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Original Article

Identification of a NodD repressible gene adjacent to *nodM* in *Rhizobium leguminosarum* biovar *viciae*

Xiao'er Yang†, Bihe Hou†, Chenzhi Zong, and Guofan Hong*

State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
†These authors contributed equally to this work.
*Correspondence address. Tel: +86-21-54921223; Fax: +86-21-54921223; E-mail: gfhong@sibs.ac.cn

The *nodFEL* and *nodMNT* operons in *Rhizobium leguminosarum* biovar *viciae* are transcribed in the same orientation and induced by NodD in response to flavonoids secreted by legumes. In the narrow intergenic region between *nodFEL* and *nodMNT*, we identified a small gene divergently transcribed from *nodM* to the 3′ end of *nodL*. Unlike the promoters upstream of *nodF* and *nodM*, the promoter of this gene is constitutively expressed. It appeared that its promoter might partially overlap with that of *nodM* and its expression was repressed by *nodD*. A deletion mutation was made and proteins produced by the mutant were compared with those by wild-type using 2D gel electrophoresis. Several protein differences were identified suggesting that this small gene influences the expression or stability of these proteins. However, the mutant nodulated its host plant (pea) normally.

Keywords *nodM*; *px3*; 2D gel electrophoresis; protein difference

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Introduction

In nitrate-poor soil, the symbiosis between rhizobia and leguminous plants leads to the development of nitrogen-fixing nodules, in which atmospheric nitrogen is fixed. Molecular signal transduction between rhizobia and their hosts is required for nodule development and root infection by rhizobia. Flavonoids secreted from legume roots induce the expression of nodulation (*nod*) genes. The products of several genes are involved in synthesis of Nod factors, which are chitin oligomers of four or five glucosamine residues carrying various substitutions including an N-linked acyl group, can induce nodule morphogenesis and are required for rhizobial entry into root hairs [1].

In *Rhizobium leguminasorum* biovar *Viciae*, there are 13 *nod* genes clustered together on large indigenous symbiosis plasmids and they lie in five transcription units (five operon), *nodD*, *nodABCIJ*, *nodFEL*, *nodMNT*, and *nodO* [2–5]. The promoters of the *nodABCIJ*, *nodFEL*, *nodMNT*, and *nodO* operons contain a highly conserved DNA motif (47 bp *nod* box) with the palindromic structure ATC-N9-GAT, which is required for binding to NodD [3,6,7]. NodD binds flavonoids and induces the high expression level of other *nod* operons [2–5]. The *nodD* gene, which is transcribed divergently from *nodABCIJ* operon [3], is differently regulated compared with other *nod* operons, because it is constitutively expressed, although its expression is negatively auto-regulated [8,9].

Regulation of the *nod* operons is not mediated exclusively by NodD in *R. leguminasorum* bv. *viciae*. For example, the *nolR* gene encoded a repressor that decreased the expression of the *nod* operons [10]. In addition, *px2*, a small gene divergently transcribed from the *nodFEL* operon specifically up-regulated the inducible expression of *nodF* [11].

In this study, a small gene (*px3*) divergently transcribed from *nodM* was identified in the intergenic region between *nodFEL* and *nodMNT*. This gene was mutated and the effects of the mutation on nodulation and the profile of produced proteins were tested by 2D gel electrophoresis. Results showed that the protein pattern was altered compared with that of wild type, which indicated that *px3* was functional, without effect on nodulation.

Materials and Methods

Bacterial strains and construction of plasmids

Bacterial strains and plasmids are listed in **Table 1**. Media and general growth conditions were used as described previously [8]. To make a deletion mutation in *px3*, the gene within a 5.7-kb region was amplified by polymerase chain reaction (PCR), using the primers 5′-CTCGCGGCCGCGGGGTTTAACTTGATCCCGCCATTGC-3′ and 5′ACTGCGGCCGCATTGGAGACAGTTTTATCGAAGAACCG-3′.

