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MucR Is Required for Transcriptional Activation of Conserved Ion Transporters to Support Nitrogen Fixation of *Sinorhizobium fredii* in Soybean Nodules

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To achieve effective symbiosis with legume, rhizobia should fine-tune their background regulation network in addition to activating key genes involved in nodulation (*nod*) and nitrogen fixation (*nif*). Here, we report that an ancestral zinc finger regulator, MucR1, other than its paralog, MucR2, carrying a frameshift mutation, is essential for supporting nitrogen fixation of *Sinorhizobium fredii* CCBAU45436 within soybean nodules. In contrast to the chromosomal *mucR1*, *mucR2* is located on symbiosis plasmid, indicating its horizontal transfer potential. A MucR2 homolog lacking the frameshift mutation, such as the one from *S. fredii* NGR234, can complement phenotypic defects of the *mucR1* mutant of CCBAU45436. RNA-seq analysis revealed that the MucR1 regulon of CCBAU45436 within nodules exhibits significant difference compared with that of free-living cells. MucR1 is required for active expression of transporters for phosphate, zinc, and elements essential for nitrogenase activity (iron, molybdenum, and sulfur) in nodules but is dispensable for transcription of key genes (*nif/fix*) involved in nitrogen fixation. Further reverse genetics suggests that *S. fredii* uses high-affinity transporters to meet the demand for zinc and phosphate within nodules. These findings, together with the horizontal transfer potential of the *mucR* homolog, imply an intriguing evolutionary role of this ancestral regulator in supporting nitrogen fixation.

Interactions between bacteria and eukaryotic hosts have driven the emergence of complex life forms and many important evolutionary innovations (Toft and Andersson 2010). Rhizobia induce the formation of new legume organs called nodules, which rhizobia colonize intracellularly and in which atmospheric nitrogen is reduced to ammonium in specialized rhizobial cells (bacteroids). In exchange for reduced nitrogen from bacteroids, the legume provides bacteroids with a carbon source and essential nutrients (Udvardi and Poole 2013). Rhizobia belong to more than 100 species from α- and β-proteobacteria and diverged much earlier than the advent of legumes (Peix et al. 2015; Turner and Young 2000). How could such diverse bacteria evolve into microsymbionts of legumes? This has been largely explained by the horizontal transfer of key symbiosis genes, including *nod* genes involved in biosynthesis of nodulation factors and *nif/fix* genes essential for adaptation to the microaerobic nodule environment and nitrogen fixation (Masson-Boivin et al. 2009; Sullivan and Ronson 1998; Sullivan et al. 1995).

It has been demonstrated that the acquisition of key symbiosis genes essential for nodulation (*nod*) and nitrogen fixation (*nif/fix*) is not enough to confer a symbiotic phenotype to recipient pathogen *Ralstonia solanacearum* (Marchetti et al. 2010). Although modifications of certain virulence regulators in the recipient *R. solanacearum* can make this bacterium rapidly evolve symbiotic traits, i.e., nodulation and intracellular infection, the evolved clones do not fix atmospheric nitrogen (Guan et al. 2013; Marchetti et al. 2010). The acquired key symbiosis genes *nod* and *nif/fix* have been well studied, but the mechanisms by which recipient genomes evolve to completely incorporate these foreign symbiosis functions remain largely unexplored (Agron et al. 1993; Batut et al. 1989; David et al. 1988; Remigi et al. 2015; Roche et al. 1996).

Sinorhizobium fredii is well-known for its ability to nodulate on diverse legume hosts and is the major microsymbiont of cultivated and wild soybeans in alkaline-saline soils in Asia (Han et al. 2009; Man et al. 2008; Pueppke and Broughton 1999; Wu et al. 2011a; Zhang et al. 2011). Moreover, *S. fredii* strains have been successfully used as soybean inoculants under these soil conditions (Albareda et al. 2009; Rodríguez-Navarro et al. 2003). Here, we report that the *mucR1* mutant of *S. fredii* forms ineffective nodules on soybean, though *nif/fix* genes are actively transcribed in bacteroids of this mutant. *mucR1* encodes a conserved C_2H_2 zinc finger–bearing transcriptional regulator, MucR/RosR, that is recently proposed as the putative ancestor of eukaryotic zinc finger structure through horizontal gene transfer (Baglivo et al. 2009; Bouhouche et al. 2000; Netti et al. 2013). Genetic evidence suggests that the observed symbiotic defects of the *mucR1* mutant is largely dependent on a frameshift mutation in *mucR2*, the second copy of the *mucR* gene localized in a plasmid region with typical features of horizontal transfer. Then, RNA-seq analysis was used to investigate the regulon of MucR1

J. Jiao and L. J. Wu contributed equally to this work.

RNA-seq data is available in the National Center for Biotechnology Information Sequence Read Archive server under accession number PRJNA302586, and the complete genome sequence of CCBAU45436 has been deposited under accession number PRJNA285929.

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under both free-living and symbiotic conditions. The implication of MucR1-regulated genes encoding ion transporters in supporting nitrogen fixation were discussed and demonstrated by reverse genetics on representative genes.

RESULTS AND DISCUSSION

Identification of *mucR1* as an essential player in symbiotic interaction between *S. fredii* CCBAU45436 and soybean.

A *mucR1*::Tn*5* mutant forming nonmucoid colonies was identified in a Tn*5* mutant library of *S. fredii* CCBAU45436, and the link of the transposon insertion and the nonmucoid phenotype was demonstrated using the *mucR1* deletion mutant (Supplementary Fig. S1). The *mucR1* deletion mutant was further inoculated on soybean to test its symbiotic performance (Fig. 1A). Nodule number induced by the *mucR1* mutant was around two times that formed by CCBAU45436 and these nodules showed reduced fresh weight per nodule (Table 1) (*t* test, *P* value < 0.001). Moreover, nodules formed by the *mucR1* mutant were ineffective, as indicated by yellow leaves and reduced shoot dry weight that was indistinguishable from the uninoculated control (Fig. 1A; Table 1). The symbiotic defects of the *mucR1* mutant could be recovered by introducing pB*mucR1* expressing wild-type *mucR1* (*mucR1*/pB*mucR1*) but not by the empty vector pBBR1MCS-2 (Fig. 1A; Table 1). Briefly, shoot dry weight and nodule fresh weight were significantly increased, while the number of nodules was significantly decreased in plants inoculated with *mucR1*/pBmucR1 compared with the *mucR1* mutant (*t* test, *P* value < 0.01; Duncan's test, $\alpha = 0.05$). Although the number of nodules induced by *mucR1*/pBmucR1 was slightly higher than that of plants inoculated with wild-type CCBAU45436 (*t* test, *P* value < 0.05; Duncan's test, *P* value > 0.05), nodule fresh weight (per nodule) and shoot dry weight were not statistically different

