Symbiotic competence in *Lotus japonicus* is affected by plant nitrogen status: transcriptomic identification of genes affected by a new signalling pathway

Selim Omrane¹, Alberto Ferrarini², Enrica D’Apuzzo¹, Alessandra Rogato¹, Massimo Delledonne² and Maurizio Chiurazzi¹

¹Institute of Genetics and Biophysics A. Buzzati Traverso, Via P. Castellino 131, Napoli, Italy; ²Università degli Studi di Verona, Strada Le Grazie 15 Cà Vignal, I–37134, Verona, Italy

**Summary**

- In leguminous plants, symbiotic nitrogen (N) fixation performances and N environmental conditions are linked because nodule initiation, development and functioning are greatly influenced by the amount of available N sources.
- We demonstrate here that N supply also controls, beforehand, the competence of leguminous plants to perform the nodulation program. *Lotus japonicus* plants preincubated for 10 d in high-N conditions, and then transferred to low N before the *Mesorhizobium loti* inoculation, had reduced nodulation. This phenotype was maintained for at least 6 d and a complete reacquisition of the symbiotic competence was observed only after 9 d.
- The time-course analysis of the change of the symbiotic phenotype was analysed by transcriptomics. The differentially expressed genes identified are mostly involved in metabolic pathways. However, the transcriptional response also includes genes belonging to other functional categories such as signalling, stress response and transcriptional regulation.
- Some of these genes show a molecular identity and a regulation profile, that suggest a role as possible molecular links between the N-dependent plant response and the nodule organogenesis program.

**Key words:** *Lotus japonicus*, nitrogen (N) supply, nodule organogenesis, nutritional status, signalling pathways, symbiotic competence.

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**Introduction**

Nitrogen (N), is the mineral element required in the greatest amount by plants, and often represents a limiting factor for growth. The production of high-yielding crops is associated with the application of a large quantity of fertilizers with a substantial increase in costs and in N pollution with the latter becoming a threat to global ecosystems. Nitrogen is available to plants in the soil in a variety of forms including ammonium (as both NH₃ and NH₄⁺), nitrate, amino acids, soluble peptides and complex insoluble nitrogen-containing compounds. Following uptake, inorganic N is reduced to ammonia before it can be incorporated into the amino acids glutamine and glutamate. Plants have evolved intricate regulatory networks for integrating N assimilation with other metabolic and developmental pathways such as root branching, leaf growth and flowering time (Marschner, 1995; Scheible et al., 1997; Zhang & Forde, 1998; Stitt & Krapp, 1999). Thus, the response to the presence of N results in important changes at the growth/phenotypic level. Nitrogen metabolites have been shown to act as regulatory signals for the transcription of genes encoding enzymes involved in various essential processes, including photosynthesis, carbon metabolism, N metabolism, and resource allocation (Raab & Terry, 1994; Scheible et al., 1997; Coruzzi & Zhou, 2001; Stitt et al., 2002; Foyer et al., 2003; Palenchar et al., 2004).

Microarray analysis has already been used to identify N-responsive genes in different plants. Most of the studies were performed on *Arabidopsis thaliana* to analyse the molecular basis of the plant response to nitrate and were designed to
determine the profiles of gene expression after plants are shifted to different nitrate conditions (Wang et al., 2000, 2003; Price et al., 2004; Scheible et al., 2004). To our knowledge, the only study regarding the transcriptional response under chronic (steady state) N-starvation conditions in A. thaliana was reported recently (Bi et al., 2007).

In leguminous plants, the environmental availability of N determines their capacity to perform a symbiotic interaction with rhizobia. Limitation of combined N in the soil is a prerequisite for symbiotic interaction and, under these conditions, a signal secreted by rhizobia cells is perceived by the elongating root hairs of a restricted, competent zone behind the root tip, where a complex transduction pathway leading to the nodule primordial formation takes place (Oldroyd & Downie, 2008). In certain genotypes of Medicago sativa, N starvation alone is sufficient to induce the development of empty (bacterium-free) nodules (Truchet et al., 1989). Nitrogen starvation is also a mandatory condition for nodule development in spontaneous nodulation mutants recently isolated in Lotus japonicus (Tirichine et al., 2006). It is also well known that external addition of excessive amounts of NO₃ as well as of other combined N sources affects the initiation, development and functioning of N-fixing nodules (Bisseling et al., 1978; Carroll & Mathews, 1990; Caetano-Anollès & Gresshoff, 1991; Barbulova et al., 2007). The analysis of the direct effects of high concentrations of combined N sources on early steps of the Nod factor-induced signalling pathway, has been recently reported in L. japonicus. This study revealed a differential effect of ammonium and nitrate sources on different early steps of the nodulation pathway identifying, by a small-scale transcriptional analysis, a direct or indirect target of the inhibitory pathways (Barbulova et al., 2007). The inhibitory effect of nitrate has been reported to act locally on nodule initiation and systemically on N fixation activity (Hinson, 1975; Caetano-Anollès & Gresshoff, 1991; Cho & Harper, 1991). An Affymetrix geneChip analysis (Affymetrix, Santa Clara, CA, USA) confirmed a strong systemic influence of the N supply on the profile of gene expression in nodulated roots of Medicago truncatula (Ruffel et al., 2008). In particular, a severe downregulation of 200 nodule-related transcripts was observed, in a split-root system, when one side of the roots was treated with 10 mM NH₄NO₃ (Ruffel et al., 2008).

However, little is known about how a different N status of the leguminous plants, acquired as a consequence of different growth conditions, may affect the symbiotic competence, before the rhizobia inoculation. Here, we analysed the nodulation response of L. japonicus plants preincubated for 10 d under a high N (10 mM NH₄NO₃) inhibitory condition, and then transferred to a low N (10 µM NH₄NO₃) permissive regime, for 0, 6 and 9 d, before the Mesorhizobium loti inoculation. This pretreatment affected the Lotus nodulation capacity for at least 6 d after the transfer on low N conditions. A gene chip-based transcriptomic analysis was performed to characterize, at the molecular level, the root response to these N-environment conditions. This analysis allowed the identification of a set of genes whose expression is regulated in response to the N supply available to the plant, and the discovery of possible molecular links between the N-status of the plants and the root pathways governing the nodulation program.