Table 1 Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
3841	*Rhizobium leguminosarum* bv. *viciae* Smr derivative of strain 300	[12]
3841ΔPX3	PX3 mutant in *Rhizobium* 3841	This work
A34	*Rhizobium* 8401 containing pRL1JI (pSym)	[13]
A34ΔPX3	PX3 mutant in *Rhizobium* A34	This work
8401	*Rhizobium leguminosarum* cured of its symbiotic plasmid; Str^r (wild type)	[14]
S17-1	*recA pro hsdR* (RP4-2 Tc::Mu Km::Tn*7*)	[15]
DH5α	recA1, Φ180 *lacZ*ΔM15	Promega
BW25113(pIJ790)	*ΔaraBAD*, *ΔrhaBAD*, λ-RED (*gam*, *bet*, *exo*), *cat*, *araC*, *rep101*^{ts}	[16]
DH5α(pRK2013)	IncColE1, helper plasmid for tripartite mating	[14]
Plasmids		
pKT230	IncQ broad-host-range plasmid; Kan^r	[17]
pKT230-nodD	1.2 kb *Bcl*I fragment containing an intact *nodD* gene from pRL1JI cloned into pKT230	[18]
pMP220	IncP broad-host-range plasmid with promterless *lacZ*; Tc^r	[3]
pMP221	Opposite multi-cloning site; pMP220 derivative; Tc^r	[19]
pMP221M	pMP221 containing *nodM* promoter	This work
pMP220px3	pMP220 containing *px3* promoter	This work
SpuerCos1	*bla*, *neo*, *cos*	Stratagene
SpuerCos1-nod5k	5.7 kb nod fragment cloned into *Not*I site of SpuerCos1	This work
SpuerCos1ΔPX3	SpuerCos1-nod5k with *px3* fragment replaced by antibiotic apramycin	This work

The PCR product was digested with *Not*I, and cloned into SuperCos1 (Stratagene, Cedar Creek, USA) to generate a plasmid called SuperCos1-5K. Then *px3* gene was replaced by the apramycin-resistance gene using the redirect protocol [16]. The apramycin-resistance gene obtained by amplifying the apramycin-resistance cassette by PCR, using primer F 5′-CACTCGGGTTGCGTCGATTAGACGTGTAGGCAGC GCATGATTCCGGGGATCCGTCGACC-3′ and primer R 5′-CATGTTTGCATGCAAAGGGGCAACTACGCCTTA GGGTCATGTAGGCTGGAGCTGCTTC-3′, which contain DNA complementary to the ends of an apramycin-resistance cassette [16] and to the DNA flanking the *px3* gene **[Fig. 1(C)]**, respectively. *Escherichia coli* strain BW25113(pIJ790) was transformed with SuperCos1-5K carrying *px3* gene and the resulting strain was then transformed using the linear amplified apramycin-resistance cassette [16]. The resulting plasmid was then isolated, introduced into *E. coli* DH5α and conjugated into *R. leguminosarum* bv. *viciae* strain A34 by triparental conjugation [14]. Apramycin and streptomycin-resistant colonies were checked for lack of kanamycin resistance to identify double crossover events. The mutation was confirmed by PCR. One such mutant was called A34ΔPX3. The mutation was then transduced into strain 3841 by transduction [20] using bacteriophage RL38 propagated on strain A34ΔPX3 making selection for apramycin resistance, and the resulting transductant was called 3841ΔPX3.

To generate reporter plasmids with *lacZ* under the control of the *nodM* and *px3* promoters, a 340-bp DNA fragment spanning the intergenic region and 5′ ends of *nodM* and *px3* was obtained by PCR, using primer E 5′-TCGGAATTCGTAGCAACGCCGGATGAATCATAG-3′ and primer P 5′-AAACTGCAGCCCACGCCCCGGTC GTAG-3′, which contained *Eco*RI and *Pst*I sites, respectively, and annealed to the 5′ ends of the coding regions of *nodM* and *px3* as indicated in **Fig. 1(B)**. The PCR product was digested with *Eco*RI and *Pst*I, and cloned into pMP221 and pMP220 to obtain pMP221M and pMP220PX3. pMP221M contained *lacZ* under the control of the *nodM* promoter and pMP220PX3 contained *lacZ* under the control of the *px3* promoter. These two plasmids were separately conjugated into strain 8401 carrying *nodD* in pKT230 (pKT230-*nodD*) or the vector control lacking *nodD* (pKT230).

β-Galactosidase activity assay

Cells were grown in TY (trypticase-yeast extract) medium in the presence or absence of 10 μM naringenin. The assays for β-galactosidase activity were carried out as previously described [21,22]. Each assay was carried out in triplicate.