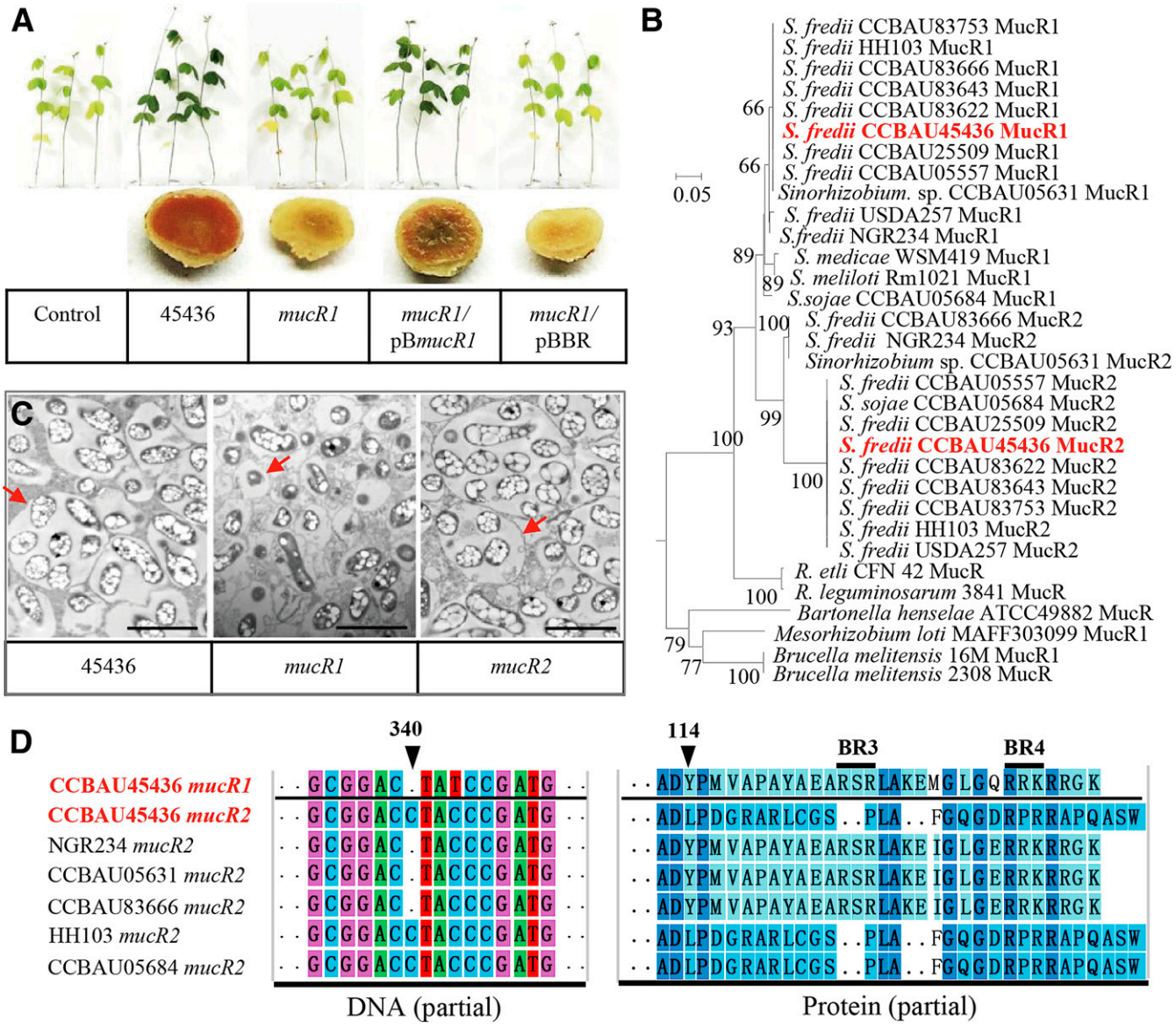


Fig. 1. *mucR1* rather than *mucR2* is essential for the symbiosis between *Sinorhizobium fredii* CCBAU45436 and soybean. **A,** Soybean shoot and nodule halves. **B,** Neighbor-joining phylogenetic tree of MucR homologs. Scale bar = 5% substitution per site. Bootstrap values above 60 are shown. **C,** Ultrathin sections of soybean nodules formed by CCBAU45436, the *mucR1*, or *mucR2* mutant. Pictures of ultrathin sections of 30–days postinoculation nodules were obtained under transmission electronic microscopy. Scale bar indicates 2 µm. Red arrows point to symbiosome membrane. **D,** Sequence analysis of MucR homologs. The triangle indicates the insertion site (340C) in certain *mucR2* genes and the start position (114L) of the corresponding frameshift event in protein sequences. BR3 and BR4 are two basic regions conserved among MucR homologs without the insertion mutation.

from those of plants inoculated with CCBAU45436 (*t* test and Duncan's test, *P* value > 0.05). Sequence analysis revealed that *mucR1* encodes a conserved C_2H_2 zinc finger–bearing transcriptional regulator MucR (or RosR), in which the C_2H_2 zinc-finger domain and four basic regions (BR), BR1 at the N-terminus and BR2-4 at the C-terminus, form a large domain essential for DNA-binding (Bouhouche et al. 2000; Malgieri et al. 2015; Netti et al. 2013). Although the MucR homolog has been reported as a pleiotropic regulator involved in motility and the production of exopolysaccharides in the model organism *S. meliloti*, its *mucR* mutant is indistinguishable from the wild-type strain in terms of symbiotic performance (Keller et al. 1995; Bahlawane et al. 2008). However, it was reported that the MucR homolog contributes to competitive nodulation of *Rhizobium etli* on *Phaseolus vulgaris* (Bittinger et al. 1997). The *mucR* mutant of *Rhizobium leguminosarum* bv. *trifolii* forms less than half the number of nodules compared with the wild-type strain on clover (Janczarek et al. 2010). Moreover, inoculated clover plants are yellowish, indicating inefficient symbiosis (Janczarek et al. 2010). These discrepancies between different species in terms of symbiotic phenotype might be partially due to the differences in their genome content, including the varying number of MucR copies as indicated in Supplementary Figure S2.

mucR2 in *S. fredii* CCBAU45436 is not functionally equivalent to *mucR1* due to a frameshift mutation.

