Nodulation enhances dark CO\textsubscript{2} fixation and recycling in the model legume \textit{Lotus japonicus}

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Abstract

During symbiotic nitrogen fixation (SNF), the nodule becomes a strong sink for photosynthetic carbon. Here, it was studied whether nodule dark CO\textsubscript{2} fixation could participate in a mechanism for CO\textsubscript{2} recycling through C\textsubscript{4}-type photosynthesis. Differences in the natural $\delta^{13}$C abundance between \textit{Lotus japonicus} inoculated or not with the N-fixing \textit{Mesorhizobium loti} were assessed. $^{13}$C labelling and gene expression of key enzymes of CO\textsubscript{2} metabolism were applied in plants inoculated with wild-type or mutant fix\textsuperscript{−} (deficient in N fixation) strains of \textit{M. loti}, and in non-inoculated plants. Compared with non-inoculated legumes, inoculated legumes had higher natural $\delta^{13}$C abundance and total C in their hypergeous organs and nodules. In stems, $^{13}$C accumulation and expression of genes coding for enzymes of malate metabolism were greater in inoculated compared with non-inoculated plants. Malate-oxidizing activity was localized in stem xylem parenchyma, sieve tubes, and photosynthetic outer cortex parenchyma of inoculated plants. In stems of plants inoculated with fix\textsuperscript{−} \textit{M. loti} strains, $^{13}$C accumulation remained high, while accumulation of transcripts coding for malic enzyme isoforms increased. A potential mechanism is proposed for reducing carbon losses during SNF by the direct reincorporation of CO\textsubscript{2} respired by nodules and the transport and metabolism of C-containing metabolites in hypergeous organs.

Key words: $^{13}$C labelling, dark CO\textsubscript{2} fixation, $\delta^{13}$C abundance, malate metabolism, symbiotic N fixation.

Introduction

The interaction between legumes and soil bacteria called rhizobia results in the formation of nodules, in which the symbiotic form of rhizobia, the so-called bacteroid, reduces molecular dinitrogen into ammonia. Both partners benefit from this symbiosis, since the plant’s requirements for N are largely covered by the rhizobia, which in turn receive organic carbon from the plant produced by photosynthesis (Voisin \textit{et al.}, 1997). The availability of photoassimilates is found to be an important factor controlling nodule growth (Voisin \textit{et al.}, 2003\textsuperscript{b}) and nitrogen fixation (Voisin \textit{et al.}, 2003\textsuperscript{c}). Sucrose from the shoot is the principal source of carbon and energy for nodule development and metabolism. Sucrose is transported to nodules via the plant vascular system and distributed apoplastically and/or symplastically to nodule cells where it is catabolized to provide energy and carbon skeletons for symbiotic nitrogen fixation (SNF), assimilation of ammonia, and export of amino acids and other nitrogenous compounds (Gordon, 1995; Voisin \textit{et al.}, 2003\textsuperscript{b}).
However, malate produced by sucrose catabolism, rather than sucrose itself, is reported to be the major carbon compound transported from the cytoplasm of colonized plant cells to the intracellular bacteroids, via the intervening plant symbiosome membrane, for bacteroid metabolism (Ronson et al., 1981; Finan et al., 1983; Vance and Heichel, 1991; Werner, 1992; Udvardi and Day, 1997). Nodule development and SNF require substantial amounts of fixed carbon, making them a strong sink for carbon (Minchin and Pate, 1973; Silsbury, 1977).

N₂ fixation in nodules comes at a high respiratory cost (Rainbird et al., 1984; Ryle et al., 1984), with concomitant loss of CO₂ (Pate et al., 1979a), which accounts for >60% of the carbon allocated to the nodules (Voisin et al., 2003a). Reincorporation of CO₂ through the combined activity of carbonic anhydrase (EC: 4.2.1.1) and phosphoenolpyruvate carboxylase (PEPC, EC: 4.1.1.31) can provide intermediates for the tricarboxylic acid (TCA) cycle and other metabolism in nodules (Vuorinen and Kaiser, 1997; Flemetakis et al., 2003). The presence of carbonic anhydrase, the enzyme responsible for the bidirectional exchange between CO₂ and HCO₃⁻, at high levels in root nodules (Kavroulakis et al., 2000; Atkins et al., 2001) further supports the potential importance of inorganic carbon in the nodules, since carbonic anhydrase can supply PEPC with HCO₃⁻ and thereby enhances dark CO₂ fixation.

It is well established that a proportion of the organic carbon originating from photosynthesis that is allocated to nodule, together with much of the N originating from N₂ fixation, cycle through nodules. These C and N pools return to the rest of the plant, via the xylem, as organic N compounds, principally amides and amino acids (e.g. Pate et al., 1979b; 1984; Atkins et al., 1980; Layzell et al., 1981). Previous studies have shown that CO₂ in nodules can, to a great extent, be fixed by PEPC (Coker and Schubert, 1981; King et al., 1986). Currently, it has been shown that dark CO₂ fixation in nodules supports bacteroid metabolism through the production of dicarboxylic acids, provides carbon skeletons for ammonium assimilation, and is thus essential for efficient nitrogen fixation (Rosendahl et al., 1990; Fischinger et al., 2010).

