Photorespiratory Metabolism and Nodule Function: Behavior of *Lotus japonicus* Mutants Deficient in Plastid Glutamine Synthetase

Margarita García-Calderón,¹ Maurizio Chiurazzi,² M. Rosario Espuny,³ and Antonio J. Márquez¹

¹Departamento de Bioquímica Vegetal y Biología Molecular, Universidad de Sevilla, C/ Profesor García González 1, 41012 Sevilla, Spain; ²Institute of Genetics and Biophysics A. Buzzati Traverso, Via Pietro Castellino, 111–80131, Napoli, Italy; ³Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes 6, 41012-Sevilla, Spain

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Two photorespiratory mutants of *Lotus japonicus* deficient in plastid glutamine synthetase (GS₂) were examined for their capacity to establish symbiotic association with *Mesorhizobium loti* bacteria. Biosynthetic glutamine synthetase (GS) activity was reduced by around 40% in crude nodule extracts from mutant plants as compared with the wild type (WT). Western blot analysis further confirmed the lack of GS₂ polypeptide in mutant nodules. The decrease in GS activity affected the nodular carbon metabolism under high CO₂ (suppressed photorespiration) conditions, although mutant plants were able to form nodules and fix atmospheric nitrogen. However, when WT and mutant plants were transferred to an ordinary air atmosphere (photorespiratory active conditions) the nodulation process and nitrogen fixation were substantially affected, particularly in mutant plants. The number and fresh weight of mutant nodules as well as acetylene reduction activity showed a strong inhibition compared with WT plants. Optical microscopy studies from mutant plant nodules revealed the anticipated senescence phenotype linked to an important reduction in starch and sucrose levels. These results show that, in *Lotus japonicus*, photorespiration and, particularly, GS₂ deficiency result in profound limitations in carbon metabolism that affect the nodulation process and nitrogen fixation.

Biological nitrogen fixation is the reduction of atmospheric dinitrogen to ammonia and is an exclusively prokaryotic process that can be, in some cases, carried out by symbiotic bacteria named rhizobia, in association with plants of the family Leguminosae. This symbiosis between rhizobia and plants is a beneficial process to both of them, because carbon and energy are supplied from the plant to the differentiated bacterial cells, termed bacteroids (Ferguson et al. 2010), in the form of dicarboxylic acids to fuel nitrogen fixation. In exchange, fixed nitrogen is transferred to the plant. Recently, it has been postulated that a more complex amino-acid cycle is essential for symbiotic nitrogen fixation by *Rhizobium* spp. in nodules (Lodwig and Poole 2003; Lodwig et al. 2003; White et al. 2007).

Glutamine synthetase (GS) is the key enzyme involved in the assimilation of ammonia derived either from nitrate reduction or N₂ fixation (Forde and Cullimore 1989). Two GS iso-

zymes, plastidic (GS₂) and cytosolic (GS₁), were identified in higher plants (Cren and Hirel 1999). The GS isoforms have specific, apparently nonredundant, physiological roles in ammonium assimilation (Coruzzi and Last 2000; Lancien et al. 2000; Lea and Ireland 1999). GS₂ is predominantly expressed in green tissues, and it has been demonstrated that this isoform has an essential role in the reassimilation of ammonium released by photorespiration (Blackwell et al. 1987; Kozaki and Takeba 1996; Orea et al. 2002; Wallsgrove et al. 1987), although its presence in nonphotosynthetic tissues of temperate legumes has been reported (Woodall and Forde 1996). A second gene encoding for GS₂ was recently shown to be exclusively expressed in developing seeds from *Medicago truncatula* (Seabra et al. 2010). In contrast, GS₁ is localized in the vascular tissue and plays an important role in the assimilation of external ammonium, the ammonia derived from N₂ fixation and other sources of nitrogen, and in the remobilization of nitrogen during senescence (Edwards et al. 1990; Kamachi et al. 1992; Kichey et al. 2005; Teixeira et al. 2005).