In the genome of CCBAU45436 or its closely related strains and species, such as *S. fredii* strains NGR234 and HH103 and *S. sojae* CCBAU05684 (Schmeisser et al. 2009; Tian et al. 2012; Weidner et al. 2012), there are two MucR copies showing high similarity to each other (such as 83% in DNA between two copies of CCBAU45436). As shown in the phylogenetic tree of MucR (Fig. 1B), MucR1/MucR2 may originate from a recent duplication event in these *Sinorhizobium* strains, in contrast to one or more other distantly related homologs found in several species or strains. Moreover, in contrast to the chromosomal *mucR1*, *mucR2* is always localized on the symbiosis plasmid and is surrounded by highly conserved sequences (Supplementary Fig. S3), including *y4aQ* coding a transposase of the IS66 family and *nodD1* required for the expression of *nod* genes (Kobayashi et al. 2004), suggesting potential horizontal transfer of *mucR2* between strains or species. In line with this hypothesis, identical *mucR2* genes were found among *S. sojae* CCBAU05684 and *S. fredii* strains CCBAU45436, HH103, and USDA257 or between *S. fredii* CCBAU83666 and *Sinorhizobium* sp. CCBAU05631. The *mucR2* gene of *S. fredii* NGR234 shows 99.1 and 99.5% similarity to these two groups, respectively.

A *mucR2* mutant and a *mucR1R2* double mutant were further constructed and tested for their symbiotic phenotype on soybean. As shown in Table 1, the *mucR1R2* double mutant showed similar symbiotic defects on soybean compared with the *mucR1* mutant. The *mucR2* mutant was indistinguishable from CCBAU45436 regarding nodule number, fresh weight per nodule, and shoot dry weight (*t* test, *P* value > 0.05). Furthermore, the symbiosome of soybean nodules formed by the *mucR2* mutant is similar to that of CCBAU45436, containing multiple bacteroids, whereas each symbiosome formed by the *mucR1* mutant contains only one bacterial cell (Fig. 1C). Sequence analysis unveiled that there is an insertion mutation (340C) in *mucR2* compared with *mucR1* in CCBAU45436 (Fig. 1D). The same insertion was also found in *mucR2* of *S. fredii* strains HH103 and USDA257 and *S. sojae* CCBAU05684 but not in *S. fredii* strains NGR234 and CCBAU83666 and *Sinorhizobium* sp. CCBAU05631. This insertion caused a frameshift event starting from the 114th amino acid of the MucR homologs (Fig. 1D) and may destroy two basic regions (BR3 and BR4) that are necessary for DNA-binding activity of MucR/RosR (Baglivo et al. 2009). Therefore, symbiotic defects of the *mucR1* mutant are likely due to *mucR2* carrying the insertion mutation being unable to fulfill the function of MucR/RosR.

To test this hypothesis, various *mucR* homologs with or without the insertion mutation at the 340th base were introduced into the *mucR1* mutant, using pBBR1MCS-2. As indicated in Table 1, *mucR* homologs without the insertion mutation, such as wild-type *mucR1* of CCBAU45436, *mucR1* and *mucR2* of NGR234, and the 340C-lacking *mucR2* derivative of CCBAU45436, could restore the symbiotic performance of the *mucR1* mutant regarding nodule weight and shoot dry weight. In contrast, the *mucR1* derivatives harboring a 340C insertion mutation, point mutations in BR3 or without the BR4 region, and wild-type *mucR2* of CCBAU45436 could not complement the symbiotic defects of the *mucR1* mutant (Table 1) (*t* test, *P* value < 0.05).

The characteristic phenotypes, under free-living conditions, associated with the *mucR* mutants in all documented bacterial species are the nonmucoid (rough outer surface) colonies

Table 1. Responses of soybean plants to inoculation of *Sinorhizobium fredii* CCBAU45436 and its derivatives[a]

Inoculant	No. of nodules[b]	Nodule fresh weight (mg)		Shoot dry weight[c]
		Per plant	Per nodule	
WT	9.1 ± 0.8	122.0 ± 9.1	14.4 ± 1.5	493.6 ± 11.9
Uninoculated control	–	–	–	414.5 ± 21.3**
mucR1	17.0 ± 1.8***	91.4 ± 7.4*	5.5 ± 0.3***	421.6 ± 22.9*
mucR2	10.3 ± 1.0	132.6 ± 5.1	13.9 ± 1.2	451.5 ± 29.7
mucR1R2	15.1 ± 1.1***	90.7 ± 3.9**	6.1 ± 0.5***	402.0 ± 15.1***
mucR1/pBBR1MCS2	17.9 ± 1.3***	93.2 ± 4.8*	5.3 ± 0.2***	441.4 ± 16.6*
mucR1/pB*mucR1*	11.4 ± 0.6*	135.7 ± 5.1*	12.3 ± 0.9	524.4 ± 16.4
mucR1/pB*mucR2*	15.6 ± 1.9**	86.8 ± 6.8**	6.0 ± 0.5***	382.8 ± 27.4**
mucR1/pB*mucR1+340C*	16.2 ± 1.4***	79.6 ± 5.0***	5.2 ± 0.4***	376.6 ± 22.3***
mucR1/pB*mucR2–340C*	10.5 ± 0.8	132.9 ± 5.6	13.4 ± 1.2	479.2 ± 29.2
mucR1/pB*mucR1-NGR234*	10.0 ± 0.7	119.2 ± 6.1	12.4 ± 1.4	483.7 ± 13.8
mucR1/pB*mucR2-NGR234*	9.8 ± 1.0	116.6 ± 4.6	12.8 ± 1.4	494.1 ± 18.6
mucR1/pB*mucR1-BR3PM*	15.0 ± 1.0***	73.8 ± 8.2***	5.2 ± 0.5***	384.3 ± 25.0***
mucR1/pB*mucR1-BR4D*	16.3 ± 1.2***	77.4 ± 6.9***	5.0 ± 0.5***	388.1 ± 20.0***
mucR1R2/pB*mucR1*	10.9 ± 0.7	128.3 ± 5.9	12.4 ± 1.1	485.3 ± 18.5
mucR1R2/pB*mucR2*	15.5 ± 1.6**	94.6 ± 7.6*	6.7 ± 0.3***	405.6 ± 25.2**

[a] Average ± standard error of the means scored from ten plants. Similar results were obtained in three independent experiments. Significant difference compared with values of CCBAU45436 wild-type strain based on *t* test is indicated. * = *P* value < 0.05; ** = *P* value < 0.01; and *** = *P* value < 0.001.
[b] Nodules per plant.
[c] Shoot dry weight in milligrams per plant.

