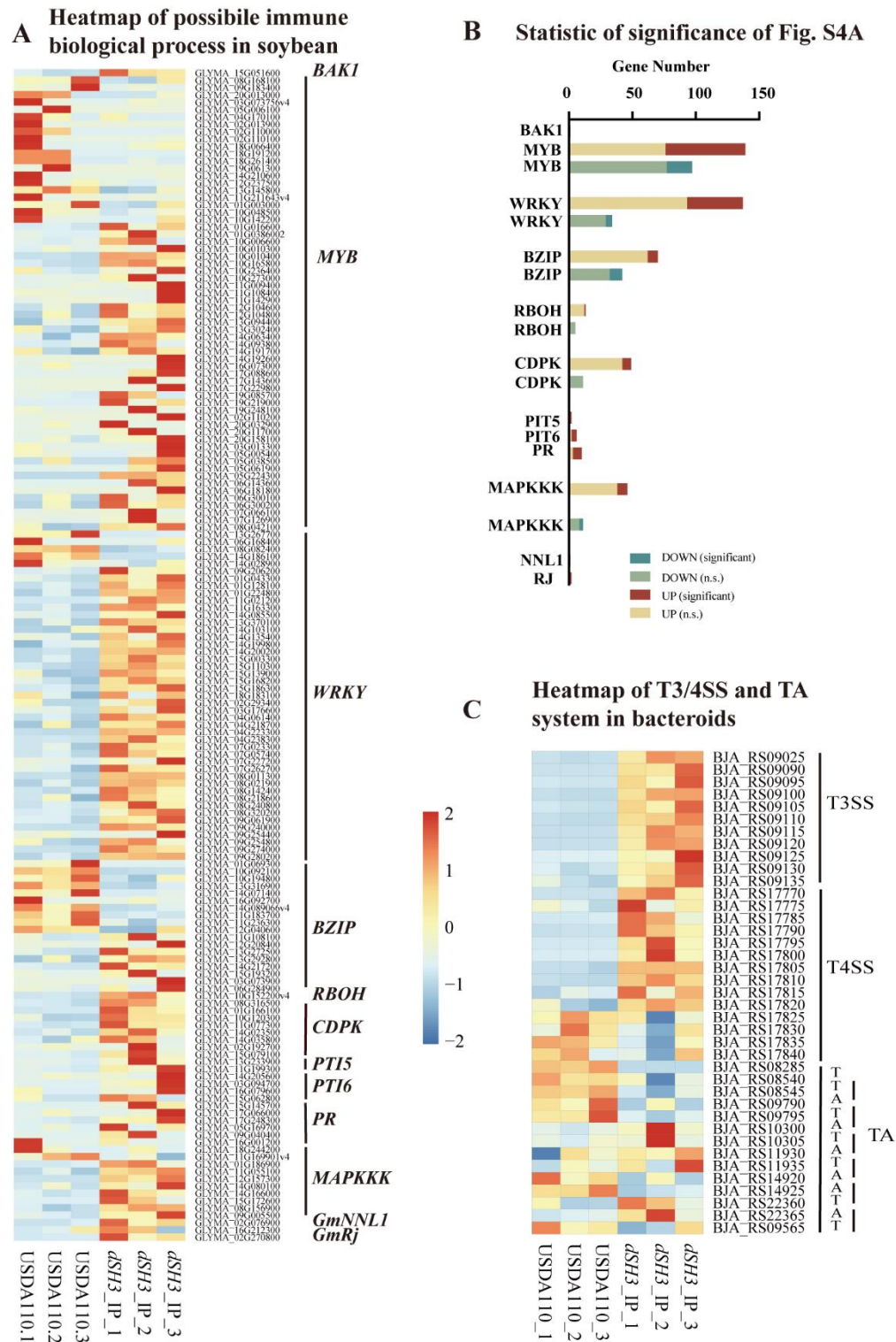


Fig. S4. Downregulated KEGG pathway enrichment analysis. (A) Downregulated pathway of root nodule. Size of circle represents number of enriched genes, colors represent significant. (B) Downregulated pathway of bacteroids in root nodule. Size of circle represents number of enriched genes, colors represent significant.