The plant *Lotus japonicus* is currently used as a model temperate legume, based on its biochemical, physiological, and genetic characteristics (Handberg and Stougaard 1992; Pajuelo and Stougaard 2005). A single *Ljgln2* locus encoding for GS₂ in this plant was mapped together with other symbiotic loci (Sandal et al. 2006). Two *L. japonicus* photorespiratory mutants deficient in GS₂, denominated *Ljgln2-1* and *Ljgln2-2* (Betti et al. 2006), were previously isolated and characterized at the molecular level in our laboratory (Orea et al. 2002; Betti et al. 2006) and have been recently used to analyze their transcriptomic response towards drought stress (Diaz et al. 2010). These mutants were specifically affected in GS₂, having normal levels of GS₁ enzyme activity. It was shown that the lack of GS₂ resulted in 70 to 80% diminution of total GS activity from mutant leaves compared with the wild type (WT). In contrast, no decrease in GS enzyme activity was obtained in roots of the mutant plants. This result would be in accordance with the fact that GS₂ constitutes most of the GS activity present in leaves, while in roots, GS enzyme activity must correspond basically to GS₁, surely as a consequence of the fact that roots have very few plastids. A single-point allelic mutation was identified on each mutant that was responsible for two different amino-acid replacements in the corresponding GS₂ proteins. Both mutations affected the stability of GS₂ polypeptide, although in a different manner. Small levels of GS₂ proteins could be detected in leaves and roots of *Ljgln2-1* mutant, but a complete lack of GS₂ protein was observed in *Ljgln2-2*. Both GS₂ mutant plants are able to grow in a

Corresponding author: A. J. Márquez; E-mail: cabeza@us.es; Telephone: +34 954 557145; Fax: +34 954 626853.

similar manner to WT on 0.7% (vol/vol) CO₂ (photorespiration suppressed conditions) but showed an air-sensitive phenotype when the plants were transferred to a normal air atmosphere (photorespiratory active conditions). Typical air-sensitive stress symptoms of photorespiratory mutants were chlorosis and necrosis of the edges of some young leaves, which turned yellow and warped after one to two weeks in air. Mutant plants accumulated significant amounts of ammonium when transferred to air as a result of GS₂ deficiency. However, the photorespiratory mutants could be rescued when transferred back to high CO₂ (Orea et al. 2002).

Ljgln2-1 and *Ljgln2-2* are the first photorespiratory mutants deficient in plastid GS₂ isolated in legumes and represent the perfect tool for the study of the possible relationship between GS₂, photorespiration, and nodulation, which has not been sufficiently studied to date. This investigation is particularly intriguing, as we report, in this paper, a significant presence of the GS₂ isoform in *L. japonicus* nodule tissues associated with 40% of the total nodular GS biosynthetic activity. Furthermore, we compared the effect of photorespiration on nodule formation and functioning in WT and GS₂ mutants subjected to different CO₂ atmospheric conditions, establishing the influence of photorespiratory metabolism and plastidic GS₂ on symbiotic performances.

RESULTS

GS enzyme activity and protein levels in WT and *Ljgln2* mutant nodules.

In order to determine if the mutant plants were affected in nodule GS enzyme activity, biosynthetic GS activity was examined comparatively in crude nodule extracts of WT and mutant plants (Fig. 1A). The results showed that mutant nodules had around 40% reduction of total nodule GS activity compared with the WT, thus indicating that GS₂ has a very significant

contribution to the total levels of GS activity present in nodules of *L. japonicus* plants. A Western blot analysis of nodule crude extracts was carried out to determine the presence of GS₁ and GS₂ polypeptides in the plant nodules. The results revealed the presence of the 45- and 39-kDa (GS₂ and GS₁, respectively) polypeptides in nodules of WT plants (Fig. 1B). A comparative Western blot analysis of *Ljgln2-1* and *Ljgln2-2* mutant nodules showed a similar level of the GS₁ polypeptide in both genotypes, analogous also to that of the WT, whereas the GS₂ polypeptide was undetectable in nodules of *Ljgln2-2* mutant and only a faint band could be observed in *Ljgln2-1*, in contrast to the WT (Fig. 1B). These results indicated that mutants *Ljgln2-1* and *Ljgln2-2* are not only deficient in the GS₂ isoform from leaves and roots as previously reported for non-nodulated plants (Orea et al. 2002) but also in the GS₂ isoform from nodules, which must be responsible for at least 40% of GS activity in this organ.

Nodulation tests of *Ljgln2-1* and *Ljgln2-2* photorespiratory mutants.

The rate of nodulation was compared in *Ljgln2-1*, *Ljgln2-2*, and WT plants. *Ljgln2-2* mutant plants grown in nonphotorespiratory conditions (0.7% CO₂) showed similar nodulation rates to WT plants (Fig. 2). However, around 30% fewer nodules were counted for *Ljgln2-1* mutant plants after 35 days postinoculation (dpi). Nodule nitrogen-fixation ability was analyzed by acetylene reduction activity (ARA), finding similar levels of this activity per gram of fresh weight (FW) in nodules of both mutant plants compared with the WT (data not shown). Similar results were obtained using different rhizobial strains (NZP2235, R7A) or plant growth systems (agar tubes, Magenta boxes) (data not shown). The fact that mutant plants were able to establish symbiosis and fix nitrogen in a similar way than WT indicated that GS₂ isoenzyme was not essential for nodulation and nitrogen-fixation processes under nonphotorespiratory conditions.