Interestingly, pulse-chase labelling experiments using ¹⁴CO₂ fed through the root system of nodulated soybean plants indicated that inorganic carbon fixed by nodules could be rapidly transported to shoots through xylem sap mainly in the form of TCA cycle intermediates (Vance et al., 1985). However, the physiological significance of this mechanism remains to be elucidated. Later studies in other C₃ plants indicated that CO₂ fixation and malate production can take place in roots with active PEPC (Cramer and Richards, 1999; Hibberd and Quick, 2002). Furthermore, it has been proposed that malate can be transported through the vascular system via the xylem sap to the leaves, where it can be decarboxylated, releasing CO₂ for photosynthesis (Hibberd and Quick, 2002). It is possible that a similar mechanism operating in nodulated plants could transfer carbon from nodules to photosynthetic plant organs, thus minimizing carbon losses associated with SNF.

In an attempt to further explore the physiological significance of dark CO₂ fixation in nitrogen-fixing nodules, we used stable isotope labelling of roots and nodules containing wild-type or mutant rhizobia, together with transcript analysis and histochemical localization of malate-oxidizing activity to assess the hypothesis that nodulation could result in the enhancement of CO₂ fixation and recycling in the model legume Lotus japonicus L., coupled with systemic changes in the metabolism of its immediate products, mainly malate, in the shoots and leaves of nodulated plants.

## Materials and methods

### Plant material and bacteria strains

*L. japonicus* (Gifu B-129) seeds were kindly provided by Dr Jens Stougaard (University of Aarhus, Denmark). Prior to germination, seeds were scarified for 5 min with H₂SO₄ and then sterilized for 20 min in a solution containing 2.0% (v/v) NaOCl and 0.02% (v/v) Tween 20. Seeds were pre-germinated at 18°C in the dark for 72 h. *Mesorhizobium loti* (wild-type strain R7A and mutant strains Δ*nifA*, Δ*nifH*) was cultivated on solid YMB medium (Hooykaas et al., 1977) with 1.5% agar for 72 h at 30°C. After germination, the *L. japonicus* plants were transferred into plastic pots (two in each pot) containing quartz sand. For inoculation with rhizobia, the plants were spot-infected with a 0.1 OD₅₆₀ suspension culture of *M. loti* (wild-type strain R7A or mutant strains Δ*nifA*, Δ*nifH*). Half of the plants used for the experiments on natural ¹³C abundance, ¹³C labelling, and gene expression were not infected and were used as controls. *M. loti* strains R7A, Δ*nifH*, and Δ*nifA* were kindly provided by Professor Clive Ronson (University of Otago, New Zealand).

### Growth conditions

*L. japonicus* plants were grown in a controlled environment characterized by 16-h day/8-h night rotation, 22°C air temperature, 65% air relative humidity, and 300 μmol m⁻² s⁻¹ light intensity (Handberg and Stougaard, 1992). Two days after transfer into plastic pots watering was initiated alternately with Hoagland solution and deionized water, every 2 d. The concentration of the Hoagland solution was gradually increased from 25% to 50% and finally to 100%. For nitrogen-fixing plants, nitrogen-free Hoagland solution was used, whereas for non-inoculated plants nitrate was used as an N source in the Hoagland solution.

### Experimental set-up

Four sets of experiments were carried out. In the first three, *L. japonicus* plants inoculated with *M. loti* and non-inoculated ones were used for determining differences in (i) natural ¹³C, (ii) patterns of ¹³C labelling, and (iii) expression levels of genes encoding key enzymes of C metabolism. Furthermore, in order to test whether the differences in the carbon balance between inoculated and non-inoculated plants were related to the nitrogen-fixing capacity of the former, an additional approach was used. In this fourth set of experiments, ¹³C labelling and gene expression analyses were performed with *L. japonicus* plants inoculated either with the wild-type strain R7A of *M. loti*, or with its mutant strains Δ*nifH* and Δ*nifA*. Inoculation with both the mutant strains resulted in the formation of ineffective nodules with no nitrogenase activity (Supplementary Fig. S2, at *JXB* online). In contrast to Δ*nifH*, nodules formed after inoculation with the Δ*nifA* strain contained no differentiated bacteroids (data not shown).
Nitrogenase activity
Nitrogenase activity of *L. japonicus* plants inoculated either with the wild-type strain R7A of *M. loti*, or with its mutant strains Δ*nifH* and Δ*nifA*, was estimated by acetylene reduction as described by Hardy *et al.* (1973) using a Perkin-Elmer 8500 gas chromatograph (PerkinElmer Life and Analytical Science, Inc., Wellesley, MA, USA) equipped with a 2-m Porapak R column and a flame ionization detector.

Natural 13C abundance
In total 20 inoculated and 20 non-inoculated 30-d-old *L. japonicus* plants were used. All plants were harvested and separated into leaves, stems, roots, and nodules (only for the inoculated plants). All organs were collected, homogenized in liquid nitrogen, and stored at −80°C until further analysis.

13C labelling
The 13C-labelling experiment was similarly performed with 30-d-old inoculated and non-inoculated *L. japonicus* through the root system of the plants. For this purpose, an experimental approach was established for labelling through nodulated roots with a 13C-enriched solution (Supplementary Fig. S1, available at JXB online). In total 108 plants were used for the labelling experiment: half of them inoculated and half non-inoculated. All plants were carefully removed from the plastic pots to limit root damage and transferred to hydroponics in vials (100-ml volume). Each vial was connected to a CO2-free air supply system. This system provided sufficient aeration to each plant’s root system, by means of moderate pumping, during a 2-h acclimation period. CO2 was removed from the air supply with soda lime CO2 traps in order to ensure sufficient carbon uptake by the roots during the subsequent 13C-labelling period.