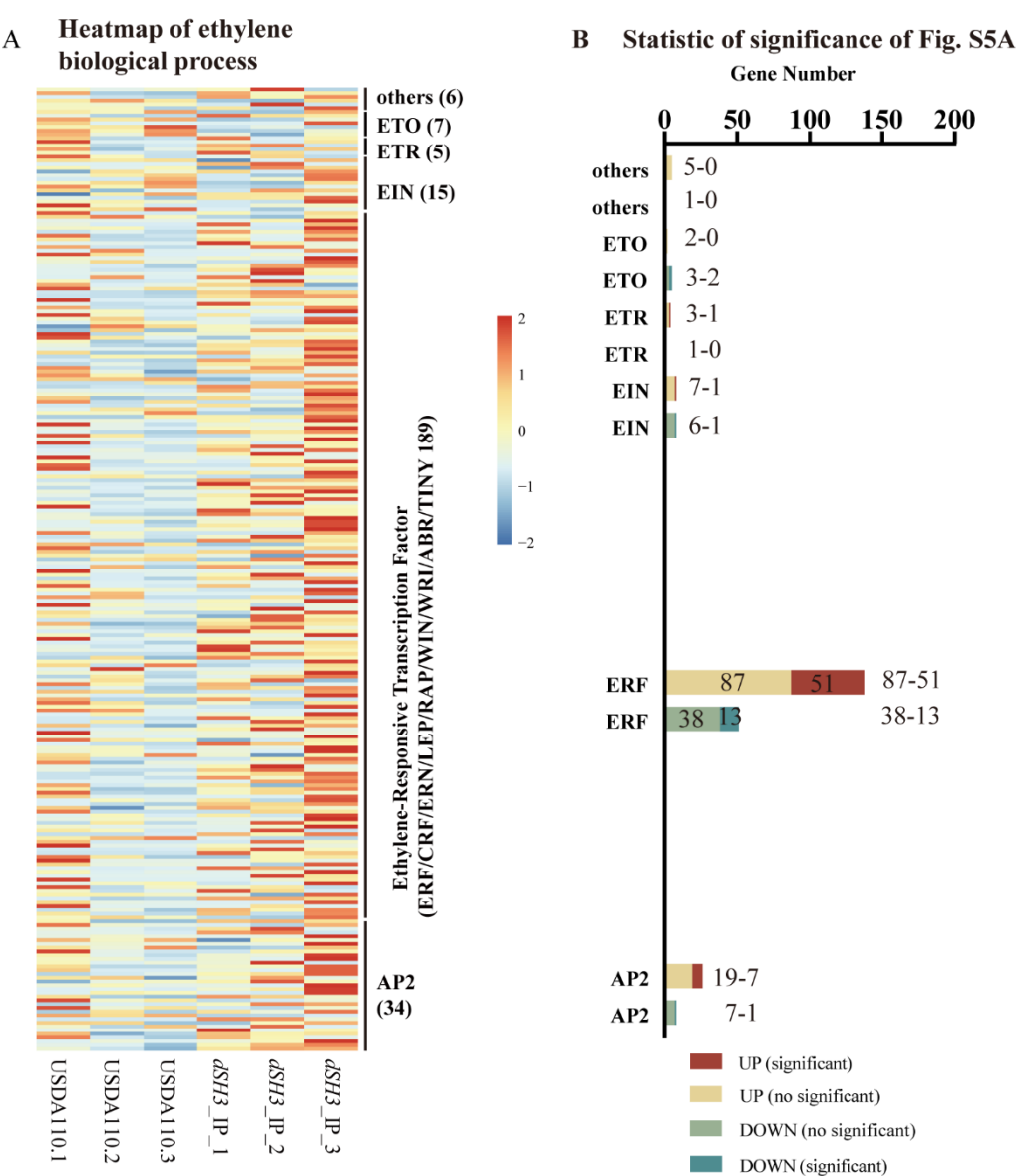
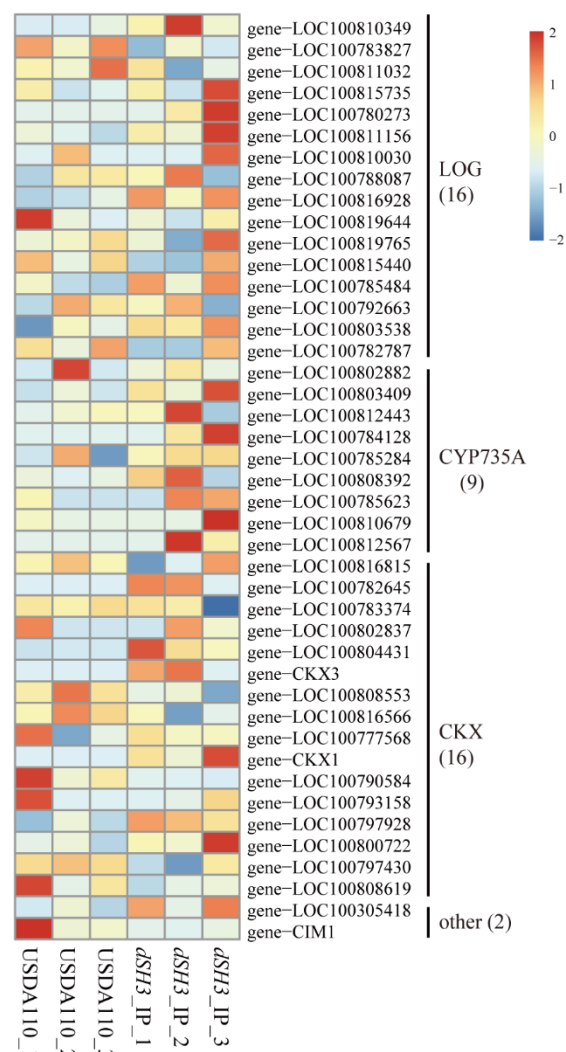