Materials and Methods

Plant material and germination procedure

All the experiments were carried out with L. japonicus (Regel) K. Larsen ecotype B129 F9 GIFU. Before germination, seeds were sterilized for 20 min with 25% commercial bleach (1% hypochlorite) and 0.1% Triton-X100, washed six times in sterile H₂O and kept overnight in water at 4°C. Thereafter, they were sown on H₂O 1% plant agar (three seeds per 140 × 10 mm Petri dish) 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 (Barbulova & Chiurazzi, 2005).

Inhibitory and permissive plant growth conditions

Seedlings (3–5 d old) were transferred and grown for 10 d on solid medium with the same composition as that of B5 medium (Gamborg, 1970), except that (NH₄)₂SO₄ and KNO₃ were omitted and replaced by 10 mM (high-N) or 10 µM (low-N) NH₄NO₃. Potassium chloride was added to the medium to replace the potassium source. This medium contained vitamins (Catalogue No. G0415; Duchefa, Haarlem, the Netherlands) and no carbon (C) source was added. The pH was adjusted to 5.7 with MES. During these 10 d of preincubation, plants were transferred twice on fresh media to avoid nutrient depletion. All plants were cultivated in growth chambers at 23°C, with a 16 h photoperiod and light intensity of 246 µE s⁻¹ m⁻². Unsynchronized seedlings were discarded at this stage.

Inoculation procedure and nodulation test

After 10 d, both high-N and low-N plants were transferred on fresh 10 µM NH₄NO₃ medium and progressively inoculated with M. loti. The M. loti strain R7A was grown to mid-log phase in liquid TY medium (Beringer, 1974) and used to inoculate the plants at a concentration of 10⁶ cells per primary root tip. Six days after inoculation, plants were transferred on 10 µM NH₄NO₃ medium with addition of cefotaxime at 50 mg l⁻¹. A killing curve was performed to identify the minimal antibiotic concentration required.

Extraction and measurement of amino acids

We followed the procedure described in Rigano et al. (1996).
RNA extraction, quantization and integrity check

Roots were detached for the RNA extraction procedure just before *M. loti* inoculation. Total RNA was prepared from *L. japonicus* roots using the procedure of Kistner & Matamoros (2005). The samples were treated with DNase I (Ambion, Austin TX, USA) to remove contaminating DNA, the absence of which was subsequently confirmed by PCR. The RNA was quantified by NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific Inc. Waltham, MA, USA). The RNA samples used for CombiMatrix array gene chip experiments (CombiMatrix, Mukilteo, WA, USA) were quantified and quality checked by Agilent 2100 BIOANALYZER (Santa Clara, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR) experiments

The RNA samples were treated with DNase I (Ambion) to remove contaminating DNA the absence of which was subsequently confirmed by PCR. One microgram of total RNA was annealed to random decamers and reverse-transcribed with reverse transcriptase (Ambion) to obtain cDNA. Real-time PCR was performed with a DNA Engine Opticon 2 System (MJ Research, Watertown, MA, USA) using SYBR Green to monitor dsDNA 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, 63°C for 15 s and 72°C for 15 s. Data were analysed using Opticon Monitor Analysis Software Version 2.01 (MJ Research). The relative level of expression was calculated with the following formula: relative expression ratio of the gene of interest is $2^{-\Delta CT} = 2^{-\Delta CT_{\text{CTUBI}}}$. Analysis of the melting curve of PCR products 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 ensure accuracy and specificity. The sequences of primers used for the qRT-PCR analysis are listed in the Supporting Information, Table S2.

Array conception

A total of 12 485 available TCs (tentative consensus sequences) were retrieved from the Lotus japonicus DFCI database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_ann.pl?gudb=Lotus japonicus). A total of 12 403 specific oligos (35- to 40-mer) were designed on 12 403 TCs by using the programme OligoArray 2.1 (Rouillard et al., 2003). Nine bacterial oligonucleotides sequences provided by CombiMatrix were used as negative controls. The Custom Array 90K was designed with seven replicates of each probe randomly distributed across the array to allow measurement of the variability within the array. Two technical and two biological replicas were used for each hybridization experiment.

Hybridization procedures

Target labelling Amino allyl aRNA synthesis and labelling with dye Cy5TM was performed by following the instructions from manufacturer (AM1796; Ambion). The purified labelled aRNA was quantified by spectrophotometer (RNA is quantified at $\lambda_{260\text{nm}}$ and incorporated Cy5 at $\lambda_{650\text{nm}}$). A sample of 4 µg of labelled RNA was first fragmented and hybridized to the array as indicated by the manufacturer (http://www.combimatrix.com/support_docs.htm).

Pre-hybridization, hybridization, washings and imaging were performed by following the protocols indicated on the CombiMatrix custom array web site (http://www.combimatrix.com/support_docs.htm).

Microarray data analysis

The array was scanned with a ScanArray 4000XL microarray scanner (Perkin–Elmer). The TIF images were exported to the Microarray Imager 5.8 (CombiMatrix) for the densitometric analysis of the spots. The probe signals that were higher than that of the negative controls plus two times the standard deviation were considered as ‘present’. Microarray data was normalized by median scaling. Data from different biological replicates showed a strong Pearson correlation ranging from 0.93 to 0.97. A significance analysis of microarray (SAM) analysis was implemented on the normalized data (type Two Class Unpaired) for the case of the low-N0 vs high-N0 experiment and a multiclass analysis for the tendency pattern of the time-points high-N6 vs high-N0 and high-N9 vs high-N0 (http://www-stat.stanford.edu/~tibs/SAM/pnassam.pdf) with a selected False Discovery Rate (FDR) < 10%.