Immediately after acclimation, 13C labelling was performed. All plants, apart from controls, were transferred from hydroponics to air-tight glass vials (100-ml volume) containing 10 mM Na2 13CO3 solution (99% enriched in 13C; pH 6). At this pH value a ratio of ~4 between HCO3− and CO2 is achieved. The vials were sealed carefully and an air exit pipe, connected to the top of each vial, was used to prevent contamination of the hypergeous plant parts with any respired 13CO2 produced during labelling. Labelling was performed by a 2-h pulse. One-third (18) of the inoculated and non-inoculated plants, respectively, were kept in hydroporics for a 1-h chase period after completion of the labelling. After each phase, plants were harvested and separated into leaves, stems, roots, and nodules. All collected tissues were ground in liquid nitrogen and stored at −80°C until further analysis.

The 13C-labelling conditions (pretreatment with CO2 traps, duration of aeration and labelling, concentration of the label) were evaluated after a series of pre-tests. Thus, the applied labelling procedure was appropriate to achieve sufficient, detectable labelling of the upper plant parts at the shortest possible duration.

Gene expression analysis
Three biological repeats of 30-d-old inoculated and non-inoculated *L. japonicus* plants were used (20 plants per repeat). Moreover, three biological repeats of *L. japonicus* plants inoculated with mutant strains (Δ*nifH*, Δ*nifA*) and the wild-type strain R7A were used. All plants were harvested and separated into leaves, stems, roots, and nodules (only for inoculated plants), and stored at −80°C until further analysis.

δ13C composition
Plant material of all tissues was oven-dried (3 d, 65°C). Samples of 0.5 mg were transferred into tin capsules (IVA Analysetechnik, Meerbusch, Germany). Subsequently, the samples were injected into a ratio mass spectrometer (Delta Plus; Finnigan MAT GmbH, Bremen, Germany) for δ13C analysis. δ13C values are expressed relative to the VPDB (Vienna Pee Dee Belemtian) standard.

Calculation of tissue and plant 13C excess
According to Simard *et al.* (1997), the 13C per tissue is the enrichment level of the tissue (mg 13C/tissue) in excess of its natural 13C abundance (mg 13Cnat).

*Excess mg 13C tissue = mg 13C tissue − mg 13C nat* 

and 

*mg 13C tissue = A tissue × mg 12C + 13C*  

where *A tissue* is the fractional abundance of 13C relative to the sum of 12C and 13C. Thus:

*A tissue = 13C / (12C + 13C) = R tissue / (R tissue + 1)*  

and 

*R tissue = ([δ13C / 1000] + 1) × R standard*  

where *R standard* is the R value of the VPDB standard, which is 0.0112372.

To determine the 13C excess per plant, the 13C excess of the separated plant tissues was summed.

Determination of transcript levels using real-time RT-qPCR
Organs of 30-d-old *L. japonicus* plants were harvested and ground in liquid nitrogen. Total RNA was isolated and quantified by spectrophotometry and agarose gel electrophoresis. Prior to RT-PCR, the total RNA samples were treated with DNase I (Promega, Madison, WI, USA) at 37°C for 45 min, in order to eliminate any traces of contaminating genomic DNA. Successful removal of genomic DNA was tested by PCR. First-strand cDNA was reverse-transcribed from 2 μg of DNase-treated total RNA. All DNase-treated total RNA samples were denatured at 65°C for 5 min followed by quick chill on ice in a 12-μl reaction mixture containing 500 ng of oligo(dT)12–18mer and 1 μl of 10 mM dNTPs. After the addition of 4 μl of 5X-first-strand buffer (Invitrogen, Paisley, UK), 1 μl (40 units) of RNaseOUT (Invitrogen) ribonuclease inhibitor, and 2 μl of 0.1 M DTT, the reaction was preheated at 42°C for 2 min before the addition of 1 μl (200 units) of SuperScript II reverse transcriptase (Invitrogen). The reaction mixture was incubated at 42°C for 50 min, followed by heat inactivation at 70°C for 15 min. The resulting first-strand cDNA was diluted to a final volume of 200 μl, and target cDNAs were amplified using gene-specific primers (Supplementary Table S1, at JXB online) designed from the transcribed region of each gene using Primer Express 1.5 software (Applied Biosystems, Darmstadt, Germany). Quantitative RT-PCR reactions were performed on the Stratagene Mx3005P using SYBR Green Master Mix (Applied Biosystems) gene-specific primers at a final concentration of 0.2 μM each and 1 μl of the cDNA as template. PCR cycling started with the initial polymerase activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primer specificity and the formation of primer dimers were monitored by dissociation curve analysis and agarose gel electrophoresis on a 4% (w/v) gel. The expression levels of a *L. japonicus* ubiquitin gene were used as internal standards to normalize small differences in cDNA template amounts. For the relative quantification of gene expression, a modification of the comparative threshold cycle method was used. In the case of symbiotic and non-symbiotic organs, relative transcript levels of the gene of interest (X) were calculated as a ratio to the ubiquitin gene transcripts (U), as 

*(1+E)−Ct*  

where *AC* was calculated as *C(U)−C(X)*, PCR efficiency (E) for each amplicon was calculated employing the linear regression method on the log (fluorescence) per cycle number data, using LinRegPCR software (Ramakers *et al.*, 2003). All real-time qPCR reactions were performed in triplicate.

Histochemistry for malate dehydrogenase/malic enzyme activity
The method used was a modification according to Colombo *et al.* (1997). Stem segments of 30-d-old inoculated *L. japonicus* plants...
were used to produce thin hand-cut sections while embedded in fixation buffer [2% (w/v) polyvinylpyrrolidone-40, 2 mM DTT, 2% (v/v) paraformaldehyde, 0.1% (w/v) BSA]. After being kept in the fixation buffer for 20 min, the sections were transferred to 1% (v/v) Triton at −20 °C overnight. After being defrosted, the sections were washed four times (30-min intervals between each wash) with cold water before being assayed. The reaction mixture contained 50 mM Tris–HCl pH 7.0, 0.5 mM NAD or NADP, 0.025% (w/v) cold water before being assayed. The reaction mixture contained NBT, 1 mM EDTA, 5 mM MgCl2, and 8 mM malate.

