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A protein phosphatase 2C gene, *LjNPP2C1*, from *Lotus japonicus* induced during root nodule development

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ABSTRACT **Symbiotic interactions between legumes and compatible strains of rhizobia result in root nodule formation. This new plant organ provides the unique physiological environment required for symbiotic nitrogen fixation by the bacterial endosymbiont and assimilation of this nitrogen by the plant partner. We have isolated two related genes (*LjNPP2C1* and *LjPP2C2*) from the model legume *Lotus japonicus* that encode protein phosphatase type 2C (PP2C). Expression of the *LjNPP2C1* gene was found to be enhanced specifically in *L. japonicus* nodules, whereas the *LjPP2C2* gene was expressed at a similar level in nodules and roots. A glutathione *S*-transferase–LjNPP2C1 fusion protein was shown to have Mg^{2+}- or Mn^{2+}-dependent and okadaic acid-insensitive PP2C activity *in vitro*. A chimeric construct containing the full-length LjNPP2C1 cDNA, under the control of the *Saccharomyces cerevisiae* alcohol dehydrogenase promoter, was found to be able to complement a yeast PP2C-deficient mutant (*pct1*Δ). The transcript level of the *LjNPP2C1* gene was found to increase significantly in mature nodules, and its highest expression level occurred after leghemoglobin (*lb*) gene induction, a molecular marker for late developmental events in nodule organogenesis. Expression of the *LjNPP2C1* gene was found to be drastically altered in specific *L. japonicus* lines carrying monogenic-recessive mutations in symbiosis-related loci, suggesting that the product of the *LjNPP2C1* gene may function at both early and late stages of nodule development.**

Interaction of legume plants with bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium, Mesorhizobium*, and *Azorhizobium* triggers a developmental program leading to the formation of a highly specialized organ, the nodule. Nodules provide a unique environment for symbiotic fixation and assimilation of dinitrogen. The early events during nodule organogenesis are set in motion by molecular cross-talk between the two symbiotic partners culminating in the synthesis of bacterially encoded lipochito-oligosaccharide signal molecules, the Nod factors (for recent reviews, see refs. 1 and 2). Morphogenic activity of Nod factors induces diverse cellular responses in the root of a compatible host plant, resulting in the redirection of a subset of the root cortical cells toward the formation of a nodule primordium (2). Invasion and subsequent intracellular colonization of the nodule primordium by the microsymbiont initiate the final differentiation process of plant and bacterial cells, which leads to the formation of a functional nitrogen-fixing nodule (3, 4). The induction of a number of plant host genes, named nodulins (5, 6), accompanies nodule organogenesis and functioning. We have been interested in studying the complexity and regulation of plant genes expressed during relatively late stages of the symbiotic interaction (late nodulin genes; refs. 7 and 8). The components of the signal transduction pathways involved in the late developmental events of nodule morphogenesis and functioning remain mostly uncharacterized, although nodule-specific MADS-box genes have been identified (9, 10). To identify novel genes with potential regulatory functions during late stages of nodule organogenesis and/or nodule functioning, we have constructed a *Lotus japonicus* late-nodulin expressed sequence tag (EST) library using the mRNA differential display procedure (8). One of the ESTs identified during our initial screening, *LjN3*, showed strong similarity to protein phosphatase 2C genes from different organisms (8). Here we describe the molecular characterization of two homologous *L. japonicus* genes, *LjNPP2C1* and *LjPP2C2*. The *LjNPP2C1* gene corresponds to EST *LjN3* and was found to encode a functional protein phosphatase 2C (LjNPP2C1). During *L. japonicus* nodule development, the expression of the *LjNPP2C1* gene was found to correlate with relatively late stages of nodule organogenesis. In contrast, the *LjPP2C2* gene, a member of a small family of related genes in the *L. japonicus* genome, was found to be expressed at a similar level in nodules and uninfected roots. Therefore, we postulate that the *LjNPP2C1* gene product may play a specific role in a signaling cascade at a late stage of nodule development or functioning.

MATERIALS AND METHODS

Plant Material. The *Lotus japonicus* GIFU B-129-S9 wild-type line and mutant lines derived thereof were grown as described previously (11, 12). *Mesorhizobium loti* wild-type strain NZP2235 was used for all nodulation experiments (12). Uninoculated control roots were harvested 7, 11, 21, and 35 days after sowing. Roots of wild-type plants inoculated with *M. loti* NZP2235 were harvested 7 and 11 days after inoculation (dai), and fully developed nodules were collected 21 and 35 dai. Inoculated roots as well as nodule-like structures from mutant lines were harvested 35 dai. Leaves and stems were harvested from 21-day-old plants, whereas flowers were obtained from mature *L. japonicus* plants.