Results

Experimental design

A high concentration of combined N source is known to arrest the nodulation response and N-fixation performance in leguminous plants upon rhizobia inoculation (Bisseling et al., 1978; Carroll & Mathews, 1990; Caetano-Anollès & Greshoff, 1991; Barbulova et al., 2007). In *L. japonicus* seedlings, 10 mM NH4NO3 blocks nodulation and the root hair deformation and cortical cell division normally induced by added Nod factors (Barbulova et al., 2007). To investigate whether this N regime can affect, beforehand, the symbiotic competence of *L. japonicus* plants before the *M. loti* inoculation, and to analyse the duration of this effect, *L. japonicus* seeds were germinated on H2O agar plates and, after 3–5 d, plantlets with unfolded cotyledons were transferred onto on 10 mM NH4NO3 (high-N) or 10 µM NH4NO3 (low-N) or 10 µM NH4NO3 blocks nodulation and the root hair deformation and cortical cell division normally induced by added Nod factors (Barbulova et al., 2007). To investigate whether this N regime can affect, beforehand, the symbiotic competence of *L. japonicus* plants before the *M. loti* inoculation, and to analyse the duration of this effect, *L. japonicus* seeds were germinated on H2O agar plates and, after 3–5 d, plantlets with unfolded cotyledons were transferred onto on 10 mM NH4NO3 (high-N) or 10 µM NH4NO3 (low-N) or 10 µM NH4NO3 blocks nodulation and the root hair deformation and cortical cell division normally induced by added Nod factors (Barbulova et al., 2007).
NH$_4$NO$_3$ (low-N) media and grown for 10 d. Plants grown in high-N or low-N were then transferred to fresh low-N medium and the primary roots inoculated with M. loti, 0, 6, 9 or 13 d after transfer (Fig. 1). To ensure that the nodulation response (nodule number per plant), was reflecting the state of symbiotic competence of the L. japonicus plants at the time of inoculation, as a direct consequence of the preincubation on high-N or low-N conditions 6 d after inoculation, inoculated plants were transferred to a 10 µM NH$_4$NO$_3$ medium containing 50 mg l$^{-1}$ cefotaxime to eliminate rhizobia cells from the medium and thus avoid additional cycles of nodule formation (Fig. 1). The 6-d time-interval and the 50 mg l$^{-1}$ drug concentration did not affect nodule formation when compared with untreated plants (see the Supporting Information, Fig. S1) indicating that the experimental design was appropriate.

Effects of the high-N preincubation on nodule formation capacity

The experimental design described in the previous paragraph permitted testing whether a high environmental N condition, that might influence the general N status of the plants, may also affect the nodulation program in L. japonicus, before M. loti inoculation. To verify that the treatment described in Fig. 1 affected the N-status of the plants, the amino acid content was assayed in roots of L. japonicus seedlings grown at high-N and low-N at the end of the preincubation period (Fig. 2a). The continuous supply of 10 mM NH$_4$NO$_3$ led to significant differences in the content of amino acids such as glutamate, asparagine and glutamine, that are key indicators of the pathways of N assimilation and transport. Consistently, the biomass data reported in Fig. 2b indicated a progressive difference in the fresh shoot weight of uninoculated L. japonicus plants incubated for increasing time on high-N or low-N conditions.

The nodulation test indicated that, at 4 wk post inoculation, the high-N0 plants had a significantly lower number of nodules (c. 40%) than Low-N0 plants (Fig. 3a). This indicated that the 10-d long preincubation in the presence of 10 mM NH$_4$NO$_3$ was sufficient to reduce the competence of Lotus seedlings for symbiotic interaction. As reported in Fig. 2c, high-N0 and low-N0 plants showed no significant differences in primary roots length; hence this growth parameter was not responsible for the observed differences in nodules number. The shoot biomass of high-N0 and low-N0 plants was not significantly changed at 4 wk postinoculation, as indicated by the analysis of fresh weight shown in Fig. 2d. To examine the nodulation phenotypes in more detail, a M. loti strain carrying a constitutively expressed hema::lacZ reporter gene fusion was used and the nodulation phenotypes were examined 6 d after inoculation of high-N0 and low-N0 plants. The dark-blue-stained nodule primordia observed on Low-N0 plants reflected the activity of the bacterially encoded β-galactosidase lacZ reporter gene and indicated a successful root colonization that was restricted to the original area of primary root inoculation (Fig. 4a,b). Nodules primordia were reduced on roots of high-N0 plants where, instead, bacteria were distributed on a larger area of the primary root inoculation zone (Fig. 4c,d).

As indicated in the experimental design shown in Fig. 1, the high-N plants were maintained at different lengths of times on 10 µM NH$_4$NO$_3$ before M. loti inoculation. We could thus monitor, in a time-dependent manner, the duration of the inhibitory effect of the high-N preincubation conditions. The number of nodules per plant indicated that even high-N6 plants, which had been on 10 µM NH$_4$NO$_3$ for 6 d before M. loti inoculation maintained a state of partial symbiotic competence when compared with the low-N6 plants maintained throughout on permissive, low-N conditions (Fig. 3b). Interestingly, a striking, complete recovery of nodulation capacity was instead observed, after longer incubation in N-starvation, in high-N9 and high-N13. By contrast, low-N9 and low-N13 plants that had been maintained 16 d and 20 d on 10 µM NH$_4$NO$_3$ before inoculation with M. loti (Fig. 1), showed a reduction of their nodulation capacity (Fig. 3c,d). As expected, an increased number of nodules per plant was observed on high-N9 and high-N13 competent plants when compared with the younger low-N0 and low-N6 plants as a consequence of the increase of the primary root size (Fig. S2). Under all the conditions tested the addition of antibiotic fixed a plateau in the nodule number that was reached c. 15 d postinoculation (dpi; Fig. 3).
Consistently with the symbiotic phenotype, analysis of the shoot biomass revealed, at 28 dpi, a significant increase of the high-N9 and high-N13 shoot fresh weights compared with low-N9 and low-N13 plants (Fig. 2d), with the latter showing shoot stress symptoms and chlorosis (data not shown).

