Evidence for Phosphate Starvation of Rhizobia without Terminal Differentiation in Legume Nodules

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Phosphate homeostasis is tightly modulated in all organisms, including bacteria, which harbor both high- and low-affinity transporters acting under conditions of fluctuating phosphate levels. It was thought that nitrogen-fixing rhizobia, named bacteroids, inhabiting root nodules of legumes are not phosphate limited. Here, we show that the high-affinity phosphate transporter PstSCAB, rather than the low-affinity phosphate transporter Pit, is essential for effective nitrogen fixation of *Sinorhizobium fredii* in soybean nodules. Symbiotic and growth defects of the *pst* mutant can be effectively restored by knocking out PhoB, the transcriptional repressor of *pit*. The *pst* homologs of representative rhizobia were actively transcribed in bacteroids without terminal differentiation in nodules of diverse legumes (soybean, pigeonpea, cowpea, common bean, and *Sophora flavescens*) but exhibited a basal expression level in terminally differentiated bacteroids (alfalfa, pea, and peanut). *Rhizobium leguminosarum* bv. *viciae* Rlv3841 undergoes characteristic nonterminal and terminal differentiations in nodules of *S. flavescens* and pea, respectively. The *pst* mutant of Rlv3841 showed impaired adaptation to the nodule environment of *S. flavescens* but was indistinguishable from the wild-type strain in pea nodules. Taken together, root nodule rhizobia can be either phosphate limited or nonlimited regarding the rhizobial differentiation fate, which is a host-dependent feature.

Inorganic phosphate (Pi) is essential for maintaining cellular structure and energy currency in all organisms (Qi et al. 2016). Pi sensing and signaling has been intensively investigated in the model unicellular microorganism *Escherichia coli*. Under high Pi conditions, Pi binding to the high-affinity Pi transporter PstSCAB can inhibit the histidine kinase PhoR, leading to the inactivation of transcription factor PhoB and subsequent repression of *pstSCAB* transcription and derepression of the low-affinity Pi transporter Pit (McCleary 2017; Taylor et al. 2014). Under Pi-limited conditions, the repression on PhoR

autophosphorylation is released, allowing activation of PhoB and PhoB-mediated modulation of Pho regulon (Santos-Beneit 2015). The extensive studies of diverse bacterial pathogens encountering either Pi-limiting or Pi-rich environments in eukaryote hosts have further revealed an adaptive role of bacterial Pi sensing or signaling in stress response and virulence (Lamarche et al. 2008; Taylor et al. 2014). However, our understanding of Pi homeostasis in bacteria symbiotically associated with eukaryotes is limited.

Rhizobia are characterized by their ability of forming nitrogen-fixing nodules on legume roots and play important roles in sustainable agriculture and global ecology. Within nodule cells, differentiated rhizobia named bacteroids can fix atmospheric nitrogen to support plant growth, while the host provides carbon sources and essential nutrients to bacteroids to support the energy-consuming process of nitrogen fixation (Udvardi and Poole 2013). The morphotype and fate of bacteroids are governed by the varying number and type of symbiotic nodule-specific cysteine-rich (NCR) peptides in different legumes (Montiel et al. 2017). Within *Medicago* root nodules, bacteroids of the model rhizobium *Sinorhizobium meliloti* undergo an irreversible (terminal) differentiation characterized by enlarged cell size and impaired reproductive ability (Van de Velde et al. 2010). The terminal differentiation of bacteroids can also be found in other legumes such as species of *Pisum*, *Trifolium*, *Aeschynomene*, and *Arachis*, but not in *Lotus*, *Glycine*, *Phaseolus*, *Vigna*, *Cajanus*, *Leucaena*, *Sophora*, and so on (Crespo-Rivas et al. 2016; Li et al. 2013; Montiel et al. 2016; Oono et al. 2010).

Despite a great diversity of rhizobium-legume symbioses (Alunni and Gourion 2016; Haag et al. 2013; Remigi et al. 2016) and the fact that approximately 70% of global cultivated land is deficient in Pi (López-Arredondo et al. 2014), our knowledge of rhizobial Pi homeostasis is restricted in *S. meliloti*, a natural microsymbiont of *Medicago sativa*. The low-affinity *orfA-pit*-encoded Pi uptake system Pit rather than the high-affinity transporters PstSCAB and PhoCDET is expressed in nodules and is sufficient for symbiosis of *S. meliloti* bacteroids (Bardin et al. 1996; diCenzo et al. 2017; Yuan et al. 2006), and it has been consequently proposed that root nodule bacteria are not Pi limited (Udvardi and Poole 2013). However, a recent transcriptomic analysis of *S. fredii* bacteroids in *Glycine max* (soybean) nodules suggested active expression of *pstSCAB* and *phoCDET* but very little expression of *orfA-pit* genes (Jiao et al. 2016), and the *pstSCAB* operon is essential for the nitrogen fixation process (Jiao et al. 2016). This highlights that bacteroids may encounter contrasting nodule environments of different legume hosts.