Northern Blot Analysis. Poly(A)$^+$ mRNA was isolated by using the PolyATtract system (Promega), following the manufacturer's instructions. Northern blot hybridization was carried out in 0.5 M phosphate buffer (pH 7.2) containing 7% SDS and 1% BSA at 65°C, essentially as described (8, 11).

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Abbreviations: PP2C, protein phosphatase type 2C; dai, days after inoculation; EST, expressed sequence tag; GST, glutathione *S*-transferase; RACE, rapid amplification of cDNA ends; ADH, alcohol dehydrogenase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accessions nos. AF092431 and AF092432 for *LjNPP2C1* and *LjPP2C2*, respectively).

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Filters were washed at high stringency: 25 min in 2× SSC/0.1% SDS, 25 min in 1× SSC/0.1% SDS, and 15 min in 0.1× SSC/0.1% SDS at 65°C. Signals from Northern blot assays were quantified by using PhosphorImager analysis (Molecular Dynamics) and were standardized to ubiquitin RNA levels.

cDNA Library Screening and 5′ Rapid Amplification of cDNA Ends (RACE) Analysis. A ^{32}P-radiolabeled *LjN3* cDNA fragment and a partial cDNA corresponding to the *LjPP2C2* gene were used to screen a *L. japonicus* nodule-specific cDNA library (kind gift from Jens Stougaard, Aarhus University, Denmark), following standard protocols (13). The 5′ RACE procedure (14) was performed using the 5′ RACE system (GIBCO/BRL), following the manufacturer's instructions. For the reverse transcription reaction, 1 μg of poly(A)$^+$ mRNA derived from *L. japonicus* nodules and a *LjNPP2C1* gene-specific primer (5′-CAACCCCGTCACGGTCTCCTC-3′) were used.

***In Vitro* Protein Phosphatase Assay.** A portion of the *LjNPP2C1* cDNA, corresponding to amino acids 96–362 of the LjNPP2C1 protein, was inserted into the pGEX-5X-1 vector (Pharmacia) to express a glutathione *S*-transferase (GST)-LjNPP2C1 fusion protein in *Escherichia coli*. Affinity purification of the resulting fusion protein was carried out using glutathione-agarose beads (product G-4510; Sigma), following the procedure described by Smith and Johnson (15). Purified protein was aliquoted and stored in 10% glycerol at −20°C.

Protein phosphatase activity was assayed by using phosphorylated casein as a substrate, essentially as described by McGowan and Cohen (16). Briefly, 5 mg of dephosphorylated bovine casein (product C-4765; Sigma) was phosphorylated using 50 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 25 units of the catalytic subunit of cAMP-dependent protein kinase (product P-2645; Sigma) for 4 hr at 30°C, as described (16). The radiolabeled casein was precipitated with 15% trichloroacetic acid (TCA), washed three times with cold 20% TCA and twice with cold acetone, air-dried, dissolved in 0.2 M Tris·HCl (pH 8.0), and stored at 4°C. The purified GST-LjNPP2C1 fusion protein was assayed for phosphatase activity in a time-course experiment in 50 μl of incubation mixture consisting of 50 mM Tris·HCl (pH 7.0), 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 20 mM magnesium acetate, 1 μM okadaic acid, ≈0.2 μg of the fusion protein, and 2 × 10^4 cpm (Cerenkov counts) of ^{32}P-labeled casein at 30°C. In the control reactions the fusion protein was replaced with 1–2 μg of purified GST protein. Divalent cation dependence was analyzed using similar conditions, except that magnesium acetate was substituted with either 20 mM $MnSO_4$, 20 mM $CaCl_2$, or 10 mM EDTA. All reactions were terminated by the addition of 50 μl cold 30% TCA. After 5 min of incubation on ice, the samples were centrifuged for 10 min at 14,000 × *g*, and the radioactivity in the supernatant and pellet was measured by Cerenkov counting. All assays were performed in triplicate.

Site-Directed Mutagenesis of the *LjNPP2C1* cDNA. An *Arabidopsis abi1–1*-type amino acid substitution (see *Results*) was created by site-directed mutagenesis of the *LjNPP2C1* cDNA after the method described by Kunkel (17). An oligonucleotide (5′-TTTGACGGTCA*TG*A*C*GGAGCTCAGG-3′) containing the *abi1–1*-type mutation (G → A) was used to change the Gly-133 residue to Asp. In addition, a silent C → T substitution was introduced 2 bp upstream from the G → A mutation to create a unique *Bsp*HI restriction site. The presence of the base pair substitutions was confirmed by DNA sequence analysis.