Comparison of nodulation properties of WT and GS₂ deficient mutants under photorespiratory-active and photorespiratory-suppressed conditions.

Since GS₂ protein is involved in photorespiratory ammonium reassimilation (Orea et al. 2002), it was of interest to analyze

Fig. 1. A, Comparison of biosynthetic glutamine synthetase (GS) activity in crude extracts from nodules of *Lotus japonicus* wild type (WT) and *Ljgln2-1* and *Ljgln2-2* mutants. Plants were inoculated with *Mesorhizobium loti* TONO JA76 and were grown in high CO₂ conditions for 6 weeks. Values are the means of two different determinations for each of three independent biological replicates. Error bars represent standard error. ** indicates significant difference between WT and mutant plants as determined by Student's test ($P < 0.01$). **B,** Immunodetection of plastidic (GS₂) and cytosolic (GS₁) polypeptides in crude extracts from *L. japonicus* WT and mutant nodules analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Total protein (10 µg) were loaded on each case.

Fig. 2. Nodulation rate of *Lotus japonicus* wild type (WT) and *Ljgln2-1* and *Ljgln2-2* mutant plants. Plants were germinated in agar and water plates, were transferred to pots after 4 days, and were inoculated with the *Mesorhizobium loti* TONO JA76 strain. The plants were grown in high CO₂ conditions and nodules were counted for 10 weeks. Values are the means of five different biological replicates and error bars represent standard error. Black circles = WT plants, white circles = *Ljgln2-1* plants, and black triangles = *Ljgln2-2* plants.

the effects of GS₂ deficiency and photorespiration on the nodulation process and nitrogen fixation. For this purpose, WT and *Ljgln2-1* and *Ljgln2-2* mutants were transferred from high CO₂ (photorespiration suppressed) to normal air (photorespiration active) conditions. Two different times of nodule development were selected, 20 and 40 dpi. In the first case, plants growing for 20 days in high CO₂ were compared with plants growing 10 days in high CO₂ that were then transferred to air for another 10 days. In the second case, the plants were maintained for 40 days in CO₂ and were compared with plants cultivated for 30 days in high CO₂ that were then transferred to air for another 10 days. Tables 1 and 2 show that the transfer of WT and mutant plants from high CO₂ to air affected, in all cases, the number of nodules obtained, the FW of nodules, as well as the levels of ARA, which were substantially reduced compared with the plants maintained in high CO₂. These data indicate that the photorespiratory activity of the plant generates a negative influence in nodule formation, development, and function. At 20 dpi, *Ljgln2-1* and *Ljgln2-2* mutants were affected in a very similar way to the WT (Table 1), thus indicating that the additional lack of GS₂ does not seem to further influence nodulation at this plant developmental stage. However, results were quite different at a later stage of plant development (40 dpi) (Table 2), since in this case, mutant nodules were considerably more affected than the WT. The ratio between nodule FW and nodule number as well as the specific ARA per nodule FW ratio were considerably lower for the mutants than for WT plants transferred to air (but not for control plants maintained in high CO₂), indicating that GS₂ deficiency affects nodule mass and function in 40-dpi plants, particularly under photorespiratory active conditions. Furthermore, the number and FW of mutant nodules showed up to a 40 to 60% decrease, and in addition, ARA was 85 to 90% inhibited with regard to the mutant plants maintained in high CO₂ conditions. These results illustrate the relationship existing between the photorespiratory activity of the plant and nodule development and functioning. Noteworthy, total number, FW, and ARA of nodules were also diminished in *Ljgln2-1* mutant plants maintained in

high CO₂ conditions at 40 dpi (Table 2) but not in mutant plants maintained in high CO₂ conditions at 20 dpi (Table 1). This indicates that mutant nodules were also affected at this later stage of growth as a result of GS₂ deficiency, independently of photorespiration, in agreement with nodulation rate data shown in Figure 2. All these results revealed that GS₂ deficiency caused deleterious effects in nodules of *Ljgln2-1* and *Ljgln2-2* mutant plants, particularly at the later stages of plant development.

Optical microscopy.