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In this study, we aimed to find a clue about the Pi homeostasis of bacteroids associated with different legume hosts. First, various single and combined deletion mutants of high- and low-affinity Pi transporters and PhoB were constructed in *S. fredii* and tested for their symbiotic performance on *G. max*. Together with in vivo measurement of the expression of different transporters in nodules induced by the wild-type *S. fredii* and the *phoB* mutant, it was demonstrated that *S. fredii* bacteroids in *G. max* nodules were Pi limited. The expression level of rhizobial high-affinity Pi transporter Pst in nodules of *Cajanus cajan* (pigeonpea), *Phaseolus vulgaris* (common bean), *Vigna unguiculata* (cowpea), *Pisum sativum* (pea), *Sophora flavescens*, *Arachis hypogaea* (peanut), and *Medicago sativa* (alfalfa) was further determined to test its potential correlation with the types of nodule development (with either persistent or transient meristems) or the differentiation fate of bacteroids (with or without terminal differentiation). By using *Rhizobium leguminosarum* bv. *viciae* 3841 (Rlv3841) inducing indeterminate nodules on both *P. sativum* and *S. flavescens*, it was further uncovered that the differentiation fate of bacteroids is related to the observed differential expression of Pst in bacteroids. The significance of our findings was further discussed in the context of coevolution of two symbiotic partners.

RESULTS

Symbiotic defects of the *pst* mutant of *S. fredii* can be recovered by the deletion of *phoB*.

The *S. fredii* CCBAU45436 (abbreviated as SF45436) genome harbors genes encoding the conserved high-affinity Pi transporters PstSCAB and PhoCDET and an *orfA-pit* encoded low-affinity Pit transport system, which showed 91.82 to 97.42, 79.45 to 95.35, and 91.59 to 93.41% identity, respectively, to their homologous proteins of *Sinorhizobium meliloti* 2011 (Fig. 1). We had previously demonstrated an impaired symbiotic performance of the *pst* mutant of SF45436 on *G. max* but a mutant lacking the key regulator PhoB was still an efficient microsymbiont (Jiao et al. 2016). To determine whether PhoCDET or Pit also contribute to the symbiosis of SF45436 in *G. max* nodules, *phoC* and *pit* were individually deleted in the wild-type SF45436 and the *pst* mutant. The *phoC* and *pit* mutants were not significantly different from SF45436 regarding shoot dry weight and nodule fresh weight of inoculated *G. max* plants at 30 days postinoculation (dpi) (P values > 0.05, t test) (Fig. 2A and B). Like SF45436 and the *phoB* mutant, multiple bacteroids within each symbiosome can be found in nodule cells infected by the *phoC* and *pit* mutants (Fig. 2C). The shoot dry weight and nodule fresh weight of plants inoculated with double mutants *pst-pit* and *pst-phoC* were significantly lower than SF45436 (P values < 0.05, t test) (Fig. 2A and B). In contrast to the *pst* mutant, many bacteroids of irregular shape were observed for the *pst-pit* and *pst-phoC* mutants, and the number of bacteroids per symbiosome was increased in nodules infected by the *pst-phoC* mutant (mean $\pm$ standard error, 3.2 ± 0.2 compared with 1.7 ± 0.1 for the *pst* mutant; P value < 0.001, t test with 100 scored symbiosomes) (Fig. 2C), indicating a contribution of Pit and PhoCDET to rhizobial adaptation to nodule environments.

Because PhoB acts as a positive regulator for *pstSCAB* and *phoCDET* but represses the transcription of *pit* (Bardin and Finan 1998; Krol and Becker 2004; Yuan et al. 2006), *phoB-pst*, *phoB-phoC*, and *phoB-phoC-pst* mutants were constructed to test whether symbiotic defects associated with *pst* could be recovered. It turned out that these mutants were as effective as SF45436 when inoculated on the test *G. max* cultivar in terms of shoot dry weight and nodule fresh weight (P values > 0.05, t test) (Fig. 2A and B). The morphology or the number of bacteroids per symbiosome were partially recovered in the *phoB-*

pst and *phoB-phoC-pst* mutants compared with *pst* and *pst-phoC* (Fig. 2C). To further verify the PhoB-dependent regulation of *pstSCAB*, *phoCDET*, and *pit* in nodules, an *lacZ* translational reporter lacking its start codon was fused after the promoter-start codon region of *pstS*, *phoC*, or *pit*, and plasmids carrying these *lacZ* reporters were individually conjugated into SF45436 and the *phoB* mutant. Within *G. max* nodules collected at 30 dpi, *pstS::lacZ* and *phoC::lacZ* were highly expressed in SF45436 while significantly downregulated in the *phoB* mutant (Fig. 3). However, *pit::lacZ* was much more highly expressed than *pst::lacZ* and *phoC::lacZ* in nodules infected by the *phoB* mutant (Fig. 3).

Deletion of *phoB* can fully recover growth defects of the *pst* mutant under excess but not limiting Pi conditions.