The colour was revealed by incubation of stem sections for 15 min at room temperature. The reaction was stopped with the addition of 4% formaldehyde. Reaction mixture without malate was used as a negative control.

Chlorophyll visualization was conducted using a Zeiss Axiolab epifluorescence microscope with a BP 450–490 excitation filter and an FT-510 chromatic beam splitter with an LP520 barrier filter.

**Statistical analysis**

All statistical analysis was carried out using SPSS 12.0 (SPSS, Inc., USA). Comparisons were performed by applying t-tests for independent samples and a Duncan post-hoc test at a 95% level of significance.

**Results**

**Nodulation results in changes in natural abundance of Δ^{13}C**

Table 1 presents a comparison of the natural Δ^{13}C abundance between the various tissues of inoculated and non-inoculated *L. japonicus* plants. Significant differences were found in the Δ^{13}C values of leaves and roots; both tissues were Δ^{13}C enriched in inoculated plants compared with the non-inoculated ones. In contrast, no significant difference due to nodulation was observed in the natural Δ^{13}C abundance of the stem of *L. japonicus* plants.

The fixation and allocation of inorganic carbon by *L. japonicus* hypogeous organs

**Non-inoculated compared with inoculated plants.** To determine whether nodules contribute to the carbon economy of *L. japonicus* by fixing CO2 and exporting the products to other plant organs, a set of experiments using Δ^{13}C-labelled Na2CO3 fed through the root system of the plants was performed.

Two hours of Δ^{13}C labelling resulted in a substantial increase in Δ^{13}C abundance in all organs of both inoculated and non-inoculated plants (Fig. 1A) compared with their natural Δ^{13}C abundance (Table 1). Interestingly, Δ^{13}C of the different plant organs increased by between 45% and 120% in non-inoculated plants, and by 50–215% in inoculated plants, compared with unlabelled plants. Although Δ^{13}C values were not significantly different between inoculated and non-inoculated plants for any of the organs, they tended to be higher for inoculated plants after 2 h of labelling (Fig. 1A).

After an additional hour of chase, i.e. in the absence of Δ^{13}C around the root system, Δ^{13}C abundance of the plants was further enhanced, especially in inoculated plants in which Δ^{13}C increased by between 145% and 310% in the various organs, relative to unlabelled plants (Fig. 1B, Table 1).

After completion of Δ^{13}C labelling (2 h pulse labelling plus 1 h chase), above-ground organs (leaves and stems) of inoculated plants were significantly more enriched in Δ^{13}C than those of non-inoculated plants (Fig. 1B).

Figure 2 shows the Δ^{13}C enrichment of each plant organ, in excess of its natural Δ^{13}C abundance, expressed in μg of Δ^{13}C per mg DW. After the 2 h of pulse labelling, Δ^{13}C enrichment was significantly greater in stems of inoculated plants than in stems of non-inoculated plants (Fig. 2A). After an additional

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**Table 1.** Natural Δ^{13}C abundance (‰) of inoculated and non-inoculated *L. japonicus* plants

<table>
<thead>
<tr>
<th>Organ</th>
<th>Non-inoculated</th>
<th>Inoculated</th>
</tr>
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<tbody>
<tr>
<td>Nodules</td>
<td>−38.30±0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−38.19±0.17</td>
</tr>
<tr>
<td>Roots</td>
<td>−37.0±0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−37.0±0.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leaves</td>
<td>−40.01±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−39.40±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stems</td>
<td>−36.73±0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>−36.64±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Statistical analysis refers to the comparison of the same organ between inoculated and non-inoculated *L. japonicus* plants. Different superscript letters indicate statistical differences at a 95% level of significance.
1 h of chase, $^{13}$C enrichment was significantly greater in inoculated plants than in non-inoculated plants not only in stems, but also in leaves (Fig. 2B).

Inoculation with fix$^{-}$ compared with wild-type strains of $M.$ loti. To test whether the export of fixed inorganic carbon from nodulated roots to shoots requires an active nitrogen-fixation process in nodules, $^{13}$C-labelling experiments were also performed with $L.$ japonicus plants inoculated with an $M.$ loti R7A $\Delta$nit$H$ mutant strain, which triggers normal nodule development but with no detectable nitrogenase activity, and an $M.$ loti R7A $\Delta$nit$A$ mutant strain that triggers aberrant nodule development, fails to differentiate into the bacteroid form, and has no detectable nitrogenase activity (Supplementary Fig. S2, at JXB online).

For plants inoculated with the mutant strains of $M.$ loti, the $\delta^{13}$C, as well as the excess $^{13}$C ($\mu$g $^{13}$C mg$^{-1}$ DW) accumulated into the biomass of each plant organ after the completion of $^{13}$C labelling (2 h pulse labelling and 1 h chase), were expressed as fold change in the respective organs compared with plants inoculated with the wild-type strain R7A (Fig. 3).

