MPMI Vol. 28, No. 4, 2015, pp. 432–442. http://dx.doi.org/10.1094/MPMI-09-14-0285-R

e-Xtra*

PII Overexpression in *Lotus japonicus* Affects Nodule Activity in Permissive Low-Nitrogen Conditions and Increases Nodule Numbers in High Nitrogen Treated Plants

Enrica D'Apuzzo,[1] Vladimir Totev Valkov,[1] Aurora Parlati,[1] Selim Omrane,[1] Ani Barbulova,[1] Maria Martha Sainz,[1] Marco Lentini,[2] Sergio Esposito,[2] Alessandra Rogato,[1] and Maurizio Chiurazzi[1]

[1]Institute of Biosciences and Bioresources, CNR, Via P. Castellino 111, Napoli, Italy; [2]Università degli Studi di Napoli Federico II, Dipartimento di Biologia, Via Cinthia 6, 80126, Napoli, Italy

Submitted 25 October 2014. Accepted 31 December 2014.

We report here the first characterization of a *GLNB1* gene coding for the PII protein in leguminous plants. The main purpose of this work was the investigation of the possible roles played by this multifunctional protein in nodulation pathways. The *Lotus japonicus LjGLB1* gene shows a significant transcriptional regulation during the light-dark cycle and different nitrogen availability, conditions that strongly affect nodule formation, development, and functioning. We also report analysis of the spatial profile of expression of Lj*GLB1* in root and nodule tissues and of the protein's subcellular localization. Transgenic *L. japonicus* lines overexpressing the PII protein were obtained and tested for the analysis of the symbiotic responses in different conditions. The uncoupling of PII from its native regulation affects nitrogenase activity and nodule polyamine content. Furthermore, our results suggest the involvement of PII in the signaling of the nitrogen nutritional status affecting the legumes' predisposition for nodule formation.

The superfamily of PII signal transduction proteins represents one of the most widely distributed signaling proteins in nature (Forchhammer 2004; Huergo et al. 2013). Many bacteria and archaea have multiple PII proteins, whereas a single copy is found in plants and most cyanobacteria. PII proteins are homotrimers composed of 12- to 13-kDa subunits, forming a compact cylinder from which three long loops (the T loops) protrude (Cheah et al. 1994; Vasudevan et al. 1994). The PII regulatory action in bacteria takes place through a protein–protein interaction mechanism controlling the activities of a wide range of targets, including enzymes, transcription factors, and membrane transporters mostly involved in nitrogen (N) metabolism. Thereafter, PII was also identified as a key C and N metabolic integrator in early studies examining glutamine synthetase (GS) GS regulation (Moorhead and Smith 2003) and, more recently, a biochemical characterization of bacterial PII proteins revealed allosteric complexes involving ATP or ADP and 2-oxoglutarate (2-OG) 2-OG, which function to sense cellular energy and carbon (C) levels (Jiang and Ninfa 2007). Layered on top of allosteric sensing of cellular C and energy availability is the covalent modification of PII in response to cellular N status. An ATPase activity has been formally proven in the PII paralog GlnK, leading to a model where the role of ATP or ADP binding is to effect a 2-OG-dependent molecular switch that drives a conformational change in the T loop (Radchenko et al. 2013). The convergence of these two types of input alters the ability of PII to interact with partner proteins which, in turn, controls the ability of these partners to modify various aspects of N metabolism, including gene transcription, membrane transporters, and metabolic enzymes (Forchhammer 2008; Moorhead and Smith 2003).

C and N requirements of plant cells can vary between tissue types, requiring precise intertissue and, therefore, cellular cooperation. N taken up by roots, mainly in the form of nitrate and ammonium, can be either used in root N metabolism or transported to photosynthetic tissues for incorporation into amino acids. Conversely, C can be fixed locally by photosynthetic processes and synthesized into the necessary C substrates to supplement chloroplast-mediated N assimilation and amino acid biosynthesis, or translocated in the form of sucrose from photosynthetic tissues to provide energy and C skeletons for N assimilation in root tissues. In the case of leguminous plants, N-fixing root nodules are the organs where atmospheric N reduction and release take place and, at the same time, are optional C sink organs that need to assimilate an energy source for the formation of nodule primordium (Complainville et al. 2003) and to provide energy for the N fixation performed by the microsymbiont (*Rhizobium*) and assimilation of the produced ammonium and starch biosynthesis (Vance 2008).

Despite the central role played as an integrator of C and N cellular metabolism in many prokaryotes, plant PII is a nuclear-encoded chloroplast protein (GLB1) that seems to have evolved secondary, tissue-specialized roles. Preliminary studies suggested a conserved role as an N or C sensor due to the lack of glutamine sensing in *Arabidopsis thaliana* PII overexpressors (Hsieh et al. 1998). Later, PII has been implicated in the control of the arginine

E. D'Apuzzo and V. T. Valkov contributed equally to this work.

Current address for A. Barbulova: Arterra Bioscience Srl, Via B. Brin 69, 80142, Napoli, Italy.

Current address for M. M. Sainz, Departamento de Nutrition Vegetal, Estacion Experimental de Aula Dei, Consejo Superior de Investigationes Cientificas, 50080, Zaragoza, Spain.

Corresponding author: M. Chiurazzi: Telephone: +1 0039-081-6132433; Fax: +1 0039-081-6132706; E-mail: maurizio.chiurazzi@ibbr.cnr.it

*The *e*-Xtra logo stands for "electronic extra" and indicates that four supplementary figures are published online.

(Arg) biosynthetic pathway through interaction with N-acetyl-L-glutamate kinase (NAGK) (Burillo et al. 2004; Ferrario-Méry et al. 2006; Sugiyama et al. 2004) to reduce feedback inhibition by high Arg concentrations (Chen et al. 2006; Ferrario-Méry et al. 2006; Maheswaran et al. 2004). The plant complex PII-NAGK only shows gradual inhibition of complex formation at 2-OG concentrations greater than 1 mM (Beez et al. 2009; Mizuno et al. 2007a). Most recently, major fluctuation of the *GLB1* transcript during seed maturation in *A. thaliana* (Uhrig et al. 2009) was associated with a crucial role played by PII in the fine tuning of fatty acid biosynthesis and partitioning in *Arabidopsis* seed (Baud et al. 2010). In addition, two PII target proteins in *A. thaliana* that are biotin carboxyl carrier subunits of the plastidial acetyl-CoA-carboxylase (ACCase) (namely, BCCP1 and BCCP2), involved in the production of the precursor of lipid biosynthesis, malonyl-CoA, have been identified (Feria Bourrellier et al. 2010). ACCase activity was inhibited by PII in chloroplast extracts, in the presence of MgATP, while the addition of 2-OG, pyruvate, or oxalacetate was sufficient to release the inhibition, thus suggesting, for the first time, the involvement of PII in the regulation of an enzyme dedicated to C metabolism (Feria Bourrellier et al. 2010).

We report here the first characterization of the *GLB1* gene in a leguminous plant, with a detailed analysis of the transcriptional regulation in different growth conditions metabolically linked to the symbiotic N fixation process. Phenotypic analysis of transgenic *Lotus japonicus* overexpressing lines revealed a putative novel role of PII in the control of nodule functioning phenotypes and involvement in the signaling pathways governing N-dependent nodulation competence.

RESULTS

Identification of the *L. japonicus* sequence encoding for the PII protein and description of its structural features.

Blast search for orthologs of the *Medicago sativa* PII protein (accession number AY027892) in the *L. japonicus* genome sequence database identified a single-copy gene (chr1. CM0122.1600.r2.m) coding for a 195-amino-acid (aa) protein with a molecular mass of 21.49 kDa sharing 70% aa identity with the *M. sativa* PII protein. The genomic locus consists of eight exons separated by seven introns (Supplementary Fig. S1) with a gene structure identical to that identified in *A. thaliana* (AT4G01900). Multiple sequence alignment recognized the region (positions 106 to 124) highly conserved between bacteria and plant PII proteins (the T-loop motif) that plays a key role in mediating interactions between PII and downstream-effector proteins (Mizuno et al. 2007b; Sant'Anna et al. 2009). In particular, amino acid residues involved in the 2-OG-dependent conformational change of the T-loop (Radchenko et al. 2013) are conserved in the *L. japonicus* sequence (Q108 and K128). Furthermore, highly conserved charged amino acids likely to be involved in homotrimer formation in *A. thaliana* are identical in the *L. japonicus* sequence corresponding to K72, E74, D101, R103, K130, and E132 (Smith et al. 2003). The N- and C-terminal plant PII signatures are also identified in the *Lotus* sequence (positions 69 to 81 and 173 to 188, respectively). The molecular phylogenetic tree (Fig. 1) based on the alignment of complete amino acid PII sequences and drawn using the neighbor-joining method (Saitou and Nei 1987) integrates the taxonomic analysis previously reported (Uhrig et al. 2009), indicating a close relationship among the PII legume sequences.

Transcriptional regulation of LjGLB1 in response to light-dark cycle and N supply.

In the literature, most of the reported transcriptional analyses of the *GLB1* gene use leaf tissues to investigate a possible mechanism of control associated with the dark-light cycle or C supply derived from photosynthesis. Our analysis of *LjGLB1* was especially aimed to investigate a possible regulation of the transcriptional profile in both photosynthetic (leaves) and nonphotosynthetic organs (roots and nodules) under conditions that may affect symbiotic performances. Using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), we first analyzed the distribution of the *LjGLB1* transcript in different organs of *L. japonicus*. The amount of *LjGLB1* transcript didn't change significantly between root and leaf tissues of plants grown on Gamborg-B5-derived media with 1 mM ammonium nitrate as N source or without N (Fig. 2A). In the latter conditions, plants were inoculated with *Mesorhizobium loti* to test *GLB1* expression in mature nodules and a comparable level of transcript was detected in nodular tissue, suggesting that PII might be performing some roles in this organ (Fig. 2A).

