Journal of Experimental Botany, Vol. 56, No. 417, pp. 1741–1749, July 2005
doi:10.1093/jxb/eri171 Advance Access publication 23 May, 2005

Journal of Experimental Botany

FOCUS PAPER

Nitrate assimilation in *Lotus japonicus*

Antonio J. Márquez[1,*], Marco Betti[1], Margarita García-Calderón[1], Peter Pal'ove-Balang[1,2], Pedro Díaz[1,3] and Jorge Monza[3]

[1] *Departamento de Bioquímica Vegetal y Biología Molecular, Facultad de Química, Universidad de Sevilla, Apartado 553, E-41080 Sevilla, Spain*

[2] *Institute of Botany, Slovak Academy of Sciences, Dúbravská cesta 14, SK-842 23-Bratislava, Slovakia*

[3] *Laboratorio de Bioquímica, Departamento de Biología Vegetal, Facultad de Agronomía, Avda. E. Garzón 780, CP 12900, Montevideo, Uruguay*

Received 6 December 2004; Accepted 31 March 2005

Abstract

This paper summarizes some recent advances in the understanding of nitrate assimilation in the model legume *Lotus japonicus*. First, different types of experimental evidence are presented that emphasize the importance of the root in the nitrate-reducing assimilatory processes in this plant. Secondly, the main results from an ethyl methanesulphonate mutagenesis programme are presented. In this programme, chlorate-resistant and photorespiratory mutants were produced and characterized. The phenotype of one particular chlorate-resistant mutant suggested the importance of a low-affinity nitrate transport system for growth of *L. japonicus* plants under nitrate nutrition. The phenotype of photorespiratory mutants, affected in all forms of plastid glutamine synthetase in leaves, roots, and nodules, indicated that plastid glutamine synthetase was not required for primary nitrate assimilation nor for the symbiotic associations of the plant (nodulation, mycorrhization), provided photorespiration was suppressed. However, the phenotype of these mutants confirmed that plastid glutamine synthetase was required for the reassimilation of ammonium released by photorespiration. Finally, different aspects of the relationship between nitrate assimilation and osmotic stress in *L. japonicus* are also discussed, with specific reference to the biosynthesis of proline as an osmolyte.

Key words: Ammonium, drought, mutants, nitrogen, osmotic stress, roots, salt, transgenic plants.

Introduction

Nitrogen is often considered to be one of the most important factors limiting plant growth in natural ecosystems and in most agricultural soils. In modern agricultural systems, where plants rely on fertilizers to meet their demand for nitrogen, inadequate practices still cause environmental problems, mainly linked to nitrate loss in the environment (Crawford and Campbell, 1990; Lawlor *et al.*, 2001). Most plant species are able to take up and assimilate nitrate (NO_3^-), ammonium (NH_4^+), urea, and amino acids, but the response to a particular form of nitrogen varies from species to species (Forde and Clarkson, 1999). The major source of inorganic nitrogen available to plants is a mixture of nitrate and ammonium, with nitrate being the predominant form in well-aerated soils as a consequence of bacterial nitrification. The nitrate present in the soil solution is then taken up by roots and assimilated within plant cells by three sequential steps (Fig. 1) as follows. (i) Transport across the plasma membranes, using a variety of high-affinity or low-affinity transport systems. The high-affinity transport system displays Michaelis–Menten kinetics saturating at 0.2–0.5 mM nitrate. The low-affinity transport system is observed at concentrations above 0.5 mM, and usually displays non-saturating uptake kinetics. Plants have multiple nitrate carriers with distinct kinetic properties and regulation. Two nitrate transporter gene families NRT1 and NRT2 have been discovered so far. The NRT2 family encodes transporters that contribute to high-affinity transport systems. The NRT1 family is more complex, including nitrate transporters with dual affinity (high and low) or just low affinity. (ii) Reduction of nitrate to nitrite and nitrite to ammonium, through the consecutive action of nitrate

* To whom correspondence should be addressed. Fax: +34 95 462 6853; E-mail: cabeza@us.es
Abbreviations: Fd, ferredoxin; GS, glutamine synthetase; GOGAT, glutamate synthase; NiR, nitrite reductase; NR, nitrate reductase.