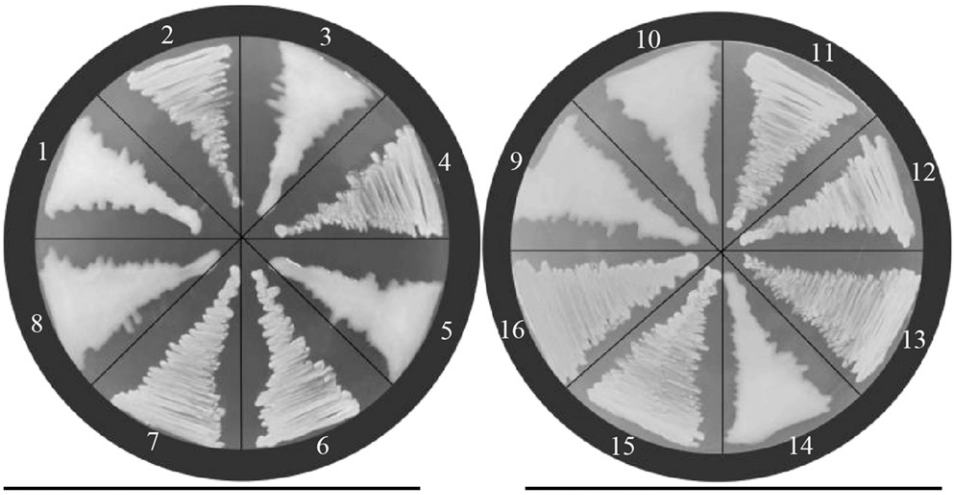
(Bertram-Drogatz et al. 1998; Bittinger et al. 1997; Caswell et al. 2013; Janczarek and Skorupska 2007). As shown in Figure 2, the *mucR1* mutant and the *mucR1R2* double mutant formed nonmucoid colonies similar to the *exoA* mutant that is unable to produce exopolysaccharides (EPS) (Rodríguez-Navarro et al. 2014). In contrast to truncated *mucR1* derivatives and the wild-type *mucR2* from CCBAU45436, the wild-type *mucR1* from CCBAU45436, *mucR1* or *mucR2* from NGR234, and various *mucR1* and *mucR2* derivatives without the frameshift mutation can successfully restore the mucoid surface. Therefore, in the genome of *S. fredii* CCBAU45436, MucR2 may have lost the typical function of MucR/RosR due to a frameshift mutation.

The MucR1 regulon in nodules does not include *nif/fix* genes despite the Fix$^-$ phenotype of the *mucR1* mutant.

To reveal the underlying mechanisms of ineffective symbiotic performance of the *mucR1* mutant, RNA-seq transcriptomic analysis was carried out for bacteroids of this mutant and the wild-type CCBAU45436. This high-resolution technique uncovered 597 differentially expressed genes (DEG) (with a false discovery rate [FDR] ≤ 0.001, $|\log_2 \text{ratio}| \geq 1$) between the *mucR1* mutant and CCBAU45436 in soybean nodule (Fig. 3A), to our knowledge, the largest regulon associated to MucR/RosR documented so far in bacteria (Bahlawane et al. 2008; Bittinger and Handelsman 2000; Caswell et al. 2013; Mueller and González 2011). Similarly, there were 621 DEG uncovered in tryptone yeast (TY) culture (Fig. 3A), resulting in a total of 1,076 DEG between the *mucR1* mutant and CCBAU45436 under test conditions (Supplementary Dataset S1). To validate RNA-seq results, we performed quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on 25 genes with different expression profiles (Supplementary Table S1). The RNA-seq results agree well with the qRT-PCR data from three independent biological replicates (Fig. 3B) and the Pearson correlation coefficient value is 0.891 (P value < 0.001).

Intriguingly, less than 10% of the DEG (106 of 1,076) showed similar transcription profiles in both TY culture and soybean nodule (Fig. 3A). Further analysis disclosed that DEG in bacteroids of the *mucR1* mutant are enriched in COG (clusters of orthologous groups) categories P (inorganic ion transport and metabolism) and U (intracellular trafficking, secretion, and vesicular transport) (Fisher's exact test, P value < 0.001) (Supplementary Table S2). By contrast, the enriched COG category is N (cell motility) under free-living conditions for the *mucR1* mutant compared with CCBAU45436 (Fisher's exact test, P value < 0.001). This indicates that MucR1 is not a local regulator and transcriptomic analysis based on free-living cells of the *mucR1* mutant, as reported earlier for other bacteria, including *S. meliloti* (Bahlawane et al. 2008; Bittinger and Handelsman 2000; Caswell et al. 2013; Mueller and González 2011), is not enough to reveal a global picture of the MucR1 regulon, which can vary when facing different environmental stimuli.

Soybean plants inoculated with the *mucR1* mutant showed the typical phenotype of nitrogen starvation (Fig. 1A; Table 1) and only a trace level of nitrogenase activity, as measured using an acetylene reduction assay, could be detected from nodules induced by the *mucR1* mutant (Fig. 4A). However, RNA-seq suggests similar expression levels of *nif/fix* genes between the *mucR1* mutant and CCBAU45436 (Log2 ratio < 1 for all *nif/fix* genes), though a slight expression difference in a few genes such as *nifD* was statistically significant (Log2 ratio = 0.59; FDR < 0.001). These results are also supported by qRT-PCR analysis of *nifD* (encoding dinitrogenase subunit), *nifA* (encoding a positive transcriptional regulator for *nif* genes), and *fixA* (encoding a component of electron transfer complex) (Fig. 4B). These



ID	Strain
1	CCBAU45436
2	*mucR1*
3	*mucR2*
4	*mucR1*/pBBR1MCS2
5	*mucR1*/pB*mucR1*
6	*mucR1*/pB*mucR2*
7	*mucR1*/pB*mucR1+340C*
8	*mucR1*/pB*mucR2-340C*

ID	Strain
9	*mucR1*/pB*mucR1-NGR234*
10	*mucR1*/pB*mucR2-NGR234*
11	*mucR1*/pB*mucR1-BR3PM*
12	*mucR1*/pB*mucR1-BR4D*
13	*mucR1R2*
14	*mucR1R2*/pB*mucR1*
15	*mucR1R2*/pB*mucR2*
16	*exoA*

Fig. 2. Rough colonies of the *mucR1* mutant depend on a frameshift mutation of *mucR2* in *Sinorhizobium fredii* CCBAU45436.

findings imply that nitrogen fixation genes are actively transcribed in bacteroids of the *mucR1* mutant and could not account for its symbiotic defects.

Downregulation of ion transporters accounts for the Fix⁻ phenotype of the *mucR1* mutant.