The *GLB1* transcriptional analysis during the dark-light cycle (8 and 16 h, respectively) in hydroponic cultures of *L. japonicus* plants is reported in Figure 2B and C. A rapid enhancement (approximately fourfold) of *GLB1* transcription in roots was revealed at 3 h from the beginning of the light cycle, with the amount of transcript remaining constant till the end of the light period and starting to decay after 2 h until the end of the dark period (Fig. 2B). A similar trend of transcriptional regulation was detected in leaves under the same experimental conditions (Fig. 2C). In both root and leaf tissues, the presence of 3% sucrose in the growth medium doesn't affect the level of expression during the light period. However, the effect of light on

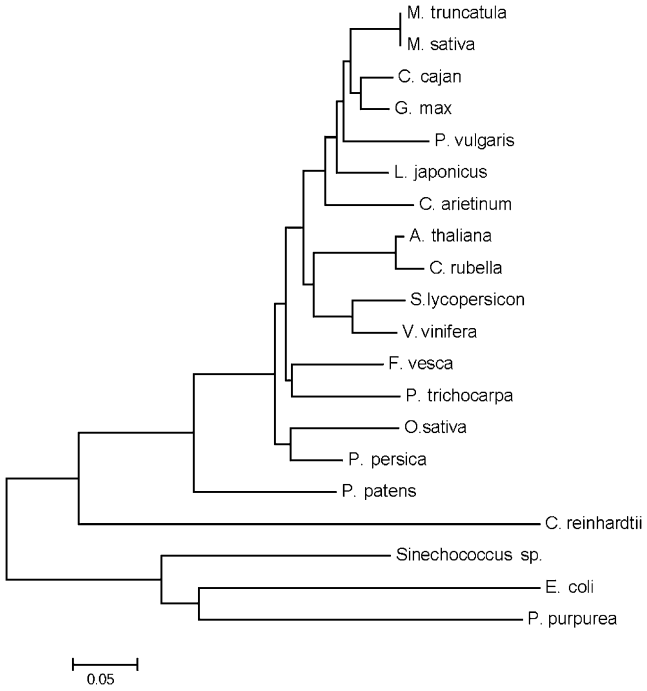

Fig. 1. Phylogenetic relationship of PII amino acid sequences. Twenty full length amino acid sequences were aligned with the ClustalW program. The optimal tree with the sum of branch length = 2.33470189 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA5.2.2 (Tamura et al. 2007). Protein sequences were obtained from NCBInr and DOEJGI online databases using BLSTP algorithms. Sequences are as follows: *Arabidopsis thaliana*, *Cajanus cajan, Capsella rubella*, *Chlamydomonas reinhardtii*, *Cicer arietinum*, *Escherichia coli*, *Fragaria vesca*, *Glycine max*, *Medicago sativa*, *M. truncatula*, *Oryza sativa*, *Phaseolus vulgaris*, *Physcomitrella patens*, *Populus trichocarpa*, *Porphyra purpurea*, *Prunus persica*, *Solanum lycopersicon*, *Vitis vinifera*, and *Synechococcus* sp. PCC7002.

GLB1 mRNA could be mimicked in both root and leaf tissues at the end of the dark period by the addition of 3% sucrose, indicating that, in *L. japonicus*, the effect of light on *GLB1* transcription is mediated by photosynthetic products (Fig. 2B and C).

GLB1 transcription was further examined in roots of *L. japonicus* plants grown in hydroponic cultures for 2 weeks in N-sufficient conditions (1 mM ammonium nitrate) and then transferred to fresh nutrient solution containing no N source. To minimize the diurnal changes in the expression level, all samples were taken in the middle of the light time (Fig. 2, 6 h) and RNA was extracted from roots at time 0, 24, 48, and 72 h and 5 days after the shift. The *LjGLB1* transcription showed a rapid downregulation (fourfold) that was maintained until at least 72 h after the shift (Fig. 3). Interestingly, the amount of *LjGLB1* transcript in roots increased again after 5 days under N-starvation conditions, to the basal level observed at 0 h (Fig. 3). As a control of the experimental conditions, we analyzed the expression profile of the high-affinity ammonium transporter *LjAMT1;1* that, as expected, was strongly induced after the shift from N-sufficient to N-starvation conditions (Fig. 3) (D'Apuzzo et al. 2004). Therefore, our results indicated a significant regulation of the *GLB1* expression at the transcriptional level that takes place in either leaves or root tissues.

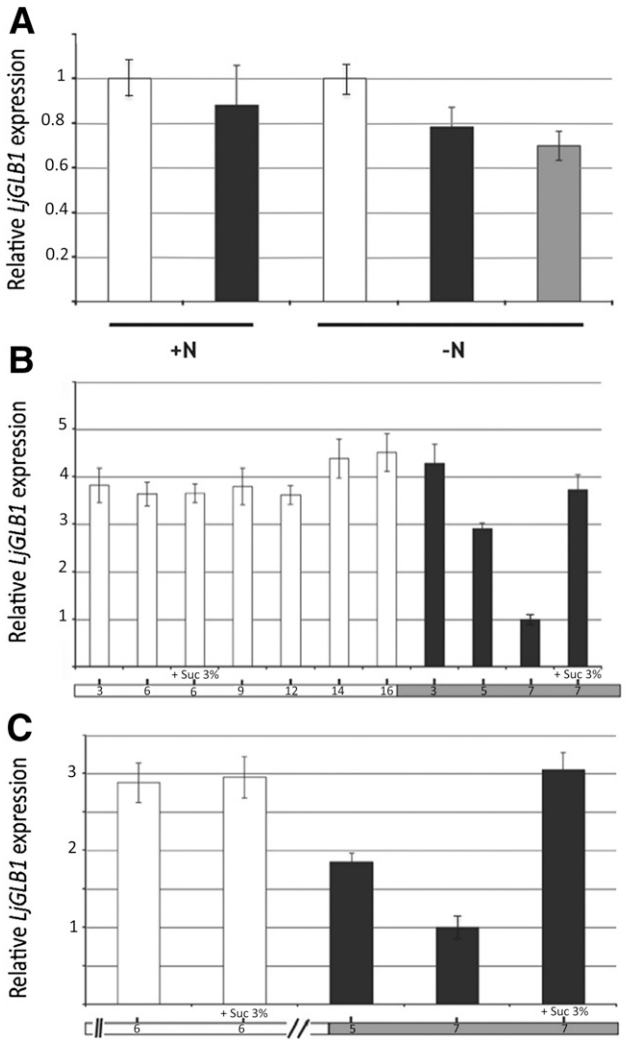

Fig. 2. *LjGLB1* transcriptional regulation in different organs and during day-night cycle. **A,** Expression in different organs. RNAs were extracted by 3-week-old plants grown on 1 mM NH_4NO_3 (+N) or without nitrogen (–N). White bars = roots, black bars = leaves, and gray bar = nodules. **B** and **C,** Expression during the day-night cycle. Plants were grown in hydroponic conditions (1 mM NH_4NO_3) and RNAs were extracted by roots (B) and leaves (C) of 3-week-old plants at different hours from the beginning of light (white) and dark (gray) periods. The presence of 3% sucrose in the growth medium is indicated. *LjGLB1* expression levels were normalized with respect to the internal control *ubiquitin* (*UBI*) gene and plotted relative to the expression from roots in A and to the 7-h dark samples in B and C. Data bars represent the mean and standard deviations of data obtained with RNA extracted from three different sets of plants and three real-time quantitative polymerase chain reaction experiments. Asterisks indicate significant differences with the levels of expression under light conditions ($P < 0.05$).

PII localization and spatial profile of expression.

Unlike other plant PII proteins, the *Lotus* sequence didn't show a clear-cut N-terminal transit peptide for chloroplast targeting according to the prediction program CHLOROP. Therefore, in order to check the PII protein subcellular localization, we fused its C-terminal end without a stop codon to the *green fluorescent protein* (*GFP*) gene, and placed the fusion downstream of the *Cauliflower mosaic virus* (CAMV)-35S promoter. Confocal laser-scanning fluorescence microscopy in stable transgenic hairy roots indicated unambiguously a plastid localization of GFP in roots, confirming previous data obtained in *Arabidopsis* and rice (Baud et al. 2010; Hsieh et al. 1998; Sugiyama et al. 2004). The specificity of the plastids as target was indicated by colocalization with the red fluorescence of the *AtrecA*-DsRed2 fusion (Supplementary Fig. S2 A to C) (Köhler et al. 1997).

To gain further information about the profile of *LjGLB1* expression in roots and nodules, we isolated the 5′ region of the gene to obtain a T-DNA construct carrying a promoter-*gus*A fusion. A PCR fragment extending up to 980 bp upstream of the ATG of *LjGLB1* and including the first 21 PII codons was subcloned in the pBI101.1 binary vector to obtain a translational fusion with the *gus*A reporter gene (Jefferson 1987). *Lotus* composite plants obtained upon transformation with *Agrobacterium rhizogenes* (Martirani et al. 1999) were used to analyze the expression of the translational p*LjGLB1-gus*A fusions in a hairy root system. In the root tissues, β-glucuronidase (GUS) activity was substantially limited to the stele, where it was detected in 100% of the stained roots (Fig. 4A). In some cases, a longer incubation time allowed the detection of the blue staining in cortical cells and cap cells, whereas no activity could be observed in meristematic regions (Fig. 4B and C). Root cross sections allowed the definition of a more precise pattern showing a distribution of GUS activity inside the endodermis cell layer, with the main staining apparently located in the primary phloematic space (Fig. 4D). The promoter activity of the *Arabidopsis thaliana GLB1* gene has been described in leaf vascular structures only in an overexpressing *Arabidopsis* WRl1 genotype (Baud et al. 2009, 2010). However, analysis of the 980-bp 5′ untranslated region *LjGLB1* region exploited for driving the *gus*A expression in transgenic hairy roots didn't reveal any of the 15-bp boxes (cAAAAGtAggggttT) reminiscent of the consensus sequence required for WRl1 induction of glycolitic and fatty acid biosynthetic genes (Baud et al. 2010), while only one putative AW-box motif ($CnTnGn_7CG$; Maeo et al. 2009) was found at position –231 relative to the translational start codon (data not shown).

The profile of *LjGLB1* promoter activity was also analyzed during different steps of nodule development or function. GUS activity was never induced during the initial stages of *M. loti* infection whereas the analysis in young and mature nodules allowed the detection of a strong blue staining in the nodule vascular bundles, with a weaker activity in the nodule parenchyma (Fig. 4E). The absence of *LjGLB1* transcriptional regulation early after inoculation was also confirmed by the qRT-PCR analysis shown in Supplementary Figure S3. In addition, the observed N-dependent transcriptional regulation

together with the reported impact of the photosynthate supply on the *LjGLB1* transcriptional profile (Figs. 2 and 3) prompted us to investigate the relationship between nodular *LjGLB1* expression and N fixation activity. Therefore, the pattern of GUS activity was evaluated in young nodules obtained with an *M. loti fix*$^-$ mutant. The transgenic nodules obtained in hairy roots inoculated with either the wild type or an *nifH* mutant didn't show any change in the quantitative and qualitative GUS activity distribution (data not shown).