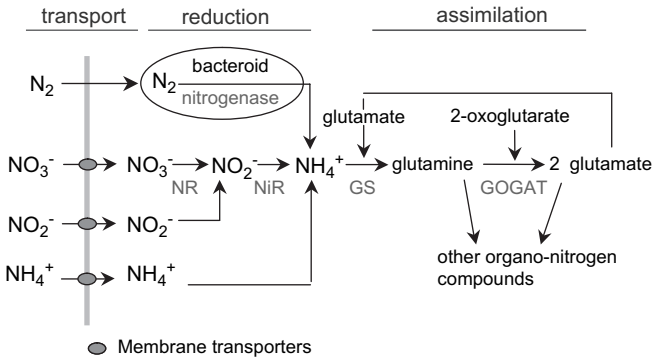

Fig. 1. The process of nitrate assimilation in plants.

reductase (NR) and nitrite reductase (NiR). (iii) Assimilation of ammonium into organic nitrogen, yielding glutamine and glutamate as the primary organic nitrogen compounds that distribute nitrogen to all other N-containing metabolites and macromolecules. Glutamine synthetase (GS) and glutamate synthase (GOGAT) are the key enzymes responsible for primary ammonium assimilation in higher plants, both of ammonium derived from nitrate as well as ammonium taken up directly from the exterior. A set of isoforms of all these enzymes, having different patterns of expression in different tissues, is a distinctive peculiarity of higher plants. Many excellent reviews are available on all these topics (Forde and Clarkson, 1999; Coruzzi and Last, 2000; Lea and Morot-Gaudry, 2001; Crawford and Forde, 2002; Foyer and Noctor, 2002; and other reviews cited or contained therein).

Plants also produce significant amounts of ammonium endogenously from processes such as photorespiration, phenylpropanoid biosynthesis, and amino acid catabolism. This ammonium is assimilated in a process called secondary ammonium assimilation, in which the GS–GOGAT pathway plays a crucial role (Lea, 1999).

Some plants, most notably legumes, can also obtain nitrogen from atmospheric N_2. The symbiotic interaction of these plants with rhizobia, which are able to reduce N_2 to NH_4^+ in the nodules by the action of nitrogenase, produces ammonium that is transferred from the microbe to the plant and assimilated by the GS–GOGAT pathway (Triplett, 2000; Gordon *et al.*, 2001). Ammonium is, consequently, the reduced form of inorganic nitrogen ultimately available to these plants, irrespective of the primary or secondary nitrogen source utilized. Despite the ability of legumes to form nitrogen-fixing symbioses there are many reasons why nodulation may not occur, and this is why inorganic nitrogen sources (NO_3^-, NH_4^+), especially nitrate, are as important for legumes as they are for non-legumes (Woodall and Forde, 1996).

In the soil solution, nitrate is carried towards the root by bulk flow and is taken up by epidermal and cortical cells of the root. Within the root symplasm, NO_3^- has four fates: (i) reduction to NO_2^- by the enzyme NR; (ii) efflux back across the plasma membrane to the apoplasm; (iii) influx and storage in the vacuole; or (iv) transport to the xylem for long-distance translocation to the shoot. Following translocation to the shoot, NO_3^- must leave the xylem and enter the leaf apoplasm to reach mesophyll cells, where NO_3^- is again absorbed and either reduced to NO_2^- or stored in the vacuole (Crawford and Glass, 1998). The proportion of nitrate that can be assimilated in the shoot and in the root of different plant species varies considerably (Andrews, 1986). Three main groups of plants exist in this respect: (i) plants that assimilate nitrate significantly both in shoots and roots (temperate annual non-legume species); (ii) plants that assimilate nitrate preferentially in leaves (tropical and subtropical legume and non-legume species); (iii) plants that assimilate nitrate preferentially in roots (temperate perennial and annual legume species). Many temperate legumes, growing in non-agricultural soils with low nitrate concentrations (1 mM) in the rhizosphere, use their roots to assimilate most of the nitrate they absorb, although shoot assimilation becomes more important at higher external concentrations. Other legumes, particularly those of tropical and subtropical origins, have been found to assimilate most of their nitrate in the shoot, regardless of the external nitrate concentration.

Theoretical considerations suggest that leaf nitrogen assimilation is energetically more efficient than root nitrogen assimilation when photosynthesis is light-saturated and competition between carbon dioxide and nitrate for photochemical energy is minimized (Smirnoff and Stewart, 1985). Thus, it is of great interest to study plants that have developed a preference for root nitrate assimilation.

Several years ago, the plant *Lotus japonicus* was proposed as a contender for the role of model legume, based on its amenable growth, propagation, genome, tissue culture, transformation, and nodulation characteristics (Handberg and Stougaard, 1992). Since then, many groups throughout the world have used this plant for their research, and resources to investigate functional genomics, including mutant collections, are being developed (Colebatch *et al.*, 2002*a*, *b*, 2004; Márquez, 2005). The use of these facilities has enabled the identification of new symbiotic genes as well as other ground-breaking achievements (e.g. Stracke *et al.*, 2002; Radutolu *et al.*, 2003, and references therein).

This paper summarizes recent research on different aspects of nitrate assimilation in *L. japonicus*. First, data are presented showing the importance of the root for nitrate assimilation in this plant. Secondly, the main results of an ongoing ethyl methanesulphonate mutagenesis programme are described briefly. Thirdly, some comments on the relationship between osmotic stress and nitrogen assimilation are described. This is of particular interest because *Lotus* species are important members of plant communities in pastures and, therefore, hay quality in many countries, and their productivity can be seriously affected by drought and

saline stress (Blumenthal and McGraw, 1999; Papadopoulos and Kelman, 1999; Diaz *et al.*, 2005*a*, *b*).