The mutants *pst*, *pst-pit*, and *pst-phoC* exhibiting an impaired symbiotic performance on *G. max* were further compared with the wild-type SF45436 regarding the growth rate in minimum medium supplied with a limiting (0.1 mM) (Fig. 4A) or excess (2 mM) (Fig. 4B) level of Pi. The *pst-phoC* double mutant lacking high-affinity Pi transporters showed the most serious delay of growth under both conditions. The *pst* and *pst-pit* mutants were indistinguishable from each other and grew more slowly than SF45436 but faster than the *pst-phoC* mutant under two conditions. The growth defect of the *pst* mutant under the excess Pi condition can be fully recovered by deleting *phoB* (Fig. 4B). Under the limiting Pi condition, the growth rate of the *phoB-pst* mutant was similar to SF45436 till the late exponential phase but slowed down at later stages (Fig. 4A), implying that the upregulated low-affinity Pi transporter Pit could only partially recover the growth defect of the *pst* mutant under the limiting Pi condition.

Relative expression level of *pst* correlates with differentiation fates of bacteroids but not nodule development.

The contrasting findings on the role of high- and low-affinity Pi transporters obtained in *S. meliloti*–*Medicago sativa* (Yuan et al. 2006) and *S. fredii*–*G. max* symbioses reminded us of their differences in nodule development and morphotype of bacteroids. To investigate whether bacteroids can be Pi limited or not in a manner depending on the type of nodule development (indeterminate versus determinate) or bacteroid differentiation (with or without terminal differentiation), the relative transcription levels of *pstS* homologs were measured in nodules of various representative symbioses using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (Fig. 5).

The *pstS* of SF45436 was expressed at similar levels in determinate nodules of *G. max* and indeterminate nodules of *C. cajan*. The *pstS1* of *R. tropici* CIAT899^T (Martínez-Romero et al. 1991) and *pstS* of *Sinorhizobium* sp. NGR234 (Pueppke and Broughton 1999) were actively transcribed in determinate nodules of *Phaseolus vulgaris* and *V. unguiculata*, respectively, while the *pstS* of *S. meliloti* 1021 (Galibert et al. 2001)

Fig. 1. Gene clusters encoding PstSCAB, PhoCDET, and Pit involved in phosphate transport in representative rhizobia. Tnp = transposase.

expressed at a relatively lower level in *M. sativa* indeterminate nodules (Fig. 5). *Bradyrhizobium arachidis* CCBAU051107^T can induce determinate nodules on *A. hypogaea* and indeterminate nodules on *Sophora flavescens* (Jiao et al. 2015; Wang et al. 2013). The transcription level of *pstS* gene of CCBAU051107^T was significantly higher in nodules of *S. flavescens* than in those of *A. hypogaea* (*P* value < 0.001, *t* test). *R. leguminosarum* bv. *viciae* strains USDA2370^T and R3841 can form indeterminate nodules on *Pisum sativum* and *S. flavescens* (Jiao et al. 2015; Young et al. 2006), and their *pstS* homologs were transcribed at a level significantly higher in *S. flavescens* than that in *P. sativum* (*P* values < 0.001, *t* test). The relative transcription levels of *pstS* homologs were apparently unrelated to the type of nodule development. Instead, as indicated in Figure 5, they correlated with the fate of bacteroids (i.e., actively transcribed in nonterminally differentiated bacteroids while expressed at a lower level in terminally differentiated ones).

Rlv3841 bacteroids are Pi limited in *S. flavescens* nodules but not in *P. sativum* nodules.

To get more insight into the host-dependent modulation of the expression of the high-affinity Pi transporter Pst, *S. flavescens* and *P. sativum*, forming indeterminate nodules

with their common microsymbiont Rlv3841, were subject to further investigation. The *lacZ* reporter lacking its start codon was fused after the *pstS* promoter-start codon region of Rlv3841, and the resulting *pstS::lacZ* translational reporter

Fig. 3. Sections of soybean nodules stained for β-galactosidase activity. Nodules were collected 30 days postinoculation from plants inoculated with derivatives of the wild-type SF45436 and the *phoB* mutant harboring promoter *lacZ* fusions, including *pstS::lacZ*, *phoC::lacZ*, and *pit::lacZ*, and the pGD926 lacking a promoter for *lacZ*.

Fig. 2. Symbiotic performance of SF45436 and its derivatives on soybean. **A**, Shoot dry weight and **B**, nodule fresh weight per plant inoculated with SF45436 strains (30 plants from three independent experiments were scored). Significant difference between the mean of each treatment and the corresponding value of the wild-type SF45436 is indicated (*t* test, * *P* value < 0.05). Error bars represent standard error of the means. **C**, Transmission electron microscopy pictures of 80-nm thin section of soybean nodules (30 days postinoculation) formed by the wild-type SF45436 and its derivatives. Scale bar indicates 2 μm. Arrows point to symbiosome membrane.

plasmid was conjugated into Rlv3841, allowing monitoring the expression level of *pstS* in vivo. At 40 dpi, the active expression of *pstS* was observed throughout the nodule section of *S. flavescens* (Fig. 6A). By contrast, *P. sativum* nodule cells generally exhibited a lower level of *pstS* expression, though those proximal infected cells corresponding to the senescent region of nodule had a relatively high β -galactosidase activity

Fig. 4. Growth of SF45436 and its derivatives in minimal medium with limiting and excess inorganic phosphate (Pi). Growth in minimal medium with **A**, 0.1 and **B**, 2 mM Pi. OD₆₀₀ = optical density at 600 nm. Each symbol indicates the mean of three cultures, and error bars indicate standard errors. Similar results were obtained in two independent experiments.