Generation of transgenic *L. japonicus* plants ectopically expressing the *GLB1* gene.

In order to test whether the *GLB1* gene in legumes plays any role in the formation or functioning of N-fixing-nodules, a process strictly dependent on a correct C/N balance, we cloned the *LjGLB1* coding sequence between a CaMV-35S promoter sequence and a tNOS terminator sequence (Hajdukiewicz et al. 1994) to obtain transgenic PII-overexpressing *Lotus* plants. Primary transformed plants were selected on hygromycin medium and allowed to self-pollinate. Independent T1 lines, germinated on hygromycin (hygromycin resistance segregation = 3:1), were tested by semiquantitative RT-PCR to analyze the level of *GLB1* transcript, and transgenic lines with various levels of overexpression were identified. T2 homozygous plants 7-13 and 8-9, showing 5- and 10-fold increase, respectively, of the *LjGLB1* transcript, were selected for further phenotypic analysis (Fig. 5A). To verify proper protein synthesis of the ectopically expressed gene, Western blot analysis was performed on crude protein extracts from the transgenic and wild-type plants, and PII was detected using a polyclonal antiserum raised against recombinant *A. thaliana* PII protein (Ferrario-Méry et al. 2005). A linear increase of the PII protein in the transformants, proportional to the observed induced amount of transcript when compared with the wild-type plant, is shown in Figure 5B.

Phenotypic characterization of PII-overexpressing plants.

The spatial profile of *GLB1* promoter activity in the nodular tissue prompted us to test whether the PII protein could play a role as an integrator of the C, N, and energy levels that are critical for a correct nodule organogenesis and functioning. A comparison of the nodulation response in *Lotus* wild-type and PII-overexpressing seedlings grown with low N supply (10 μM NH_4NO_3) and inoculated with *M. loti* indicated equivalent phenotypes in terms of nodule numbers (Fig. 6A), nodule mass (data not shown), and structural organization (Supplementary Fig. S4A). On the other hand, analysis of N fixation activity at 28 days after inoculation indicated a significant 30% reduction in nodules of PII-overexpressing plants (Fig. 6B). However, this deficiency was not sufficient to cause a significant difference in terms of fresh shoot weight that was comparable in the two plant genotypes (Supplementary Fig. S4B). Because Ferrario-Méry and associates (2006) reported a reduction of several products of the ornithine/arginine biosynthetic pathway in *A. thaliana* PII knock-out mutants in response to ammonium resupply after N starvation, we tested the content of polyamines, which are the final products of this biosynthetic pathway and have been involved in the regulation of symbiotic efficiency, in PII-overexpressing plants. Polyamines were extracted in mature nodules of plants grown in 10 μM NH_4NO_3 and their relative amounts compared in wild-type and 8-9 plants. The content of spermidine and spermine were much higher than that of citrulline and didn't change significantly between wild-type and overexpressing plants, whereas the citrulline level was strongly increased (6.5-fold) in nodules of the 8-9 plants (Fig. 6C and D).

N depletion in the soil is a prerequisite for nodule development and function, and high concentrations of N as nitrate and ammonia might abolish nodulation. We reported a link between the *L. japonicus* N nutritional status and nodule capacity predisposition, showing that the competence for nodulation of *L. japonicus* plants preincubated, prior to the *M. loti* inoculation, on high N (10 mM NH_4NO_3) conditions was significantly reduced (approximately 50%) when compared with plants maintained in low N (10 μM NH_4NO_3) permissive conditions (Omrane et al. 2009). The inhibitory effect was maintained for at least 6 days in *Lotus* plants preincubated on high N, indicating that it was correlated with a systemic change of the general N nutritional state of the plants that was transduced to the root nodulation machinery (Omrane et al. 2009). Therefore, in order to analyze any possible involvement of the

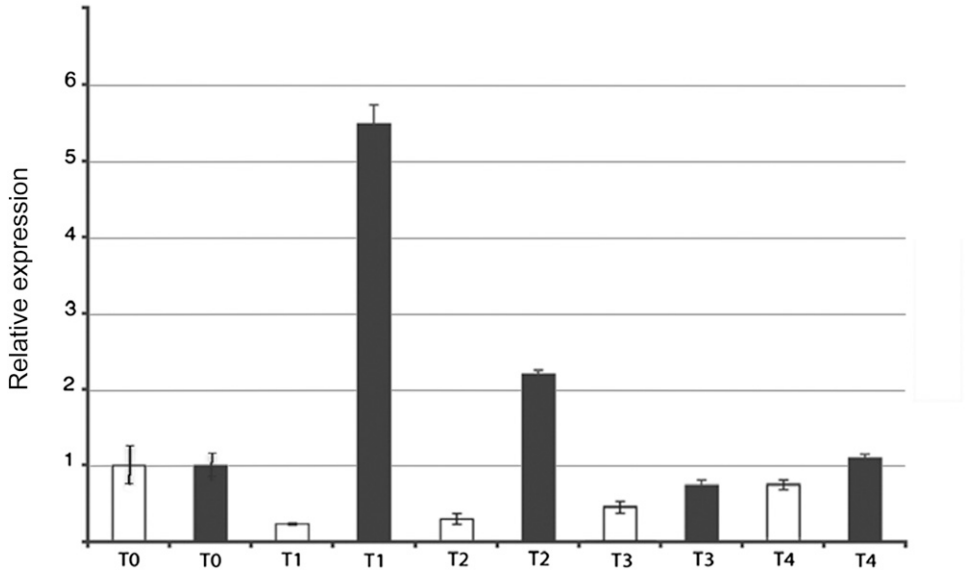

Fig. 3. Effect of N deficiency treatment on *LjGLB1* and *LjAMT1;1* expression. RNAs were extracted from roots of plants grown in presence of 1 mM NH_4NO_3 (T0) and after shifting to –N conditions (24 h = T1, 48 h = T2, 72 h = T3, and 5 days = T4). Expression levels were normalized with respect to the internal control *ubiquitin* (*UBI*) gene and plotted relative to the expression of T0. White bars = *LjGLB1* and black bars = *LjAMT1;1*. Data bars represent the mean and standard deviations of data obtained with RNA extracted from three different sets of plants and three real-time polymerase chain reaction experiments. Asterisks indicate significant differences with T0 conditions ($P < 0.05$).

PII protein in the signal transduction route connecting N nutritional status and nodulation capacity, we compared the nodule formation phenotype of wild-type and PII-overexpressing plants following the experimental scheme reported in Omrane and associates (2009). Wild-type *L. japonicus* plants preincubated on 10 mM ammonium nitrate showed a reduced number of nodules when compared with plants maintained on 10 μM ammonium nitrate (Fig. 7A; 3.37 versus 1.75 nodules/plant). Strikingly, both overexpressing 7-13 and 8-9 PII lines were able to rescue this inhibitory effect, showing the same level of nodulation capacity in low and high N conditions (Fig. 7A). In *L. japonicus*, a central role of the CLAVATA3/ESR-related glycopeptide (CLE) CLE glycopeptide LjCLERS2 in the nitrate-induced systemic suppression of nodulation through interaction with the hypernodulation aberrant root formation (HAR1) receptor kinase has been reported (Okamoto et al. 2009, 2013). In order to test whether PII overexpression could induce a deregulation of *LjCLERS2*, we compared the amount of transcript in wild-type and PII-overexpressing plants incubated for 10 days in low and high NH_4NO_3 conditions. Consistently with data reported in the literature, *LjCLERS2* was induced in *Lotus* wild-type plants incubated on higher N concentrations when compared with plants grown on 10 μM NH_4NO_3 permissive conditions, and a similar pattern was observed in the 8-9 plants (Fig. 7B).

DISCUSSION

How PII is regulated in higher plants and, hence, how this transduction protein can sense the C/N status of a plant is still a matter of debate. A posttranslational control of its activity similar to that of PII from prokaryotes has not yet been demonstrated (Smith et al. 2004). In oxygenic photosynthetic organisms, the PII interaction with NAGK, the key enzyme of the arginine biosynthesis pathway, enhances the catalytic kinase activity and leads to the relief of the feedback inhibitory effect by the final pathway product, arginine (Chen et al. 2006; Ferrario-Méry et al. 2006). PII in the ATP-ligated state has a very high affinity for NAGK, whereas ADP and 2-OG display a nonantagonizing inhibitory effect on the formation of the PII-NAGK complex which, therefore, seems not governed by the intrinsic ATPase activity of PII (Maheswaran et al. 2004; Zeth et al. 2014). 2-OG also has been proposed as the main signal controlling the PII inhibition of ACCase in chloroplast extracts, thereby expanding plant PII function beyond N regulation (Feria Bourrellier et al. 2010).