Optical microscopy analyses were carried out in nodule sections from WT and mutant plants in order to check for any possible morphological changes associated to nodule development, either in plants maintained in high CO₂ or after transfer to air conditions. No major differences regarding size and development of nodules were observed in nodule sections from WT and mutant plants in high CO₂ at 20 or 40 dpi (Fig. 3). In contrast, the analysis of nodule sections from mutant plants cultivated in high CO₂ and transferred to air conditions showed significant alterations. A slight disappearance of starch granules in mutant nodules was observed in nodules of mutant plants cultivated 10 days in CO₂ and transferred to air for 10 days more, as compared with WT grown under the same conditions (data not shown). Furthermore, severe differences were obtained in older nodule sections from plants growing 30 days in high CO₂ and transferred to an air atmosphere for another 10 days. In this case, reduced size and a complete lack of starch granules (Fig. 4A to F) were two important differences observed in mutant nodules when compared with WT nodules. In addition, a larger vacuolated area was observed in the infected cells of nodule sections from both mutant plants at 40 dpi as compared with WT (Fig. 4D to F) and, in the *Ljgln2-2* nodules, regions of infected cells with less densely packed bacteroids, which have previously been reported in *L. japonicus fix* mutants, could be clearly recognized (Fig. 4G) (Hossain et al. 2006). These results revealed that mutant plant nodules showed a higher extent of cellular lysis, possibly produced as a result of an anticipated senescence.

Table 1. Nodulation parameters of *Lotus japonicus* wild type (WT) and *Ljgln2-1* and *Ljgln2-2* mutants at 20 days postinoculation^a

Parameter	Set 1			Set 2		
	WT	<i>Ljgln2-1</i>	<i>Ljgln2-2</i>	WT	<i>Ljgln2-1</i>	<i>Ljgln2-2</i>
Nodule number (plant ⁻¹)	10.7 ± 1.3	10.3 ± 0.7	10.2 ± 0.9	6.8 ± 0.2	5.5 ± 0.6‡‡	7.4 ± 1.1
Nodule FW (mg plant ⁻¹)	6.4 ± 1.1	5.5 ± 1.0	6.3 ± 1.3	2.6 ± 0.4‡	1.7 ± 0.0*‡	2.7 ± 0.3‡
Nodule FW to nodule number ratio	0.6 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.0‡	0.4 ± 0.0
ARA (plant ⁻¹)	101.7 ± 17.1	135.8 ± 29.3	135.6 ± 21.0	15.8 ± 1.7‡	11.1 ± 1.5‡‡	21.6 ± 2.2‡‡
ARA to nodule FW	15.9 ± 0.5	24.7 ± 1.8*	21.5 ± 1.7*	6.1 ± 1.6‡‡‡	6.5 ± 0.8‡‡	8.0 ± 1.7‡‡

^a For Set 1, inoculated plants were maintained in high CO₂ for 20 days, and for Set 2, plants were cultivated 10 days in high CO₂ and were then transferred to air for 10 days more. Acetylene reduction activity (ARA) is expressed as nanomole ethylene per hour per plant. ARA to nodule fresh weight (FW) is expressed as nanomole ethylene per milligram of FW nodules per hour. Values are the means of three determinations for each of three different biological replicates ± standard error. * indicates significant differences between WT and mutant plants in each condition, ‡ indicates significant differences between CO₂ and air conditions for each plant type as determined by Student's test. * *P* < 0.05; ** *P* < 0.01; ‡ *P* < 0.05; ‡‡ *P* < 0.01.

Table 2. Nodulation parameters of *Lotus japonicus* wild type (WT) and *Ljgln2-1* and *Ljgln2-2* mutants at 40 days postinoculation^a

Parameter	Set 1			Set 2		
	WT	<i>Ljgln2-1</i>	<i>Ljgln2-2</i>	WT	<i>Ljgln2-1</i>	<i>Ljgln2-2</i>
Nodule number (plant ⁻¹)	47.5 ± 7.5	33.0 ± 1.0*	40.0 ± 3.8	34.0 ± 3.1	19.7 ± 4.1*	21.0 ± 5.1*‡
Nodule FW (mg plant ⁻¹)	80.7 ± 9.7	45.6 ± 7.4*	68.0 ± 12.0	49.8 ± 4.6‡	17.7 ± 3.6**‡‡	23.1 ± 4.8**‡‡
Nodule FW to nodule number ratio	1.7 ± 0.1	1.4 ± 0.2	1.7 ± 0.2	1.5 ± 0.1	0.9 ± 0.2*	1.1 ± 0.1*‡
ARA (plant ⁻¹)	801.5 ± 182.3	409.3 ± 42.4*	645.9 ± 7.4	294.1 ± 36.7‡	61.1 ± 21.0**‡‡‡	65.1 ± 11.4**‡‡‡
ARA to nodule FW	9.9 ± 1.1	9.0 ± 0.5	9.5 ± 0.7	5.9 ± 0.6‡	3.4 ± 0.5*‡‡‡	2.8 ± 0.1**‡‡‡