Lotus japonicus: a root nitrate assimilator

The activities of nitrate-assimilatory enzymes have been assayed in different organs of *Lotus japonicus*, under different nitrogen regimes and experimental conditions. Although different types of NADH-specific (EC 1.6.6.1) and NAD(P)H-bispecific (EC 1.6.6.2) NR isoenzymes have been reported in the literature, NR activity in *L. japonicus* leaf or root extracts uses NADH as reductant, and no significant activity can be obtained using NADPH. The enzyme is encoded by a single *Nia* gene that has been sequenced (accession number X80670) and is expressed predominantly in roots (IM Prosser, A Massonneau, AJ Smyth, RN Waterhouse, BG Forde, and DT Clarkson, unpublished results). Anti-peptide antibodies addressed against the deduced *L. japonicus* NR sequence were able to recognize a band of around 100 kDa on SDS–PAGE (Pajuelo *et al.*, 2002), which is consistent with the size of NR enzymes described in other plants (Meyer and Caboche, 1998; Campbell, 1999). The level of NR enzymatic activity in crude extracts from *L. japonicus* root tissue, expressed per unit of fresh weight, is usually 10–20-fold higher than in leaves, and this difference is even larger when expressed per unit of tissue protein. This is also true, although to a lesser extent, when NR enzyme activities are expressed on a per plant basis (integrated NR activity). Under standard growth conditions, total root NR activity per plant may be double that of the shoot (leaves + stems) (Pajuelo *et al.*, 2002; Orea *et al.*, 2005*a*). Increasing the nitrate concentration in the solution bathing the roots up to 24 mM (the concentration required for maximum nitrate accumulation in leaves, stems, and roots) produces a concomitant increase in NR activity in all plant tissues. However, it does not alter the partitioning of nitrate assimilation between the roots and the shoot significantly. Nitrate assimilation is still higher in the roots than in the stems and leaves. These results were confirmed at the NR protein level by western blots (Pajuelo *et al.*, 2002). This characteristic behaviour of *L. japonicus* differs from other plant species that assimilate nitrate in their roots, including many temperate legumes. In other species, a shift in the partitioning of nitrate assimilation occurs at high nitrate supply, when leaves become the main tissue for nitrate assimilation (Andrews, 1986; Gojon *et al.*, 1994).

The behaviour of NiR, the second enzyme required for nitrate reduction, has also been studied. A full-length NiR cDNA was isolated from an *L. japonicus* root library and sequenced (accession number AJ293240). This encoded a precursor protein of 582 amino acid residues with a predicted molecular mass of 64.8 kDa and a transit peptide consisting of 25 amino acid residues, corresponding to a mature protein of 62.1 kDa. This is consistent with SDS–PAGE and immunoblot analyses that indicate a size of 63 kDa for this protein (Orea *et al.*, 2001). The NiR gene (*Nii*) is present as a single copy in *L. japonicus* and is expressed in both roots and leaves, although its expression is much higher in roots (Orea *et al.*, 2001). This parallels the expression of NR, and is consistent with the assimilation of nitrate predominantly in roots of *L. japonicus*. Nevertheless, NiR enzyme activity is much higher than NR enzyme activity in leaves of *L. japonicus* (Orea *et al.*, 2001; Pajuelo *et al.*, 2002).

Root nitrate assimilation in *L. japonicus* is also influenced greatly by the age of the plants and plant growth conditions. NR and NiR enzyme activities, as well as nitrate accumulation, are much higher in roots of young plants, and decrease in mature plants grown in seed trays, irrespective of the abundance of nitrate in the nutrient medium. When plants are grown in larger pots, the decrease in NR activity and nitrate accumulation occurs at a later stage of growth, suggesting that the diminution of nitrate assimilation in mature plants could be related to both ageing and any restriction of root growth. Roots of *L. japonicus* respond to both these situations by a progressive decline of nitrate assimilation capability, possibly as a way to slow down the rate of root growth. A decrease in the rate of root growth was observed concomitantly with a decrease of integrated NR activity per plant (Pajuelo *et al.*, 2002; Márquez *et al.*, 2004). Although NR activity can reach very low levels in roots of mature plants, ageing does not produce a shift of nitrate assimilation from the roots to the leaves in *L. japonicus*, since leaf NR activity also decreases with plant age (Pajuelo *et al.*, 2002). Curiously, the amount of inactive NR protein, detected in roots according to currently known NR post-translational regulatory mechanisms (Campbell, 1999), can reach very high values (>50%) and is greater in roots of mature *L. japonicus* plants (Pajuelo *et al.*, 2002). However, a high proportion of inactive NR has also been reported for roots from other plant species (Glaab and Kaiser, 1993; Botrel and Kaiser, 1997) and in nitrate-induced nodulated roots of *L. japonicus* (Kato *et al.*, 2003).