(Fig. 6A). The qRT-PCR measurement of the transcription of *pstS*, *phoC*, and *pit* in free-living cultures of Rlv3841 under either limiting (0.1 mM) or excess (2 mM) Pi conditions demonstrated that *pstS* of Rlv3841 can be highly induced upon Pi starvation (Fig. 6B).

The legume nodule has been demonstrated to be a strong phosphorus sink compared with other organs under phosphorus-deficient conditions (Suliman and Tran 2015). Here, the total phosphorus content of nodules, roots, or shoots was determined for *S. flavescens* and *P. sativum* plants grown under conditions supplied with 0, 1, or 2 mM Pi (Fig. 6C). No detectable difference between two legume species in the phosphorus content of nodules could be observed under conditions with 0 or 1 mM Pi. When 2 mM Pi was supplied, a notable increase of phosphorus content in nodule, root, and shoot was detected for two legume species compared with plants grown under conditions of lower Pi levels (Fig. 6C). *P. sativum* contained a higher concentration of phosphorus than *S. flavescens* in nodules and roots under the 2 mM Pi condition (P values < 0.001, t test) (Fig. 6C), indicating a higher Pi utilization efficiency by *P. sativum*. Moreover, a significant increase in shoot dry weight was observed under the 2 mM Pi condition compared with the 0 mM Pi condition for *P. sativum* (P value < 0.001, t test) but not for *S. flavescens* (P value > 0.05, t test).

The size of Rlv3841 bacteroids associated with *P. sativum* but not those associated with *S. flavescens* was significantly larger than that of free-living cells (P value < 0.001, t test) (Fig. 6D). The number of CFU within *P. sativum* nodules was less than 5% of the number of CFU within *S. flavescens* nodules,

Fig. 5. Expression of *pstS* in bacteroids with and without terminal differentiation. Quantitative reverse-transcription polymerase chain reaction analysis of relative transcription levels of *pstS* versus 16S ribosomal RNA (rRNA) gene. The *pstS2* of *R. tropici* CIAT899^T was expressed at a basal level and only the result of *pstS1* is shown herein. Means were based on nodule samples prepared from three independent inoculation experiments. Error bars represent standard error of mean, and significant difference is indicated (t test, ***, P < 0.001). The y-axis is presented in two segments of different major ticks intervals (left segment = 0.01 and right segment = 0.0005).

either counted per nodule (Fig. 6E) or normalized by nodule fresh weight (mean $\pm$ standard error, 0.32 ± 0.09 and 8.76 ± 2.62 million CFU/mg in *P. sativum* and *S. flavescentis* nodules, respectively) (P values < 0.01 , t test on $\log_{10}$ -transformed data).

To compare whether the *pstS* mutant of Rlv3841 would perform differentially within nodule environments of *S. flavescentis* and *P. sativum*, ultrathin sections of 40-dpi nodules induced by the *pstS* mutant and the wild-type Rlv3841 were obtained (Fig. 6F). The *pstS* mutant was indistinguishable from the wild-type Rlv3841 in nodule cells of *P. sativum*, forming enlarged bacteroids. By contrast, the number of bacteroids harbored by each symbiosome membrane was significantly lower in *S. flavescentis* nodules induced by the *pstS* mutant compared with that in nodules formed by the wild-type Rlv3841 (P value < 0.001 , t test) (Fig. 6F and G), indicating impaired adaptation of the *pstS* mutant. Under the test conditions, no significant differences between the *pstS* mutant and the wild-type Rlv3841 could be detected regarding the shoot dry weight of inoculated *S. flavescentis* and *P. sativum* plants (P values > 0.05 , t test).

DISCUSSION

It was proposed that root nodule bacteria are not Pi limited based on the findings obtained in *Sinorhizobium meliloti*–*Medicago sativa* symbiosis (Udvardi and Poole 2013; Yuan et al. 2006). By contrast, this study uncovered that symbiotic

bacteroids can be Pi limited in *G. max* nodules. However, the host-dependent expression profiles of the high-affinity Pi transporter *Pst*, sensing the extracellular Pi level, were independent of the type of nodule development (i.e., determinate or indeterminate). More generally, *Pst* was expressed lower in terminally differentiated bacteroids than in those without terminal differentiation. This was further highlighted by the expression pattern of Rlv3841 *Pst* across longitudinal nodule sections of *P. sativum* and *Sophora flavescentis*, both of which form indeterminate nodules but show different Pi use efficiency. Moreover, the *pstS* mutant of Rlv3841 was impaired in adaptation to nodule environment of *S. flavescentis* but not to that of *P. sativum*. These findings shed new light on our understanding of Pi homeostasis of bacteroids within legume nodules.