With regard to the role played by a transcriptional regulation on the *GLB1* gene, recently, a striking observation reporting the upregulation of the PII transcript (10-fold) in the early- to mid-stages of *A. thaliana* developing seed strongly supported an involvement of such a mechanism of regulation in the control of plant PII function (Uhrig et al. 2009). Partially inconsistent data were reported for the transcriptional level of regulation of the *GLB1* gene expression in leaves (Ferrario-Mèry et al. 2005; Hsieh et al. 1998). A strong and rapid activation of *GLB1* transcription was first reported in dark-adapted *Arabidopsis* plants after a shift in light conditions and this derived, at least in part, from photosynthetic products (Hsieh et al. 1998). On the other hand, a stable amount of the *GLB1* transcript was observed in *Arabidopsis* rosette leaves during most of the day-night cycle, with a slight increase only at the end of the day (Ferrario-Mèry et al. 2005). We analyzed the profile of *LjGLB1* expression in both *Lotus* root and leaf tissues and found a rapid induction of the *LjGLB1* transcript during the light period. This effect could be mimicked, during the dark period, by the addition of 3% sucrose, indicating that induction of the transcription is mediated by the sensing of photosynthetic products (Fig. 2). We also observed an N-dependent regulation of transcription with a rapid and transient decrease of the amount of *LjGLB1* transcript followed by a progressive increase in roots of plants transferred into N-starvation conditions (Fig. 3). This latter result might be consistent with data reported in *A. thaliana,* where the *AtGLB1* transcript level was analyzed only 5 days after a shift to an

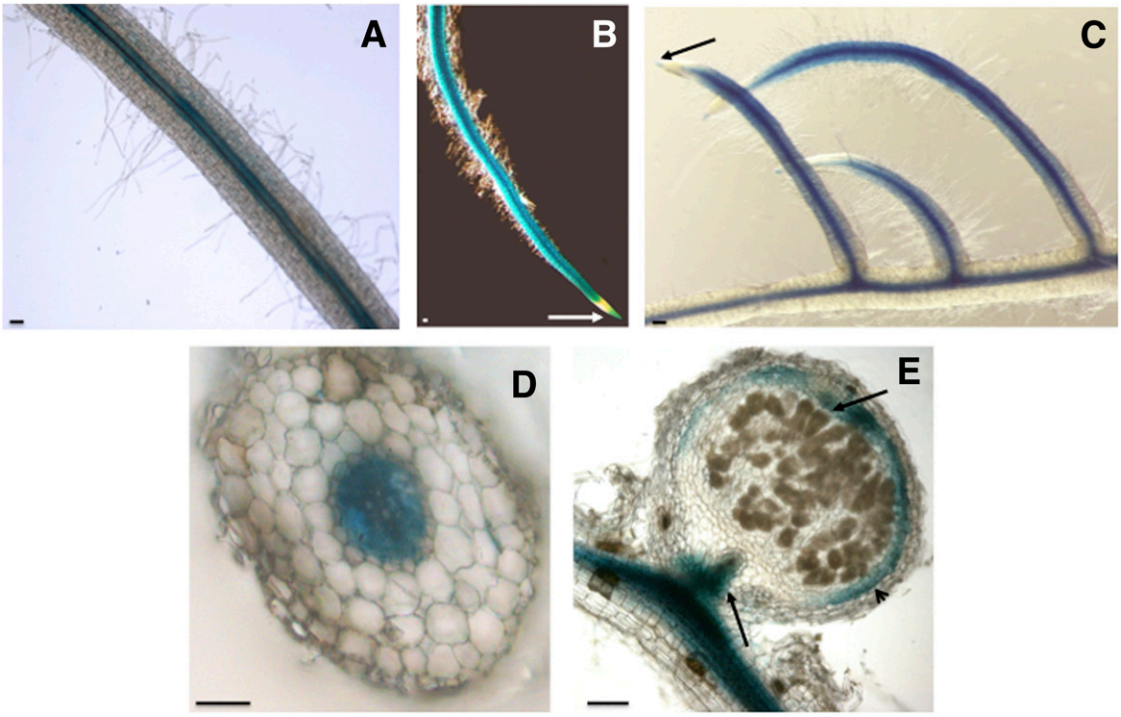

Fig. 4. Spatial profile of expression of the *LjGLB1* gene in root and nodular tissues. **A** to **C,** Whole-mount staining of *Lotus japonicus* hairy roots transformed with the *pLjGLB1-gus*A construct. **A,** β-Glucuronidase (GUS) activity in the root vascular bundle; **B** and **C,** GUS activity in the root vascular bundle, cortical cells, and root cap (arrows); **D,** cross section of atransgenic root with GUS activity detected into the root stele; **E,** longitudinal section of a transgenic mature representative nodule. GUS activity is detected in the nodule vascular bundle (arrows) and parenchyma (arrowhead). Bars on the left = 50 μm.

N-deficiency condition and showed no significant variation (Ferrario-Mèry et al. 2005). Thus, our results indicate that a significant part of the regulation of the *LjGLB1* profile of expression takes place at the transcriptional level.

The growth conditions tested in this work (availability of different N and C sources) significantly affecting the regulation of *LjGLB1* gene expression (Figs. 2 and 3) might strongly impact the efficiency of symbiotic N-fixation processes (nodule formation, development, and function). Photosynthate resources must be allocated to the nodules where C derived from the metabolism of sucrose is used for several physiological processes, including plant and bacterial respiration, assimilation of fixed N_2, and starch and cellulose biosynthesis (Tsikou et al. 2013). Furthermore, a strict interdependence exists between the level of nitrogenase activity and the efficiency of C source flux providing energy for the bacteroid-mediated N_2 reduction (Pathirana et al. 1992; Schulze et al. 1998). On the other hand, limitation of combined N in the soil is a prerequisite for initiation, development, and functioning of N-fixing nodules (Barbulova et al. 2007; Bisseling et al. 1978; Caetano-Anollés and Gresshoff 1991; Matamoros et al. 1999), and legumes employ a number of regulatory mechanisms to avoid nodulation under N-replete conditions, when sufficient mineral or organic N is available in the soil, restricting nodulation to sustainable levels only when soil N is limiting (Omrane and Chiurazzi 2009).

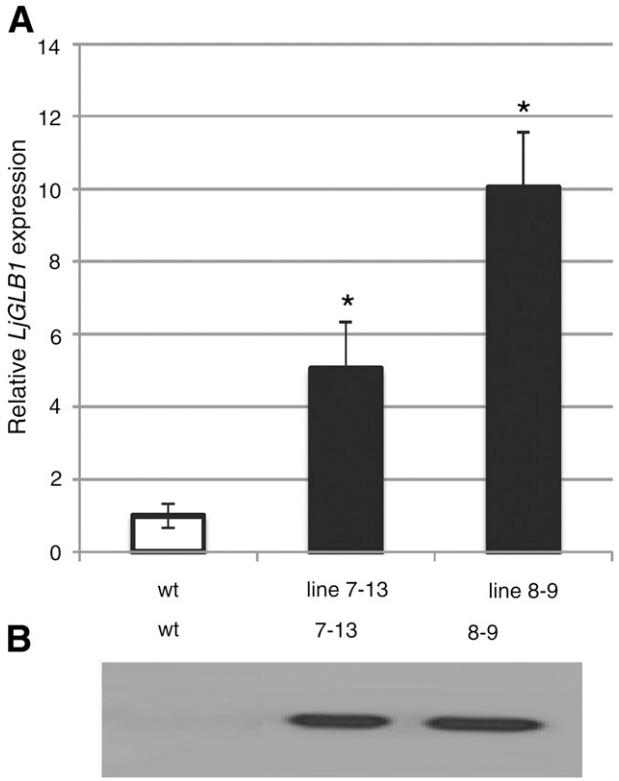

Fig. 5. Molecular characterization of 35S-*LjGLB1* overexpressing lines. **A,** Semiquantitative analysis of *GLB1* transcript level in roots of the wild type (wt) (white bar) and the two overexpressing lines 7-13 and 8-9 (T2 homozygous plants; black bars). Expression levels were normalized with respect to the internal control *ubiquitin* (*UBI*) gene and plotted relative to the expression in wild-type plants. Data bars represent the mean and standard deviations of data obtained with RNA extracted from two different sets of plants and three reverse-transcriptase polymerase chain reaction experiments. **B,** Corresponding analysis of PII protein levels by western blot using *A. thaliana* PII specific antibody. The lanes contain 20 µg of soluble proteins extracted from three *Lotus* plants. Asterisks indicate significant differences with the level of expression in wild-type plants ($P < 0.05$).

As preliminary tool to initiate studies on the putative role played by the PII protein during the nodulation process, we exploited two transgenic overexpressing lines in which *LjGLB1* expression is uncoupled from its native regulation (Fig. 5). PII-overexpressing plants grown under low N permissive conditions do not show differences in terms of nodule formation capacity, whereas a significant 30% reduction of nitrogenase activity measured as acetylene reduction activity (ARA) was observed when compared with wild-type plants (Fig. 6A

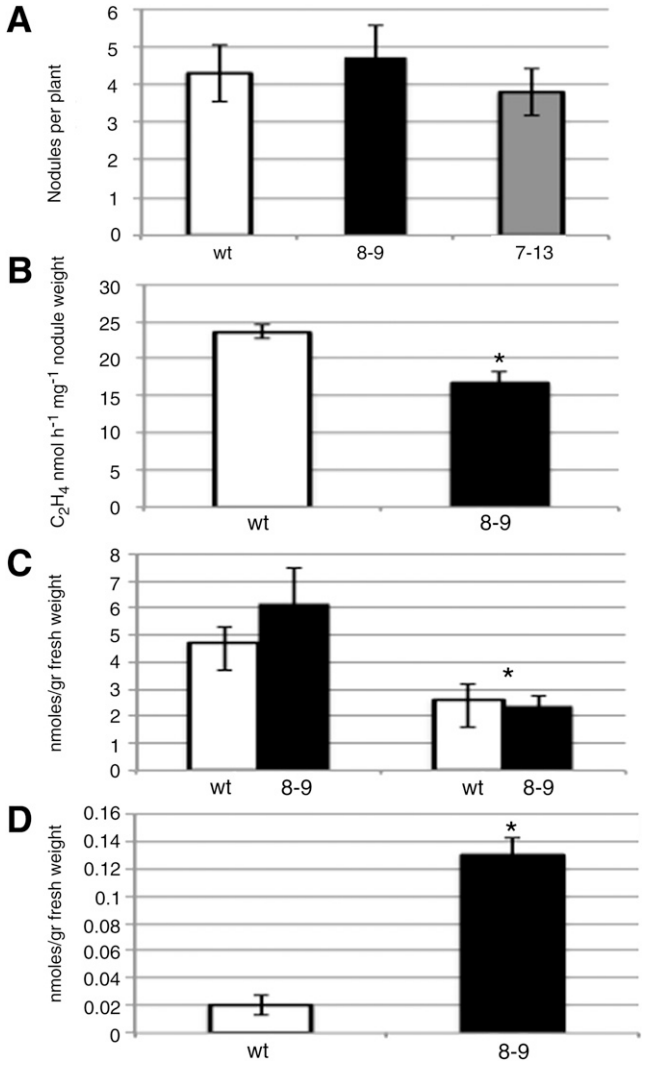

Fig. 6. Nodular phenotypical analysis of wild-type (wt) and PII-overexpressing plants grown in low N conditions. **A,** Number of nodules per plant. Plants were germinated on H_2O agar, and 7-day-old seedlings were transferred on Gamborg B5 derived medium with 10 µM NH_4KNO_3 as sole N source and inoculated with *Mesorhizobium loti*. Nodules were scored at 4 weeks after inoculation; wt (white bars), 8.9 (black bars), and 7.13 (gray bar). Data represent the mean and standard error obtained from three independent experiments (15 plants per experiment). Asterisks indicate significant differences with the number of nodules observed without putrescine. **B,** Acetylene reduction activity per nodule weight of wt and 8-9 plants. Data bars indicate the mean and standard error of three independent experiments ($n = 2$ plants per experiment). Asterisk indicates significant difference ($P < 0.02$). **C,** Spermine (left) and spermidine (right) content in nodules of wt and 8-9 plants. Polyamines were extracted from 4-week-old nodules. Data represent the mean and standard error obtained from two independent experiments (20 plants per experiment). Asterisk indicates significant difference ($P < 0.01$). **D,** Citrulline content in nodules of wt and 8-9 plants. Polyamines were extracted from 4-week-old nodules. Data represent the mean and standard error obtained from two independent experiments (20 plants per experiment). Asterisk indicates significant difference ($P < 0.01$).

and B). However, in the exploited experimental system where 10 μM KNO_3 is present as an N source, this nodular activity defect is not sufficient to affect normal shoot growth phenotype (Supplementary Fig. S4B).