The importance of roots for nitrate assimilation in *L. japonicus* was also examined using transgenic plants, in collaboration with IM Prosser and DT Clarkson (IACR-Long Ashton, Bristol). The levels of NR protein and enzyme activity were determined in roots and leaves from a collection of transgenic *L. japonicus* with altered NR expression produced by means of constitutive, leaf-specific, or root-specific/preferentially expressed promoters. Although NR expression was effectively modified in some of the transgenic lines, none of them showed higher levels of NR activity in leaves than in roots (Orea *et al.*, 2005*b*).

All these results emphasize the importance of the root for nitrate assimilation in *L. japonicus* and suggest the existence of a global regulatory mechanism for nitrate assimilation in *L. japonicus* that prevents a shift of nitrate

assimilation from the roots to the leaves. It is possible that some adaptation may be operating in *L. japonicus* to maintain low levels of NR enzyme activity in its leaves. An adaptation that allows nitrate assimilation primarily in roots may be of importance for forage legumes such as *L. japonicus* as a way to overcome the loss of photosynthetic tissue due to grazing animals. Interestingly, significant amounts of low-molecular-weight NR-inactivating compounds have been detected in crude extracts from *L. japonicus* leaves (Márquez *et al.*, 2005). Other approaches have also indicated the relative importance of the roots, compared with shoots, for nitrate assimilation in this plant. For example, Limami *et al.* (1999) found that overexpressing GS activity in roots of transgenic plants led to a decrease in plant biomass production, and suggested that this decrease was likely due to a lower nitrate uptake accompanied by a redistribution to the shoots of the newly absorbed nitrogen where it cannot be reduced. Later work showed that higher shoot NO_3^- content is mainly due to an increase in the uptake of this anion regardless of the plant biomass production (Harrison *et al.*, 2004). The higher stability of the GS enzyme in the roots of control and transformed *L. japonicus* plants was also taken as evidence that the main site of nitrogen assimilation may be the roots (Ortega *et al.*, 2004). It is also clear that asparagine is a major N-transport compound from roots to shoots in *L. japonicus* plants (Waterhouse *et al.*, 1996).

Mutagenesis of nitrate assimilation in *L. japonicus*

Several methods have been reported in the literature for the selection of nitrate assimilation-deficient mutants in plants. Nitrate reductase and nitrate uptake mutants have been identified by screening for chlorate resistance, lack of NR activity, nitrate utilization auxotropy, chlorate hypersensitivity, and resistance to elevated nitrate concentrations (Pelsy and Caboche, 1992). A conditional-lethal nitrite accumulation mutation associated with a deficiency in nitrite reductase has also been reported (Duncanson *et al.*, 1993). Mutants in GS and/or GOGAT have been obtained by means of an air-sensitive phenotype (photorespiratory mutants), observed in plants which are able to grow in a high-CO_2 (photorespiration suppressed) atmosphere but which die or show stress symptoms when transferred to ordinary air (active photorespiration). The latter mutants result from the essential role of plastidic GS and Fd–GOGAT in the reassimilation of the ammonia released by decarboxylation of glycine in the mitochondrion as a consequence of the C_2 C-N photorespiratory cycle (Lea and Forde, 1994).

So far, two of these mutant screens have been explored in *L. japonicus* to increase the number of mutants available for this plant. The ethyl methanesulphonate mutagenesis programme, using seed produced as described by Webb *et al.* (2005), is outlined in Fig. 2. The main results from this programme are summarized as follows.

(i) *Search for chlorate-resistant mutants*. Chlorate is toxic to *L. japonicus*, as it is to many other plants. This is the reason why chlorate is a powerful and commonly used herbicide. The molecular reason for this toxicity is not yet fully understood, but it is thought to be related to the fact that chlorate is an analogue of nitrate and can be reduced by NR to form chlorite, a highly toxic compound. Selection conditions were optimized that guaranteed the presence of fully induced NR, at an appropriate chlorate to nitrate ratio to produce sufficient levels of chlorate toxicity without nitrate protection. Using these conditions, around 100 000 M2 or M3 seed corresponding to 6000 M1 families were examined for symptoms of chlorate resistance. About 250 putative mutant plants were selected, most of which gradually died or showed only a minor inheritance of the chlorate-resistance trait. However, two plants, namely *Ljchl-001* and *Ljchl-002*, showed 100% inheritance of chlorate resistance in their progeny. The resistance to chlorate was only partial in the sense that they were sensitive to higher concentrations of chlorate than those used for the screening (Orea, 1999). NR activities in both *Ljchl-001* and *Ljchl-002* mutant plants were absolutely