Expression profiling and identification of differentially expressed genes

In order to obtain information about gene determinants involved in the response to low versus high N concentrations and putatively linked to the nodulation competence phenotype, the time-course analysis of the change of symbiotic phenotype reported in Fig. 3 was coupled to a large-scale analysis of the gene expression profiling. The comparison of gene expression in low-N0 versus high-N0 plants represents an analysis of the baseline gene expression of plants grown in differential N steady-state conditions. Plants were harvested after the 10 d preincubation under 10 µM NH₄NO₃ or 10 µM NH₄NO₃ conditions (Fig. 1) and total RNA was extracted from roots for transcriptome analysis. To minimize possible diurnal changes of the expression level of genes involved in *L. japonicus* C and N metabolism, all samples were taken in the middle of the light period. RNA was hybridized to a custom-designed *L. japonicus* CombiMatrix Oligonucleotide Microarray, containing 12 485 probes representing the released *L. japonicus* TC sequences (http://compbio.dfci.harvard.edu/tgi/plant.html). Significance analysis of microarray (Tusher et al., 2001) was used to identify genes differentially expressed in low-N0 vs high-N0 plants. The final number of significant
Fig. 3 Nodulation kinetics of low-N (open circles) and high-N *Lotus japonicus* plants (closed circles). The arrows indicate the time of cefotaxime addition. Data represent the mean and standard deviations obtained from three independent experiments (16 plants per experiment).

Fig. 4 Representative infection events at 6 d postinoculation of high-N0 and low-N0 *Lotus japonicus* plants. Plants were stained for β-galactosidase activity to detect the infecting *Mesorhizobium loti* strain NZP2235 (*hemA::lacZ*). (a,b) Nodule primordia are visible on low-N0 roots and the area of infection is restricted. (c,d) Nodule primordia are not visible on high-N0 roots and bacteria are diffused on the inoculated area.
TCs was based on a median FDR < 10% and a minimum of a twofold expression difference. We found 319 differentially regulated sequences, 189 being upregulated and 130 downregulated in Low-N0 roots compared with High-N0 roots (Table S1). The blast analysis of the TC sequences versus the *L. japonicus* genome structure permitted to identify the corresponding predicted genes in 88% of the cases (Table S1; Sato et al., 2008). To confirm the results of the chip analysis, the expression of 14 genes was tested by qRT-PCR and found to be consistent with the chip data in every case (Table S2).

### Functional classification of differentially expressed genes in low-N0 vs high-N0 steady-supply conditions

The functional annotation of the N-regulated genes identified in the chip analysis, was obtained by comparison of all translated TC sequences to the *A. thaliana* genome. 260 probes (81.5%) had a significant hit (*E* value < 1e⁻⁵; Table S1). These identifications were used to visualize the functional classification using Mapman software (Fig. S3; Usadel et al., 2005).

### Metabolism overview

The overall picture of the expression changes for genes involved in the main metabolic pathways, on low-N0 vs high-N0 plants, is shown in Fig. 5a. The figure shows a general over-representation of downregulated genes (with the exception of the secondary metabolism pathways). Among these, genes involved in nitrate assimilation such as nitrate reductase (*Lj* T01E03.160.nd) and nitrite reductase (chr4.CM0227.280.nd), were severely repressed 7.5- and 4.4-fold, respectively (Table S1). Thus, as expected, the level of expression of these crucial N assimilatory genes correlates with the amount of N available. Similarly, among the differentially expressed sequences, most of the genes involved in sucrose and starch synthesis and degradation were found to be downregulated in the roots of Low-N0 plants. This coordination between N and C metabolism genes may be crucial for the maintenance of the correct C:N ratio. A coordinated response can also be revealed for genes involved in amino acid metabolism. Genes for amino acid synthesis were induced whereas the opposite occurred for those involved in amino acid degradation (Fig. 5a). The overall picture suggested that the low-N0 plants, for an efficient response to the N-demand,
prefer the ex novo synthesis from newly assimilated ammonium rather than its recycling from amino acid catabolism. This response appears also consistent with a general upregulation trend observed for genes involved in nitrate, ammonium and amino acid transporters (Fig. S4). The overview also showed that among the subcategories for phytohormone synthesis and phenolics synthesis, as well as flavonoid metabolism (anthocyanin synthesis), upregulated genes were over-represented (Fig. 5a). This upregulation probably reflects the change from primary to secondary metabolism occurring in N starvation conditions. The expression of other genes involved in growth-related processes such as synthesis of cell wall precursor decreased (Table S1; chr4.CM0025.390.nc; LjSGA_006616.2) whereas transcripts of genes involved in cell wall degradation increased (Table S1; chr5.CM0077.200.nd; chr4.CM0061.140.nd). A plant response to severe N deficiency may also include growth arrest through downregulation of genes involved in protein biosynthesis, as suggested by the inhibition of the expression of genes coding for structural constituents of ribosome and translation elongation factors (Table S1; chr1.CM032.460.nc; LjSGA_014733.1; LjSGA_037921.1). Within the category of phytohormone metabolism, 83% of the differentially expressed genes were upregulated, including two TC sequences corresponding to auxin responsive genes (Table S1; chr1.CM0105.370.nd; chr2.CM0191.290.nc).

Transcriptional regulators  Transcription factors are of special interest since they are potentially capable of coordinating the expression of many downstream target genes. In our analysis we could expect to identify TF controlling different types of plant responses to N-starvation conditions, hence involving metabolic pathways, stress response, root development, nodule organogenesis, etc.

Twelve differentially expressed sequences included in the transcription and DNA processing category, identified L. japonicus genes highly similar to A. thaliana factors involved in transcriptional regulation (Table 1). The potential transcription factors were verified at http://datf.cbi.pku.edu.cn/index.php. Two out of the 12 identified sequences (chr1.CM0017.80.nc; chr4.CM0680.260.nc) belong to the NAC family whose members have been involved in the SAM specification network (Souer et al., 1996) as well as in plant hormonal control and stress response (Iwata & Koizumi, 2005). Both Chr2.CM0168.430.nd and chr3.CM1144.80.nc are similar to zinc finger-like proteins. LjSGA_032996.1 is a member of the bZIP family and is 63% identical to the soybean SBZ1 protein, a transcription factor that binds to the promoter of the chalcone synthase gene (Yoshida et al., 2008).