^a For Set 1, inoculated plants were maintained in high CO₂ for 40 days, and for Set 2, plants were cultivated 30 days in high CO₂ and were then transferred to air for 10 days more. Acetylene reduction activity (ARA) is expressed as nanomole ethylene per hour per plant. ARA to nodule fresh weight (FW) is expressed as nanomole ethylene per milligram of FW nodules per hour. Values are the means of three determinations for each of three different biological replicates ± standard error. * indicates significant differences between WT and mutant plants in each condition, ‡ indicates significant differences between CO₂ and air conditions for each plant type as determined by Student's test. * *P* < 0.05; ** *P* < 0.01; ‡ *P* < 0.05; ‡‡ *P* < 0.01.

These results are also in agreement with the reduction in FW and ARA of nodules of mutant plants observed in these conditions (Table 2), confirming the restrictions on nodule function when the plants were transferred from high CO₂ to an air atmosphere.

Measurement of carbohydrate content in nodules.

In order to confirm the reduction of starch content observed by optical microscopy in nodules of mutant plants transferred to air conditions, starch content assays were carried out in *L. japonicus* nodules from WT and *Ljgln2-1* and *Ljgln2-2* mutant

Fig. 3. Optical microscopy of representative nodule sections of *Lotus japonicus* wild type (WT) and mutant *Ljgln2-1* plants. **A to D,** Plants grown in high CO₂ conditions for 20 days. **E to H,** Plants grown in high CO₂ conditions for 40 days. **A, B, E, and F,** WT nodules. **C, D, G, and H,** *Ljgln2-1* nodules. *Ljgln2-2* data were very similar to *Ljgln2-1* data. The image magnification increases from left to right. Arrows indicate starch granules. Black bars indicate a length of 150 μm. Sections were stained with 1% basic toluidine blue.

plants. The analysis revealed a strong diminution of starch content in nodules of plants transferred to air, this effect being more remarkable in nodules from the mutants, which presented a reduction of about 85 to 90% of starch content in plants cultivated in high CO₂ conditions (Fig. 5). Thus, these data confirmed the reduction of starch granules observed in the nodule sections by optical microscopy. Interestingly, a reduction in starch nodule content that was not revealed by the optical microscopy analysis was observed between WT and mutant plants grown for 40 days in high CO₂ conditions (about 50% less in the *Ljgln2-2* background), indicating a photorespiration-independent effect.

We further studied the levels of other carbon compounds, such as glucose, fructose and sucrose, in the plant nodule extracts (Fig. 6). In nodules of WT plants growing in high CO₂ conditions, the levels of sucrose were considerably higher than those of glucose and fructose (about 20 and 50 times higher, respectively). Interestingly, under these photorespiration-suppressed conditions, the relative levels of sucrose were considerably lower in nodules from mutant plants with respect to WT, showing around 60% reduction. When the plants were transferred to air, a major reduction in sucrose was observed in nodules from mutant plants, which showed around 20 to 35%

Fig. 4. Optical microscopy of representative nodule sections of *Lotus japonicus* wild type (WT) and mutant plants grown in high CO₂ conditions for 30 days and were transferred to air for 10 days more. **A** and **D**, WT nodule. **B** and **E**, *Ljgln2-1* nodule. **C** and **F**, *Ljgln2-2* nodule. **G**, Enlargement of the square in **F**. Black arrows indicate starch granules. Black arrowheads indicate largely vacuolated infected cells. White arrowheads indicate infected cells that exhibit disintegration with less-dense and aggregated bacteroids. Black bars indicate a length of 150 μm. Sections were stained with 1% basic toluidine blue.

of the sucrose levels obtained in high CO₂ conditions. These results revealed the existence of alterations in carbon metabolism of mutant nodules, under both photorespiratory-active and -suppressed conditions.

DISCUSSION

Photorespiration is a consequence of the oxygenation of ribulose-1,5-bisphosphate and the release of glycolate, catalyzed by Rubisco (Keys 2006). This process has been extensively studied. To date however, how photorespiratory metabolism affects nodulation has not been described. Our work demonstrates that the transfer of high CO₂-grown nodulated plants (photorespiration-suppressed condition) to an ordinary air atmosphere (photorespiration-active condition) results in a dramatic reduction in nodule FW (40 to 60% decrease) and ARA activity (60 to 85% decrease) for the WT *L. japonicus* plants (Tables 1 and 2).