Nitrogenases of rhizobia contain two components, the iron (Fe) protein with a [4Fe-4S] cluster and the molybdenum-iron (MoFe) protein with an [8Fe-7S] cluster and the FeMo cofactor ($MoFe_7S_9$·homocitrate) (Dixon and Kahn 2004). Earlier proteomic and biochemical analysis of symbiosome membrane in nodules uncovered the transport of iron, molybdenum, and sulfur (Clarke et al. 2014). In line with these findings, rhizobial genes encoding transporters of iron (*afuABC,* $\log_2$ ratio above 6.0) (Chao et al. 2005; Chin et al. 1996), molybdenum (*modABC,* $\log_2$ ratio = 1.0 to 2.7) (Delgado et al. 2006), aliphatic sulfonate (*ssuABC*, $\log_2$ ratio above 4.2), and aminoethane sulfonate (*tauABC*, $\log_2$ ratio above 5.0) (Sugawara et al. 2011; van der Ploeg et al. 1997) were up-regulated in bacteroids of CCBAU45436 compared with free-living cells (FDR < 0.0001). However, in bacteroids of the *mucR1* mutant, these transporters were significantly down-regulated (Fig. 4C), implying that the defect of the *mucR1* mutant in nitrogen fixation might be partially due to the shortage of key elements essential for the functioning of nitrogenase.

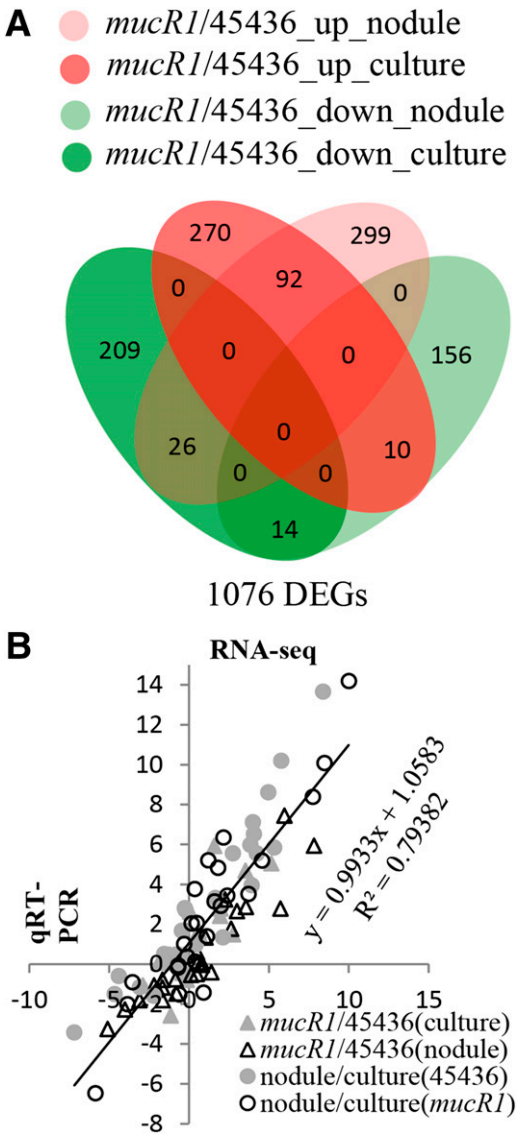


Fig. 3. Summary and validation of RNA-seq results. **A,** Summary of RNA-seq results. **B,** Validation of RNA-seq results using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) from three biological replicates. The $\log_2$-transformed expression ratios from RNA-seq (vertical axis) and qRT-PCR (horizontal axis) are shown.

The symbiosome isolated from soybean nodules has been demonstrated to uptake zinc from the medium, and GmZIP1, a soybean zinc transporter, is localized on symbiosome membrane (Moreau et al. 2002). *znuA*, *znuB*, and *znuC* encode the conserved bacterial zinc transport system importing zinc under low zinc conditions (Patzer and Hantke 1998; Vahling-Armstrong et al. 2012), and they were strongly induced in CCBAU45436 bacteroids compared with free-living cells ($\log_2$ ratio above 4, FDR < 0.0001). In bacteroids of the *mucR1* mutant, however, the expression level of *znuA*, *znuB*, and *znuC* was significantly down-regulated by onefold compared with CCBAU45436 bacteroids (FDR < 0.0001). Soybean plants inoculated with the *znuA* mutant showed reduced shoot dry weight (*P* value < 0.05) and increased nodule number (*P* value < 0.01) (Fig. 4D and E), implying that the reduced supply of zinc, due to downregulation of *znuA*, *znuB*, and *znuC*, might also contribute to the symbiotic defect of the *mucR1* mutant.

pstSCAB encoding a high-affinity phosphate transporter is involved in adaptation to phosphate starvation (Becker et al. 2004; Yuan et al. 2006) and is down-regulated in bacteroids of the *mucR1* mutant (Fig. 4C). Its deletion mutant, *pst*, exhibited symbiotic defects (Fig. 4D and E) (*P* value < 0.05) similar to the *mucR1* mutant (Table 1). Other genes involved in phosphate starvation adaptation were also down-regulated in bacteroids of the *mucR1* mutant (Fig. 4C), such as regulatory genes *phoU* and *phoB* (Geiger et al. 1999), transporter genes *ugpB*, *ugpA*, *ugpE*, and *ugpC* (sn-glycerol 3-phsphate) and *phoC*, *phoD*, *phoE*, and *phoT* (phosphonate) (Hsieh and Wanner 2010), and *btaA* and *btaB* involved in the biosynthesis of phosphorus-free lipids diacylglyceryl-N,N,N-trimethylhomoserine (DGTS) (Geiger et al. 1999; Zavaleta-Pastor et al. 2010). Upregulation of these phosphate starvation genes has also been reported in *S. fredii* NGR234 bacteroids in both determinate nodules of *Vigna unguiculata* and indeterminate nodules of *Leucaena leucocephala* (Li et al. 2013). It has been proposed that *pstS*, *pstC*, *pstA*, and *pstB* and downstream regulatory genes *phoU* and *phoB* may form a putative operon (Yuan et al. 2006). *phoU* and *phoB* encode a chaperone and a key transcriptional regulator in phosphate starvation, respectively (Hsieh and Wanner 2010), and an in-frame deletion mutant of *phoB* was constructed. However, the *phoB* mutant of CCBAU45436 was not symbiotically defective compared with wild-type strain (*t* test, all *P* values > 0.05) regarding nodule number (mean ± standard error of the mean, 9.7 ± 0.65 per plant) and shoot dry weight (448.4 ± 24.62 mg per plant) of inoculated soybean plants. PhoB positively regulates the transcription of phosphate starvation genes, including those involved in DGTS synthesis and *pstS*, *pstC*, *pstA*, and *pstB*, while it represses the expression of a low-affinity phosphate transporter encoded by *orfA-pit* operon (Becker et al. 2004; Yuan et al. 2006). In bacteroids of the *mucR1* mutant in soybean nodules, *phoB* and *pstS*, *pstC*, *pstA*, and *pstB* were down-regulated, while the *orfA-pit* operon was up-regulated. This is in contrast with the expression profiles of their homologs in bacteroids of *S. meliloti* in alfalfa nodules, in which the low-affinity transporter encoded by *orfA-pit* alone could support normal symbiotic functions of bacteroids (Yuan et al. 2006). These findings imply that bacteroids in nodules of soybean and alfalfa may face different levels of phosphate supply.