Both the nodules and the roots of plants inoculated with the mutant strains were characterized by substantially lower $\delta^{13}$C abundance than plants inoculated with the wild-type strain (~10% and 20% of normal levels in nodules and roots, respectively; Fig. 3A). The $\delta^{13}$C in leaves of the plants harbouring defective nodules was 25% lower compared with $\delta^{13}$C of the leaves of the plants containing the R7A wild type. In contrast, $\delta^{13}$C of stems was slightly higher (~20%) in the stems of plants with defective nodules than in plants with nitrogen-fixing nodules. There were no significant differences in $^{13}$C abundance between plants inoculated with the $\Delta$nit$H$ and $\Delta$nit$A$ strains.

Excess of $^{13}$C in defective nodules was less than half that in nitrogen-fixing nodules (Fig. 3B). Interestingly, $^{13}$C enrichment was significantly lower in nodules formed by the $\Delta$nit$A$ strain than in those containing the $\Delta$nit$H$ strain (Fig. 3B). Furthermore, enrichment of $^{13}$C in leaves of plants with non-fixing nodules was only 25% that of leaves from nitrogen-fixing plants. In contrast, $^{13}$C enrichment was similar in both roots and stems of plants with functional or defective nodules (Fig. 3B).

Expression profiling of genes involved in dark CO$_2$ fixation and malate metabolism

To gain insight into the molecular basis and physiological implications of the increased levels of CO$_2$ fixation and...
export from nodules, we measured the expression level of the following genes involved in CO2 fixation and malate metabolism in different organs of inoculated and non-inoculated plants: PEPC (EC: 4.1.1.31; \textit{LjPEPC1} and \textit{LjPEPC2}), cytosolic NAD-malate dehydrogenase (MDH; EC: 1.1.1.37; TC7830), (B) \textit{LjPEPC2} (TC14124), (C) cytoplasmic MDH (TC7834), (D) chloroplastic NADP-MDH (TC8525), (E) mitochondrial MDH (TC7960), (F) NADP-ME (TC17304), (G) NADP-ME (TC8784), (H) NAD-ME (TC16102), (I) PEPC (TC18110), and (J) PEPC (TC9282) gene transcripts in symbiotic and non-symbiotic organs of \textit{L. japonicus}. Total RNA was isolated from nodules, roots, leaves, and stems of inoculated and non-inoculated plants, reverse transcribed to cDNA and subjected to qRT-PCR. Relative mRNA level was calculated with respect to the level of ubiquitin transcript. Bars represent means (+SE) of three biological replications. Significant differences ($P$=0.05) are indicated by different letters.

Fig. 4. Accumulation of (A) \textit{LjPEPC1} (TC7830), (B) \textit{LjPEPC2} (TC14124), (C) cytoplasmic MDH (TC7834), (D) chloroplastic NADP-MDH (TC8525), (E) mitochondrial MDH (TC7960), (F) NADP-ME (TC17304), (G) NADP-ME (TC8784), (H) NAD-ME (TC16102), (I) PEPC (TC18110), and (J) PEPC (TC9282) gene transcripts in symbiotic and non-symbiotic organs of \textit{L. japonicus}. Total RNA was isolated from nodules, roots, leaves, and stems of inoculated and non-inoculated plants, reverse transcribed to cDNA and subjected to qRT-PCR. Relative mRNA level was calculated with respect to the level of ubiquitin transcript. Bars represent means (+SE) of three biological replications. Significant differences ($P$=0.05) are indicated by different letters.
MDH; EC: 1.1.1.37; TC7960), NADP-dependent malic enzyme (NADP-ME; EC: 1.1.1.40; TC17304 and TC8784), NAD-dependent malic enzyme (NAD-ME; EC: 1.1.1.37; TC16102), and phosphoenolpyruvate carboxykinase (PEPCK; EC: 4.1.1.49; TC18110 and TC9282). The respective genes include all the isoforms represented in the *Lotus* expressed sequence tags public databases.

qRT-PCR assays were performed on cDNA derived from total RNA of nodules, roots, stems, and leaves of inoculated and non-inoculated 30-d-old *L. japonicus* plants. All primer pairs yielded a single product of the expected size confirming their efficacy and gene specificity.

Transcript levels of *LjPEPC1* were highest in nitrogen-fixing nodules, lower in roots, and lower still in stems and leaves (Fig. 4A). *LjPEPC1* transcript levels were significantly higher in roots of inoculated plants than in roots of non-inoculated plants. In contrast, *LjPEPC2* transcripts were lowest in nodules and not affected by the nodulation status in other organs (Fig. 4B). Transcript levels of a cytosolic MDH isofrom (TC7834) were almost 2-fold higher in the stems of inoculated than non-inoculated plants (Fig. 4C). However, genes coding for a chloroplastic NADP-MDH precursor (TC8525) and a mitochondrial MDH precursor (TC7960) exhibited similar expression patterns between inoculated and non-inoculated plants (Fig. 4D, E). Interestingly, transcripts for the two NADP-ME isoforms (TC17304 and TC8784) were found to be up-regulated in stems from inoculated plants, and a NAD-ME isofrom (TC16102) showed higher accumulation of gene transcript levels in inoculated plants, which was significant in roots and stems but not in leaves (Fig. 4F–H). Finally, two genes coding for the PEPCK isoforms TC18110 and TC9282 showed significantly higher expression (almost 2-fold and 4-fold in TC18110 and TC9282, respectively) in the roots of inoculated compared with non-inoculated plants (Fig. 4I, J). Expression of all genes tested in other plant organs showed no significant differences between inoculated and non-inoculated plants.