Protein extraction for gel electrophoresis

Bacterial cultures were grown at 28°C under aeration in TY medium (with a final concentration of 50 μg/ml streptomycin) for ~20 h up to stationary phase, and then 3 ml of the cultures

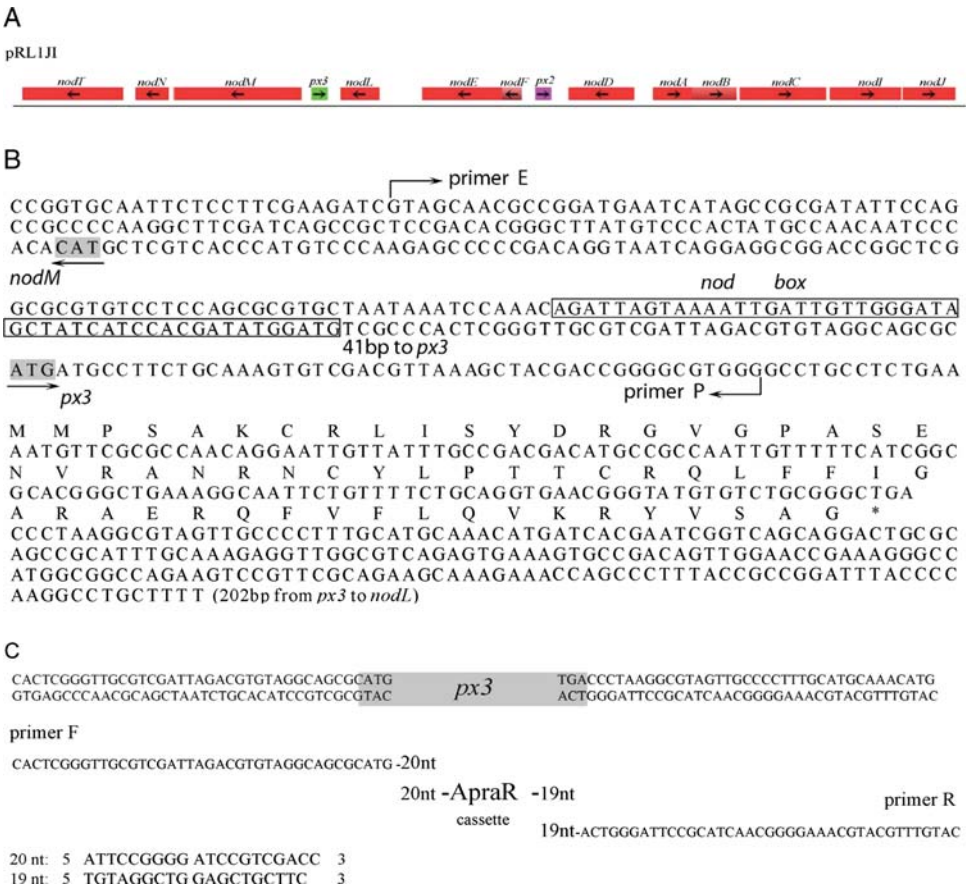

Figure 1 The location and sequence of *px3* (A) The location of *px3* in *nod* operons in pRL1JI. (B) *px3* and its flanking sequence. The deduced amino acids sequence for *px3* is below its DNA sequence. The start codons of *nodM* and *px3* were indicated by horizontal arrow and shadowed. The boxed regions are the highly conserved '*nod* box'. Primers E and P were indicated, which were used for cloning the promoters of *px3* and *nodM*. (C) Primers designed for making a complete deletion of *px3*. Primers F and R contain DNA complementary to the ends of an apramycin-resistance cassette and to the DNA flanking the *px3* gene.

were transferred into 300 ml fresh TY. The new cultures were grown until OD600 = 0.5 and the cells were harvested by centrifugation. The cell pellets were washed four times with rhizobium washing buffer (1.5 mM KH_2PO_4, 9 mM Na_2HPO_4, 3 mM KCl, and 68 mM NaCl, pH 7.5) [23]. They were then suspended in 6 ml of SDS sample solubilization buffer [1% (*w/v*) SDS, 100 mM Tris-HCl (pH 9.5)] for lysis and heated at 95°C for 10 min. Then 1.5 ml of cell lysate was transferred into microfuge tubes, which were centrifuged in an Eppendorf microfuge at maximum speed (14,000 *g*) for 30 min at 4°C. The protein concentration was determined with Bradford kit (Bio-Rad, Hercules, USA). The supernatant was then frozen in aliquots of 50 μl at −70°C. The extraction was diluted to 1 μg/μl with buffer E [2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 25 mM Tris-HCl, pH 8.5] before use [24].

2D gel electrophoresis

For some experiments, protein extracts were labeled with NHS-Cy5 [24]. This was carried out using 50 μl of the protein extract (1 μg/μl), which was diluted with buffer E to 350 μl, and 5 μl of 0.4 mM NHS-Cy5 [NHS-Cy5 dissolved in DMF (99.8%)] was added. The mixture was then put on ice and the reaction was carried out in the dark for 30 min. Then 1 μl of 10 mM lysine was added and the mixture was incubated on ice in the dark for 10 min to remove the excess NHS-Cy5. For other experiments, the proteins were not labeled with NHS-Cy5, but detected using Coomassie brilliant blue G-250.