Functional Complementation Assay in *Saccharomyces cerevisiae*. The *LjNPP2C1* cDNA fragment was cloned into the yeast expression vector pDBL2 in the sense (construct pDBL3) and antisense (construct pDBL5) orientation, with respect to the yeast alcohol-dehydrogenase promoter ADH1 (18). In addition, a mutant *LjNPP2C1* construct was prepared by inserting the *LjNPP2C1* cDNA, containing the *abi1–1*-type substitution, into the pDBL2 vector in the sense orientation to generate plasmid pDBL3M.

Plasmids were introduced into yeast strain TM126 (19), carrying a disruption in the *PTC1* locus encoding yeast protein phosphatase 2C, using a modified lithium acetate/polyethylene glycol method (20). The pDBC1 construct, containing the wild-type yeast *ptc1* gene under the control of the ADH1 promoter (19), as well as the pDBL2 vector alone also were introduced into strain TM126 and used as positive and negative controls, respectively. Complementation assays were performed essentially as described by Bertauche *et al.* (21). Each transformant was grown to saturation at 28°C in liquid synthetic SD medium (22), lacking uracil and leucine. For the complementation assay, 3 μl of the yeast suspension culture, containing approximately 10^4 cells/μl, was replica-plated onto yeast extract/peptone/dextrose media (22). Replica plates were incubated at either 28°C (permissive) or 37°C (nonpermissive) for 30–36 hr. Subsequently, the phenotype of the individual strains was analyzed carefully to examine the ability of the recombinant plasmids to complement the temperature-sensitive growth phenotype of strain TM126 (19).

RESULTS

The *L. japonicus LjNPP2C1* Gene Shares Significant Similarity with Protein Phosphatase 2C Genes. Using the mRNA differential display procedure (23), we previously isolated a range of *L. japonicus* ESTs associated with relatively late stages of nodule development and/or nodule functioning (8). One of these ESTs, *LjN3*, was found to share significant similarity with a number of protein phosphatase 2C genes from different organisms (8). Subsequent screening of a nodule-specific cDNA library, using radiolabeled *LjN3* cDNA as a probe, facilitated the cloning of the corresponding full-length cDNA. The nucleotide sequence of the longest cDNA clone (*LjNPP2C1–16*) was shown to be 1,235 bp in length and was found to contain a 1,086-bp-long ORF, starting with an ATG codon at base pair 25. Because the ATG_{25} codon was not preceded by an in-frame stop codon, the 5′ RACE procedure was used to further characterize the 5′-terminal sequence of the *LjNPP2C1* mRNA. The sequence of the longest 5′ RACE cDNA product extended the 5′ end of the *LjNPP2C1–16* cDNA by 83 bp (data not shown). However, no additional in-frame ATG codons were found in the 5′ sequence. Moreover, the length of the *LjNPP2C1* cDNA correlates well with the observed mRNA size on Northern blots (≈1,300 nt). Based on these observations, we propose that the ATG_{25} of *LjNPP2C1–16* cDNA (*A*TG_{108} in the extended sequence) represents the initiation codon of the *LjNPP2C1* gene and that the *LjNPP2C1–16* cDNA contains the entire coding region for the LjNPP2C1 protein.

The amino acid sequence of the deduced LjNPP2C1 protein was found to correspond to a polypeptide of 362 residues and a predicted molecular mass of ≈39.5 kDa. In agreement with our earlier observations (8), the deduced protein was found to share a high level of similarity with a number of PP2C proteins from different eukaryotes, including the ABI1 and ABI2 proteins from *Arabidopsis thaliana* (Fig. 1).

The *L. japonicus LjPP2C2* Gene. In the course of an independent research project, a distinct partial cDNA clone encoding a putative *L. japonicus* PP2C was fortuitously identified (data not shown). A corresponding full-copy cDNA clone (*LjPP2C2)* was isolated from the *L. japonicus* cDNA library and was found to encode a polypeptide (LjPP2C2) of 282 residues and a predicted molecular mass of ≈30.8 kDa. Similar to the LjNPP2C1 protein, the predicted amino acid sequence of the LjPP2C2 was found to share a high level of similarity with a number of PP2C proteins from different eukaryotes, including *L. japonicus* LjNPP2C1 and *A. thaliana* ABI1 (Fig. 1). Based on these results, we conclude that the *LjNPP2C1* and