The spatial profile of the *LjGLB1* promoter activity shown in Figure 4 which, to our knowledge, represents the first information reported in the literature about the PII distribution in root tissues, and indicates a main localization in vascular bundles (Fig. 4). Root vascular structures mediate long-distance transport of compounds such as N metabolites and sucrose from root to shoot and vice versa. Hence, this spatial profile of PII localization might be consistent with both transcriptional patterns shown in Figures 2 and 3, where regulation of the *LjGLB1* expression was reported to be controlled by photosynthetic products and N supply conditions.

However, the expression pattern of *LjGLB1* promoter activity in nodule vascular bundles and parenchima (Fig. 4E) might provide an additional clue about a possible link between PII function and the observed ARA defective phenotype, because this partially overlaps those of *L. japonicus* genes encoding spermidine synthase, spermine synthases, arginine decarboxylase, and ornithine decarboxylase (*LjSPDS*, *LjSPMS, LjADC*, and *LjODC*, respectively), involved in the synthesis of polyamines spermidine and spermine from putrescine and mainly expressed in the nodule parenchima and vascular bundles (Efrose et al. 2008; Flemetakis et al. 2004). A high content of putrescine, spermine, and spermidine in mature nodules, with an increased amount compared with other legume organs, has been already reported (Flemetakis et al. 2004; Fujihara et al. 1994). The *LjODC* gene encodes ornithine decarboxylase, which catalyzes the synthesis of ornithine to putrescine, the obligate precursor of spermidine and spermine (Fig. 8), and a root- and nodule-specific expression profile for *LjODC* has been detected, with a strong induction observed during nodule development (Flemetakis et al. 2004). Our analysis of polyamine content in wild-type nodules with the lower level of citrulline compared with spermine or spermidine (Fig. 6C and D) suggests the occurrence in nodular tissue of a metabolic shunt of ornithine into putrescine for polyamine biosynthesis through the action of ornithine decarboxylase, rather than the alternative pathway catalyzed by ornithine transcarbamylase that utilizes the same substrate (ornithine) for the synthesis of citrulline (Fig. 8), and this hypothesis is consistent with the previously reported *LjODC* profile of expression (Flemetakis et al. 2004). The high level of spermine and spermidine was not affected in nodules of PII-overexpressing plants (Fig. 6C), where we observe a clear-cut 10-fold increase of the citrulline content when compared with the wild type (Fig. 6D). The biosynthetic pathway involving the enzymes ornithine decarboxylase, arginine decarboxylase, spermidine synthase, and spermine synthases is controlled by the major plant PII-interacting protein NAGK (Fig. 8) (Burillo et al. 2004; Ferrario-Méry et al. 2006; Sugiyama et al. 2004) and the increased level of citrulline observed in nodules of the PII-overexpressing plants (Fig. 6D) is consistent with the reduced ornithine, citrulline, and arginine accumulation observed in *Arabidopsis* PII knock-out mutants (Ferrario-Méry et al. 2006). The reason we

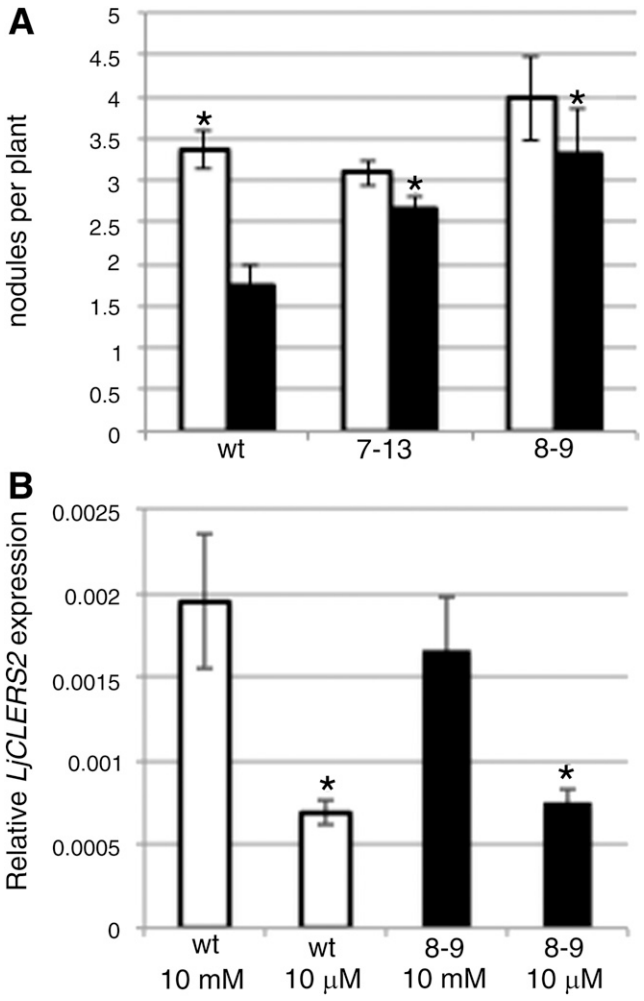

Fig. 7. N-dependent nodule formation phenotypes in wild-type (wt) and *Lotus japonicus* PII-overexpressing 7-13 and 8-9 plants. **A,** White bars represent number of nodules observed in plants preincubated for 10 days on 10 μM NH_4NO_3 and then shifted on the same condition and inoculated with *Mesorhizobium loti*. Black bars represent nodule numbers observed in plants preincubated for 10 days on 10 mM NH_4NO_3 and then shifted onto 10 μM NH_4NO_3 and inoculated with *M. loti*. Nodules were counted at 4 weeks post inoculation. Data represent the mean and standard error obtained from three independent experiments (20 plants per experiment). Asterisks indicate significant differences from the number of nodules observed in wt plants preincubated on 10 mM NH_4NO_3 ($P < 0.05$). **B,** Relative quantification of *LjCLERS2* mRNA measured by quantitative reverse-transcriptase polymerase chain reaction in roots of wt and 8-9 plants incubated for 10 days with 10 mM NH_4NO_3 or 10 μM NH_4NO_3 conditions. *L. japonicus UBI* was used as reference gene to normalize the expression of *LjCLERS2*. Data bars represent means and standard deviation of data obtained with RNA extracted from two different sets of plants and three technical repeats. Asterisks indicate significant differences with the level of expression in 10 mM conditions ($P < 0.05$).

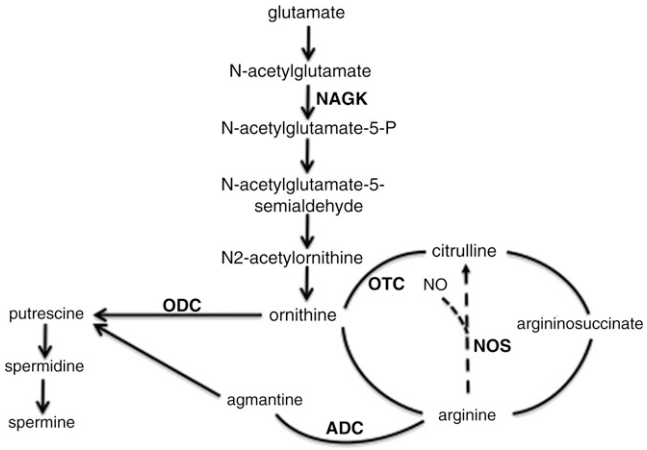

Fig. 8. Pathways of polyamines biosynthesis. Dotted lines indicate the putative nitric oxide synthase (NOS)-dependent pathway for nitric oxide (NO) biosynthesis. ADC = arginine decarboxylase, NAGK = N-acetyl-L-glutamate kinase, ODC = ornithine decarboxylase, and OTC = ornithine transcarbamylase.

didn't observe a parallel increase of the spermine and spermidine levels could be a consequence of the hypothesized metabolic shunt leading to a high basal level of these polyamines in wild-type nodules. Polyamines are important components for the establishment of optimal symbiotic performance, and a negative effect of high concentration of exogenous putrescine on ARA activity has been reported (Wisniewski and Brewin 2000). Therefore, the reduced N-fixation activity observed in PII-overexpressing plants could be related to the increase of nodular polyamines through the ornithin biosynthetic pathway.

The other clear-cut phenotype scored in the PII-overexpressing lines is the capacity of responding very effectively to a preliminary high N treatment, prior to *M. loti* inoculation, that causes an approximately 50% reduction in the nodulation rate of wild-type *L. japonicus* plants, completely rescuing this deficient phenotype (Fig. 7A) (Omrane et al. 2009). Therefore, our results suggest that the PII protein in legumes may serve as part of a complex signal transduction network involved in perceiving the N status of the plant and regulating nodulation capacity predisposition. In legumes, a systemic adaptation mediated by plant N status regulating nodule formation has been demonstrated (Jeudy et al. 2010) and Affymetrix GeneChip analyses indicate a strong systemic influence of the N supply on the profile of gene expression in roots before and after inoculation (Omrane et al. 2009; Ruffel et al. 2008). The reported effect of PII overexpression on the ornithine/arginine biosynthetic pathway (Fig. 6D) also could be correlated with the improved nodulation response of *Lotus* plants to high N conditions (Fig. 7A) because polyamines might also affect nodule formation capacity and, in particular, a positive effect of citrulline supply on the formation of infection threads structures in *L. japonicus* nodules has been reported (Mishima et al. 2008). Another intriguing correlation to the increased level of citrulline could be a positive effect of PII overexpression on the arginine-dependent synthesis of nitric oxide (Fig. 8) that plays a direct role on the nodule formation process, because a depletion of this signal causes downregulation of plant genes involved in nodule development and formation (Boscari et al. 2013; del Giudice et al. 2011) and a significant decrease of nodule number (del Giudice et al. 2011; Pauly et al. 2011).