Inductions of strong phosphorus sink in nodules have been reported for different legumes, including *M. sativa*, *M. truncatula*, *G. max*, and *Phaseolus vulgaris*, under phosphorus-deficient conditions (Kouas et al. 2005; Sa and Israel 1991; Sulieman et al. 2013). This was further supported by this study of phosphorus content in different organs of *Pisum sativum* and *S. flavescentis* plants (i.e., approximately twofold higher phosphorus concentration can be observed in nodules compared with roots and shoots under 0 mM Pi conditions). Although there was no significant difference between *P. sativum* and *S. flavescentis* in their total nodule phosphorus content under 0 and 1 mM Pi conditions, the expression patterns of Rlv3841 *pstS::lacZ* in nodules

Fig. 6. Rlv3841 bacteroids in nodules of *Sophora flavescentis* and *Pisum sativum*. **A**, Longitudinal sections of nodules stained for β -galactosidase activity. Nodules were collected 40 days postinoculation from *P. sativum* and *S. flavescentis* inoculated with Rlv3841 harboring the promoter fusion *pstS::lacZ*. **B**, Quantitative reverse-transcription polymerase chain reaction analysis of transcriptional changes of *pstS*, *phoC*, and *pit* genes of the wild-type Rlv3841 in the minimum medium with limiting inorganic phosphate (Pi) (0.1 mM Pi) compared with the culture with excess Pi supply (2 mM Pi). The means of Log2R were based on results of three cultures. **C**, Total phosphorus content of nodules, roots, and shoots from *S. flavescentis* and *P. sativum* plants grown in the vermiculite containing 2, 1, and 0 mM KH_2PO_4 . Means were obtained from three to six independent experiments. **D**, Size of bacteroids and free-living cells of Rlv3841 (100 bacteroids or free-living cells were scored). **E**, Million CFU per nodule (three nodules were used in each experiment, and the mean of three independent experiments is shown). **F**, Transmission electron microscopy pictures of 80-nm thin section of nodules (40 days postinoculation) formed by the wild-type Rlv3841 and the *pstS* mutant. Scale bar indicates 2 μm . **G**, Number of bacteroids per symbiosome in *S. flavescentis* nodules (84 symbiosomes were scored for each treatment). Error bars represent standard error of mean, and significant difference is indicated (t test, * and *** indicate $P < 0.05$ and 0.001 , respectively).

collected under 1 mM Pi conditions suggested that the *P. sativum* nodule has a relatively lower Pi concentration in its proximal infected cells than other infected cells (Fig. 6A). Moreover, it has been shown that phosphorus in *G. max* nodules was higher in the infected region than in the cortical region but most heavily accumulated in the closely associated root cortex cells (Mizukoshi et al. 1995; Ohyama 2017). Therefore, the level of phosphorus content can be heterogeneous in the well-organized nodule structure.

In the model system of *Sinorhizobium meliloti*–*Medicago sativa*, the low-affinity Pi transporter Pit was highly expressed in nodules formed by the wild-type *S. meliloti* 2011 or its *phoB* mutant, whereas very little expression of PstSCAB and PhoC-DET was detected (Yuan et al. 2006). By contrast, within *G. max* nodules induced by SF45436, high expression levels of Pst and Pho but limited Pit expression were observed. Reverse genetics suggested that any one of three Pi transporters was sufficient for effective symbiosis of *S. meliloti* with *Medicago sativa* (Yuan et al. 2006), while the *pst* mutant rather than the *pit* or *phoC* mutant of SF45436 was symbiotically defective on *G. max*. That is to say, three Pi transporters of rhizobia were complementary within *Medicago* nodules but not within *G. max* nodules. Therefore, it is reasonable to conclude that *S. meliloti* bacteroids are not Pi-limited in *Medicago* nodules (Yuan et al. 2006) but a subsequent general proposal that root nodule bacteria are not Pi limited and the Pit is the main system in planta (Udvardi and Poole 2013) should be updated.

Despite the differences between *S. meliloti* and *S. fredii* in the expression profiles of Pi transporters in nodules, the PhoB-dependent activation of Pst or Pho expression and repression of Pit is conserved in these two species, as demonstrated in earlier studies of *S. meliloti* (Bardin and Finan 1998; Bardin et al. 1998; Voegelé et al. 1997; Yuan et al. 2006) and herein for *S. fredii*. Moreover, derepressing Pit by the mutation of *phoB* can be hypothesized to restore the symbiotic defects (shoot dry weight) of the *pst-phoC* mutant of *S. meliloti* (Bardin and Finan 1998; Yuan et al. 2006) and the *pst* or *pst-phoC* mutants of *S. fredii*. However, the growth defects under the limiting Pi condition and the reduced number of bacteroids per symbiosome for the *pst* mutant of *S. fredii* could not be fully recovered in the *phoB-pst* double mutant. This is in line with the evidence that Pst is a high-affinity, high-velocity Pi transporter, compared with the nonspecific Pho and Pit systems (Voegelé et al. 1997; Yuan et al. 2006). Although Pst is expressed lower than Pit to avoid toxic levels of Pst-mediated Pi accumulation under excess Pi conditions (diCenzo et al. 2017), accumulating data show that Pst rather than Pit serves as the primary Pi transporter when Pi is in excess (Hsieh and Wanner 2010; McCleary 2017). This view is further supported by a similar growth rate of *pst* and *pst-pit* mutants of SF45436 in the medium supplied with 2.0 mM Pi (Fig. 4B). The slower growth rate of the *pst-phoC* mutant than the *pst* mutant indicated that PhoCDET also contributes to the adaptation to excess Pi conditions. It should be noted that loss-of-function mutations in the *pst* genes can lead to the constitutive phosphorylation of PhoB and subsequent induction of Pho regulon, including *phoCDET* and repression of *pit* (Hsieh and Wanner 2010; Yuan et al. 2006). However, *pit* can be deregulated in the *phoB-pst* mutant, which exhibited a similar growth rate compared with the wild-type SF45436 under the Pi excess condition.