To gauge the effect of SNF on expression of the genes described above, total RNA was isolated from nodules, roots, stems, and leaves of *L. japonicus* plants inoculated with Δ*nifA* or Δ*nifH* mutant strain, or the wild-type *M. loti* R7A strain. qRT-PCR assays revealed that transcript levels of *LjPEPC1* (TC7830) were significantly lower in defective nodules formed by both *M. loti* mutant strains (Fig. 5A). However, expression of *LjPEPC2* showed no significant difference between plants inoculated with the mutant *M. loti* strains and the wild-type strain R7A in all organs tested (Fig. 5B). In contrast to chloroplastic NADP-MDH (TC8525), which showed similar gene expression patterns in plants inoculated with mutant and wild-type strains (Fig. 5D), genes coding for cytosolic and mitochondrial MDH isoforms (TC7834 and TC7960, respectively) were down-regulated in both nodules and leaves of plants inoculated with the mutant strains (Fig. 5C, E). Interestingly, both genes coding for NADP-ME isoforms (TC17304 and TC8784) showed significantly higher transcript levels in the stems of plants inoculated with the mutant strains, compared with stems from plants inoculated with the wild-type strain. Gene induction for TC17304 was even more profound in the stems from plants inoculated with the Δ*nifA* mutant strain (Fig. 5F, G). Similarly, transcript levels of the NAD-ME isofrom (TC16102) were significantly higher in stems of plants harbouring the mutant *M. loti* strains (Fig. 5H). Both PEPCK isoforms (TC18110 and TC9282) were expressed at similar levels in equivalent organs of plants inoculated with the mutant *M. loti* strains and the wild-type strain (Fig. 5I, J). Interestingly, in roots inoculated with the mutant strains, both genes were found to be slightly down-regulated although the observed differences were not statistically significant.

**Malate-oxidizing activity is localized in the photosynthetic tissues in stems**

To determine the spatial organization of the enhanced malate metabolism, inferred from up-regulation of ME gene expression observed in the stems of inoculated plants, we used a histochemical assay that couples NAD⁺ or NADP⁺ reduction to the reduction of NBT, which results in the formation of coloured blue–purple formazans precipitate. The chromogenic reaction was carried out in the presence of either NAD⁺ or NADP⁺ in the assay buffer. Strong localized staining appeared immediately and was more intense in the presence of NADP⁺ than NAD⁺. MDH/ME activity was detected mainly in the stem vascular tissue, especially in xylem parenchyma and sieve tubes, and in the photosynthetic outer cortex parenchyma (Fig. 6A, B). Negative control assays were performed in the absence of malate. No purple staining was observed in the absence of added malate (Fig. 6C). Chlorophyll autofluorescence was greatest in the cortical parenchyma, as expected, but was also evident within vascular structures (Fig. 6D). Interestingly, strong chlorophyll fluorescence was seen in xylem parenchyma cells, coincident with high MDH/ME activity. No significant differences were detected in the spatial localization of malate-oxidizing activity and chlorophyll fluorescence between inoculated and non-inoculated plants (data not shown).

**Discussion**

**Enhanced dark CO₂ fixation and export by nodulated *L. japonicus* roots**

Several studies in the past have demonstrated that non-photosynthetic plant organs, including N₂-fixing nodules, are capable of fixing CO₂. PEPC appears to be the main enzyme responsible for dark CO₂ fixation in nodules, while the oxaloacetate produced could be used either for the synthesis of malate or as a source of carbon skeletons for the assimilation of the symbiotically reduced nitrogen (Chollet et al., 1996). Studies using ¹⁴CO₂ have demonstrated that one of the physiological roles of dark CO₂ fixation in nodules is to supply the bacteroids with carbon
skeletons, mainly in the form of organic acids, for respiration and other biochemical processes (Rosendahl et al., 1990; Vance and Heichel, 1991). Furthermore, it has been shown that a decrease in nodule PEPC activity results in nitrogen deficiency and changes in carbon/nitrogen metabolic fluxes in *L. japonicus* nodules (Nomura et al., 2006). Interestingly, organic acids containing dark-fixed CO$_2$ were found to be transported from nodules to shoots.
of legumes (Vance et al., 1985), although their physiological significance is not clear yet.

Analysis of the natural abundance of $^{13}$C isotopic in various organs revealed significant differences between nodulated and unnodulated *L. japonicus* plants. PEPC activity in nodules, which enhances dark CO$_2$ fixation by nodules, together with the fact that PEPC discriminates less against $^{13}$C compared with RuBisCO (Farquhar et al., 1989) could account for the $^{13}$C-enriched roots of inoculated plants. In contrast, $^{13}$C discrimination during root respiration is generally very small (Klumpp et al., 2005) and is, thus, less probable as an explanation of the differences in natural $^{13}$C between inoculated and non-inoculated roots. The $^{13}$C-enriched leaves of inoculated compared with non-inoculated plants may be the result of basipetal transport of the C fixed during nodule dark CO$_2$ fixation.

To gain further insight into the molecular and physiological basis of CO$_2$ fixed by the nodules and exported to the shoots we combined gene expression analysis and Na$_2$ $^{13}$CO$_3$ labelling experiments. Our data revealed that N$_2$-fixing nodules and both inoculated and non-inoculated roots of this species incorporate $^{13}$CO$_2$ into their dry mass. Interestingly, inoculated plants exported significantly higher amounts of $^{13}$C from roots to the shoots (Figs 1, 2). These results are in agreement with previous reports indicating that pulse-labelling experiments with $^{14}$CO$_2$ in nodulated roots of pea, birdsfoot trefoil, *Vigna angularis*, soybean, and alfalfa, resulted in rapid transport of the fixed CO$_2$ to shoots (Minchin and Pate, 1973; Maxwell et al., 1984; Vance et al., 1985). Transport of fixed CO$_2$ was mainly dependent on the presence of N$_2$-fixing nodules, since removal of nodules resulted in a 90% decrease in radioactivity in the xylem sap (Vance et al., 1985). These experiments showed that nodules of amide-transporting plants contributed relatively more carbon to shoots than did nodules of ureide-transporting legumes. Furthermore, label in the xylem sap of nodulated soybeans and *V. angularis* was found primarily in the acid fraction, while label in the xylem sap of nodulated alfalfa was found mainly in the basic (amino acid) fraction. Despite several attempts, the technical limitations of using a model plant as small as *L. japonicus* precluded the use of techniques such as that of Passioura (1987) for obtaining sufficient amounts of xylem sap and, thus, determining the partitioning of $^{13}$C into specific metabolites of the xylem sap from inoculated and non-inoculated plants.