However, our data indicate that one of the actors playing a crucial role in the nitrate-dependent control of nodule initiation, *LjCLERS2* (Okamoto et al. 2009; Okamoto et al. 2013), is likely not to be a target of the PII action, because its transcriptional profile did not change in overexpressing plants (Fig. 7B). Nitrate may affect lateral root development (Zhang et al. 1999) as well as nodule formation (Omrane and Chiurazzi 2009) by acting both as a nutrient through the assimilation pathways and consequent change of the systemic general nutritional status and as a local signal independent of its assimilation. *LjCLERS2* is a root-specific gene and its nitrate-dependent induction could be mediated by a local signaling pathway followed by the systemic transmission through xylem of the arabinosylated form of the peptide to the shoot where the interaction with the HAR1 receptor kinase takes place, controlling the process of nodule formation autoregulation (Okamoto et al. 2013). The *LjGLB1* gene is ubiquitously expressed in *Lotus* plants (Fig. 2A) and PII protein likely acts by perceiving the general N nutritional change determined by nitrate supply conditions. Therefore, LjCLERS2 and PII might be part of two different nitrate-dependent regulatory pathways controlling the nodule formation capacity; this would explain the conserved *LjCLERS2* profile of expression observed in wild-type and PII-overexpressing plants (Fig. 7B).

In conclusion, our data suggest a possible novel role of the legume PII protein in the control of nodule functioning and signaling pathways linking N nutritional status and nodulation competence. Ultimately, a global metabolomic analysis in the PII-overexpressing plants and the use of RNAi constructs driven by the *LjGLB1* own promoter, vascular bundle-specific, or nodule-specific promoters will allow us to gain further insights into such functional roles of PII.

MATERIALS AND METHODS

Plant material and growth conditions.

All experiments were carried out with *Lotus japonicus* ecotype B-129 F12 GIFU. Sterilized seed were sown on H_2O agar plates and left over night at 4°C cap-side down. After 24 h in the dark in the growth chamber, Petri dishes were exposed to light and kept in a vertical position. Care was taken to maintain the young emerging roots in contact with the filter paper. For the analysis of nodulation capacities, unsynchronized seedlings were discarded at this stage. Plants were cultivated in a growth chamber with a light intensity of 200 μmol m^{-2} s^{-1} at 23°C with a day and night cycle of 16 and 8 h, respectively. Solid growth substrate had the composition of B5 medium (Gamborg 1970), except that, when needed, $(NH_4)_2SO_4$ and KNO_3 were omitted or replaced by ammonium nitrate (NH_4NO_3). KCl was added to the medium to replace the potassium source. The media containing vitamins (Duchefa catalog G0415) were buffered with 2.5 mM 2-(N-morpholino)-ethanesulfonic acid (MES) (Duchefa, MIS03.0250) and pH was adjusted to 5.7 with KOH.

Experimental conditions for the analysis of the high N preincubation effect on nodulation have been described by Omrane and associates (2009). Seedlings (3 to 5 days old) were transferred and grown for 10 days on B5 derived medium where $(NH_4)_2SO_4$ and KNO_3 were omitted and replaced by 10 mM (High-N) or 10 μM (Low-N) NH_4NO_3. KCl was added to the medium to replace the potassium source. During these 10 days of preincubation, plants were transferred twice on fresh media to avoid nutrient depletion. After 10 days, both High-N and Low-N plants were transferred onto fresh 10 μM NH_4NO_3 medium and inoculated with *M. loti*. Six days after inoculation, plants were transferred onto 10 μM NH_4NO_3 medium with addition of cefotaxime at 50 mg $liter^{-1}$.

Hydroponic culture conditions for the analysis of N- and light-dark cycle-dependent *LjGLB1* regulation have been described by D'Apuzzo and associates (2004). One-week-old plants germinated on solid medium were transferred into vessels harboring eight plants in approximately 100 ml of the 1 mM NH_4NO_3 B5 derivative medium. In the N-free solution, the NH_4NO_3 was omitted. The pH of the media was adjusted to 5.7 with MES. To avoid depletion, the nutrient solution was renewed every 3 days during the 10 days of growth in hydroponic cultures. The pH of the medium was checked daily and it was maintained within close limits (5.8 to 5.6) in all the conditions of hydroponic growth. Plants were cultivated in a growth chamber with a light intensity of 200 μmol m^{-2} s^{-1} at 23°C with a day and night cycle of 16 and 8 h, respectively.

M. loti strain R7A was used for the inoculation experiments and was grown in liquid Luria Broth (LB) medium supplemented with rifampicin (20 mg/liter). The *M. loti nifH*$^-$ strain was kindly provided by Dr. Clive Ronson (University of Otago, New Zealand) and was grown in the same medium supplemented with rifampicin and gentamicin (10 mg/liter). The inoculation procedure (10^7 cells per root tip) for the in vitro nodulation assay has been reported elsewhere (Barbulova et al. 2005).

L. japonicus transformation procedures.

Agrobacterium tumefaciens and *rhizogenes*–mediated *L. japonicus* transformations were performed as described by Lombari and associates (2003) and Martirani and associates (1999), respectively.

T-DNA constructs preparation.

To obtain the PII-overexpressing construct, the *LjGLB1* cDNA was amplified with the two oligonucleotides 5′-GAA GATCTATGGCGATTGCGAGAACGCAC-3′ (including a *Bgl*II site) and 5′-ACGCGTCGACTCATACAGTAGATAA TATGTC-3′ (including a *Sal*I site) and subcloned as a *Bgl*II-*Sal*I fragment into pCAMBIA1300 *BglII-Sal*I double digested.

The 35S-*GLB1-GFP* fusion was prepared in the following way: the *LjGLB1* cDNA sequence was amplified with the two oligonucleotides 5′-GAGGATCCATGGCGATTGCGAGAA CGCAC-3′ (including a *Bam*HI site) and 5′-GCGGTACCTA CAGTAGATAATATGTCAGT-3′ (including a *Kpn*I site) and cloned into the double-digested *Bam*HI-*Kpn*I β-GFP plasmid (Duby et al. 2001), and the correct sequence for the LjGLB1-GFP translational fusion was verified by sequencing. The *LjGLB1-GFP* cassette was then cloned as a *Bam*HI-*Sac*I fragment into the double-digested *Bgl*II-*Sac*I pCAMBIA1300 vector.

The pLjGLB1-*gus*A fusion was prepared in the following way: a PCR-amplified fragment was obtained on genomic DNA with two specific oligonucleotides—5′-GCGTCGACACCGTTTTT CCCCAGTAACCG-3′ (containing a *Sal*I site) and 5′-CGCG GATCCCATTGGAGCTTCGTTGAGCTG-3′ (containing the *Bam*HI site) —and subcloned as a *Sal*I-*Bam*HI fragment into the pBI101.1 vector to obtain a translational fusion.

Confocal analysis.

Confocal microscope analyses were performed using a Nikon PCM2000 (Bio-Rad, Germany) laser-scanning confocal imaging system. For GFP and red fluorescent protein detection, excitation was at 488 nm and detection between 515 and 530 nm. The images acquired from the confocal microscope were processed using ImageJ bundle software.

Quantitative real-time RT-PCR.

Total RNA was prepared from *Lotus* tissues using the procedure of Kistner and Matamoros (2005). The samples were treated with DNAse I (Ambion) to remove contaminating DNA, the absence of which was subsequently confirmed by PCR. Total RNA (1 μg) was annealed to random decamers and reverse-transcribed with reverse transcription (Ambion) to obtain cDNA. Real-time PCR was performed with a DNA Engine Opticon 2 System (MJ Research, MA, U.S.A.) using SYBR to monitor double-stranded DNA synthesis. The *ubiquitin* (*UBI*) gene (AW719589) was used as an internal standard. The concentration of primers was optimized for each PCR reaction and each amplification was carried out in triplicate. The PCR program used was as follows: 95°C for 13 min and 39 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 15 s. Data were analyzed using Opticon Monitor Analysis Software (version 2.01; MJ Research). The qRT-PCR data were analyzed using the comparative cycle threshold (Ct) method. The relative level of expression was calculated with the following formula: relative expression ratio of the gene of interest is $2^{-\Delta CT}$, with $\Delta CT = Ct_{AMT1} - CT_{UBI}$. Analysis of the melting curve of PCR product at the end of the PCR run revealed a single narrow peak for each amplification product, and fragments amplified from total cDNA were gel purified and sequenced to assure accuracy and specificity. The oligonucleotides used for the qRT-PCR were the following: PII-forw 5′-GCAGAGGAAATGCCATGATT-3′, PII-rev 5′-CACCACGGATTCCCATATTC-3′, CLERS2-forw 5′-GCTCGTAATCTCCAAATCATTCACA-3′, and CLERS2-rev 5′-GGTGAGAGTCTTTGCTGTTGATATCC-3′

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis.

Tissue powder from 0.5-g aliquots was prepared by grounding fresh tissue in liquid N. The powder was then resuspended in 1 ml of 50 mM potassium phosphate buffer, 10 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride PMSF, pH 7.2, prechilled at 4°C. The cell-free crude extract samples were centrifuged for 15 min at 12,000 × *g* to remove insoluble material. Aliquots containing 20 μg of protein from a given supernatant preparation were separated by electrophoresis on a 12% (wt/vol) sodium dodecyl sulfate polyacrylamide gel. The separated proteins were then electroblotted onto polyvinylidene fluoride membranes and rinsed with Tris-buffered saline (20 mM Tris-HCl and 0.5 mM NaCl, pH 7.5). The membrane was stained with red Ponceau to check for an equally loaded amount of protein. PII protein was detected using the polyclonal antiserum raised against recombinant *Arabidopsis thaliana* PII protein (Ferrario-Méry et al. 2005). Western blot analysis was done according to the manufacturer's instruction (ECL Plus Western Blotting Detection System, Amersham, Uppsala, Sweden).