Contrasting expression patterns of the high-affinity Pi transporter Pst in determinate nodules of *G. max* and indeterminate nodules of *Medicago sativa* inspired us to further quantify *pst* transcription levels in nitrogen-fixing bacteroids within indeterminate nodules of *C. cajan*, *Sophora flavescens*, and *P. sativum*, or within determinate nodules of *Phaseolus vulgaris*, *V. unguiculata*, and *A. hypogaea*, despite potential

variation in their nodulation dynamics. The transcription profiles of *pst* were independent of whether the nodule is determinate or indeterminate, and correlated well with differentiation types of bacteroids (either terminally or nonterminally differentiated) reported earlier for these legumes (Oono et al. 2010). These transcriptional patterns of *pst* are supported by transcriptomic data collected for bacteroids within nodules of *G. max* (Jiao et al. 2016; Pessi et al. 2007), *P. vulgaris* (Vercruyssen et al. 2011), *V. unguiculata* (Li et al. 2013), and *Leucaena leucocephala* (Li et al. 2013). In these transcriptomes of nonterminally differentiated bacteroids, the high-affinity Pi transporter Pst rather than Pit was significantly upregulated in bacteroids compared with free-living cultures. Notably, *L. leucocephala* nodules are indeterminate ones harboring a consistent meristem similar to those of *Medicago sativa* (Li et al. 2013), in contrast to those determinate nodules with a transient meristem. Different transcriptional profiles of *pst* in bacteroids with either terminal or nonterminal differentiations (Fig. 5) are also supported by expression patterns of *pstS::lacZ* reporters in nodules of *G. max* (Fig. 3), *S. flavescens* (Fig. 6), and *Pisum sativum* (Fig. 6) or a *pstS::gusA* reporter in *Medicago* nodules (Yuan et al. 2006). These findings constitute an indication of the correlation between Pi homeostasis and differentiation of bacteroids.

Terminally differentiated bacteroids, characterized by an irreversible loss of cell division ability, genome amplification, and enlarged cell size, are modulated by host-derived NCR peptides such as those found in the model system *Sinorhizobium meliloti*–*Medicago* spp. (Mergaert et al. 2006; Van de Velde et al. 2010; Wang et al. 2010). Notably, it has been reported that many prokaryotes, including *Shewanella* and *Vibrio* spp., can use extracellular DNA as the sole phosphate source (McDonough et al. 2016; Pinchuk et al. 2008). The ploidy level of *Haloferax volcanii* and *Synechocystis* sp. PCC 6803 was elevated at higher phosphate concentrations while a rapid reduction in genome copy number was observed during the growth under limited phosphate conditions (Zerulla et al. 2014, 2016). These findings could raise intriguing hypotheses regarding the contribution of phosphate homeostasis to the ploidy level of bacteroids in legumes such as *Medicago sativa* and *P. sativum*, though nodule environment is much more complex than free-living cultures. On the other hand, host legumes such as *G. max* and *Sophora flavescens* may restrict phosphate supply to avoid overproliferation of bacteroids without terminal differentiation in nodules.

Host-dependent morphologies of bacteroids such as swollen, elongated, spherical, and elongated-branched correlated well with the number and type of symbiotic NCR peptides (Czernic et al. 2015; Montiel et al. 2017). Moreover, CFU of rhizobial populations isolated from nodules have been demonstrated to be an effective indicator for the relative reproductive ability of bacteroids with either terminal or nonterminal differentiations (Montiel et al. 2016). However, NCR-induced differentiation and survival of rhizobia are strongly affected by rhizobial surface polysaccharides (Montiel et al. 2017), the structures of which can be different among diverse rhizobial species (Downie 2010). To avoid the influence of differences in rhizobial strains, Rlv3841 bacteroids within nodules of *S. flavescens* and *P. sativum* were compared in this study. Reproductive ability was significantly higher for Rlv3841 bacteroids from nodules of *S. flavescens* than those from *P. sativum*. This is in line with the evidence that Rlv3841 bacteroids have complete metabolic dependence on *P. sativum* and behave like organelles (Prell et al. 2009). The stronger induction of high-affinity Pi transporter Pst in Rlv3841 bacteroids in *S. flavescens* than in *P. sativum* may represent one of the largely unexplored adaptive routes, which are crucial for the persistence of bacteroids without terminal differentiation in nodules, as experimentally demonstrated using