Fig. S5. Genes expression of cytokinin synthesis. (A) Genes expression heatmaps of cytokinin synthesis. (B) Genes expression distribution of significant or not in root nodule($|\log_2(FC)| > 1$, FDR < 0.001).

A Heatmap of cytokinin biological process



B Statistic of significance in Fig.S6A

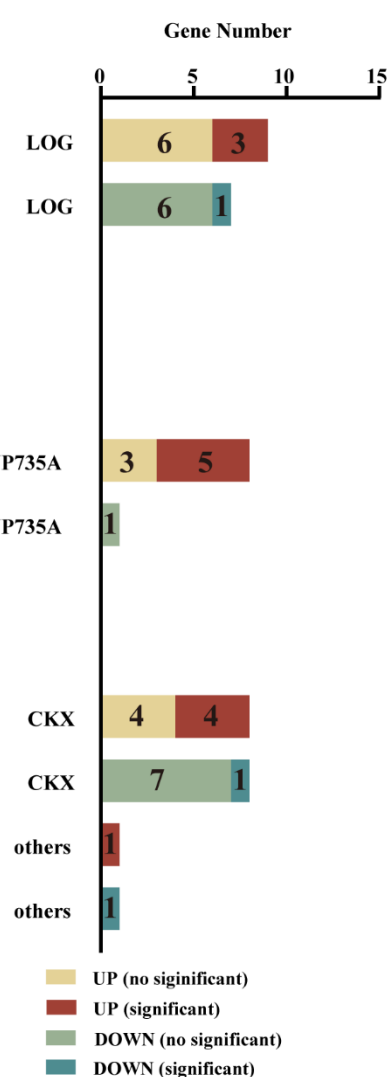
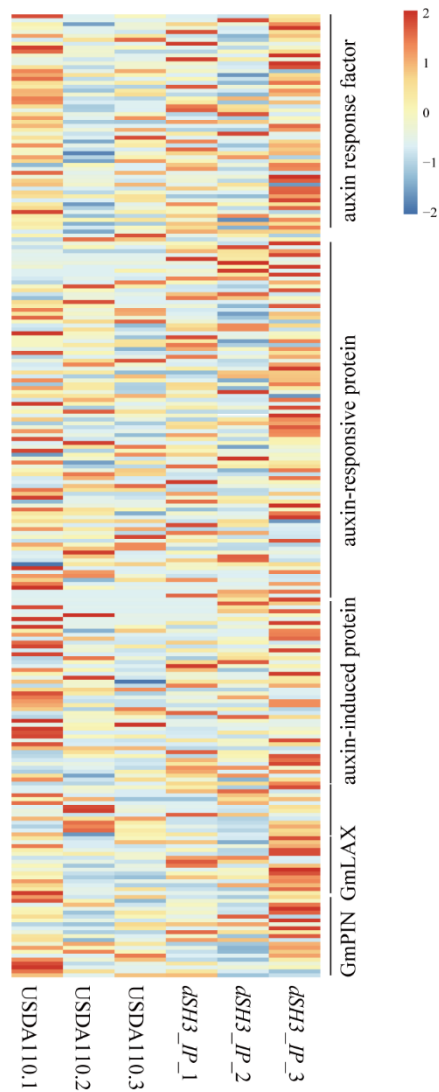


Fig. S6. Genes expression of ethylene response. (A) Genes expression heatmaps of ethylene response. (B) Genes expression distribution of significant or not in root nodule ($|\log_2(FC)| > 1$, $FDR < 0.001$).