For electrophoresis, dithiothreitol and pH 3–10 ampholyte to their final concentration of 2% for isoelectric focusing were added to the protein samples. Electrophoresis was carried out as described in the 2D instruction manual (Bio-Rad). In the first dimension, IPG gel strips (17 cm, linear pH 4–7; Bio-Rad) were used. In the second dimension, 12% SDS gels were used. After electrophoresis, the fluorescent gels were scanned by Fuji FLA-9000 (Tokyo, Japan) and the Coomassie blue stained gels were scanned by GS 800 Calibrated Densitometer (Bio-Rad). Images were analyzed by PDQuest software (Bio-Rad).

Quantitative RT-PCR

The transcription levels of three genes were analyzed using real-time reverse transcriptase (RT)-PCR using *ftsZ* as a control. The primers used were shown in **Table 2**.

Table 2 Primers used for quantitative RT-PCR

Gene	Primer sequence (5′→3′)
ftsZ1	CACCGTGTTCGGCGTTGGCGG
	CGCCGACCGTCAGGATACCC
pRL80142 (*trbB*)	CGAGCACCTTTGCATTGCCCTC
	TCGAAGCCTGAGCTTCGGTCATG
pRL120713	CGGCCTTGCCGCAAATCCGTC
	CGAAATCTTCGCGCTTGGCGATC
RL4089 (*ibpA*)	CCGGCTCTTCACCATGCTCGAC
	GGGGCTTCATGGCCTCGGG

For the PCR, the bacteria were grown as described for the protein analysis. Total 10 ml of cells were harvested and RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA). For the RT reaction, 4 μg of RNA was incubated with a mixture of random primers for 10 min at 95°C before addition of RT and incubation for 1 h at 42°C. The reaction was completed by incubation at 72°C for 10 min. A 0.2-μl aliquot from the RT reaction was used for PCR. Each reaction contained 10 μl of an SYBR® Premix Ex Taq™ Green I (TaKaRa, Dalian, China) in a final volume of 20 μl. PCR conditions are as follows: 95°C for 30 s, and then 40 cycles with steps of 95°C for 3 s, 60°C for 30 s ,68°C for 30 s. The reaction was run on an ABI 7500 and the data were analyzed using the ABI software (Foster City, USA).

Results

Identification of a potential gene divergently transcribed from *nodM*

In view of the observation that upstream of the *nodFEL* operon in *R. leguminosarum* bv. *viciae* there was a small divergently transcribed gene (*px2*), required for full induction of the *nodFEL* promoter [11], we analyzed the sequence upstream of *nodM* to determine whether there was an equivalent gene. We identified a 61-amino acid coding region that could correspond to a gene transcribed divergently from *nodM* [**Fig. 1(B)**]. The predicted translation site is only 41 bp from the end of the *nodM nod* box [**Fig. 1(B)**] suggesting that if this is an expressed gene, the *px3* and *nodM* promoters would be likely to overlap. Database searches of translated nucleotide sequences revealed that the predicted PX3 protein coding region was highly conserved in *R. leguminosarum* bv. *viciae* strains A34 and 3841, but there were no other homologs or strongly similar proteins in the database.

Px3 is expressed constitutively

To determine whether the expression of the *px3* gene is affected by *nodD*, plasmids expressing *lacZ* under the control of the *nodM* promoter (pMP221M) and *px3* promoter (pMP220PX3) were transferred into *R. leguminosarum* strain 8401, which lacked a symbiosis plasmid and hence all *nod* genes. To test the effect of *nodD* on *px3* expression, one derivative of 8401 carried *nodD* (pKT230-*nodD*), whereas the control (lacking *nodD*) carried the vector pKT230. Incubated in the presence and absence of the *nod* gene inducer naringenin, the expression of *lacZ* was assayed by measuring β-galactosidase activity. Firstly, it is clear that the region upstream of *px3* has strong *nodD*-independent promoter activity, because pMP220PX3 confers a high level of activity to strain 8401(pKT230) (**Fig. 2**). Unlike *nod* genes such as *nodM* (**Fig. 2**), the expression of the *px3* promoter was not increased by *nodD* and naringenin, but decreased by about one-third. This decreased expression required *nodD* but not naringenin and may be similar to the NodD-dependent repression of the *nodD* promoter [9], which is divergently transcribed from *nodABC* operon. A possible explanation is that the binding of NodD to the *nodM* promoter reduces the access of the transcription factor required for expression of *px3*. The expression level of *px3* in the absence of *nodD* is similar to the fully induced expression level of *nodM* in the presence of *nodD* and naringenin (**Fig. 2**). Therefore, we can conclude that *px3* is relatively strongly expressed, and that *nodD* reduces this expression.