the *pstS* mutant of Rlv3841 and the *pst* mutant of *Sinorhizobium fredii* associated with *G. max*. Notably, the impaired adaptation of the *pstS* mutant in *Sophora flavescens* nodules didn't lead to a significant decrease of symbiotic efficiency regarding shoot dry weight of inoculated plants under test conditions. Although the underlying mechanisms remain unexplored, it has been demonstrated that the Pho regulon involved in phosphate starvation adaptation is upregulated in the *pst* mutant of diverse bacteria (Hsieh and Wanner 2010; Yuan et al. 2006). This regulon includes genes encoding transporters for secondary phosphorus sources such as phosphonates (*phoCDET*) and organophosphates (*ugpBAECQ*). There could be some variation in the pool of secondary phosphorus sources in nodules of different legumes where phosphate-starved bacteroids without terminal differentiation may perform differentially in symbiotic efficiency.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

E. coli strains, rhizobia, and plasmids used in this study are listed in Supplementary Table S1. Tryptone-yeast (TY) medium (tryptone at 5 g liter⁻¹, yeast extract at 3 g liter⁻¹, and CaCl₂ at 0.6 g l⁻¹), or minimum medium (MOPS at 10 g/liter, mannitol at 10 g/liter, sodium glutamate at 3.55 g/liter, 1 mM MgSO₄, 250 μM CaCl₂, FeCl₃ at 10 mg/liter, H₃BO₃ at 3 mg/liter, MnSO₄·4H₂O at 2.23 mg/liter, biotin at 1 mg/liter, ZnSO₄·7H₂O at 0.3 mg/liter, NaMoO₄·2H₂O at 0.12 mg/liter, and CoCl₂·6H₂O at 0.065 mg/liter; pH 7.2) (Krol and Becker 2004) containing 0.1 mM Pi or 2 mM Pi was used to grow rhizobial strains at 28°C. Luria-Bertani medium was used to grow *E. coli* strains at 37°C. The concentrations of antibiotics used were 30 μg/ml for nalidixic acid, 10 μg/ml for trimethoprim, 50 μg/ml for kanamycin, 30 μg/ml for gentamycin (Gm), and 10 μg/ml for tetracycline.

Construction of the deletion mutants and the *lacZ* reporters.

The primers used in this study are shown in Supplementary Table S2. The plasmid pRK2013 (Ditta et al. 1980) was used as the helper plasmid in all conjugation experiments. All cloned fragments harbored by engineered plasmids described below were verified by Sanger sequencing, and the resultant rhizobial mutants were confirmed by PCR.

The SF45436 mutants $\Delta pstSCAB::Gm$ and $\Delta phoB::Gm$ had been constructed previously using the *cre-lox* system (Jiao et al. 2016; Marx and Lidstrom 2002). To obtain the *phoB-pst* double mutant, the Gm resistance gene flanked by two *loxP* sites in the $\Delta phoB::Gm$ mutant was removed by introducing the plasmid pCM157 expressing Cre recombinase (Marx and Lidstrom 2002). Briefly, pCM157 was introduced into the *phoB* mutant by conjugation using pRK2013 as the helper plasmid. Transconjugants sensitive to Gm were further screened for pCM157-cured strains, which are tetracycline sensitive. The *pstSCAB* operon was deleted in this $\Delta phoB$ mutant by introducing a pCM351 derivative containing PCR fragments encompassing the upstream N-terminal coding region and the downstream C-terminal coding region of the *pstSCAB* operon amplified using *pstUL-pstUR* and *pstDL-pstDR* primers. To construct the *phoB-phoC* double mutant, the *phoC* coding region in the $\Delta phoB$ mutant was inactivated by site-specific insertion mediated by a pVO155 (Oke and Long 1999) derivative harboring a *phoC* internal fragment amplified using *phoCF-phoCR* primers. To obtain the *phoB-phoC-pst* mutant, the *pstSCAB* operon in this *phoB-phoC* mutant was further deleted using the pCM351 derivative, as described above. To construct the *pst-pit* mutant, the *pit* gene in the $\Delta pstSCAB::Gm$ mutant was inserted by a pVO155 derivative containing *pit* internal fragment

amplified using primers *pitF* and *pitR*. The *pst-phoC* mutant was obtained by introducing the abovementioned pVO155 derivative harboring the *phoC* internal fragment into the $\Delta pstSCAB::Gm$ mutant. The single mutants *phoC* and *pit* were constructed by mobilizing the same pVO155 derivatives described above into the wild-type SF45436.

To construct the *pstS* deletion mutant of Rlv3841, a seamless cloning strategy was used to engineer a pJQ200SK (Quandt and Hynes 1993) derivative containing the PCR fragments encompassing the upstream N-terminal coding region and the downstream C-terminal coding region of *pstS* amplified using primers 3841pstUF-UR and 3841pstDF-DR. The primers 3841pstUF and 3841pstDR contained 5' sequences corresponding to two ends of pJQ200SK produced by *SmaI* digestion, and the primers 3841pstUR and 3841pstDF had reversely complemented 5' sequences of 20 bp in length. PCR fragments amplified using these two sets of primers and the *SmaI*-digested pJQ200SK were subject to seamless assembly using Master Assembly Mix (Clone Smarter) at 50°C for 15 min. Cooled products were transformed into *E. coli*, then mobilized into the wild-type Rlv3841 by conjugation. Gm-resistant transconjugants with a single crossover were subject to further sucrose counterselection for double recombinants using 5% sucrose.