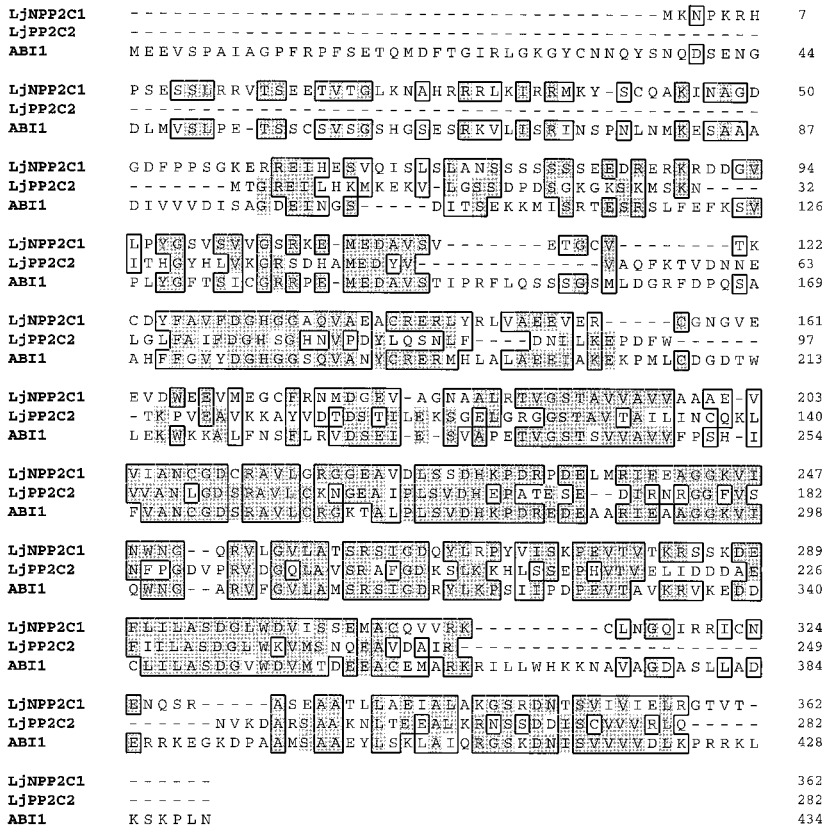

FIG. 1. Amino acid sequence alignment of *L. japonicus* LjNPP2C1 and LjPP2C2 and *A. thaliana* ABI1 (25, 26) proteins. The alignment was performed using the PILEUP algorithm from the GCG software package (GCG). Identical amino acids are shaded and conservative substitutions are boxed using the SEQVU 1.1 program. Gaps introduced to allow an optimal alignment are represented by dashes.

LjPP2C2 genes are likely to encode two similar but distinct *L. japonicus* PP2C proteins.

***LjNPP2C1*, but Not *LjPP2C2*, Transcripts Accumulate Preferentially in *L. japonicus* Nodules.** We reported previously that *LjNPP2C1* mRNA levels were enhanced in *L. japonicus* nodules versus uninoculated control roots (8). To address the question of whether the *LjPP2C2* gene also was expressed preferentially in nodules, a Northern blot analysis was performed. Poly(A)$^+$ mRNA derived from uninfected *L. japonicus* control roots and nodules was hybridized sequentially with [α-^{32}P]dATP-labeled *LjNPP2C1*, *LjPP2C2*, and *Sesbania rostrata* ubiquitin cDNA probes (Fig. 2). A significantly higher level of *LjNPP2C1* mRNA was detected in nodules than in uninfected *L. japonicus* control roots, confirming and extending our earlier observations (8). In contrast, the level of *LjPP2C2* mRNA was found to be equal in both tissues. Based on these results, we conclude that the *LjNPP2C1* is likely to have a unique function(s) associated with nitrogen-fixing nodules. Therefore, our further analysis concentrated on a more detailed characterization of the *LjNPP2C1* gene.

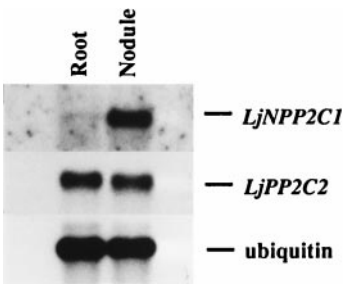

FIG. 2. Northern blot analysis of *LjNPP2C1* and *LjPP2C2* expression. Two micrograms of poly(A)$^+$ mRNA from uninoculated roots and mature nodules were separated under denaturing conditions and hybridized sequentially with α-^{32}P-labeled probes corresponding to the *L. japonicus LjNPP2C1*, *LjPP2C2*, and *Sesbania rostrata* ubiquitin cDNAs.