Genes involved in the assembly of pilus and three secretion systems (T1SS, T3SS-I, and T4SS-III) were up-regulated in the *mucR1* mutant (Fig. 4C). The T1SS is thought to simply transport

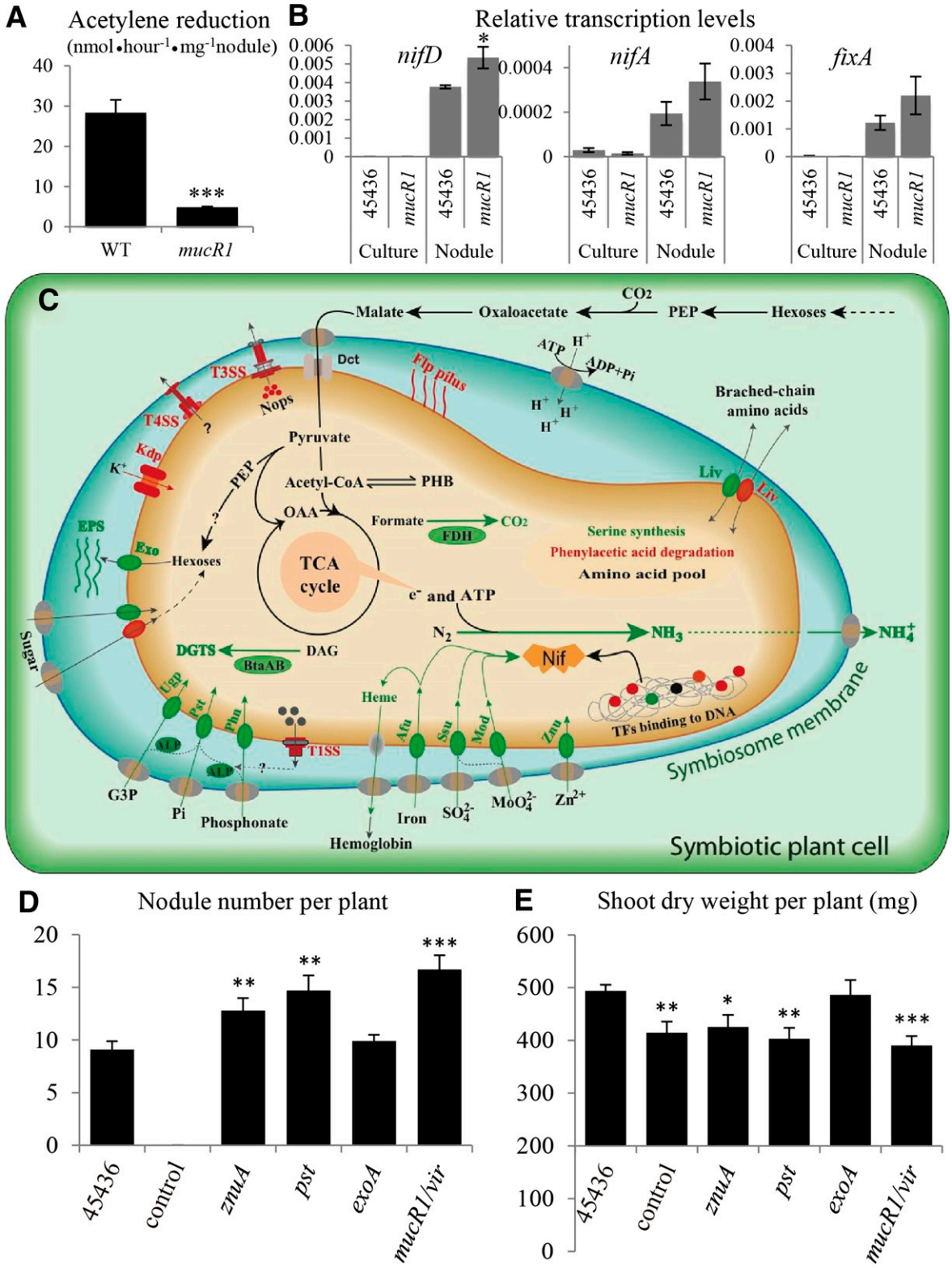


Fig. 4. MucR1 maintains the conditions essential for nitrogen fixation but not the expression of *nif/fix* genes. **A,** Nitrogenase activity as measured using an acetylene reduction assay on three biological replicates. **B,** Relative transcription level of *nifD*, *nifA*, and *fixA* compared with the 16S ribosomal RNA gene (measured using quantitative reverse transcription-polymerase chain reaction on three biological replicates). **C,** Enrichment of differentially expressed genes in the inorganic ion transport and secretion system in bacteroids of the *mucR1* mutant compared with CCBAU45436. Up- and down-regulated genes are represented in red and green, respectively. EPS = exopolysaccharide, ALP = alkaline phosphatase, G3P = sn-glycerol 3-phosphate, FDH = formate dehydrogenase, DGTS = diacylglyceryl-N,N,N-trimethylhomoserine, DAG = diacylglycerol, and PHB = polyhydroxybutyrate. **D,** Nodule number and **E,** shoot dry weight per plant inoculated with *Sinorhizobium fredii* strains (10 plants were scored in each treatment and similar results were obtained in multiple independent experiments). Significant difference between the mean of each treatment and the corresponding value of CCBAU45436 is indicated (*t* test: * = *P* value < 0.05, ** = *P* value < 0.01, *** = *P* value < 0.001). Error bars represent standard error of the means.

proteins outside of the cell (Nelson and Sadowsky 2015; Schmeisser et al. 2009). In *S. fredii*, the influence of T3SS-I on symbiosis with soybean happens at an early interaction stage, i.e., either increasing or reducing nodulation (Krishnan et al. 2003; Yang et al. 2010). The T4SS system can translocate DNA or protein substrates across the cell envelope (Cascales and Christie 2003), and the closest homolog of *S. fredii* T4SS-III has been reported to be required for conjugation but not for symbiosis of *S. meliloti* (Jones et al. 2007; Sugawara et al. 2013). In contrast to T1SS and T3SS-I, both of which were actively transcribed in CCBAU45436 bacteroids, T4SS-III were rarely transcribed in bacteroids of CCBAU45436 (reads per kilobase per million mapped reads [RPKM] below 25) but expressed in bacteroids of the *mucR1* mutant at levels (RPKM up to 6,294) comparable to *nif/fix* genes. Consequently, to relieve this burden in the *mucR1* mutant, the T4SS-III (*vir*) was deleted in the *mucR1* mutant. The *mucR1/vir* double mutant was still symbiotically defective, similar to the *mucR1* mutant regarding nodule number and shoot dry weight (Fig. 4D and E). Therefore, the unexpected high expression of T4SS-III might not be the key determinant of the symbiotic defect of the *mucR1* mutant.