To construct *lacZ* reporters with promoter regions of *pstS*, *phoC*, and *pit* from SF45436, or that of *pstS* from Rlv3841, the in-frame *lacZ* translational fusion plasmid pGD926 (Ditta et al. 1985) was used. PCR fragments amplified using primers GD45436pstSF-R, GD45436phoCF-R, GD45436pitF-R, or GD3841pstSF-R were individually assembled with the *BamHI-HindIII*-digested pGD926 through the seamless assembly procedure described above. The resultant derivatives of pGD926 were mobilized into wild-type SF45436, the *phoB* mutant, or wild-type Rlv3841 by conjugation.

Plant assays, CFU from nodules, and electronic microscopy.

Seed of legumes were surface sterilized in 3% (vol/vol) NaClO solution, allowed to germinate, and inoculated with 1 ml of culture of the indicated rhizobial strain at an optical density at 600 nm (OD₆₀₀) = 0.2. Plants were grown in vermiculite moistened with low-N nutrient solution in Leonard jars at 24°C with 12-h day and night cycles (Vincent 1970). At 30 dpi, plant shoots were dried at 65°C for 5 days; then, dry weight was determined. The number of rhizobial cells in nodules being able to form colonies was determined by plating the rhizobial population from 40 dpi nodules of *S. flavescens* and *P. sativum*. Three nodules were used in each experiment, and three independent experiments were performed.

Ultrathin sections of root nodules collected at 30 or 40 dpi as indicated were prepared using a Leica Ultracut C6i and observed in a JEM-1230 transmission electron microscope (TEM), as described earlier (Li et al. 2013). The size of Rlv3841 bacteroids in *P. sativum* and *S. flavescens* were scored from 100 bacteroids. To determine the number of Rlv3841 or *pstS* bacteroids per symbiosome in *S. flavescens* nodules, 84 symbiosomes from seven TEM views were scored. Similarly, the number of *Sinorhizobium fredii* bacteroids per symbiosome in *G. max* nodules was scored from 100 symbiosomes.

Expression of *lacZ* reporters in nodules.

Plants of *G. max*, *P. sativum*, and *Sophora flavescens* were inoculated with corresponding rhizobial strains carrying the pGD926 derivatives, which express *pstS::lacZ*, *phoC::lacZ*, or *pit::lacZ* reporter gene fusion. Nodules were collected at 30 or 40 dpi as indicated, and fixed with 2% (vol/vol) glutaraldehyde in Z' buffer (0.1 M potassium phosphate buffer, pH 7.4; 1 mM

MgSO₄; and 10 mM KCl). Then, 80-μm-thick longitudinal nodule sections were prepared using a vibrating-blade microtome (VT1000S; Leica), stained for 1 h at 28°C, and observed under a light microscope.

qRT-PCR.

For bacteroid samples, nodules collected at 30 dpi were subject to RNA extraction using the QIAGEN RNeasy mini kit. For free-living cultures, rhizobial cells were first cultured in TY medium, then inoculated, at a starting density of OD₆₀₀ = 0.05, into minimal medium containing either 0.1 or 2 mM Pi. Cultures were collected at midlog phase (OD₆₀₀ = approximately 0.6 to 0.7), and RNA was then extracted using the QIAGEN RNeasy mini kit according to the manufacturer's instructions. RNA quality was assessed by using Nanophotometer Pearl. Single-strand cDNA was synthesized by using the StarScript II First-Strand cDNA Synthesis Mix (Genstar). qPCR was performed by using 2× RealStar Green Mixture (Genstar) and LightCycler 480 II. The mean of crossing point values for the control gene 16S ribosomal RNA was used as the reference for normalization of gene expression, as described earlier (Li et al. 2013). Three independent biological replicates were analyzed in all qRT-PCR experiments.

Phosphorus content determination.

Seed of *P. sativum* and *S. flavescentis* were surface sterilized as mentioned above, then inoculated with Rlv3841. Plants were grown in vermiculate moistened with low-N nutrient solution containing 2, 1, or 0 mM KH₂PO₄. After 40 dpi, plant nodules, roots, and shoots were collected and dried at 65°C for 5 days. These dry samples were ground into powder, 0.2 to 0.3 g of which was then weighed and placed in a digestion vessel of the CEM Microwave Accelerated Reaction System (CEM-MARS-6; CEM Corporation) with 8 ml of concentrated nitric acid. Overnight samples were digested at 160°C till the volume reached 2 ml. The cooled samples were diluted with water to 50 ml, the phosphorus content of which was analyzed using a Thermo-iCAP6300 inductively coupled plasma radial spectrophotometer (Thermo Fisher Scientific) at National Energy R&D Center for Biomass, China Agricultural University, Beijing, China. Three to six replicated biological samples were determined.

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