The *LjNPP2C1* Gene Encodes a Functional PP2C Protein. To investigate whether the *LjNPP2C1* gene encoded a functional PP2C protein, an *in vitro* phosphatase assay was carried out. For this purpose, a recombinant protein was created containing the C-terminal catalytic domain of LjNPP2C1 fused to the GST protein. The GST-LjNPP2C1 fusion protein was expressed in *E. coli*, purified, and found to display a time-dependent phosphatase activity using phosphorylated casein as a substrate (Fig. 3*A*). The phosphatase activity of the GST-LjNPP2C1 fusion protein was found to be insensitive to 1 μM okadaic acid, a potent inhibitor of protein phosphatases other than type 2C (24), and to be dependent on the presence of the divalent cation Mg^{2+} or Mn^{2+} (Fig. 3*B*). The GST protein alone, expressed and purified under similar conditions, was found to be inactive in the phosphatase assay (Fig. 3*B*).

The biochemical evidence for PP2C activity of the *LjNPP2C1* gene product was supported further by heterologous genetic complementation experiments in yeast. *S. cerevisiae* strain TM126 carries a disrupted PP2C gene (*ptc1*Δ), resulting in a temperature-sensitive growth phenotype (*ptc1*Δ mutant cells grow slower at 37°C then at 28°C; ref. 19). The *LjNPP2C1* cDNA fused to the yeast ADH1 promoter was introduced into *S. cerevisiae* strain TM126 on plasmid pDBL3 and indeed was found to complement the yeast *ptc1* mutant phenotype (Fig. 4). A plasmid carrying the wild-type *LjNPP2C1* cDNA in the antisense orientation (pDBL5) failed to complement the temperature-sensitive growth phenotype of *ptc1*Δ yeast cells (Fig. 4).

To provide further evidence for the designation of the *LjNPP2C1* gene product as a PP2C, we made use of the

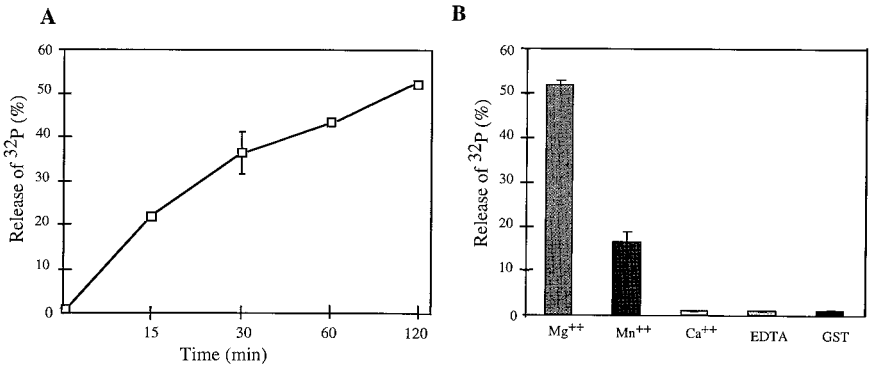

FIG. 3. Protein phosphatase type 2C activity of a GST-LjNPP2C1 protein. (*A*) Experiments showing a time-dependent dephosphorylation of ^{32}P-labeled casein by a recombinant GST-LjNPP2C1 protein in the presence of 20 mM magnesium acetate and 1 μM okadaic acid. The GST protein alone was ineffective in this assay (data not shown). (*B*) Divalent cation requirement for the protein phosphatase activity of LjNPP2C1. The recombinant GST-LjNPP2C1 protein was assayed for its PP2C activity in the presence of Mg^{2+}, Mn^{2+}, or Ca^{2+} or in the absence of a divalent cation and in the presence of EDTA. "GST" denotes the assay performed in the presence of the GST protein alone.

phenotype of an *A. thaliana* mutant PP2C protein. The *A. thaliana abi1–1* mutation is caused by a single base pair transition that substitutes amino acid residue Gly-180 with an Asp residue, resulting in a significant decrease of PP2C activity *in vitro*, as well as the generation of a dominant-negative mutation leading to abscisic acid insensitivity (21, 25, 26). An *abi1–1*-type mutation (Gly-133 → Asp) was created in the wild-type *LjNPP2C1* gene by site-directed mutagenesis. The resultant mutant LjNPP2C1 protein was found to be unable to complement the *ptc1*Δ mutant phenotype of yeast strain TM126 (Fig. 4).

***LjNPP2C1* mRNA Accumulates During Relatively Late Stages of Nodule Development.** To correlate the expression pattern of the *LjNPP2C1* gene with specific phase(s) in nodule development, a time-course Northern hybridization analysis was performed. *LjNPP2C1* mRNA was found to be present at a low level in uninoculated *L. japonicus* roots and to be slightly increased at 7 and 11 days after inoculation with *M. loti*. However a ≈20-fold increase of *LjNPP2C1* mRNA was observed in nodules harvested 21 days after inoculation (Fig. 5). Interestingly, the most dramatic enhancement of *LjNPP2C1* mRNA level was found to occur after the induction of *L. japonicus* leghemoglobin (*lb*) gene expression (a molecular marker for late stages of nodule development; ref. 7), which was found to occur in nodules 11 days after inoculation (Fig. 5; see also ref. 12).