In addition to above-mentioned genes encoding ion transporters and secretion systems, DEG also include those involved in the biosynthesis of EPS and serine, formate metabolism, phenylacetic acid degradation, and more (Fig. 4C). A *Bradyrhizobium japonicum* mutant lacking formate dehydrogenase activity was found to be capable of nodulating soybean and fixing nitrogen (Manian et al. 1982). EPS is important for rhizobial adaptations to diverse stresses and crucial for *S. meliloti–Medicago* symbiosis (Deaker et al. 2004; Skorupska et al. 2006). Although the expression of *exo* genes was down-regulated in bacteroids of the *mucR1* mutant compared with CCBAU45436, the *exoA* mutant defective in producing EPS (Fig. 2) formed an effective symbiosis with soybean plants (Fig. 4D and E). This indicates that the defect in producing EPS by the *mucR1* mutant, a typical phenotype observed in diverse bacteria (Bertram-Drogatz et al. 1998; Bittinger et al. 1997; Caswell et al. 2013; Janczarek and Skorupska 2007), may not lead to its impaired symbiotic performance.

Conclusions.

The rhizobium-legume symbiosis is one of the model systems for the study on facultative intracellular host adaptation. The effectiveness of nitrogen fixation not only depends on the active transcription of key symbiosis genes (*nif/fix*) but, also, relies on fine-tuning of the background regulation network in bacteroids. Here, we demonstrated that, in soybean nodules elicited by *S. fredii*, an ancestral zinc finger transcriptional factor MucR1 is dispensable for transcription of *nif/fix* genes but is required for active expression of transporters for essential elements of nitrogenase (iron, molybdenum, and sulfur), phosphate, and zinc. Soybean plants inoculated with mutants of high-affinity transporters for phosphate or zinc exhibited a typical phenotype of nitrogen starvation, similar to plants inoculated with the *mucR1* mutant. Taken together with the horizontal transfer potential of the *mucR* homolog localized on the symbiosis plasmid (containing key symbiosis genes *nod* and *nif/fix*), we hypothesized that MucR might have been recruited in activating conserved local ion transporters to support nitrogen fixation processes in soybean nodules. These findings shed new light on the evolutionary importance of zinc finger–bearing MucR transcriptional factor in symbiotic adaptation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

S. fredii and *Escherichia coli* strains and plasmids used in this study are listed in Table 2. *S. fredii* strains were grown at 28°C in TY medium or on yeast extract-mannitol agar medium (Vincent 1970). *E. coli* strains were cultured in Luria-Bertani medium at 37°C. The concentrations of antibiotics

Table 2. Strains and plasmids used in this study

Names	Descriptions[a]	Source
Sinorhizobium fredii		
CCBAU45436	Wild type, Nal^r,Gen^r	Tian et al. 2012
mucR1::Tn*5*	CCBAU45436 Tn*5* insertion in *mucR1* coding for MucR1	This work
mucR1	CCBAU45436 Δ*mucR1::Gm*, Nal^r,Gen^r	This work
mucR2	CCBAU45436 Δ*mucR2::Gm*, Nal^r,Gen^r	This work
mucR1/R2	CCBAU45436 Δ*mucR1,* Δ*mucR2*::*Gm*, Nal^r,Gen^r	This work
exoA	CCBAU45436 Δ*exoA*, Nal^r,Gen^r	This work
mucR1/vir	CCBAU45436 Δ*mucR1,* Δ*virB2-10::Gm*, Nal^r,Gen^r	This work
znuA	CCBAU45436 Δ*znuA::Gm*, Nal^r,Gen^r	This work
pst	CCBAU45436 Δ*pstSCAB::Gm*, Nal^r,Gen^r	This work
phoB	CCBAU45436 Δ*phoB::Gm*, Nal^r,Gen^r	This work
Escherichia coli		
DH5α	*fhuA2 Δ(argF$^-$lacZ)U169 phoA glnV44 Φ80Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Bethesda Research
Plasmids		
pRK2013	ColE1, Tra$^+$, Km^r	Ditta et al. 1980
pCM157	IncP plasmid that provides expression of the Cre recombinase, Tet^r	Marx and Lidstrom 2002
pCM351	Allelic exchange vector bearing a gentamicin cassette flanked by *loxP* sites, Gen^r, Tet^r	Marx and Lidstrom 2002
pRL1063a	Carrying Tn5-1063a, Km^r, Bm^r and Sm^r	Wolk et al. 1991
pBBR1MCS-2	Broad-host cloning vector, Km^r	Kovach et al. 1995
pB*mucR1*	pBBR1MCS-2 expressing the *mucR1* gene of *S. fredii* CCBAU45436	This work
pB*mucR2*	pBBR1MCS-2 expressing the *mucR2* gene of *S. fredii* CCBAU45436	This work
pB*mucR1+340C*	pB*mucR1*-derived vector carrying a C-insertion at N_{340} of *mucR1* ORF	This work
pB*mucR2–340C*	pB*mucR1*-derived vector carrying a C deletion at N_{340} of *mucR2* ORF	This work
pB*mucR1-BR3PM*	pB*mucR1*-derived vector expressing MucR1 (R125A, R127A)	This work
pB*mucR1-BR4D*	pB*mucR1*-derived vector expressing MucR1 (1 to 136)	This work
pB*mucR1-NGR234*	pBBR1MCS-2 expressing the *mucR1* gene of *S. fredii* NGR234	This work
pB*mucR2-NGR234*	pBBR1MCS-2 expressing the *mucR2* gene of *S. fredii* NGR234	This work

[a] Nal^r,Gen^r, Km^r, Tet^r, Bm^r, and Sm^r indicate resistance to nalidixic acid, gentamycin, kanamycin, tetracycline, bleomycin, and streptomycin. ORF = open reading frame.

used, when required, for *S. fredii* and *E. coli* cultures were 30 μg/ml for nalidixic acid, 10 μg/ml for trimethoprim, 10 μg/ml for tetracycline, 50 μg/ml for kanamycin, and 30 μg/ml for gentamicin.