Construction of a *px3* mutant and assays of its phenotype

To determine whether the *px3* gene conferred a phenotype to *R. leguminosarum* strains, we generated a deletion mutation in which the *px3* coding region was replaced by an antibiotic resistance cassette [**Fig. 1(C)**]. This mutation was

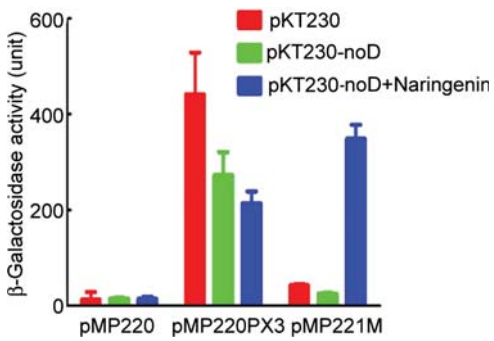

Figure 2 The promoter activity of *nodM* and *px3* Plasmid pMP221M contains *lacZ* under the control of the *nodM* promoter and plasmid pMP220PX3 contains *lacZ* under the control of the *px3* promoter. pKT230-*nodD* carried *nodD* and the vector control pKT230 lacked *nodD*. *Rhizobium* 8401 (pMP220), 8401 (pMP221M), and 8401 (pMP220*px3*), harboring pKT230 or pKT230-nodD were grown separately in TY medium at 28°C. β-Galactosidase activities were assayed as described at the absence (−) or presence (+) of 10 μM naringenin. All values were the means of three separate experiments and the error bars showed the standard deviations.

introduced by homologous recombination into strain A34 (the derivative of 8401 carrying *px3* on the indigenous symbiosis plasmid) and the mutation was then transduced into strain 3841, whose genome has been completely sequenced. The resulting mutants A34ΔPX3 and 3841ΔPX3 grew normally and had colony morphologies indistinguishable from the wild-type parents. Both mutants were inoculated onto peas, but the mutation had no effect on pea nodulation (data not shown).

Proteins in the mutant 3841ΔPX3 are altered compared with wild type

In an effort to gain an insight into whether *px3* plays a role in *R. leguminosarum* bv. *viciae*, protein extracts of 3841ΔPX3 and 3841 were labeled with the fluorescent dye NHS-Cy5 and separated by 2D electrophoresis using narrow (pH 4–7) isoelectric focusing in the first separation. Images of the scanned gels obtained are shown in **Fig. 3(A)** and several spots that were different in the mutant 3841ΔPX3 compared with the wild-type 3841 are circled. There are several clear differences, thus indicating that *px3* was actively involved in the protein expression or stability. We tried to isolate these proteins and analyze them by mass spectroscopy, but were unsuccessful, because the protein levels were too low.

We repeated the 2D analysis using the narrow range (pH 4–7) isoelectric focusing using higher levels of proteins and after the second dimension, the gels were stained with Coomassie brilliant blue G250. Again there were clear differences between the mutant 3841ΔPX3 and the wild-type 3841 [**Fig. 3(B)**]. Three regions of the gel are boxed showing two