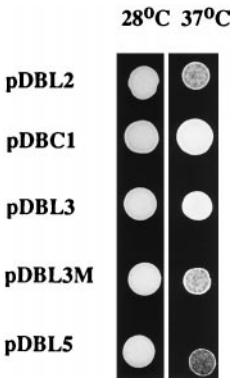

FIG. 4. Complementation of the PP2C-deficient yeast mutant strain TM126 (*ptc1*Δ). For each strain, 3 μl of cell suspension of each yeast strain, containing approximately 3×10^4 cells, was spotted onto agar plates (see *Materials and Methods*) and incubated at either 28°C or 37°C for 2 days. Strain TM126 was transformed with the yeast expression vector pDBL2 as a control; plasmid pDBC1 contains the wild-type yeast *PTC1* gene (19), plasmids pDBL3 and pDBL3M contain either wild-type or mutant *L. japonicus LjNPP2C1* genes, and plasmid pDBL5 contains the *LjNPP2C1* gene in the antisense orientation with respect to the yeast ADH promoter. The poor growth phenotype of strain TM126 at the nonpermissive temperature (37°C) is evidenced by a less dense (partially translucent) spot; complementation of the temperature-sensitive growth phenotype is evidenced by denser (nontranslucent) spots.

Tissue-Specific Expression of the *LjNPP2C1* Gene. To gain further insight into the tissue specificity of *LjNPP2C1* gene expression, poly(A)$^+$ mRNA isolated from different *L. japonicus* tissues was probed with radiolabeled *LjNPP2C1* cDNA. *LjNPP2C1* mRNA was found to accumulate to the highest level in nodules and flowers (Fig. 6). A low level of *LjNPP2C1* mRNA also could be found in uninfected root and leaf tissues (Fig. 6), whereas no hybridization signal, even upon a prolonged exposition period, was detected in *L. japonicus* stems (data not shown). Therefore, we conclude that *LjNPP2C1* gene expression is not nodule-specific, but that expression of this gene is enhanced substantially in fully developed nodules and flowers.

***LjNPP2C1* Gene Expression Is Altered in Symbiotic Mutants of *L. japonicus*.** The expression pattern of the *LjNPP2C1* gene was analyzed further by examining six nonallelic *L. japonicus* symbiotic mutants. Four nonnodulating lines (Nod$^-$; LjEMS34, LjEMS46, LjEMS70, and LjEMS76), which fail to display macroscopically visible signs of nodulation, and two distinct mutant lines (LjEMS88 and LjEMS75), forming white, mostly ineffective (Nod$^+$Fix$^{-/+}$) nodules (12), were examined for *LjNPP2C1* expression.

Mutant lines LjEMS88 and LjEMS75 differ with respect to their ability to support late stages of nodule development. Mutant line LjEMS88 forms only small, white nodule-like

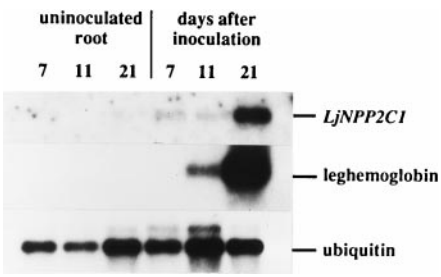

FIG. 5. Developmental Northern blot analysis of *LjNPP2C1* gene expression. Two micrograms of poly(A)$^+$ mRNA isolated from uninoculated roots, harvested 7, 11, and 21 days after sowing, and roots harvested from *L. japonicus* plants 7 and 11 dai, as well as 21-day-old nodules were analyzed. The blot was sequentially probed with the radiolabeled *LjNPP2C1* cDNA, the *LjN77* EST corresponding to an *L. japonicus* leghemoglobin gene (8) and the *S. rostrata* ubiquitin cDNA as a loading control.

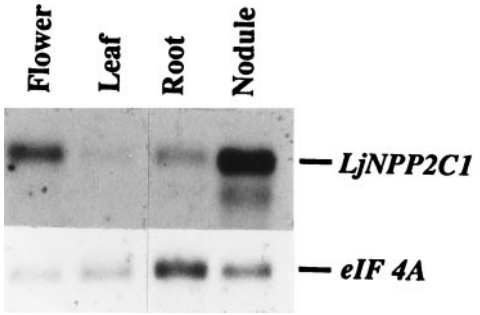

FIG. 6. Northern blot analysis of *LjNPP2C1* expression in different tissues of *L. japonicus*. Four micrograms of poly(A)$^+$ RNA isolated from flowers, leaves, uninoculated roots, and mature nodules of *L. japonicus* were separated under denaturing conditions and hybridized with radiolabeled probes corresponding to the *L. japonicus LjNPP2C1* and *A. thaliana eIF4A* cDNAs.