Sucrose was the predominant sugar quantified in nodules of *L. japonicus* WT and mutant plants (Fig. 6), as also found by other researchers for WT plants (López et al. 2008). This carbohydrate is the first photosynthate supplied to nodules and, consequently, nitrogen fixation in legume nodules is highly dependent on the supply of sucrose delivered from the phloem (Gordon et al. 1999). Carbohydrate analysis revealed a diminution of these compounds in WT nodules of plants grown in high CO₂ that were then transferred to air (in particular, starch and sucrose contents). Since the levels of sucrose diminished, the reduction of starch content can also be explained by the sucrose reduction. Starch is the main storage compound when carbon levels are high. In fact, electron micrographs have previously suggested the presence of higher starch content in nodules grown at elevated CO₂ (Phillips et al. 1976). When *L. japonicus* plants are transferred to air, photorespiration begins and CO₂ assimilation is consequently diminished, as it is also the available photosynthate content that can be directed to nodules. This situation leads to alterations of carbon metabolism, and therefore, bacteroids are limited in carbon compound levels, causing reduction of atmospheric nitrogen fixation (Tables 1 and 2). So, our data indicate that the photorespiratory activity of the plant influences nitrogen fixation negatively by means of a limitation of carbon flux.

Furthermore, the availability of two different photorespiratory mutants with ordinary levels of GS₁ but specifically lack-

ing in GS₂, *Ljgln2-1* and *Ljgln2-2*, allowed us to show that the lack of GS₂ substantially increased the negative effect of photorespiration on the nodulation process and nitrogen fixation in the mutant plants (Table 2). In addition, optical microscopy data revealed alterations in mutant nodules as development restrictions, disappearance of starch granules in the uninfected cells, increase in size of vacuoles, and appearance of regions with lower bacteroid density in the cytoplasm of infected cells, indicative of a cell lysis process (Fig. 4). The data indicate an anticipated senescence in mutant nodules in air atmosphere, concluding that the symbiotic process was highly affected under active photorespiratory conditions. Considering that *Ljgln2-1* and *Ljgln2-2* mutants are defective in photorespiratory ammonium assimilation, our results emphasize how a "nitrogen" assimilation defect affects "carbon" metabolism and nodule function in *L. japonicus*. Other recent reports established that antisense inhibition of NADH-GOGAT impairs carbon and nitrogen metabolism in nodules of alfalfa (Cordoba et al. 2003).

Fig. 5. Starch content of nodules from *Lotus japonicus* wild type (WT) and mutants cultivated in CO₂-enriched atmosphere and transferred to air. Plants at 40 days postinoculation were analyzed. Values are the means of two independent determinations for each of three biological replicates. Error bars represent standard error. * indicates significant differences between WT and mutant plants in each condition, ‡ indicates significant differences between CO₂ and air conditions for each plant type as determined by Student's test. * *P* < 0.05; ** *P* < 0.01; ‡ *P* < 0.05; ‡‡ *P* < 0.01.

Fig. 6. Glucose, fructose, and sucrose content of nodules from *Lotus japonicus* wild type (WT) and mutants cultivated in CO₂-enriched atmosphere and transferred to air. Plants were analyzed at 40 days postinoculation. Values are the means of four independent determinations. Error bars represent standard error. * indicates significant differences between WT and mutant plants in each condition, ‡ indicates significant differences between CO₂ and air conditions for each plant type as determined by Student's test. * *P* < 0.05; ** *P* < 0.01; ‡ *P* < 0.05; ‡‡ *P* < 0.01.

The requirement of carbon skeletons for ammonium condensation and the supply of reducing equivalents as products of photosynthesis, respiration, and photorespiration pathways is well known (Kinoshita et al. 2011; Masclaux-Daubresse et al. 2010). It was also recently shown that the general nitrogen nutritional status of *L. japonicus* plants can strongly affect the competence for nodule formation (Omrane et al. 2009). Therefore, the reduced number of nodules observed in the *Ljgln2-1* and *Ljgln2-2* mutants after the shift in air conditions could be explained by the increased amount of nonassimilated ammonium representing an inhibitory signal for the nodule formation pathway (Omrane and Chiurazzi 2009; Omrane et al. 2009).