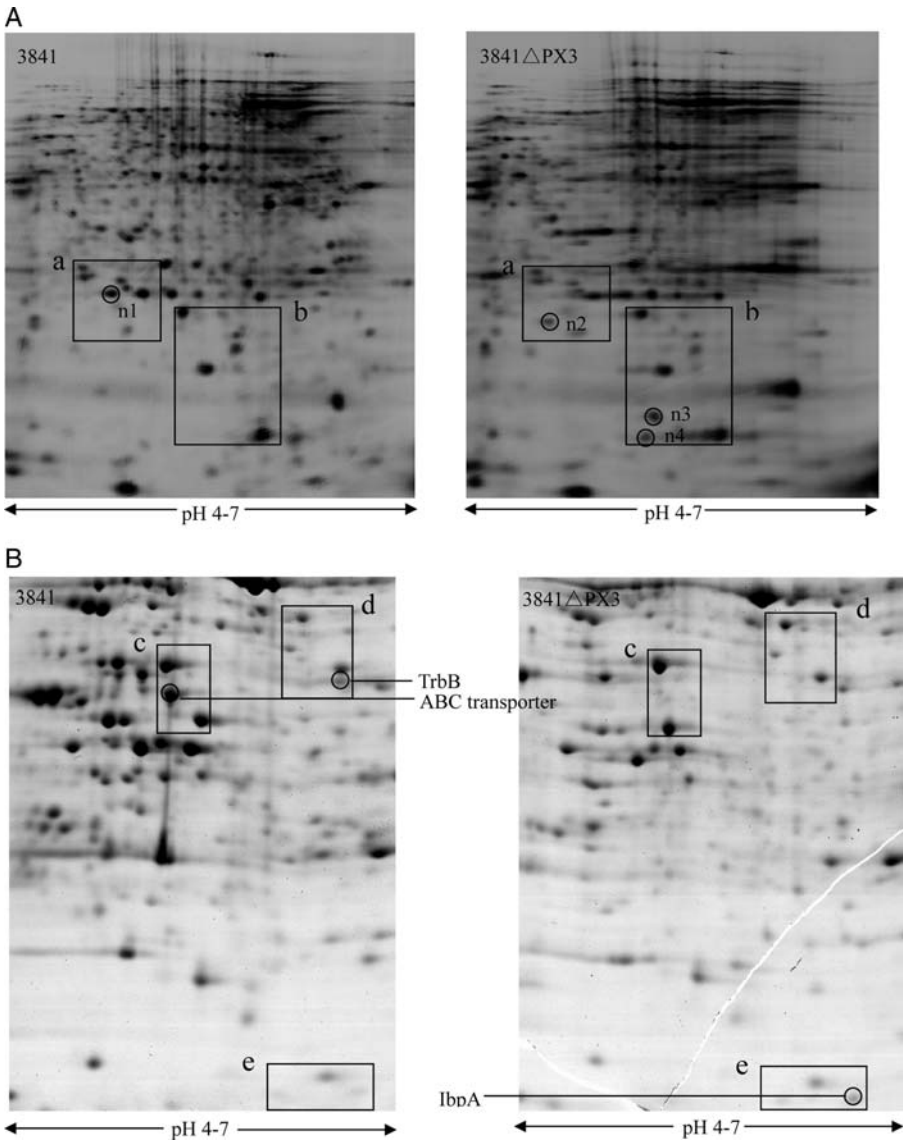

Figure 3 Representative 2D gel images of *Rhizobium* 3841 and its *px3*-deleted mutant (A) Total protein extracts labeled by Cy5 were separated on pH 4–7 linear IPG strips in the first dimension followed by 12% SDS–PAGE in the second dimension. Some differentially expressed spots are circled and marked with numbers. (B) The proteins on gels were staining with Coomassie brilliant blue G-250. Three proteins circled in box c, box d and box e were analyzed by mass spectroscopy, and their identities were indicated.

Table 3 Differentially expressed mRNAs between 3841 and px3 mutant

Sample name	Target name	RQ	RQ Min	RQ Max	Ct	Ct mean	Ct SD	ΔCt mean	ΔCt SE	ΔΔCt
3841	ftsA				28.038418	27.981924	0.1555009			
3841	trbB	24570.051	2777.466	217351.8	23.094751	24.09844	1.9558346	−3.8834858	1.132765	−14.58461
3841	ibpA	0.1657111	0.076719	0.357934	24.901604	24.143236	0.6754286	−3.8386886	0.40016	2.5932579
3841	pRL120713	5.8265529	0.264956	128.1297	30.050688	27.240677	2.7772286	−0.7412484	1.605945	−2.542643
px3 mutant	ftsA				26.605787	26.419388	0.3292305			
px3 mutant	trbB	1	0.645832	1.54839	37.064362	37.120514	0.0794141	10.701127	0.198202	0
px3 mutant	ibpA	1	0.543992	1.838264	19.964224	19.987442	0.4380066	−6.4319463	0.316356	0
px3 mutant	pRL120713	1	0.51635	1.936669	27.999769	28.220781	0.4954661	1.8013941	0.343453	0

proteins (circled in boxes c and d) that were reduced in the mutant and one (circled in box e) that was increased in the mutant. Mass spectroscopy revealed that the protein circled in box e as being increased in the mutant corresponds to the putative heat-shock protein A (ibpA) (significance score 60.2) corresponding to RL4089 in the genome sequence. The protein in box c that is reduced in the mutant corresponds to the predicted periplasmic solute binding protein pRL120713 (significance score 76.2) that is most probably associated with an ABC transporter; the downstream genes pRL120712 and pRL120711 in genome sequence encode the predicted membrane spanning and ATP-binding components of a predicted ABC transporter. The other protein (circled in box d) reduced in the mutant corresponds to the conjugal transfer protein TrbB (pRL80142) (significance score 80.2) encoded on the indigenous plasmid pRL8.