To test whether export of CO$_2$ fixed in nodules is directly linked with the N$_2$-fixation process, and to estimate its contribution to the provision of carbon skeletons for the assimilation and transport of the symbiotically fixed N$_2$, we performed pulse-labelling experiments in *L. japonicus* plants harbouring ineffective nodules formed by the *M. loti* R7A Δ*nifA* and Δ*nifH* mutant strains. Both types of ineffective nodules had significantly lower $^{13}$C and $^{13}$Ce excess when compared with the N$_2$-fixing nodules formed by the wild-type rhizobial strain (Fig. 3A, B). Interestingly, nodules formed by the Δ*nifA* strain showed slightly, but significantly, lower accumulation of label, compared with the Δ*nifH* strain (Fig. 3B). Thus, accumulation of label in nodules was dependent not only on active N$_2$ fixation, but also on the process of differentiated bacteroids, which are lacking from nodules formed by the Δ*nifA* strain. The decrease in $^{13}$C accumulation in ineffective nodules could be explained by a down-regulation of nodule CO$_2$ fixation, as we observed a 3-fold decrease in the transcript levels of *LjPEPC1* in both types of ineffective nodule (Fig. 5). These results are in agreement with $^{14}$CO$_2$-labelling experiments in wild-type and fix$^{-}$ pea nodules formed by the mutant *Rhizobium leguminosarum* MNF 3080 strain defective in dicarboxylic transport (Rosendahl et al., 1990). $^{14}$C incorporation was ~3-fold lower in these fix$^{-}$ nodules, compared with those harbouring the wild-type rhizobia. As in the case of the *M. loti* Δ*nifH* strain, *R. leguminosarum* MNF 3080 was able to differentiate into bacteroids, although these ineffective bacteroids accumulated 7-fold less $^{14}$C, mainly in the form of amino acids, than the wild-type rhizobial strain, which accumulated label in the form of organic acids. Apart from the decrease in $^{13}$C in ineffective *L. japonicus* nodules, we observed a significantly lower label accumulation in the roots of plants inoculated with either mutant strain, compared with roots inoculated with wild-type rhizobia (Fig. 3). The lower $^{13}$C accumulation in these roots was not associated with a decrease in transcripts levels of either *LjPEPC* isoforms in roots (Fig. 5). Similarly, a significant decrease was detected in the excess $^{13}$C in the leaves of the plants harbouring the fix$^{-}$ nodules (Fig. 3). Therefore, it is likely that the decline in root and leaves resulted from the decrease in CO$_2$ fixed in nodules,
although other factors, such as phosphorylation status, could be involved in PEPCK regulation. As *L. japonicus* is an amide-transporting legume (Tajima et al., 2004), the decrease in $^{13}$C accumulation in leaves may reflect the lack of amide amino acid export from the fix$^{-}$ nodules, in contrast to the wild-type nodules, in which the biosynthesis of the exported amino acids constitutes a large sink for the carbon skeletons produced by dark CO$_2$ fixation. Amides have been shown to account for up to 80% of the fixed $^{14}$CO$_2$ label transported through xylem sap in alfalfa (Vance et al., 1985). Interestingly, we did not detect any decrease in $^{13}$C labelling in the stems of plants inoculated with either of the mutant rhizobial strains (Fig. 3). Although we could not directly measure the xylem sap composition of fix$^{-}$ plants, the observed high accumulation of label in the stems of inoculated plants (Fig. 2B) was independent of active N$_2$ fixation, indicating that compounds other than amides, may account for the $^{13}$C label in stems. Organic acids, we suspect, may be responsible since significant amounts of these compounds are present in the xylem sap of amide transport in legumes (Vance et al., 1985).

**Genes involved in malate metabolism are up-regulated in the stems of nodulated *L. japonicus* plants**

Previous studies have demonstrated that organic acids translocated from roots to the shoots can be decarboxylated, with the released CO$_2$ being reassimilated through photosynthesis to form carbohydrates (Cramer and Richards, 1999; Hibberd and Quick, 2002). This C$_4$-type photosynthesis has been proposed to operate in cells of the vascular bundles in the stems of C$_3$ plants (Hibberd and Quick, 2002). To test whether a similar mechanism could operate in the stems of nodulated *L. japonicus* plants, we compared transcript accumulation for several isoforms of the three main decarboxylation enzymes indicative of the three C$_4$ subtypes, namely NAD-ME, NADP-ME, and PEPCK. Our data revealed that transcripts for these enzymes are present in all organs tested. Interestingly, transcript levels of an NAD-ME (TC16012) and two NADP-MEs (TC17304 and TC8784) were found to be at least 2-fold higher in the stems of inoculated plants than in the stems of non-inoculated plants (Fig. 4F–H). In addition, all ME genes tested were found to be more highly expressed in stems of plants with ineffective nodules than in those with effective ones, indicating that gene induction was dependent on the presence of nodules rather than on the process of symbiotic nitrogen fixation and export. Histological analyses revealed that most of the malate-oxidizing activity was localized in specific cell types, including xylem parenchyma. Hibberd and Quick (2002) reported that high ME activity is associated with photosynthetic cells in the stem vascular bundles of celery and tobacco, and that this decarboxylating activity is responsible for the metabolism of $^{14}$C-malate into the xylem stream, which led to the refixation of released $^{14}$CO$_2$ in the veins of petioles and stems in tobacco. Furthermore, it was found that inorganic $^{14}$C supplied to roots led to the accumulation of $^{14}$C in insoluble material, mainly starch, in cells associated with the stem vascular bundles.