Its expression is repressed in low-N0 plants (2.8-fold) and this is consistent with the expression profile of the chalcone synthase gene orthologue (Table S1; chr2.CM0018.700.nc), the only sequence involved in flavonoids metabolism to be down-regulated (Table S1 and Fig. 5a). A L. japonicus bZIP member (Ljbzf) was also reported to negatively regulate nodule development (Nishimura et al., 2002). LjSGA_085595.1 and LjSGA_020116.2 belong the GRAS-SCARECROW-LIKE family that has been involved in the control of radial root patterning (Scheres et al., 1996). More recently, a crucial role for NSP1 and NSP2, two members of the large legumes GRAS family (Udvardi et al., 2007; http://bioinfo.noble.org/gene-atlas/), was demonstrated in the formation of nodule primordia (NSP1 and NSP2; Kalò et al., 2005; Smit et al., 2005). The AP2/EREBP family is represented by the LjT01F24.60.nd gene, belonging to the subgroup VII B-2 (Riechmann et al., 2000), which has been involved in a variety of processes throughout the plant life cycle such as root elongation and has been proposed as part of the mechanisms used by plants to respond to various types of biotic and environmental stress. Remarkably, another member of the AP2/EREBP family, chr2.CM0608.10.nd encodes for the L. japonicus RAP2.4 transcription factor (Asamizu et al., 2008). This is part of a group of 20 putative nodulation-associated transcription factor genes (Asamizu et al., 2005), phylogenetically distinct from the M. truncatula AP2/EREBP transcription factors that are also involved in the nodulation process (Andriankaja et al., 2007; Middleton et al., 2007; Verniè et al., 2008). LjRap2.4 was found to be induced in roots of L. japonicus seedlings as early as 3 h after M. loti inoculation, and after treatment with ethylene or jasmonic acid (JA), which are known to be important regulators of the nodulation process (Penmetsa & Cook, 1997; Nakagawa et al., 2006).

Signalling, stress-response and nodulation-related sequences  Fifteen L. japonicus sequences, orthologous to annotated A. thaliana genes encoding modifying proteins, are expressed differentially in low-N0 versus high-N0 conditions (Signalling category of Table 1). These include protein kinases, phosphatases and calcium-binding proteins, suggesting a significant role exerted by signalling pathways in the plant response to chronic N environmental conditions (Sheen, 1996). Remarkably, in the signalling category are represented L. japonicus genes involved in early steps of nodule organogenesis that were not previously annotated as regulated by the N-supply conditions. LjT02O17.60.nc, which is upregulated 2.7-fold in low-N-0 plants, encodes for a calcium–calmodulin-binding protein (LjCCAMK), extensively characterized in L. japonicus. This protein plays a crucial role in the Nod factor-dependent transduction pathway, likely through a decodification of the calcium spiking early signal (Tirichine et al., 2006). Chr3.CM0282.970.nc, which is downregulated in Low-N0 conditions (2.69-fold), encodes a L. japonicus protein phosphatase type 2C (LjPMP2C1) that has been implicated in both early and late stages of nodule development (Kapranov et al., 1999). Moreover, chr1.CM0104.190.nc, annotated as a L. japonicus Nod factor binding lectin-nucleotide phosphohydrolase, was strongly upregulated in the Low-N0 condition (4.2-fold). This sequence codes for a member of the apyrase family and orthologous sequences have been reported to play...
a crucial role in nodule initiation and development in *Dolichos biflorus* and *Glycine max* (Etzler et al., 1999; Day et al., 2000; Govindarajulu et al., 2009). These genes might represent molecular links between pathways involved in the plant N status and nodule organogenesis. In addition, the correlation between N-regulated and nodulation-related sequences is confirmed by the differential expression of genes coding for nodulin-like proteins (Table S1; LjSGA_137301.1.1, LjSGA_067657.1, chr3.CM0005.600.nc, LjSGA_005472.1). Chr5.LjT18P14.20.nd, showing a high degree of upregulation (3.8-fold), encodes a *L. japonicus* phosphoenolpyruvate carboxylase kinase protein (LjPEPC-PK) that is expressed both in emerging and mature nodules (Nakagawa et al., 2003).

It is part of a large kinase family, which represent c. 9% of all the protein kinase in the *Arabidopsis* genome and is a Ca$^{2+}$ independent enzyme that is activated by a process involving protein synthesis in response to a range of signals in different plant tissues (Hartwell et al., 1999).

**Table 1** Sublist of genes differentially expressed in low-N0 vs high-N0 plants

<table>
<thead>
<tr>
<th>Lotus Affimex ID</th>
<th>Putative annotation</th>
<th>CombiMatrix data</th>
<th>Quantitative real-time PCR data</th>
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<td>Low N0 vs High-N0</td>
<td>High-N9 vs High-N0</td>
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<td>High-N9 vs High-N0</td>
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<td>Transcription factors</td>
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Data bold type indicate the sequences maintaining the same profile of expression in high-N9 vs high-N0 plants. When performed, the validation values obtained by quantitative real-time PCR are indicated. For the quantitative real-time PCR (as for the array hybridization) two technical and biological replicates were used.
and high-N9 differentially regulated genes had a significant hypersensitive response (Breton et al., 2003). The lowest level of expression was found for the CM1983.110.nc gene that encodes for a trehalose phosphatase catalysing the dephosphorylation of trehalose-6-phosphate to trehalose and orthophosphate (Table 1). Trehalose is a common disaccharide of bacteria, fungi and invertebrates that appears to play a major role in desiccation tolerance. The trehalose biosynthetic pathway has also been identified in plants where it is involved in stress protection response (Vogel et al., 1998). Interestingly, two molecular chaperones (LjSGA_106569.0.1 and chr5.CM0260.550.nd) showed a severe level of downregulation (Table 1; 7.6- and 3.3-fold). Molecular chaperones are a diverse family of proteins that function to protect proteins in the intracellular milieu from irreversible aggregation during synthesis and in times of cellular stress.