Transcription alterations in the identified genes

To determine whether deletion of *px3* is more likely to cause a transcriptional change in gene expression compared with a change in the stability of the identified proteins, the relative expression levels of the three genes identified above were compared by quantitative real-time RT-PCR using the constitutively expressed *ftsZ* gene as a control. Data were shown in **Table 3** and **Fig. 4**. Results showed that the relative expression of RL4089 was increased ~4 folds in the mutant compared with wild type in agreement with the increased protein level observed. Conversely, pRL120713 and pRL80142 were expressed at significantly lower levels in the mutant than wild type corresponding with their decreased protein levels in the mutant. Therefore, it appears that the observed effects of mutating *px3* are due to changes in transcription of the genes.

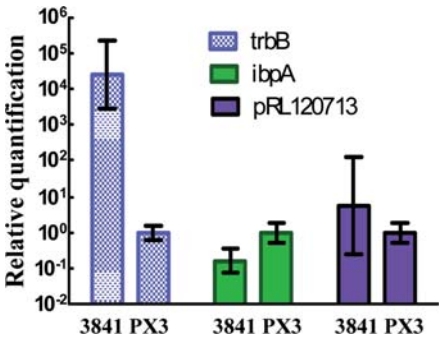

Figure 4 Quantitative RT-PCR levels of *trbB*, *ibpA* and pRL120713 from 3841 wild type and *px3* mutant The quantitative values from the PCR were normalized to the *ftsA* (endogenous control) and presented as fold recruitment, compared with the control (*px3* mutant) defined as 1. The error bars display the calculated maximum (RQ Max) and minimum (RQ Min) expression levels that represent standard error of the mean expression level (RQ value). Levels of the *trbB* and pRL120713 were significantly higher and level of the *ibpA* was significantly lower in *rhizobium* 3841 wild type compared with *px3* mutant.

Discussion

It is evident that there is a small gene, *px3*, which is strongly expressed and divergently transcribed from *nodM* in strains of *R. leguminosarum* bv. *viciae*. This gene seems to be specific for this bacterium, but is conserved in two different strains. The location of *px3*, within the *nod* gene cluster implied a possible role in nodulation. Instead of being induced by NodD, *px3* appeared to be slightly repressed by NodD. This repression could be due to NodD binding to the *nodM* promoter and occluding the access of transcription factors to the *px3* promoter. Mutation of *px3* gene did not have an observed effect on nodulation. Unlike *px2*, which was observed to have positive effects on *nodF* expression [11], *px3* was not observed to have any significant effects on *nod* cluster's genes expression (data not shown). In agreement with this, *px3* mutation did not affect nodulation. These data all indicate that *px3* is unlikely to play a role in nodulation despite its location in the *nod* gene cluster.

Although it does not play a role in nodulation, *px3* is relatively strongly expressed and mutation of the *px3* gene

alters the levels of several proteins produced by strain 3841. We identified one of the proteins with increased expression in the mutant that was predicted as the heat-shock protein A encoded on the chromosome (RL4089). Two proteins whose expressions were decreased in the mutant correspond to TrbB, a conjugation protein, and pRL120713, a predicted ABC-transporter solute binding protein. The different expression levels of corresponding proteins were due to the changes of the transcription through the analysis of cDNA levels.

Since the product encoded by *px3* shows no similarity to other proteins in sequence databases, it is not clear how it acts. Given the observations that mutation of the gene causes both increased and decreased protein and transcript levels of different genes, it seems unlikely that the protein acts independently. Since it also lacks any recognizable DNA-binding domain, it seems more likely that it affects the stability or activity of another regulator. Perhaps identification of such a regulator would shed more light on the role of this protein.