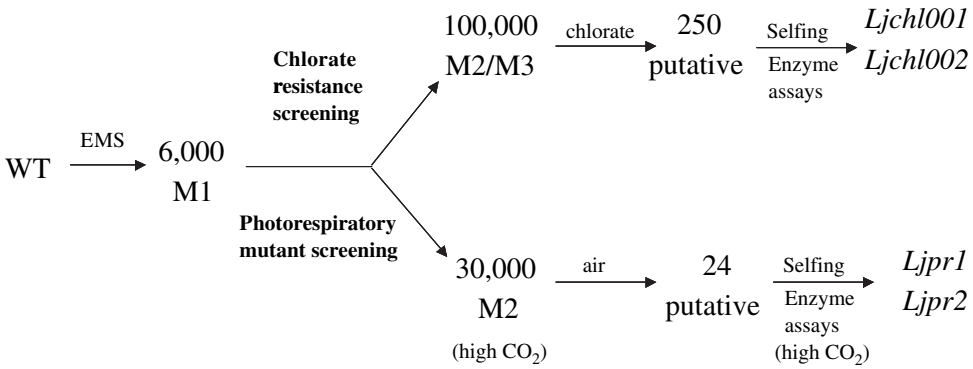

Fig. 2. The search for nitrate assimilation mutants in *Lotus japonicus*.

normal. Combined nitrate depletion and electrophysiological measurements of high-affinity and low-affinity nitrate transport systems in these mutants indicated that one of the mutants (*Ljchl-001*) was specifically affected in low- affinity transport system(s) for nitrate and chlorate. The growth of this mutant was dramatically reduced compared with the wild-type plants when grown in the presence of either nitrate alone or nitrate plus ammonium, but it was not significantly different from wild-type plants when grown on ammonium (Pal'ove-Balang *et al.*, 2003). Little work has yet been published on nitrate transporters in *L. japonicus*, except for the cloning of an *Nrt*2 gene. Interestingly, a strong co-regulation of both *Nrt*2 and *NR* genes has been shown: both genes were switched-off after 72 h of nitrate starvation, and rapidly induced within 30 min of re-supplying of nitrate (Forde, 1997; Forde and Clarkson, 1999).

(ii) *Search for photorespiratory mutants*. Screening approximately 30 000 M2 plants, corresponding to 660 independent M1 families, yielded 24 putative photorespiratory mutants that showed symptoms of stress in air but could be recovered when placed in a high-CO_2 atmosphere. In only three of these mutants (*Ljpr1*, *Ljpr2*, and *Ljpr3*) did 100% of their progeny exhibit the same mutant phenotype as their parents. Further genetic analysis confirmed that these mutants were allelic and affected in a single Mendelian recessive trait. Measurements of GS and ferredoxin (Fd)–GOGAT activities in crude extracts from these mutants showed that they all had GS activities between 20% and 30% of the wild-type. DEAE–Sephacel fractionation of crude extracts and chloroplast fractions, followed by western blot analysis demonstrated that the mutant plants were specifically affected in the plastidic isoform of GS, having normal levels of cytosolic GS (Orea *et al.*, 2002). Interestingly, by contrast to most plant species, plastid GS from *L. japonicus* elutes earlier than cytosolic GS from DEAE–Sephacel columns (Orea *et al.*, 2002). Both biosynthetic and transferase enzyme activities of GS are affected in the mutants. Molecular analysis of these mutants indicates that they are affected post-translationally. Single base point mutations in the structural frame of the *LjGln2* gene were identified in the mutants, affecting the quaternary structure and/or stability of the corresponding polypeptides (Márquez *et al.*, 2002).

The mutants isolated from *L. japonicus* are the first of their type isolated from legumes, since similar mutants have only been reported in barley (Lea and Forde, 1994). The accumulation of ammonium in the mutants when they are transferred from high-CO_2 to air conditions indicates that in root-assimilating legumes there is also an essential role for plastid GS in the reassimilation of ammonium released by photorespiration (Orea *et al.*, 2002). A connection between photorespiration and ammonium transporters in *L. japonicus* has been reported recently (D'Apuzzo *et al.*, 2004). It is quite likely that in *L. japonicus*, as in other plant species, plastid GS is expressed in different cell types than cytosolic GS, and therefore both isoenzymes perform non-overlapping functions *in vivo* (Coruzzi and Last, 2000). This would explain why the normal level of cytosolic GS detected in the *L. japonicus* mutants is unable to compensate for the loss of plastid GS, and leads to the accumulation of ammonium under photorespiratory conditions.