Expression profiling during the time-course experiment

The analysis of the symbiotic phenotypes during the time-course experiment shown in Fig. 3 identified two states of nodulation competence: high-N0 and high-N6 plants have a reduced nodulation capacity when compared with low-N0 and high-N9 plants. In order to analyse the profiles of gene expression (looking for putative gene determinants that could be involved in the nodulation phenotype change) the transcriptome analysis was repeated with RNAs extracted from high-N6 and high-N9 and these transcriptomes compared with that of high-N0 plants. The comparison identified 135 (43 upregulated and 92 downregulated) and 212 (119 upregulated and 93 downregulated) differentially regulated genes in high-N6 vs high-N0 and high-N9 vs high-N0 plants, respectively (Table S3). The functional annotation of the differentially expressed sequences was obtained by comparison with the A. thaliana genome. In total, 113 and 172 high-N6 and high-N9 differentially regulated genes had a significant hit in the A. thaliana genome (Table S3; 84% and 81%, respectively). Use of the mapman software (Usadel et al., 2005) helped to reveal a similarity in the expression profile of genes involved in the general metabolic pathways (Fig. 5b,c). In particular, strikingly similar in the two groups was the over-representation of downregulated transcripts involved in the photosynthetic pathways. A significant downregulation of photosynthetic gene transcripts in A. thaliana seedlings grown under N starvation has been previously reported (Martin et al., 2002; Scheible et al., 2004; Bi et al., 2007). Remarkably, 60% and 50% of the photosynthetic transcripts downregulated in the high-N6 vs high-N0 and high-N9 vs high-N0 samples were also found to be significantly downregulated in A. thaliana roots after being shifted to low nitrate conditions (Wang et al., 2003). However, the general views of metabolic pathways in these two transcriptomes reveal important differences when compared with that of the low-N0 vs high-N0 sample, suggesting significant differences in the respective general nutritional status. A striking example is represented by the different regulation of genes coding for nitrate and nitrite reductase (Fig. 5).

The Venn diagram shown in Fig. S5, takes into account the genes found differentially expressed in each condition (low-N0, high-N6 and high-N9 vs high-N0). The whole list of genes showing a similar profile of expression in the three transcriptomes comparisons is reported in Table S4 and, as expected, the high-N6 and high-N9 samples had the highest number of genes sharing a common behaviour (Fig. S5). This analysis also indicated that only seven genes showed a similar response throughout the time-course experiment (Fig. S5; Table S4). Among these, chr2.LjT17O14.70.nc, was strongly downregulated throughout the experiment. This gene is an orthologue of the A. thaliana sulphate transporter SULTR3;5 that was also reported to be inhibited by N-starvation conditions (Scheible et al., 2004). The reduction of the long-distance transport of sulphate through the xylem (Kataoka et al., 2004) might be integrated in a general plant response program that determines a downregulation of sulphate as well as of other metabolic pathways. A different transporter gene that was found to be constantly upregulated in all the comparisons, is the L. japonicus high-affinity ammonium transporters LjAMT1;1 (Table S4), which has been reported to be induced in conditions of low N availability (D’Apuzzo et al., 2004). Finally, Chr5.CM0239.440.nd, which encodes for a hydrolase enzyme involved in cell wall metabolism (Goujon et al., 2003) was, similarly to other genes involved in growth-related processes, downregulated in low N conditions.

We particularly focused on genes showing a common regulative profile between low-N0 vs high-N0 and high-N9 vs high-N0 samples, because of the consistency between their change of expression and the change of symbiotic phenotype. Interestingly, we found that some of the genes included in the transcription factors (bZIP and AP2/EREBP), signalling (apprase, carboxylase kinase) and biotic and abiotic stress (trehalose phosphatase, xylose isomerase) functional categories can be included in this sublist of genes (Table 1). The profile of expression of these six genes was further checked by qRT-PCR and the obtained results were consistent with the array data (Table 1).

Discussion

Developmental consequences of variations in N supply include effects on flowering time, root and shoot branching as well as nodulation (Marschner, 1995; Scheible et al., 1997; Zhang & Forde, 1998; Sitt & Krapp, 1999; Babulova et al., 2007). Therefore, a mechanism must exist for integrating information about external N availability and internal N status so as to modulate in a coordinated fashion a number of developmental pathways, including the specific signalling pathway leading to nodule formation. Recently, Ruffel et al. (2008) showed that systemic signalling is likely to have a strong impact on nodule development and reported a strong
transcriptome response in N-fixing roots, when one side of the split root system was shifted to 10 mM NH$_4$NO$_3$. The observed downregulated transcripts, were involved in nodule structure, function and N fixation and remarkably, some genes were correlated to the early stages of signalling in the nodule organogenesis pathway.

However, the link with N availability has always been analysed by testing the direct effects of N sources addition on the execution of nodule formation, development and functioning programs (Bisseling et al., 1978; Carroll & Mathews, 1990; Caetano-Anollès & Gresshoff, 1991; Barbulova et al., 2007). The only data relying on the analysis of a preliminary effect of a high N condition on the Nod factor-dependent pathway, after the transfer of Vicia sativa seedlings to N-free medium, was reported by Heidstra et al. (1994). They showed a block of root hairs deformation after a 36 h preincubation in the presence of 10 mM NH$_4$NO$_3$ and that the ability to deform was not regained after the shift in N-free medium (Heidstra et al., 1994). Furthermore, it was shown that the high-N treatment did not affect the expression of genes involved in the Nod factor-dependent transduction pathway (Heidstra et al., 1997).