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References

1 Relic B, Perret X, Estrada-Garcia MT, Kopcinska J, Golinowski W, Krishnan HB and Pueppke SG, *et al*. Nod factors of Rhizobium are a key to the legume door. Mol Microbiol 1994, 13: 171–178.
2 Shearman CA, Rossen L, Johnston AW and Downie JA. The R*hizobium leguminosarum* nodulation gene *nodF* encodes a polypeptide similar to acyl-carrier protein and is regulated by nodD plus a factor in pea root exudate. EMBO J 1986, 5: 647–652.
3 Spaink HP, Okker RJH, Wijffelman CA, Pees E and Lugtenberg BJ. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol Biol 1987, 9: 27–39.
4 Surin BP and Downie JA. Characterization of the *Rhizobium leguminosarum* genes *nodLMN* involved in efficient host-specific nodulation. Mol Microbiol 1988, 2: 173–183.
5 de Maagd RA, Wijfjes AH, Spaink HP, Ruiz-Sainz JE, Wijffelman CA, Okker RJ and Lugtenberg BJ. nodO, a new nod gene of the *Rhizobium leguminosarum* biovar *viciae* sym plasmid pRL1JI, encodes a secreted protein. J Bacteriol 1989, 171: 6764–6770.
6 Fisher RF, Egelhoff TT, Mulligan JT and Long SR. Specific binding of proteins from Rhizobium meliloti cell-free extracts containing NodD to DNA sequences upstream of inducible nodulation genes. Genes Dev 1988, 2: 282–293.
7 Rostas K, Kondorosi E, Horvath B, Simoncsits A and Kondorosi A. Conservation of extended promoter regions of nodulation genes in *Rhizobium*. Proc Natl Acad Sci USA 1986, 83: 1757–1761.
8 Hu H, Liu S, Yang Y, Chang W and Hong G. In *Rhizobium leguminosarum*, NodD represses its own transcription by competing with RNA polymerase for binding sites. Nucleic Acids Res 2000, 28: 2784–2793.
9 Rossen L, Shearman CA, Johnston AW and Downie JA. The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the nodA,B,C genes. EMBO J 1985, 4: 3369–3373.
10 Kiss E, Mergaert P, Olah B, Kereszt A, Staehelin C, Davies AE and Downie JA, *et al*. Conservation of nolR in the *Sinorhizobium* and *Rhizobium* genera of the *Rhizobiaceae* family. Mol Plant-Microbe Interact 1998, 11: 1186–1195.
11 Yang Y, Hu HL and Hong GF. px(2), the newly identified gene in *Rhizobium leguminosarum*, is characterized to enhance its adjacent nodF expression. Biochem Biophys Res Commun 2000, 275: 91–96.
12 Johnston AW and Beringer JE. Identification of the *Rhizobium* strains in pea root nodules using genetic markers. J Gen Microbiol 1975, 87: 343–350.
13 Downie JA, Knight CD, Johnston AW and Rossen L. Identification of genes and gene products involved in the nodulation of peas by *Rhizobium leguminosarum*. Mol Gen Genet 1985, 198: 255–262.
14 Lamb JW, Hombrecher G and Johnston AW. Plasmid-determined nodulation and nitrogen-fixation abilities in Rhizobium phaseoli. Mol Gen Genet 1982, 186: 449–454.
15 Simon R, Priefer U and Pühler A. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Biotechnology 1983, 1: 784–791.
16 Gust B, Challis GL, Fowler K, Keiser T and Chater KF. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA 2003, 100: 1541–1546.
17 Bagdasarian M, Lurz R, Rückert B, Franklin FC, Bagdasarian MM, Frey J and Timmis KN. Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene 1981, 16: 237–247.
18 Hou BH, Li FQ, Yang XE and Hong GF. A small functional intramolecular region of NodD was identified by mutation. Acta Biochim Biophys Sin 2009, 41: 822–830.
19 Chang WZ and Hong GF. Two functional regions were discovered within nodA promoter. Chin J Biotechnol 1997, 13: 83–87.
20 Buchana-wollaston V. Generalized transduction in *Rhizobium leguminosarum*. J Gen Microbiol 1979, 112: 135–142.
21 Mao CJ, Downie JA and Hong GF. Two inverted repeats in the nodD promoter region are involved in nodD regulation in *Rhizobium leguminosarum*. Gene 1994, 145: 87–90.
22 Mill JH. Experiments in Molecular Genetics. New York: Cold Spring Harbor Laboratory, 1972, 325–355.
23 Guerreiro N, Redmond JW, Rolfe BG and Djordjevic MA. New *Rhizobium leguminosarum* flavonoid-induced proteins revealed by proteome analysis of differentially displayed proteins. Mol Plant-Microbe Interact 1997, 10: 506–516.
24 Görg AG, Klaus A, Lück C, Weiland F and Weiss W. Two-dimensional electrophoresis with immobilized pH gradients for proteome analysis. A Laboratory Manual, 2007. http://www.weihenstephan.de/blm/deg.