A Heatmap of auxin biological process



B Statistic of significance in fig.S7A

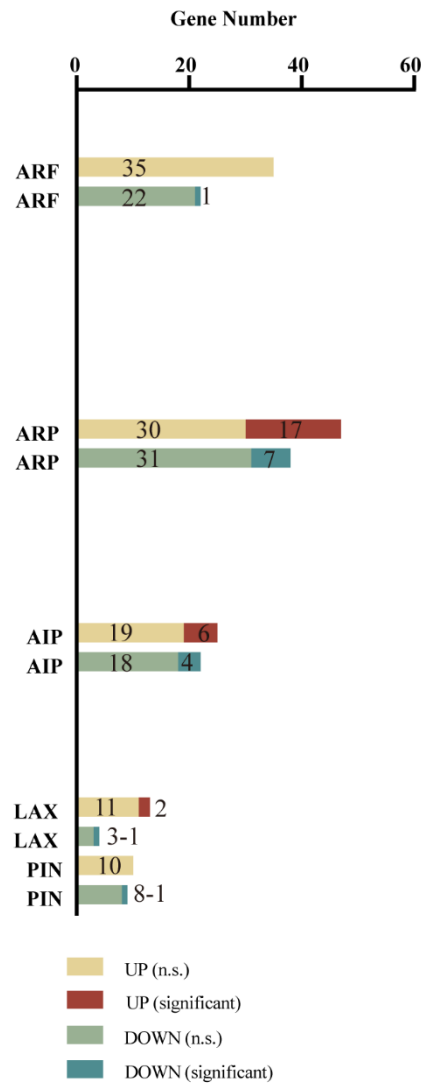
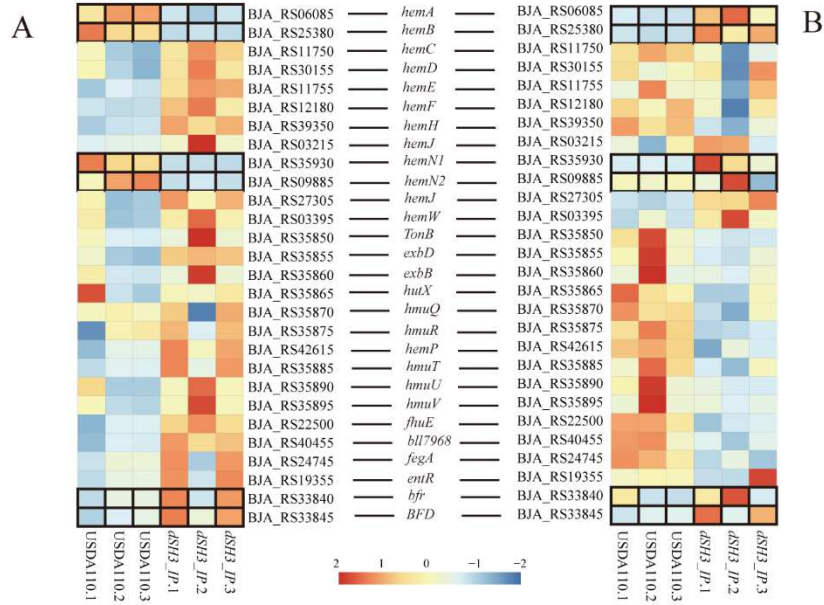


Fig. S7. Genes expression of auxin response. (A) Genes expression heatmaps of auxin response. **(B)** Genes expression distribution of significant or not in root nodule ($|\log_2(FC)| > 1$, $FDR < 0.001$).