In the literature it has been reported that nodulated plants growing at atmospheric CO₂ enrichment show enhanced whole-plant growth and increased nodule biomass, and the nodules showed higher sugar and starch contents as well as enhancement of some activities related to nodule carbon metabolism and increased ARA (Cabrero et al. 2001; Finn and Brun 1982; Ortega et al. 1992). These results indicate that under CO₂ enrichment a higher level of carbon must be fixed and directed to nodules. In other studies, plants with CO₂ application tended to form fewer and bigger nodules, but in this case, the CO₂ was applied around the root and nodule compartment (Fischinger et al. 2010) and not around the shoots, as in our and other studies. However, neither a short- nor a long-term effect on nodule ARA-specific activity has been observed in these plants (Cabrero et al. 2001; Vance and Heichel 1991). By contrast, it has recently been described that increases in nodule ARA-specific activity in nodules of plants subjected to high CO₂ treatment applied only to the root and nodule compartment (Fischinger et al. 2010). Studies concerning the effect of CO₂ enrichment on legume productivity and nodulation are of much interest because of the CO₂-mediated greenhouse effect and associated climate change.

It is generally assumed that GS₁ is the GS isoenzyme responsible for primary assimilation of the NH₄⁺ released by bacteroids (Forde and Cullimore 1989). Different approaches have been utilized to study the physiological impact of altered GS activity in the nodules, using transgenic plants showing a reduction or overexpression in GS₁ enzyme activity (Harrison et al. 2003; Ortega et al. 2004). Our results with mutants lacking GS₂ confirmed the current view that cytosolic GS₁ is sufficient for efficient assimilation of ammonium derived from N₂ fixation. A 10 to 30% reduction of sucrose and glucose nodule content has been reported in *L. japonicus* plants with reduced nodular GS₁ activity (Harrison et al. 2003), but this was correlated to an increase in nodule FW, as a consequence of the induction of an alternative route of ammonium assimilation through asparagine synthetase activity, leading to an increase of Asn content and a more active utilization of C skeletons exported from the shoot (Carvalho et al. 2003; Harrison et al. 2003).

The presence of plastid GS₂ isoform in nodules, as reported for *Medicago truncatula* (Carvalho et al. 1997; Melo et al. 2003), has also been shown here for *L. japonicus*. The availability of GS₂ mutants from this plant has enabled us to determine that GS₂ accounts for up to 40% of total nodule GS activity (Fig. 1), a relatively unexpected result, since no decrease was observed in the mutant plants with regard to total GS activity from roots (Orea et al. 2002), a closely related organ to nodules. Our analysis of the symbiotic behavior in mutant plants under nonphotorespiratory conditions indicated a number of phenotypes that could be correlated with the lack of GS₂ activity. In mutant plants maintained in high CO₂ conditions for 40 days after *M. loti* inoculation, we observed a clear-cut deficiency in carbon metabolism, with a strong reduction in the sucrose nodule content as well as a significant decrease in

starch, glucose, and fructose levels compared with WT plants (Figs. 5 and 6). Thus a role of GS₂ in the C/N balance of *L. japonicus* plants, independent of the photorespiratory activity of the plant, can be established. Furthermore, several differences were found between WT and *Ljgln2-1* mutant plants maintained in high CO₂ (photorespiration-suppressed conditions) at 40 dpi with regard to nodule number, nodule FW, and ARA activity per plant, thus suggesting that the small amount of inactive GS₂ protein present in the *Ljgln2-1* mutant may have an effect on nodule function, independently of its main role in photorespiration.

In conclusion, since *Ljgln2-1* and *Ljgln2-2* mutants are the first photorespiratory mutants lacking GS₂ isolated in legumes, the work described in this paper gives new insights into the influence of photorespiratory metabolism and GS₂ on nodule function and demonstrates the utility of these mutant plants for investigation in this area.

MATERIALS AND METHODS

Plant growth and rhizobial inoculation.

L. japonicus (Regel) Larsen cv. Gifu were initially obtained by J. Stougaard (University of Aarhus, Denmark) and were then self-propagated at the University of Seville (Spain). *Ljgln2-1* and *Ljgln2-2* mutants were isolated from photorespiratory mutant screening as described previously (Márquez et al. 2005; Orea et al. 2002). The homozygous recessive mutant progeny from two consecutive backcrosses of the original mutants was used in this work. WT and mutant seeds were scarified and surface-sterilized, were germinated in agar and water in petri dishes, and were transferred to sterilized pots with vermiculite as solid support. Five seedlings were planted in each plot and were grown in SANYO or IBERCEX growth chambers under 16-h-day and 8-h-night cycles (20 and 18°C, respectively), with a photosynthetic flux density of 250 μmol m⁻² s⁻¹ and a constant humidity of 70%. WT and mutant plants were grown in the presence of 0.7% (vol/vol) CO₂ atmosphere (normally referenced as high CO₂), obtained by automatic injection and continuous monitoring of CO₂, using an infrared gas analyzer in conjunction with a series of solenoid valves. One set of plants was maintained in CO₂ (photorespiration-suppressed conditions) during the indicated time and a second set was transferred for a fixed length of time to a growth chamber with normal air (photorespiratory-active conditions). Plants were watered with 40 ml of sterile Hornum nutrient solution (Handberg and Stougaard 1992) without a nitrogen source and supplemented with 3 mM KCl.