A peculiarity of *L. japonicus*, is the presence in roots, as well as in leaves, of a 45 kDa plastid GS polypeptide, quite distinct in size from the 39 kDa polypeptide characteristic of cytosolic GS. Nevertheless, while the plastidic GS is the most abundant polypeptide in leaves, the cytosolic GS is the most abundant polypeptide in roots. The photorespiratory mutants mentioned above are also deficient in this root plastid GS polypeptide, thus confirming the existence of a single *Gln2* locus in this plant encoding both the leaf and root forms of plastid GS (Orea *et al.*, 2002). Recently, cDNA clones for plastid GS were obtained by RT–PCR and sequenced (accession number AY187004). These encode a precursor protein of 430 amino acids containing an N-terminal transit peptide of 43 residues that produces a mature protein of 42.7 kDa, which is consistent with the 45 kDa band detected both in roots and in leaves (Márquez *et al.*, 2002; Orea *et al.*, 2002).

The roots of most plant species contain only cytosolic GS, but roots of some plant species, like *L. japonicus*, also contain a plastid GS. Woodall and Forde (1996) found a remarkably strong correlation between the climatic origin of the species and the presence or absence of the plastid GS polypeptide in the root. Plastid GS was found in roots of all 31 temperate species examined, but was not detected in roots of any of the 21 tropical species. The fact that the plastid GS isoform has been shown to be induced by nitrate but not by ammonium, suggests that plastid GS in the roots of at least some plant species may have a specific role in the nitrate assimilatory pathway. Such a role would be consistent with the plastid location of NiR, the enzyme responsible for producing ammonium from primary nitrate assimilation. This hypothesis has been examined further using the photorespiratory mutants of *L. japonicus* mentioned above. The growth of wild-type and mutant plants supplied with nitrate, ammonium, or mixed N sources was compared under high-CO_2 (photorespiration suppressed) conditions. One of the mutants (*Ljpr2*) produced almost the same yield of shoot and root fresh weight as wild-type plants under all conditions. However, the other mutant (*Ljpr1*) showed some delay in growth, particularly when supplied nitrate or mixed N sources. Neither high-affinity nor low-affinity nitrate uptake transport systems, nor nitrate-reducing enzyme activities, seem to be affected in these mutants (Márquez *et al.*, 2004). Therefore, it can be concluded that plastid GS activity is not an essential requirement for the primary assimilation of nitrate or ammonium in roots of *L. japonicus*. Woodall and Forde (1996) found no clear correlation between the presence of plastid GS in roots and a preference for root nitrate

assimilation. Instead they discussed the possibility that expression of plastid GS in the root could be part of a more extensive physiological adaptation to root nitrate assimilation that evolved in temperate species to suit alkaline, nitrate-rich soils found in the centres of origin in temperate latitudes. Consequently, cytosolic GS appears to be sufficient for ammonium assimilation in the roots of *L. japonicus* plants. Nevertheless further work will be required to explain the delayed growth phenotype of *Ljpr1*, which may be related to the observation that some plastid GS protein, although fully inactive, can be detected in this mutant, which contrasts with the undetectable amounts in *Ljpr2* (Orea *et al.*, 2002). Amino acid analysis and metabolome studies of the mutants are now in progress in collaboration with G Desbrosses and M Udvardi (Golm, Germany).

Plastid GS has also been found in nodules from *L. japonicus*. There is less plastid GS in nodules from *Ljpr1* and *Ljpr2* plants than in nodules from wild-type plants, which accounts for a 40% reduction in the total GS activity in nodules of these mutants. Despite these differences, the GS-deficient mutants are able to produce nodules quite efficiently when grown in high CO_2 (non-restrictive conditions), showing similar nodulation kinetics and nitrogen fixation ability as the wild type. Thus, the lack of plastid GS apparently does not interfere with the nodulation process or nitrogen fixation in the mutant plants (Márquez *et al.*, 2002). Plastid GS is, therefore, not essential for the nodulation process. However, a deleterious effect was observed in nodules when high-CO_2-grown nodulated plants were transferred from high-CO_2 to an ordinary air atmosphere (M García-Calderón and AJ Márquez, unpublished results). By contrast with leaves, no ammonium accumulation was detected in roots of GS-mutant plants in air conditions (Orea, 1999), and it is unlikely that this deleterious effect observed in the nodules can be due to an indirect toxic effect of ammonium coming from the leaves. Nevertheless, the data suggest that active photorespiration and lack of plastid GS must somehow be connected with nodule function. Recent reports show that plastid GS is stimulated in nitrogen-fixing nodules from *Medicago truncatula* (Melo *et al.*, 2003). Other transgenic approaches have examined the role of cytosolic GS and asparagine synthetase in *L. japonicus* nodule function (Harrison *et al.*, 2003; Suárez *et al.*, 2003; Ortega *et al.*, 2004).

For comparative purposes, the possible consequences of the lack of plastid GS in other symbiotic processes like mycorrhization were also studied in collaboration with C Azcón (Granada, Spain). The mutant plants were effectively colonized by the symbiotic fungus in a high CO_2 atmosphere, thus indicating that the lack of plastid GS does not interfere with the ordinary function of the root in the metabolic exchange taking place in this type of symbiosis (M García-Calderón, C Azcón, and AJ Márquez, unpublished results).