Heatmap of rhizobial heme and iron metabolism in free living (A) and symbiosis (B)



Heatmap of rhizobial TCA enzymes in free living (C) and symbiosis (D)

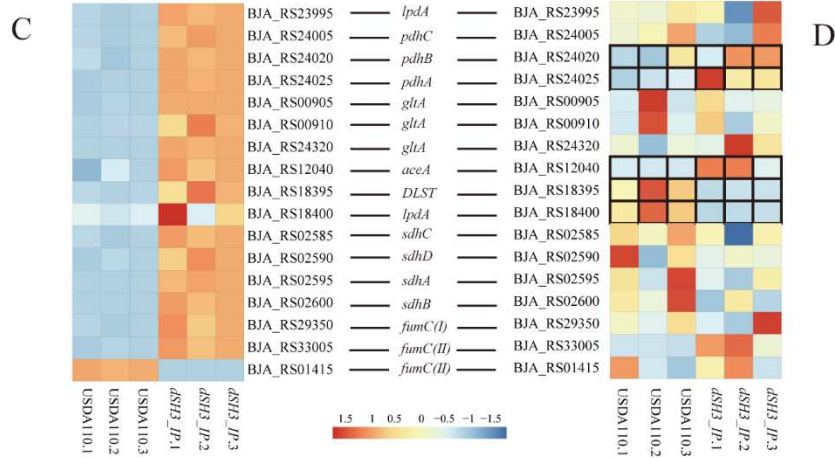


Fig. S8. Genes expression of metabolism in bacteroids and rhizobia. (A) Partial genes expression heatmaps of heme and iron metabolism in rhizobia. (B) Partial genes expression heatmaps of heme and iron metabolism in bacteroids. (C) Partial genes expression heatmaps involving in TCA cycle in rhizobia. (D) Partial genes expression heatmaps involving in TCA cycle in bacteroids.

Supplementary Table S1. Primers used in this study.

Primers	Sequence (5'-3')
GLYMA_08G162400 qF	CATCATCATCATCATCAC
GLYMA_08G162400 qR	AATCTCATTAGGTATTCTCCAT
GLYMA_16G214500 qF	AGAGCAAGAATACAGAGA
GLYMA_16G214500 qR	TAACCTTACTTCGTCAGA
GLYMA_13G210300 qF	AGTGCTTGTCGTATCTTATTATG
GLYMA_13G210300 qR	CCGTCTCCTTATCTCTTGAA
GLYMA_16G214800 qF	GGATGATGATTATACCAAGTGT
GLYMA_16G214800 qR	ATCTTATCTTATGCTTAGCCAAT
GLYMA_13G076700 qF	GAAAGGGGTGTGAGAGGAGAG
GLYMA_13G076700 qR	CGCCACTCAAGAAAGAATGTT
GLYMA_15G051600 qF	GGACATATAGCACCAGAGTA
GLYMA_15G051600 qR	GTTATTAGTTCAAGAAGCATCAC
GLYMA_06G120400 qF	GGAAGTGAAGAGGAGGAA
GLYMA_06G120400 qR	GAGAGGCAATGAATGAGAG
GLYMA_08G021900 qF	CCATAATTCAAAGAAAAGAAAGATAAC
GLYMA_08G021900 qR	ACCTATAGTAACTTCTGGGAT
GLYMA_12G040000 qF	TGCTCATCGATCCAGAATCA
GLYMA_12G040000 qR	GGGGAAAATCCTTCGAATTACTT
GLYMA_12G073300 qF	CAATGGGCAGGAAAGAGC
GLYMA_12G073300 qR	ATGGCAGTCGATGGAAAGGT
GLYMA_08G044400 qF	ACACCTAACGCCTATACA
GLYMA_08G044400 qR	GTAACCTCATCACTTGACATA
GLYMA_02G205600 qF	TGCTTACTTCAACGACTC
GLYMA_02G205600 qR	GCCTTCTTATCCAATCCAT
GLYMA_08G127000 qF	TGGCTACTGTTCGTAATATG
GLYMA_08G127000 qR	GGCAATAACTAACTCCTGAA
GLYMA_08G200100 qF	TGTTGGAATCATTGGAGAC
GLYMA_08G200100 qR	GCATATCAGCATACCTTATTAC
GLYMA_11G097300 qF	TAACTGTCTCAATGTCAATATCT
GLYMA_11G097300 qR	CTAACCTCGCTAATCAATCA
GLYMA_08G076300 qF	TCGTAGCAGATTACAGAAC
GLYMA_08G076300 qR	CTATCCATCCTCCAAGAAG
GLYMA_08G195100 qF	AGACCATAGAGGACATTGA
GLYMA_08G195100 qR	AGAGAAGCATCGTTACATAC
GLYMA_20G191200 qF	TTCACTGAGAAGCAAGAG
GLYMA_20G191200 qR	AACGCTGTATTGAGGAAT
GLYMA_01G178800 qF	TACTCTCGCAGGTATAGC
GLYMA_01G178800 qR	TGATAAGTGGCAACAACAT
GLYMA_06G312600 qF	GGAAGTGAAGAGGAGGAA
GLYMA_06G312600 qR	GAGAGGCAATGAATGAGAG
GLYMA_13G168500 qF	ACGAGGCATTCAAGTAAG
GLYMA_13G168500 qR	AGGAGCAGGAAGATAGAG
GLYMA_01G037100 qF	TCAAGAAGATTGGTGAGAAT