In our study we first described the differences in the general N status of L. japonicus plants grown throughout under high-N (10 mM NH$_4$NO$_3$) or low-N (10 µM NH$_4$NO$_3$) supply by measuring amino acid content and shoot biomass (Fig. 2a,b). A nodulation assay experiment was then used to test the influence on symbiotic competence. Importantly, high-N0 plants, that were grown for 10 d on 10 mM NH$_4$NO$_3$ condition, before transfer to 10 µM NH$_4$NO$_3$, followed by immediate M. loti inoculation, exhibited a clearly reduced nodulation capacity (c. 40%) when compared with low-N0 plants that were maintained in 10 µM NH$_4$NO$_3$ permissive conditions. This result indicates that N availability not only directly affects the capacity of the plants to execute the various symbiotic steps (root infection, nodule development, nodule functioning; Bisseling et al., 1978; Carroll & Mathews, 1990; Caetano-Anollès & Gresshoff, 1991; Barbulova et al., 2007) but is also modulating, beforehand, the general competence of leguminous plants for symbiosis. Shoot biomasses of high-N0 and low-N0 plants are not significantly different at 4 wk after inoculation (Fig. 2d). This could be the result of a long-term effect of the preincubation on 10 mM NH$_4$NO$_3$ and/or because the few nodules observed on high-N0 plants were sufficient to satisfy, at least for that interval of time, the plant N demand to grow in the presence of 10 µM NH$_4$NO$_3$ as sole N source. Moreover, the experiment shown in Fig. 3 indicated a long-term effect of the preincubation on 10 mM NH$_4$NO$_3$ on the *Lotus* symbiotic competence. After transferring the plants to the low-N, permissive conditions (10 µM NH$_4$NO$_3$), it was 9 d before they showed full nodulation capacity (Fig. 3). The long time required for the reacquisition of full nodulation competence strongly suggests that, in *L. japonicus*, the capacity of the local nodule organogenesis pathway is regulated by the general N status of the plants. Interestingly, low-N9 and low-N13 plants did not show a complete competence for nodulation, probably because of the long incubation under N-starvation stress conditions (Fig. 2b,c).

Despite several studies on the plant responses to N, based on microarray gene expression profiling, the molecular mechanisms and factors involved in the regulation of N acquisition by the roots are still largely unknown. Most studies have focused on the effects of nitrate as N source and gene expression was monitored to analyse the response after shifting the plants to different nitrate concentrations for different intervals (Wang et al., 2003; Scheible et al., 2004). To our knowledge, only one microarray gene expression profiling study, aimed at obtaining a comprehensive evaluation of the plant responses to the availability of different N sources, has been reported for leguminous plants. Ruffell et al. (2008) reported a transcriptomic analysis in *M. truncatula* to delineate the gene networks responding systemically to different N sources. The study confirmed the existence of negative feedback pathways that regulate N acquisition in the roots on the basis of the N status of the whole plant. That study also revealed some specificities of the *M. truncatula* response to different N sources.

The large-scale transcriptomic analyses provided in this study has shown a remarkable consistency between our and previously reported results, relative to genes involved in metabolic pathways in *A. thaliana* plants shifted to low-nitrate conditions (Wang et al., 2003; Scheible et al., 2004; Bi et al., 2007). In particular, low-N0 plants, when compared with high-N0 plants, showed a downregulation of genes involved in pathways of N and C primary metabolism (nitrate assimilation, sucrose and starch synthesis), in agreement with a plant response strategy correlated to the amount of available N (Table S1; Fig. 5a). The gene expression profiling over time revealed that the number of differentially expressed genes in high-N6 and high-N9 vs high-N0 plants was reduced compared with those of low-N0 vs high-N0 plants (Table S3; Fig. S5). This result is not unexpected since these plants were initially grown under the same conditions as high-N0 plants (10 d in the presence of 10 mM NH$_4$NO$_3$) and their general nutritional status was probably still reflecting this preincubation treatment (Figs 1, 5b,c).

In Table 1 we listed several genes, differentially expressed in low-N0 vs high-N0 plants that could play an important role in the control of the plant response to N conditions and might identify direct or indirect targets of N-dependent pathways. These include transcription, stress-response and signalling factors. The comparisons visualized in Fig. S5 indicated the similarities between high-N6 and high-N9 plants, where the highest number of genes showing a common transcriptional response was found (Table S4). However, high-N6 and high-N9 plants differ strongly in their symbiotic performances, as only the latter showed full nodulation capacity similar to the low-N0 plants. The subgroup of genes showing a common
expression profile in low-N0 and high-N9 plants, which also share the same symbiotic phenotype, was of particular interest (Table S4). In particular six genes included in the Table 1, maintained the same profile of expression in the high-N9 vs high-N0 transcriptomes comparison and two of these are included in the transcription factors functional category. Chr2.CM0608.10.nd, induced in our conditions, encodes for the LjRAP2.4 factor, a member of the AP2/EREBP transcription factor family (Asamizu et al., 2008). In L. japonicus (LjERF1) and M. truncatula, AP2/EREBP genes have been shown to be essential for the nodulation process (Andriankaja et al., 2007; Middleton et al., 2007; Vernière et al., 2008; Asamizu et al., 2008). LjRAP2.4 was slightly induced in roots, 3 h after M. loti inoculation and after ethylene or JA treatment. Based on this profile, the authors suggested a role in the regulation of downstream genes, including defence response genes, or in the crosstalk between ethylene and calcium spiking signalling pathways, and/or in the JA-mediated systemic suppression of nodulation (Asamizu et al., 2008). The second TF showing a common expression profile (downregulation) in the low-N0 vs high-N0 and high-N9 vs high-N0 transcriptomes comparisons is the LjSGA_032996.1 coding for a basic leucine zipper (b-ZIP) transcription factor. The predicted protein shows an extensive level of identity with the soybean SBZ1 protein, a transcription factor that binds to the promoter of the chalcone synthase gene (Yoshida et al., 2008). Chalcone synthase regulates the production of flavonoids that play a central role in the early stages of symbiosis and are possibly involved in the balance between symbiotic and defence response that modulates the nodule organogenesis program (Lawson et al., 1994). A member of this L. japonicus bZIP family (ASTRAY gene) has been involved in the autoregulatory control of nodulation and the corresponding mutant showed an increased number of nodules as well as nonsymbiotic phenotypes (Nishimura et al., 2002).