Nitrate assimilation and osmotic stress in *L. japonicus*

Drought and salinity are the major limitations to crop productivity (Boyer, 1982). Both these stresses have common metabolic consequences, because both of them decrease water activity inside the cell. When plants are stressed, highly complex biochemical and physiological mechanisms are switched on to protect major processes such as cell respiration, photosynthetic activity, nutrient transport, and nitrogen metabolism (Bray, 2002). Stressful conditions associated with high light intensity promote the production of reactive oxygen species and oxidative stress. Biochemical responses include the accumulation of compatible osmolytes (Zhu, 2002), such as proline, glycine betaine, and poliols (sucrose, manitol), which may act as scavengers of reactive oxygen species, thus playing a protective role against oxidative damage on macromolecules. The synthesis of these osmolytes has clear interconnections with nitrogen metabolism.

Drought and saline stress have been identified among the main factors affecting growth rate and dry matter production of *Lotus* species used as forage for animal production in many pastures in temperate regions (Blumenthal and McGraw, 1999; Papadopulous and Kelman, 1999). This situation prompted an examination of some of the possible interconnections between N metabolism and osmotic stress in *L. japonicus*, and a comparison of this model legume with closely related *Lotus* species used in the field.

Drought and salt stress resulted in up to a 12-fold increase in proline concentrations in *L. japonicus*. In parallel, both drought and salt stress led to an increased level of oxidative damage in *L. japonicus* as evaluated through measurement of thiobarbituric acid reactive substances (Díaz *et al.*, 2005*b*). Since total free amino acids and protein content did not change significantly when the plants were subjected to osmotic stress, this suggests that proline accumulation was not derived from protein degradation but rather as a consequence of an increase in the *de novo* proline biosynthesis:degradation ratio. An increase in proline is also observed in other *Lotus* species, such as *L. corniculatus* (Borsani *et al.*, 1999; P Diaz, O Borsani, AJ Márquez, and J Monza, unpublished results), *L. glaber*, *L. subbiflorus*, and *L. uliginosus* (P Diaz, O Borsani, AJ Márquez, and J Monza, unpublished results), used agronomically.

It has also been examined whether the stress-related accumulation proline is associated with changes in N-assimilatory enzymes. No change in GS biosynthetic activity was observed in leaves of *L. japonicus* plants subjected to drought or salt stress for 4 d. However, western blot analysis of GS showed that the abundance of cytosolic and chloroplastic GS was influenced by drought stress. An increase in cytosolic GS, and a concomitant decrease in plastid GS, was observed (Díaz *et al.*, 2002). These results are consistent with studies on tobacco plants indicating that

cytosolic GS in the phloem plays a major role in regulating proline production, and are also consistent with proline being both a nitrogen source and a key metabolite synthesized in response to water stress (Brugière *et al.*, 1999). An increase in proline was detected in *L. japonicus* plants over-expressing a cytosolic GS gene (Ortega *et al.*, 2004). The use of *L. japonicus* mutants deficient in plastid GS (*Ljpr1* and *Ljpr2* mutants) showed that, under osmotic stress, more oxidative damage occurred in the mutants compared with wild-type plants, and that this was inversely related to the proline content of the plants (Márquez *et al.*, 2002). This suggests a significant role for plastid GS in stress-induced proline biosynthesis in *L. japonicus*. Also consistent with a role of plastid GS in acclimation to osmotic stress, is the observation that transgenic rice over-expressing chloroplast GS show enhanced tolerance to salt stress (Hoshida *et al.*, 2000).

There are also reports of changes in GOGAT activity in response to salt and drought stress. A 2-fold increase in Fd–GOGAT protein and activity content was observed in *Lycopersicum esculentum* exposed to salt (Berteli *et al.*, 1995) and a similar situation was detected in *Lotus corniculatus* under drought stress (Borsani *et al.*, 1999). The increase in Fd–GOGAT has been associated with a higher demand for glutamate as a precursor of proline biosynthesis in this situation (Borsani *et al.*, 1999). Curiously, *L. japonicus* plants failed to produce this increase in Fd–GOGAT protein and/or activity in response to either salt or drought stress, indicating that the changes in Fd–GOGAT observed in *L. corniculatus* may be peculiar to this species (Diaz *et al.*, 2002). Supporting this conclusion, it has been reported that salt stress in *Mesembryanthemum crystallinum* produced a decrease in Fd–GOGAT (Popova *et al.*, 2002).

In plants, proline can be synthesized from either glutamate or ornithine precursors (Rossens *et al.*, 1998; Verma and Zhang, 1999). Significant differences in the levels of proline and other amino acids have been found in response to drought stress in *L. japonicus* plants grown under NH_4^+ or NO_3^- as single N source. These results have been also reproduced in other *Lotus* species (P Diaz, O Borsani, AJ Márquez, and J Monza, unpublished results). The metabolic pathways available for proline biosynthesis appear to contribute differently in response to different types of plant nitrogen nutrition. The lower levels of proline detected under NO_3^- nutrition suggests also a potential role of NO_3^- in the osmotic and nutritional response of *Lotus* plants, which is in accordance with studies using other plant species (McIntyre, 1997).