structures that do not fix nitrogen and accumulate only background levels of leghemoglobin mRNA (≤50-fold less than wild-type level; ref. 12). In contrast, the LjEMS75 line produces a mixed population of well developed white nodules, in addition to pink wild-type-like nodules, that fix nitrogen, albeit at a significantly diminished level (12). Both types of the LjEMS75 nodules were harvested and analyzed separately. White nodules of line LjEMS75 were found to contain a significant level of *L. japonicus lb* mRNA (only ≈3-fold lower than the wild type; Fig. 7*A*), which clearly distinguishes them from white nodules formed on line LjEMS88 (see above).

Thirty-five-day-old wild-type nodules and pink nodules of mutant line LjEMS75 showed similar, 15- to 20-times-elevated levels of *LjNPP2C1* mRNA, as compared with uninfected control roots. White nodules of LjEMS75 were found to have a 2-fold-lower level of *LjNPP2C1* transcripts than wild-type nodules, but an approximately 7-times-higher transcript level than uninfected control roots (Fig. 7*A*). Analysis of *LjNPP2C1* gene expression in white nodule-like structures formed on the LjEMS88 mutant line showed only a background level of the corresponding mRNA (Fig. 7*B*). A background level of *LjNPP2C1* mRNA also was detected in wild-type uninfected roots and infected roots of lines LjEMS70 and LjEMS76. In contrast, the infected roots of the nonnodulating mutant line LjEMS34 showed a relatively modest increase (two times higher than the wild-type uninoculated roots) of *LjNPP2C1* transcript levels, whereas LjEMS46 was found to display a significant level of *LjNPP2C1* mRNA: ≈50% of that present in nitrogen fixing nodules.

DISCUSSION

Reversible phosphorylation of proteins has been implicated in the regulation of cellular processes as diverse as metabolism, transcription and translation, cell division, membrane transport and secretion, stress response, fertilization, and memory (27). The role of protein kinases in controlling the level of phosphorylation of proteins has been well documented (28). The finding that the activity of protein phosphatases is regulated in specific cases has led to the hypothesis that cellular responses to external stimuli might result from direct activation or inhibition of protein phosphatases rather then through the action of protein kinases (24, 28). So far, three of the four known major classes of Ser/Thr protein phosphatases have been described in plants (PP1, PP2A, and PP2C), although additional protein phosphatases unrelated to these major groups are thought to exist (24). In general, PP2C is the least well characterized class of protein phosphatases. However, recent studies have suggested an intriguing connection between PP2C enzyme activity and several cellular processes in different eukaryotes (for recent reviews see refs. 24, 28, and 29). Plant members of this group of protein phosphatases include four different PP2Cs from *A. thaliana*: KAPP (30), AtPP2C (31), ABI1 (25, 26), ABI2 (32) and a protein phosphatase 2C (MP2C) from alfalfa (33).

Here we report the identification and characterization of a plant gene, *LjNPP2C1*, encoding a protein with amino acid similarity to PP2C proteins, the expression of which is enhanced significantly during *L. japonicus* root nodule organogenesis. The following biochemical and genetic experiments have led us to the conclusion that the *LjNPP2C1* gene encodes a functional PP2C. (*i*) A recombinant GST-LjNPP2C1 protein is capable of dephosphorylating phosphorylated casein, a commonly used artificial substrate for measuring PP2C activity (16). The activity of the LjNPP2C1 catalytic domain present in the GST-LjNPP2C1 fusion is insensitive to inhibition by okadaic acid and dependent on the presence of divalent cations (Mg^{2+} or Mn^{2+}). (*ii*) The *LjNPP2C1* gene is able to complement a yeast PP2C-deficient mutant (*pct1*Δ.) (*iii*) The *LjNPP2C1* cDNA, carrying an *abi1–1*-type single-amino acid substitution (Gly → Asp), is unable to complement the temperature-sensitive phenotype of yeast strain TM126, thereby mimicking the behavior of the *A. thaliana* ABI1 mutant protein used in similar complementation experiments (21). We also describe a second gene, *LjPP2C2,* which encodes a protein sharing significant amino acid similarity with LjNPP2C1, as well as other PP2C proteins from different eukaryotes. Unlike in the case of the *LjNPP2C1* gene, the *LjPP2C2* transcript appears to accumulate to a similar level in both uninoculated *L. japonicus* roots and nodules, suggesting that two different regulatory mechanisms are involved in the expression of these otherwise similar genes. Moreover, *LjNPP2C1* gene is represented by a single- or low-copy gene number in the *L. japonicus* genome, whereas *LjPP2C2* gene is a member of a small family