Construction of plasmids and mutant strains.

Primers used for DNA amplification are listed in Supplementary Table S3. For construction of the in-frame gene deletion mutants, we used the *cre-lox* system (Marx and Lidstrom 2002). PCR fragments encompassing the upstream N-terminal coding region and the downstream C-terminal coding region of the one or more genes to be deleted were sequentially amplified and cloned into each of the two multiple cloning sites of pCM351 by digestion-ligation-transformation manipulation. The resultant pCM351 derivatives with correct cloned sequences, verified by Sanger sequencing (Beijing Genomic Institute [BGI]), were then conjugated into *S. fredii* CCBAU45436 or its derivatives using pRK2013 as the helper plasmid (Ditta et al. 1980; Marx and Lidstrom 2002). To obtain the mutant lacking the gentamicin-resistance gene flanked by two *loxP* sites, the plasmid pCM157 expressing Cre recombinase was introduced into rhizobial cells by conjugation. Transconjugants sensitive to gentamicin were selected for subsequent screening of pCM157-cured strains, which are tetracycline sensitive (Marx and Lidstrom 2002). *S. fredii* mutants obtained in this study are listed in Table 2 and mutations for selected DEG are visualized in Supplementary Figure S4. Briefly, in-frame deletion mutations were constructed for *mucR1* and *mucR2*, both of which are monocistrons. To inactivate the production of EPS and T4SS-III, *exoA* within *exo* gene cluster and *virB2-virB10* within the T4SS-III gene cluster were replaced with a gentamicin cassette. To block the formation of the high-affinity transporter for phosphate and zinc, in-frame deletion mutants of *pstSCAB* and the monocistron *znuA*, respectively, were constructed. Moreover, an in-frame deletion mutant of the phosphate starvation regulator gene *phoB*, which is downstream of *pstSCAB*, was constructed to check whether the symbiotic defect of the *pstSCAB* mutant was due to a potential polar effect on the expression *phoB*.

To construct the plasmids expressing *mucR* homologs, the fragments containing coding sequences of *mucR* homologs from *S. fredii* strains CCBAU45436 and NGR234 and upstream promoter regions were cloned into a multiple cloning site of pBBR1MCS-2 (Kovach et al. 1995). Four plasmids were obtained, i.e., pB*mucR1*, pB*mucR2*, pB*mucR1-NGR234*, and pB*mucR2-NGR234*. On the basis of pB*mucR1* and pB*mucR2*, site-directed mutagenesis was performed using a Phusion site-directed mutagenesis kit (Thermo Scientific) to introduce the single-nucleotide insertion or deletion or truncation of MucR1 at C-terminal basic regions. This resulted in pB*mucR1+340C* (insertion), pB*mucR2–340C* (deletion), pB*mucR1-BR3PM* (point mutations at the third basic region, R125A and R127A), pB*mucR1-BR4D* (deletion mutation of the fourth basic region). All constructs were verified by PCR and Sanger sequencing in *E. coli* and by PCR in *S. fredii*. Plasmids were transferred from *E. coli* to *S. fredii* by triparental mating using pRK2013 as the helper plasmid.

Plant assays, nitrogenase activity assays, and electron microscopy.

Symbiotic performance of *S. fredii* strains was tested on *Glycine max* cv. JD17 as previously described (Wang et al. 2015). Seeds of *G. max* cv. JD17 were surface-sterilized in 3% NaClO (wt/vol) solution, were germinated, and were inoculated with 1 ml of physiological saline (0.8% NaCl) suspension of rhizobia with an optical density at 600 nm (OD_{600}) equivalent to 0.2. Plants were grown for 30 days at 24°C with 12-h day and night cycles, in vermiculate moistened with low-N nutrient solution in Leonard jars (Vincent 1970). Nitrogenase activity of nodules was measured using acetylene reduction method as descried previously (Buendia-Claveria et al. 1989). For electron microscopy, ultrathin sections of nodules of 30–days postinoculation (dpi) plants were prepared as described earlier (Li et al. 2013) and were observed with a JEM-1230 transmission electron microscope.

RNA-seq and real-time PCR.

Cultures of free-living cells in TY medium were collected by centrifugation when the OD_{600} reached about 1.2. Isolation of bacteroids from 30-dpi nodules of *G. max* cv. JD17 was performed using the approach described earlier (Li et al. 2013). RNA from free-living bacteria or bacteroids was extracted using the Qiagen RNeasy mini kit. RNA samples were sent to BGI-Shenzhen for further treatments, library construction, and strand-specific RNA sequencing. Briefly, residual DNA was removed using RNase-free DNase I (30 min at 37°C). To remove the ribosomal (r)RNA, total RNA was then treated with Ribo-Zero rRNA removal kit (gram-negative bacteria), according to the manufacturer's instructions. The mRNA-enriched RNA was chemically fragmented to 150 to 200 bp, using divalent cations under elevated temperature. The first-strand cDNA was synthesized, using reverse transcription and random primers based on the cleaved RNA fragments. During the synthesis of the second strand, nonincorporated nucleotides were removed and dTTP was substituted by dUTP, as described earlier (Parkhomchuk et al. 2009). Then, products with ligated adapters were purified and digested with N-glycosylase (Applied Biosystems) to remove the second-strand cDNA. Fifteen cycles of PCR with phusion polymerase were carried out and the resultant products were sequenced on an Illumina Hiseq 2000 platform. To validate the results of RNA-seq, real-time qPCR was performed for 25 genes with different transcription profiles. Three independent biological replicates were tested. The experimental procedure for qRT-PCR was the same to the one described earlier (Li et al. 2013).

Bioinformatics analyses.

For RNA-seq data, clean reads in fastq files (90-bp paired end reads) were mapped to the *S. fredii* CCBAU45436 reference sequence using HISAT (–no spliced alignment; –rna-strandness) (Kim et al. 2015). Unique mapped reads for each protein coding gene were extracted from sorted bam files by HTseq-count (Anders et al. 2015). Then, the DEG (FDR ≤ 0.001, $|\log_2 \text{ratio}| \geq 1$) were identified by DEGseq, using the normalized gene expression data (RPKM) as inputs (Wang et al. 2010). COG annotation for *S. fredii* CCBAU45436 proteins was obtained using a reserved position-specific BLAST against the COG database integrated in WebMGA (Wu et al. 2011b).

Availability of supporting data.

Raw sequence data from our RNA-seq analysis can be accessed via the National Center for Biotechnology Information Sequence Read Archive server under accession number PRJNA302586, and the complete genome sequence of CCBAU45436 has been deposited under accession number PRJNA285929.

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