Histochemical GUS analysis.

Histochemical staining of whole-plant and section material was performed as described by D'Apuzzo and associates (2004).

Determination of ARA.

Detached roots with comparable numbers of nodules were placed in glass vials. The vials were filled with an acetylene-air mixture (C_2H_2–air = 1:9 [vol/vol]). After 1 h of incubation at 25°C, the amount of ethylene in the gas phase was determined using a gas chromatograph (PerkinElmer Clarus 580).

Measurement of polyamine levels.

For polyamine extraction and high-performance liquid chromatography (HPLC) analysis, a benzoylation method was performed as described previously (Flores and Galston 1982; Smith and Davies 1985), with some modifications. Fresh tissue (1 g) was homogenized in 10 ml of cold 0.2 N per-chloride acid containing 1 μmol of hexanediamine as an internal standard. The samples are incubated on ice for 40 min, then centrifuged at 4°C for 20 min. Aliquots of 0.5 ml of supernatant were added to 1 ml of 2 N NaOH with 10 μl of benzoyl chloride. The mixtures were incubated at room temperature for 20 min, and the reaction was terminated by the addition of 2 ml of saturated NaCl. Benzoylamines were extracted with 2 ml of diethyl ether. After centrifugation, the ether layer was collected and dried under N gas. The powdered samples were stored at −20°C until HPLC measurements. For HPLC analysis, the residues were redissolved in 120 μl of methanol. Standards were treated in a similar way, with 1 μmol of putrescine, cadaverine, hexanediamine, spermidine, and spermine in the reaction mixture. HPLC analysis was performed with a programmable Kratos dual-pump liquid chromatograph with detector 773. The solvent system consisted of methanol and water, run at 65% (vol/vol) methanol at a flow rate of 0.7 ml/min. Benzoylated extract (5 μl) was eluted at room temperature through a 4.6 x 250 mm, 5-mm particle size reverse-phase (C18) column (Varian, Walnut Creek, CA) and detected at 254 mm. The peak areas were recorded on a pen recorder and calculated by a computer with NIH Image software (National Institutes of Health, Bethesda, MD).

Phylogenetic studies.

The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are measured in units of the number of amino acid substitutions per site. The analyses involved 20 amino acid sequences. All positions containing gaps and missing data were eliminated. Phylogenetic analyses were conducted in MEGA5.2.2 (Tamura et al. 2007).

Statistical analysis.

Statistical analyses were performed using the VassarStats analysis of variance program.

ACKNOWLEDGMENTS

This work was supported by grants from the Italian Ministry of Education (Progetti di Rilevanza Nazionale, PRIN 2010/2011, PROROOT, Prot. 20105XLAXM); Progetto CISIA, Integration of Knowledge for sustainability and Innovation in the Agrofood Made in Italy; and Progetto POR CAMPUS, Qualità delle Produzioni Tipiche Campane ed il suo Territorio: Approcci Innovativi ed Integrati per rafforzare la Competitività del Sistema Agroalimentare—QUARC. We thank the facility of Integrated Microscopy of the Institute of Genetics and Biophysics for supporting the microscopy analysis; S. Salvia and D. Maiello for technical assistance; and S. Ferrario-Méry and M. Hayashi for providing PII antibody and *AtrecA*-DsRed2 construct, respectively.

LITERATURE CITED

Barbulova, A., D'Apuzzo, E., Rogato, A., and Chiurazzi, M. 2005. Improved procedures for in vitro regeneration and for phenotypical analysis in the model legume *Lotus japonicus*. Funct. Plant Biol. 32:529-536.

Barbulova, A., Rogato, A., D'Apuzzo, E., Omrane, S., and Chiurazzi, M. 2007. Differential effects of combined N sources on early steps of the Nod factor-dependent transduction pathway in *Lotus japonicus*. Mol. Plant-Microbe Interact. 20:994-1003.

Baud, S., Wuillème, S., To, A., Rochat, C., and Lepiniec, L. 2009. Role of WRINKLED1 in the transcriptional regulation of glycolytic and fatty acid biosynthetic genes in *Arabidopsis*. Plant J. 60:933-947.

Baud, S., Feria Bourrellier, A. B., Azzopardi, M., Berger, A., Dechorgnat, J., Daniel-Vedele, F., Lepiniec, L., Miquel, M., Rochat, C., Hodges, M., and Ferrario-Méry, S. 2010. PII is induced by WRINKLED1 and fine-tunes fatty acid composition in seeds of *Arabidopsis thaliana*. Plant J. 64:291-303.

Beez, S., Fokina, O., Herrmann, C., and Forchhammer, K. 2009. N-acetyl-L-glutamate kinase (NAGK) from oxygenic phototrophs: P(II) signal transduction across domains of life reveals novel insights in NAGK control. J. Mol. Biol. 389:748-758.

Bisseling, T., van den Bos, R. C., and van Kammen, A. 1978. The effect of ammonium nitrate on the synthesis of nitrogenase and the concentration of leghemoglobin in pea root nodules induced by *Rhizobium leguminosarum*. Biochim. Biophys. Acta 539:1-11.

Boscari, A., del Giudice, J., Ferrarini, A., Venturini, L., Zaffini, A. L., Delledonne, M., and Puppo, A. 2013. Expression dynamics of the *Medicago truncatula* transcriptome during the symbiotic interaction with *Sinorhizobium meliloti*: Which role for nitric oxide? Plant Physiol. 161:425-439.

Burillo, S., Luque, I., Fuentes, I., and Contreras, A. 2004. Interactions between the nitrogen signal transduction protein PII and N-acetyl glutamate kinase in organisms that perform oxygenic photosynthesis. J. Bacteriol. 186:3346-3354.

Caetano-Anollés, G., and Gresshoff, P. M. 1991. Plant genetic control of nodulation. Annu. Rev. Microbiol. 45:345-382.

Cheah, E., Carr, P. D., Suffolk, P. M., Vasudevan, S. G., Dixon, N. E., and Ollis, D. L. 1994. Structure of the *Escherichia coli* signal transducing protein PII. Structure 2:981-990.

Chen, Y. M., Ferrar, T. S., Lohmeier-Vogel, E. M., Morrice, N., Mizuno, Y., Berenger, B., Ng, K. K. S., Muench, D. G., and Moorhead, G. B. G. 2006. The PII signal transduction protein of *Arabidopsis thaliana* forms an arginine-regulated complex with plastid N-acetyl glutamate kinase. J. Biol. Chem. 281:5726-5733.

Complainville, A., Brocard, L., Roberts, I., Dax, E., Sever, N., Sauer, N., Kondorosi, A., Wolf, S., Oparka, K., and Crespi, M. 2003. Nodule initiation involves the creation of a new symplasmic field in specific root cells of *Medicago* species. Plant Cell 15:2778-2791.

D'Apuzzo, E., Rogato, A., Simon-Rosin, U., El Alaoui, H., Barbulova, A., Betti, M., Dimou, M., Katinakis, P., Marquez, A., Marini, A. M., Udvardi, M. K., and Chiurazzi, M. 2004. Characterization of three functional high-affinity ammonium transporters in *Lotus japonicus* with differential transcriptional regulation and spatial expression. Plant Physiol. 134:1763-1774.

del Giudice, J., Cam, Y., Damiani, I., Fung-Chat, F., Meilhoc, E., Bruand, C., Brouquisse, R., Puppo, A., and Boscari, A. 2011. Nitric oxide is required for an optimal establishment of the *Medicago truncatula–Sinorhizobium meliloti* symbiosis. New Phytol. 191:405-417.

Duby, G., Oufattole, M., and Boutry, M. 2001. Hydrophobic residues within the predicted N-terminal amphiphilic alpha-helix of a plant mitochondrial targeting presequence play a major role in in vivo import. Plant J. 27:539-549.

Efrose, R. C., Flemetakis, E., Sfichi, L., Stedel, C., Kouri, E. D., Udvardi, M. K., Kotzabasis, K., and Katinakis, P. 2008. Characterization of spermidine and spermine synthases in *Lotus japonicus*: Induction and spatial organization of polyamine biosynthesis in nitrogen fixing nodules. Planta 228:37-49.

Feria Bourrellier, A. B., Valot, B., Guillot, A., Ambard-Bretteville, F., Vidal, J., and Hodges, M. 2010. Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit. Proc. Natl. Acad. Sci. U.S.A. 107:502-507.

Ferrario-Méry, S., Bouvet, M., Leleu, O., Savino, G., Hodges, M., and Meyer, C. 2005. Physiological characterisation of *Arabidopsis* mutants affected in the expression of the putative regulatory protein PII. Planta 223:28-39.

Ferrario-Méry, S., Besin, E., Pichon, O., Meyer, C., and Hodges, M. 2006. The regulatory PII protein controls arginine biosynthesis in *Arabidopsis*. FEBS (Fed. Eur. Biochem. Soc.) Lett. 580:2015-2020.

Flemetakis, E., Efrose, R. C., Desbrosses, G., Dimou, M., Delis, C., Aivalakis, G., Udvardi, M. K., and Katinakis, P. 2004. Induction and spatial organization of polyamine biosynthesis during nodule development in *Lotus japonicus*. Mol. Plant-Microbe Interact. 17: 1283-1293.

Flores, H. E., and Galston, A. W. 1982. Analysis of polyamines in higher plants by high performance liquid chromatography. Plant Physiol. 69: 701-706.

Forchhammer, K. 2004. Global carbon/nitrogen control by PII signal transduction in cyanobacteria: From signals to targets. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 28:319-333.

Forchhammer, K. 2008. P(II) signal transducers: Novel functional and structural insights. Trends Microbiol. 16:65-72.

Fujihara, S., Abe, H., Minakawa, Y., Akao, S., and Yoneyama, T. 1994. Polyamines in nodules from various plant-microbe symbiotic associations. Plant Cell Physiol. 35:1127-1134.

Gamborg, O. L. 1970. The effects of amino acids and ammonium on the growth of plant cells in suspension culture. Plant Physiol. 45: 372-375.

Hajdukiewicz, P., Svab, Z., and Maliga, P. 1994. The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol. Biol. 25:989-994.