The near-completion of the sequencing of the L. japonicus genome (Sato et al., 2008) allowed the identification of c. 1500 genes that encode putative transcription factors (c. 5% of all annotated genes) and similar numbers were deduced in M. truncatula (Udvardi et al., 2007). To date, a small number of host transcription factors have been genetically characterized in terms of their crucial function in the nodulation process (Schauer et al., 1999; El Yahyaoui et al., 2004; Kalb et al., 2005; Smit et al., 2005; Combier et al., 2006; Lohar et al., 2006; Andriankaja et al., 2007; Middleton et al., 2007; Asamizu et al., 2008; Vernière et al., 2008). None of these functionally characterized TF was revealed by our transcriptomic analysis but this was not unexpected since our analysis was performed on RNA extracted before Rhizobium inoculation and hence before the developmental switch leading to nodule organogenesis. This is, for example, the case for the NIN gene, a true nodulin, not significantly expressed in uninoculated roots and strongly induced 24 h after inoculation, which was reported to be inhibited by the concomitant presence of high N concentration in the medium (Barbulova et al., 2007). In this same article, we reported a slight reduction of the expression of NSP1 and NSP2, two transcriptional activators of the GRAS-SCARECROW family involved in the nodule primordial formation, in uninoculated roots of L. japonicus seedlings grown under high- and low-N conditions (Barbulova et al., 2007). However, the regulatory level reported is under the threshold of significance that we chose in our analysis.

Two genes belonging to the signalling category maintain the same regulative profile in low-N0 and high-N9, the two classes of plants that are symbiotically competent. Chr5.LjT18P14.20.nd encodes for the LjPEPC-PK that is expressed in all types of nodule and root cells (Nakagawa et al., 2003). In symbiotic nodules PEPC-PK is involved in the phosphorylation of PEPCs that play different roles in energy supply to bacteroids and maintenance of the charge balance in xylem sap (Deroche & Carrayol, 1988). Phosphoenolpyruvate carboxylase kinases are activated in response to a range of signals in different plant tissues playing a key role in the control of plant metabolism (Hartwell et al., 1999). This large kinase family participates in the coupling of cellular responses to environmental and developmental signals, hence representing a good candidate for linking N conditions and the nodule organogenesis program. Furthermore, it was of particular interest to find the apyrase, Nod factor-binding lectin (chr1.CM0104.190.nc) among the few genes displaying a common response (Table 1). The lectin nucleotide phosphohydrolase was first characterized in D. biflorus (as Db-Lectin-nucleotide phosphohydrolase (LNP) protein). It was shown that antisera raised against this protein inhibited root nodulation induced by rhizobia (Etzler et al., 1999) and that the protein was present on the surface of root hair tips after addition of rhizobia (Kalsi & Etzler, 2000). Remarkably, the Db-LNP root induction-dependence by NO3− and NH4+ starvation was directly demonstrated in D. biflorus (Kalsi & Etzler, 2000). Similar results were obtained in G. max by Day et al. (2000) who showed that a specific antiserum against this protein (GS52) inhibited nodulation and by McAvin & Stacey (2005) who showed that, in L. japonicus, overexpression of the soybean LNP leads to an increase in infection threads and nodule numbers. These observations strongly support a role for LNPs during legume nodulation. More recently the role of the GS52 ecto-apyrase in the early infection process, initiation of nodule primordium development and subsequent nodule organogenesis was demonstrated in soybean (Govindarajulu et al., 2009). Its catalytic activity might probably regulates ATP and ADP levels at the root hair surface (Govindarajulu et al., 2009).

Biotic and abiotic stress factors are involved in a variety of plant responses to different environmental conditions such as N starvation, and hence might be involved in linking these responses to specific developmental programs. A trehalose phosphatase (CM1983.110.nc) is downregulated in the low-N0 vs high-N0 and high-N9 vs high-N0 transcriptomes. Trehalose is regularly found in plant roots interacting with
antagonistic fungi and mycorrhizal fungi, and in nitrogen-fixing nodules, probably as a microbial substance. An *A. thaliana* trehalose phosphatase mutant (*zip1*) shows a recessive embryo lethal phenotype and is involved in the regulation of sugar metabolism/embryo development pathways (Eastmond *et al.*, 2002). Another stress response gene highlighted by our analysis is the LjSGA_017485.1 coding for a xylose isomerase that plays a role in the carbohydrate metabolism and it is probably involved in the recovery from dehydration.

In conclusion, we have introduced the concept of ‘competence’ in the analysis of the links occurring between N availability and N-fixing symbiotic interaction. We have shown that excess N not only affects the execution of the symbiotic performances of leguminous plants during the interaction with the bacteria, but also their predisposition for symbiosis and that such an noncompetent state is maintained for several days. The inhibition of nodulation competence represents *per se* a novel N-dependent symbiotic phenotype that must be taken into account when designing experimental procedures. It also has practical applications in agriculture, particularly with regard to the exploitation of legumes, such as soybean or alfalfa, for crop rotation programs. We also identified *L. japonicus* genes that are differentially expressed in low-N and high-N conditions. The molecular identities of some of the genes differentially expressed at the same time as the symbiotic phenotype changes suggest possible links with the modulation of the nodule organogenesis competence and will be further investigated through the analysis of overexpression and/or RNAi transgenic lines.

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References


Research 15


Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Nodule numbers in *Lotus japonicus* plants at 4 wk post inoculation.

**Fig. S2** Kinetic of primary root elongation of low-nitrogen (N) and high-N plants.

**Fig. S3** Functional categories of genes differentially expressed in low-N0 vs high-N0 according to the mapman software.

**Fig. S4** MapMan transport overview window of low-N0 vs high-N0 differentially expressed *Lotus japonicus* genes.

**Fig. S5** Venn diagram of genes identified as differentially expressed in the time course experiment.

**Table S1** Transcriptomic profile data of low-N0 vs high-N0 plants

**Table S2** Comparison of CombiMatrix and real time reverse transcriptase polymerase chain reaction fold ratios

**Table S3** Transcriptomic profile data of high-N6 vs high-N0 and high-N9 vs high-N0 plants

**Table S4** Sublist of genes showing a common profile of expression in low-N0, high-N6 and high-N9 plants vs high-N0 plants

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