Primers	Sequence (5'-3')
GLYMA_01G037100 qR	GAAGGTAGCCGAAGTTAG
ENOD40 qF	GAAAGGGGTGTGAGAGGAGAG
ENOD40 qR	CGCCACTCAAGAAAGAATGTT
cons6_qRT_F	AGATAGGGAAATGGTGCAGGT
cons6_qRT_R	CTAATGGCAATTGCAGCTCTC
modB qF	CTTCTATCTGCTGGTCCT
modB qR	GAACGGCATGGAATAGAC
phasin qF	GACCACTGAAACCAACAC
phasinR qR	CGGTCTTCTTGACGAATT
nifH qF	GAGCCTACGAGAACATTG
nifH qR	TTCACCAGACATCACGAT
nifA qF	ATCGTATGGCAAGACAGA
nifA qR	CATTCTCGCACCCTAAC
dctA qF	TTCAATCTTCAAGTTCCTTATCT
dctA qR	GATATTGGTGCCGTCAG
SafE qF	GCTTATTCTACTTGGCTGTT
SafE qR	CCAGAAGAAGAACGAGTC
dnaK qF	GCAGAAGATGAAGGAGAC
dnaK qR	TCTTGTCCAGACCATAGG
pstS qF	GCATCTATCTCGGCAAGA
pstS qR	AGGTAGTTGGTGAAGTTGA
fixK2 qF	GACCCAGACACTCAAGAC
fixK2 qR	ACGACCTGGTAGACATATTC
napE qF	GAGATTTTCGCCTTCCTG
napE qR	CGGCAAACATCTGGTAGA
cybH qF	GAGCCGAAGATGTATGTC
cybH qR	CCTGACCTTCCGAATAGA
cyt c552 qF	AGAGGAATTTGCCTGGTA
cyt c552 qR	TTCTCCGAAGTGAAGTCC
groL qF	GTTGAAGAAGCCAAATCG
groL qR	CGTTGATGAGGATGTAGG
nhaA qF	CTGTACCAGTCCATTGTG
nhaA qR	GCAGTGATAGAGGAGTTG
nirA qF	GACCAGGAGAAGTTCAAG
nirA qR	CATAGAAGATGCCGTAGTAG
isocitrate lyase qF	GATGAAGGCGGAATATGAC
isocitrate lyase qR	ATCAGGTGGTGGAAGATG
fixH qF	AAGTCTTCCTGATGTTGGT
fixH qR	GCGTTGACCTTCCATTTG
hemN qF	CTACGGCGAAGAAAGACT
hemN qR	GCAATACCAGCACATCTC
efflux RND related qF	GACCAGACACTGCCTTAT
efflux RND related qR	GCTCTCCAACGTAAGAAG
ccoN qF	CGGCTACAACCTTCTCATC
ccoN qR	AAGACGAGCAGATAGACC
hemB qF	CCCGAACAATCTGGTCTG

Primers	Sequence (5'-3')
hemB qR	GTCTCGTCGTTTCAGGATC
ppk2 qF	GATGGATCTGGAATCCTATC
ppk2 qR	ACCTTGTCCTTCTTGATG
phbB qF	CTTCCACAAGATGAGCCT
phbB qR	CGAGGAGATGTTGATGATG
ccmC qF	CTGATTCTGTTCTGATGTAT
ccmC qR	CCGAGAACTTGATGATGG
tssC qF	ACCTGGATCAACGAATAC
tssC qR	AAAGTGTGGACGAAGAAA
coxB qF	CGGTGATGGACAATATCG
coxB qR	CGTTGAACTTGATGACCA
hypothetical protein qF	AAGTTGAAGCTTGTAGCC
hypothetical protein qR	GCCGTAATTGTGCAGATA
16S qF	AATCGCTAGTAATCGTGGATCAG
16S qR	CGCACCGTCTTCAGGTAAA