M. loti TONO JA76 (Kawaguchi et al. 2002) was grown in YM liquid medium (Vincent 1970) at 28°C to an optical density at 600 nm = 1, and was then collected by centrifugation for 30 min at 2,408 × g and was resuspended in 0.75% (wt/vol) NaCl. Once sown in the pots, the plants were inoculated by the addition of 2 ml of this bacterial solution. A control with non-inoculated plants was carried out to check for the absence of nodules.

Enzyme assays.

GS enzyme activity levels in different organs of the plants were determined by the biosynthetic reaction assay as described previously (Montanini et al. 2003), using plant extracts as the enzyme source. Leaves, roots, and nodules were harvested 6 h after the beginning of the light period. Whole healthy leaves, roots, and nodules from WT and mutant plants were pooled, were quickly frozen in liquid nitrogen, were ground in a mortar, and then, were kept at -80°C until used. Extracts were prepared by grinding frozen tissues (100 mg wet weight), suspending them in 0.5 ml of extraction buffer (100

mM Tris-HCl, pH 7.8, 5 mM EDTA, pH 8, 14 mM β -mercaptoethanol), and then, centrifuging for 15 min at $20,800 \times g$. The standard reaction mixture (final volume 100 μ l) contained 10 μ mol of Tris-HCl (pH 7.50, at 25°C), 100 μ mol of L-glutamate, 5 μ mol of NH_4Cl , 5 μ mol of MgCl_2 , 0.75 μ mol of ATP (from a 100 mM stock solution, pH 7.5) and the corresponding amount of crude extract. Following incubation at 37°C for 15 min, inorganic phosphate released by ATP hydrolysis was determined, using the malachite green method (Lanzetta et al. 1979). One unit of enzyme activity corresponds to the formation of 1 μ mol P_i per min.

Nitrogenase enzyme activity was determined by the acetylene reduction (ARA) assays (Buendía-Clavería et al. 1986) using a Hewlett Packard 5890 II Plus gas chromatograph.

Carbohydrate determinations.

Frozen powdered leaf, root, or nodule materials (0.10 to 0.15 g) were extracted for 30 min at 80°C in screw-cap tubes as follows: twice with 1 ml of 80% aqueous ethanol (buffered with 5 mM HEPES-KOH, pH 7.5), once with 1 ml of 50% aqueous ethanol (buffered as before), and once with 1 ml of distilled water. Extracts from each step were centrifuged ($20,800 \times g$, 5 min), and the four supernatants were collected, combined, and kept at -20°C, until carbohydrate determinations were accomplished. Glucose, fructose, and sucrose in the extracts were measured as described by Stitt and associates (1989). Starch determinations were carried out using the insoluble material obtained in the former extraction procedures. The precipitates were incubated with 1 ml of distilled water for 5 h at 100°C and were cooled at room temperature, and 50 μ l of each sample was added to 950 μ l of a reaction mixture containing 80 mM sodium acetate, pH 4.9, 1.4 U amyloglucosidase, and 2 U α -amylase, with further incubation for 12 h at 37°C. These preparations were centrifuged at $20,800 \times g$ for 5 min, and 100 μ l from each supernatant were used for glucose determination, as described above. Starch content was expressed as micromole glucose equivalents per gram of FW of tissue.

Electrophoresis and Western blot.

Crude extracts containing 10 μ g of total protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% (wt/vol) acrylamide gels (Laemmli 1970) and were transferred onto polyvinylidene difluoride sheets (Immobilon-P; Millipore, MA, U.S.A.), as described by Pajuelo and associates (1993). Western blot was performed using the ECL Western blotting system (Amersham Biosciences, Piscataway, NJ, U.S.A.) according to Betti and associates (2006).

Optical microscopy.

L. japonicus nodules were harvested and fixed immediately in 4% (vol/vol) paraformaldehyde, 3% (vol/vol) glutaraldehyde, 4% (wt/vol) sucrose, and 50 mM sodium phosphate buffer, pH 7.4, applying vacuum for 2 h, and incubating overnight in fixative solution. The nodules were rinsed twice in 50 mM sodium phosphate buffer and were embedded in Historesin (Poly/bed 812; Polysciences, Warrington, PA, U.S.A.). Fixed nodule sections were cut into 2 μ m in thickness and stained with 1% (wt/vol) basic toluidine blue for light microscopy enabling the detection of starch grains.

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