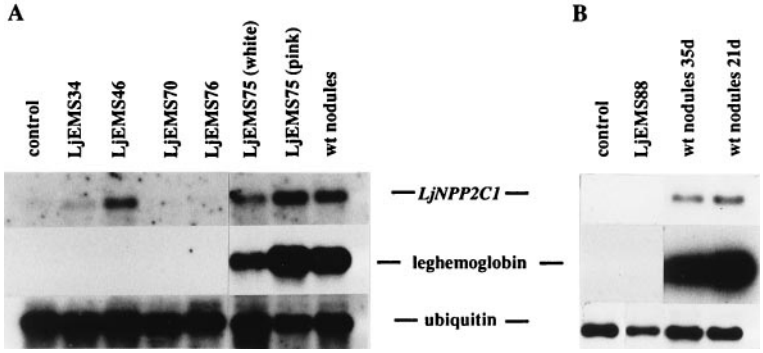

FIG. 7. Comparison of *LjNPP2C1* transcript levels in wild-type uninoculated roots (control), inoculated roots of nonnodulating mutant lines LjEMS34, LjEMS46, LjEMS70, and LjEMS76, white (LjEMS75 white) and pink (LjEMS75 pink) nodules of mutant line LjEMS75, and 35-day-old wild-type nodules (*A*), and wild-type uninoculated roots (control), white nodule-like structures induced on the mutant line LjEMS88, and 35- and 21-day-old wild-type nodules (*B*). The blots shown in *A* and *B* were sequentially hybridized with [α-^{32}P]dATP-labeled probes corresponding to the *LjNPP2C1* cDNA, the *L. japonicus* leghemoglobin gene (EST *LjN77*; ref. 8), and a *S. rostrata* ubiquitin cDNA, as loading control.

of related genes, as evidenced by Southern blot hybridization (data not shown).

LjNPP2C1 appears to be the only plant protein phosphatase 2C reported thus far whose expression is enhanced during plant–microbe interactions in general and symbiotic root nodule formation in particular. Moreover, the temporal expression pattern of the *LjNPP2C1* gene during rhizobial infection and nodule formation appears to be unusual in comparison with that of other nodulin genes. Only a limited level of *LjNPP2C1* mRNA accumulation is observed at relatively early stages of infection and nodule morphogenesis. In contrast, a 20-fold-higher level of *LjNPP2C1* transcripts, as compared with the control uninoculated roots, is observed in 21-day-old nodules. The prominent increase in *LjNPP2C1* mRNA levels observed is preceded by the developmental transition between nodule ontogeny and the establishment of a functional, nitrogen-fixing nodule, as indicated by the expression pattern of the *L. japonicus* leghemoglobin gene, which serves as a molecular marker for this transition (Fig. 5; see also ref. 12). This observation is supported by results obtained from studies of *LjNPP2C1* gene expression in symbiotic mutants of *L. japonicus*. The highest levels of *LjNPP2C1* transcripts were found in nodules, or nodule-like structures, already containing a substantial level of leghemoglobin mRNA. For example, the white and pink nodules formed on mutant line LjEMS75 accumulate a high level of both *lb* and *LjNPP2C1* gene transcripts, whereas only a low level of these mRNA species are found in white, ineffective, nodule-like structures formed on line LjEMS88 (Fig. 7; see also ref. 12).

On the basis of these results, it appears that the LjNPP2C1 protein has a specific function(s) in nodules that are in the process of initiating nitrogen fixation and have already passed the developmental time point at which expression of a late nodulin molecular marker gene *lb* is activated. The intriguing observation that the roots of two particular nonnodulating *L. japonicus* mutant lines, LjEMS34 and LjEMS46, contain an elevated level of *LjNPP2C1* mRNA is difficult to interpret. Phosphatases may function to positively or negatively affect signal transduction pathways. Therefore, it is conceivable that the mutations in lines LjEMS34 and LjEMS46 cause a deregulated *LjNPP2C1* expression pattern, resulting in elevated levels of mRNA at an inappropriate developmental stage of nodulation. It is even possible that altered levels of *LjNPP2C1* in these mutant lines contribute to or cause the observed Nod^- phenotype. Further analysis of *LjNPP2C1* gene expression, the phenotype(s) of transgenic plants (over)expressing the *LjNPP2C1* transcript in sense and antisense orientation, and characterization of the relevant *in vivo* substrate(s) of LjNPP2C1 will be essential to resolve the function of the *LjNPP2C1* gene during nodulation or symbiotic nitrogen fixation.

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