Hsieh, M. H., Lam, H. M., van de Loo, F. J., and Coruzzi, G. 1998. A PII-like protein in *Arabidopsis*: Putative role in nitrogen sensing. Proc. Natl. Acad. Sci. U.S.A. 95:13965-13970.

Huergo, L. F., Chandra, G., and Merrick, M. 2013. P(II) signal transduction proteins: Nitrogen regulation and beyond. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 37:251-283.

Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5:387-405.

Jeudy, C., Ruffel, S., Freixes, S., Tillard, P., Santoni, A. L., Morel, S., Journet, E. P., Duc, G., Gojon, A., Lepetit, M., and Salon, C. 2010. Adaptation of *Medicago truncatula* to nitrogen limitation is modulated via local and systemic nodule developmental responses. New Phytol. 185:817-828.

Jiang, P., and Ninfa, A. J. 2007. *Escherichia coli* PII signal transduction protein controlling nitrogen assimilation acts as a sensor of adenylate energy charge in vitro. Biochemistry 46:12979-12996.

Kistner, C., and Matamoros, M. 2005. RNA isolation using phase extraction and LiCl precipitation. Pages 123-124 in: *Lotus japonicus* Handbook. A. J. Márquez, ed. Springer, Berlin.

Köhler, R. H., Cao, J., Zipfel, W. R., Webb, W. W., and Hanson, M. R. 1997. Exchange of protein molecules through connections between higher plant plastids. Science 276:2039-2042.

Lombari, P., Ercolano, E., El Alaoui, H., and Chiurazzi, M. 2003. A new transformation-regeneration procedure in the model legume Lotus japonicus: Root explants as a source of large numbers of cells susceptible to Agrobacterium-mediated transformation. Plant Cell Rep. 21:771-777.

Maeo, K., Tokuda, T., Ayame, A., Mitsui, N., Kawai, T., Tsukagoshi, H., Ishiguro, S., and Nakamura, K. 2009. An AP2-type transcription factor, WRINKLED1, of *Arabidopsis thaliana* binds to the AW-box sequence conserved among proximal upstream regions of genes involved in fatty acid synthesis. Plant J. 60:476-487.

Maheswaran, M., Urbanke, C., and Forchhammer, K. 2004. Complex formation and catalytic activation by the PII signaling protein of N-acetyl-L-glutamate kinase from *Synechococcus elongatus* strain PCC 7942. J. Biol. Chem. 279:55202-55210.

Martirani, L., Stiller, J., Mirabella, R., Alfano, F., Lamberti, A., Radutoiu, S. E., Iaccarino, M., Gressohff, P. M., and Chiurazzi, M. 1999. T-DNA tagging of nodulation- and root-related genes in Lotus japonicus: Expression patterns and potential for promoter trapping and insertional mutagenes. Mol. Plant-Microbe Interact. 12:275-284.

Matamoros, M. A., Baird, L. M., Escuredo, P. R., Dalton, D. A., Minchin, F. R., Iturbe-Ormaetxe, I., Rubio, M. C., Moran, J. F., Gordon, A. J., and Becana, M. 1999. Stress-induced legume root nodule senescence. Physiological, biochemical, and structural alterations. Plant Physiol. 121:97-112.

Mishima, E., Hosokawa, A., Imaizumi-Anraku, H., Saito, K., Kawaguchi, M., and Saeki, K. 2008. Requirement for *Mesorhizobium loti* ornithine transcarbamoylase for successful symbiosis with *Lotus japonicus* as revealed by an unexpected long-range genome deletion. Plant Cell Physiol. 49:301-313.

Mizuno, Y., Berenger, B., Moorhead, G. B., and Ng, K. K. 2007a. Crystal structure of *Arabidopsis* PII reveals novel structural elements unique to plants. Biochemistry 46:1477-1483.

Mizuno, Y., Moorhead, G. B., and Ng, K. K. 2007b. Structural basis for the regulation of N-acetylglutamate kinase by PII in *Arabidopsis thaliana*. J. Biol. Chem. 282:35733-35740.

Moorhead, G. B. G., and Smith, C. S. 2003. Interpreting the plastid carbon, nitrogen, and energy status. A role for PII? Plant Physiol. 133: 492-498.

Okamoto, S., Ohnishi, E., Sato, S., Takahashi, H., Nakazono, M., Tabata, S., and Kawaguchi, M. 2009. Nod factor/nitrate-induced CLE genes that drive HAR1-mediated systemic regulation of nodulation. Plant Cell Physiol. 50:67-77.

Okamoto, S., Shinohara, H., Mori, T., Matsubayashi, Y., and Kawaguchi, M. 2013. Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase. Nat. Commun. 4:2191.

Omrane, S., and Chiurazzi, M. 2009. A variety of regulatory mechanisms are involved in the nitrogen-dependent modulation of the nodule organogenesis program in legume roots. Plant Signal. Behav. 4:1066-1068.

Omrane, S., Ferrarini, A., D'Apuzzo, E., Rogato, A., Delledonne, M., and Chiurazzi, M. 2009. Symbiotic competence in *Lotus japonicus* is affected by plant nitrogen status: Transcriptomic identification of genes affected by a new signalling pathway. New Phytol. 183:380-394.

Pathirana, S. M., Vance, C. P., Miller, S. S., and Gantt, J. S. 1992. Alfalfa root nodule phosphoenolpyruvate carboxylase: Characterization of the cDNA and expression in effective and plant-controlled ineffective nodules. Plant Mol. Biol. 20:437-450.

Pauly, N., Ferrari, C., Andrio, E., Marino, D., Piardi, S., Brouquisse, R., Baudouin, E., and Puppo, A. 2011. MtNOA1/RIF1 modulates *Medicago truncatula–Sinorhizobium meliloti* nodule development without affecting its nitric oxide content. J. Exp. Bot. 62:939-948.

Radchenko, M. V., Thornton, J., and Merrick, M. 2013. P(II) signal transduction proteins are ATPases whose activity is regulated by 2-oxoglutarate. Proc. Natl. Acad. Sci. U.S.A. 110:12948-12953.

Ruffel, S., Freixes, S., Balzergue, S., Tillard, P., Jeudy, C., Martin-Magniette, M. L., van der Merwe, M. J., Kakar, K., Gouzy, J., Fernie, A. R., Udvardi, M., Salon, C., Gojon, A., and Lepetit, M. 2008. Systemic signaling of the plant nitrogen status triggers specific transcriptome responses depending on the nitrogen source in *Medicago truncatula*. Plant Physiol. 146:2020-2035.

Saitou, N., and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Sant'Anna, F. H., Trentini, D. B., de Souto Weber, S., Cecagno, R., da Silva, S. C., and Schrank, I. S. 2009. The PII superfamily revised: A novel group and evolutionary insights. J. Mol. Evol. 68:322-336.

Schulze, J., Shi, L., Blumenthal, J., Samac, D. A., Gantt, J. S., and Vance, J. P. 1998. Inhibition of alfalfa root nodule phosphoenolpyruvate carboxylase through an antisense strategy impacts nitrogen fixation and plant growth. Phytochemistry 49:341-346.

Smith, M. A., and Davies, P. J. 1985. Separation and quantitation of polyamines in plant tissue by high performance liquid chromatography of their dansyl derivatives. Plant Physiol. 78:89-91.

Smith, C. S., Weljie, A. M., and Moorhead, G. B. G. 2003. Molecular properties of the putative nitrogen sensor PII from *Arabidopsis thaliana*. Plant J. 33:353-360.

Smith, C. S., Morrice, N. A., and Moorhead, G. B. G. 2004. Lack of evidence for phosphorylation of *Arabidopsis thaliana* PII: Implications for plastid carbon and nitrogen signaling. Biochim. Biophys. Acta 1699:145-154.

Sugiyama, K., Hayakawa, T., Kudo, T., Ito, T., and Yamaya, T. 2004. Interaction of N-acetylglutamate kinase with a PII-like protein in rice. Plant Cell Physiol. 45:1768-1778.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

Tsikou, D., Kalloniati, C., Fotelli, M. N., Nikolopoulos, D., Katinakis, P., Udvardi, M. K., Rennenberg, H., and Flemetakis, E. 2013. Cessation of photosynthesis in *Lotus japonicus* leaves leads to reprogramming of nodule metabolism. J. Exp. Bot. 64:1317-1332.

Uhrig, R. G., Ng, K. K. S., and Moorhead, G. B. G. 2009. PII in higher plants: A modern role for an ancient protein. Trends Plant Sci. 14:505-511.

Vance, C. P. 2008. Carbon and nitrogen metabolism in legume nodules. Pages 293-320 in: Nitrogen-Fixing Leguminous Symbioses. M. J. Dilworth, E. K. James, J. I. Sprent, and W. E. Newton, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Vasudevan, S. G., Gedye, C., Dixon, N. E., Cheah, E., Carr, P. D., Suffolk, P. M., Jeffrey, P. D., and Ollis, D. L. 1994. *Escherichia coli* PII protein: Purification, crystallization and oligomeric structure. FEBS (Fed. Eur. Biochem. Soc.) Lett. 337:255-258.

Wisniewski, J. P., and Brewin, N. J. 2000. Construction of transgenic pea lines with modified expression of diamine oxidase and modified nodulation responses with exogenous putrescine. Mol. Plant-Microbe Interact. 13:922-928.

Zeth, K., Fokina, O., and Forchhammer, K. 2014. Structural basis and target-specific modulation of ADP sensing by the *Synechococcus elongatus* PII signaling protein. J. Biol. Chem. 289:8960-8972.

Zhang, H., Jennings, A., Barlow, P. W., and Forde, B. G. 1999. Dual pathways for regulation of root branching by nitrate. Proc. Natl. Acad. Sci. U.S.A. 96:6529-6534.

Zuckerkandl, E., and Pauling, L. 1965. Evolutionary divergence and convergence in proteins. Pages 97-166 in: *Evolving Genes and Proteins*. V. Bryson and H. J. Vogel, eds. Academic Press, New York.

AUTHOR-RECOMMENDED INTERNET RESOURCES

ChloroP 1.1 server: www.cbs.dtu.dk/services/ChloroP
Department of Energy Joint Genome Institute website: jgi.doe.gov
ImageJ bundle software: rsb.info.nih.gov/ij/
L. japonicus genome sequence database: www.kazusa.or.jp/lotus
National Center for Biotechnology Information website: www.ncbi.nlm.nih.gov
VassarStats: faculty.vassar.edu/lowry/VassarStats.html