In the case of PEPCK, we did not observe any significant differences in transcript level accumulation for either isoform tested in the aerial organs between inoculated and non-inoculated plants (Fig. 4). Interestingly, in contrast to the increased ME transcripts in ineffective nodules, expression of both PEPCK isoforms tended to be higher in roots inoculated with wild-type rhizobia, contrary to the roots of plants harbouring the fix$^{-}$ nodules (Fig 5). Apart from its function as a decarboxylase in the photosynthetic CO$_2$-concentrating mechanism in the leaves of some C$_4$ and CAM plants, PEPCK is also widely expressed in many other organs and participates in diverse physiological and biochemical processes in C$_3$ plants (Leegood and Walker, 2003; Malone et al., 2007). Well-established roles of PEPCK include an essential role in the conversion of lipids and some amino acids to sugars by gluconeogenesis during seed germination, and a role in the metabolism of nitrogenous compounds (Delgado-Alvarado et al., 2007). PEPCK participates in the dissimilation of organic acids such as citrate and malate (Leegood and Walker, 2003), and it is involved in pH homeostasis during the transport and metabolism of different forms of amino acids and amides (Walker and Chen, 2002). Thus, it would be of interest to study the possible roles of PEPCK in nodulated roots, especially concerning the transport and metabolism of amides and organic acids produced in nodules. It has been proposed that the use of amino acids, both as a respiratory substrate and for gluconeogenesis, could be a process that is far more widespread than is generally considered (Leegood and Walker, 2003). The possible catabolic function of PEPCK could account for the reduced accumulation of $^{13}$C label in the roots inoculated with both the Δ$nifA$ and Δ$nifH$ *M. loti* strains, compared with wild-type rhizobia (Fig. 3).

**Dark CO$_2$ fixation in nodules may fulfill multiple physiological functions**

The influence of root-assimilated inorganic carbon on carbon balance and its contribution to the enhancement of plant growth has been demonstrated in several studies (Cramer and Richards, 1999; Viktor and Cramer, 2005). The influence of CO$_2$ concentration and fixation in the rhizosphere has been reported for several nodulated legumes (Grobbleaer et al., 1971; Yamakawa et al., 2004; Fischinger et al., 2010). However, little is known about the significance of non-nitrogenous products of nodule CO$_2$ fixation, including malate, that are exported from the nodule (Vance et al., 1985). The observation that nodulation induces the expression of genes encoding malate decarboxylating enzymes in the stems of *L. japonicus*, points to an interesting additional role for the malate produced and exported from nodules. In this respect, malate formed by refixation of CO$_2$ respired or taken up by nodules could be translocated to stems, where after decarboxylation it could supply the photosynthetic cells in the stems with CO$_2$ in a C$_4$-type mechanism. This process could be particularly
important for the photosynthetic cells in vascular bundles, as CO₂ diffusion to these cells is restricted by the relatively few stomata in stem epidermal cells and the presence of many surrounding cell layers (Hibberd and Quick, 2002). The existence of this mechanism could have a positive impact on the carbon budget of the plant as a whole, by at least partially reducing the carbon costs of nodules. In addition, nodulated plants would be able to reassimilate through photosynthesis, a proportion of the CO₂ respired by nodules, while maintaining relatively low stomatal conductance and thus conserving water. The export and recycling of surplus malate produced in nodules could also have a role in maintaining a constant pool of this metabolite in nodules, as organic acid accumulation is suggested to have a negative regulatory impact on nitrogenase activity (Le Roux et al., 2008). Indeed, elevated CO₂ in the rhizosphere of nodulated alfalfa did not result in any significant increase in malate levels in nodules (Fischinger et al., 2010).

In conclusion, we used natural isotope abundance, stable isotope labelling, gene expression studies, and histochemical localization of enzyme activities to probe new physiological functions of dark CO₂ fixation in nitrogen-fixing nodules. From our data we propose that nodulation of the model legume L. japonicus could result in the enhancement of dark CO₂ fixation in nitrogen-fixing nodules coupled with systemic changes in the metabolism of its immediate products, mainly malate, in the shoots and leaves of nodulated plants. In addition to its generally accepted role in providing carbon skeletons for nitrogen fixation and assimilation, this mechanism could reduce carbon losses during SNF by CO₂ recycling through C₄-type photosynthesis. Future work will aim to quantify the contribution of such a mechanism to the total plant C budget, especially in relation to photosynthesis and nodule respiration, and thus to assess its relative significance for the mitigation of C losses during SNF on a whole-plant basis.

Supplementary data

Supplementary Fig. S1 shows the experimental set-up used for ¹³C pulse labelling.

Supplementary Fig. S2 shows the nitrogenase activity of L. japonicus plants inoculated with the M. loti wild-type strain R7A and with the mutant strains R7A::ΔnifH and R7A::ΔnifA.

Supplementary Table S1 lists the primers used for real-time qRT-PCR.

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