Acknowledgements

The authors are grateful for financial support given by AECI and research projects BMC2001-3162 and BFU2004-02753 from MCYT/MEC-FEDER (Spain), as well as BIO2-CT93-0400, HPRN-CT2000-00086, MRTN-CT-2003-505227, and FP6-2003-INCO-DEV2-517617 from the European Union, and support given by Junta de Andalucía to group CVI-163.

References

Andrews M. 1986. The partitioning of nitrate assimilation between root and shoot of higher plants. *Plant, Cell and Environment* **9,** 511–586.

Berteli F, Corrales E, Guerrero C, Ariza M, Pliego F, Valpuesta V. 1995. Salt stress increases ferredoxin-dependent glutamate synthase activity and protein level in the leaves of tomato. *Physiologia Plantarum* **93,** 259–264.

Blumenthal M, McGraw R. 1999. *Lotus* adaptation, use and management. In: Beuselinck P, ed. *Trefoil: the science and technology of* Lotus. Madison, WI: American Society of Agronomy, 97–120.

Borsani O, Díaz P, Monza J. 1999. Proline is involved in water stress responses of *L. corniculatus* nitrogen fixing and nitrate fed plants. *Journal of Plant Physiology* **155,** 269–273.

Botrel A, Kaiser WM. 1997. Nitrate reductase activation state in barley roots in relation to the energy and carbohydrate status. *Planta* **201,** 496–501.

Boyer J. 1982. Plant productivity and environment. *Science* **218,** 443–448.

Bray E. 2002. Classification of genes differentially expressed during water-deficit stress in *Arabidopsis thaliana*: an analysis using microarray and differential expression data. *Annals of Botany* **89,** 803–811.

Brugière N, Dubois F, Limami AM, Lelandais M, Roux Y, Sangwan RS, Hirel B. 1999. Glutamine synthetase in the phloem plays a major role in controlling proline production. *The Plant Cell* **11,** 1995–2011.

Campbell WH. 1999. Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 277–303.

Colebatch G, Desbrosses G, Ott T, Krusell L, Kloska S, Kopka J, Udvardi MK. 2004. Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *The Plant Journal* **39,** 487–512.

Colebatch G, Trevaskis B, Udvardi M. 2002*a*. Functional genomics: tools of the trade. *New Phytologist* **153,** 27–36.

Colebatch G, Trevaskis B, Udvardi M. 2002*b*. Symbiotic nitrogen fixation research in the postgenomics era. *New Phytologist* **153,** 37–42.

Coruzzi G, Last R. 2000. Amino acids. In: Buchanan B, Gruissem W, Jones R, eds. *Biochemistry and molecular biology of plants*. Rockville, MD: American Society of Plant Physiologists, 358–410.

Crawford NM, Campbell WH. 1990. Fertile fields. *The Plant Cell* **2,** 829–835.

Crawford NM, Glass ADM. 1998. Molecular and physiological aspects of nitrate uptake in plants. *Trends in Plant Sciences* **3,** 389–395.

Crawford NM, Forde BG. 2002. Molecular and developmental biology of inorganic nitrogen nutrition. In: *The Arabidopsis book*. American Society of Plant Biologists. http://www.aspb.org/publications/Arabidopsis.

D'Apuzzo E, Rogato A, Simon-Rosin U, *et al.* 2004. Characterization of three functional high-affinity ammonium transporters in *Lotus japonicus* with differential transcriptional regulation and spatial expression. *Plant Physiology* **134,** 1763–1774.

Díaz P, Borsani O, Monza J. 2005*a*. *Lotus japonicus* related species and their agronomic importance. In: Márquez AJ, ed. Lotus japonicus *handbook.* Dordrecht: Springer (in press).

Díaz P, Monza J, Márquez AJ. 2005*b*. Drought and saline stress. In: Márquez AJ, ed. Lotus japonicus *handbook*. Dordrecht: Springer (in press).

Díaz P, Orea A, Monza J, Márquez A. 2002. Cambios de las actividades en el ciclo GS/GOGAT en plantas de *Lotus japonicus* sometidas a estrés hídrico. *Actas XI Reunión Latinoamericana de Fisiología Vegetal*, Ediciones del Copista, Argentina.

Duncanson E, Gilkes AF, Kirk DW, Sherman A, Wray JL. 1993. A conditional-lethal mutation in barley causing a defect in nitrite reduction. *Molecular and General Genetics* **236,** 219–226.

Foyer CH, Noctor G. 2002. *Photosynthetic nitrogen assimilation and associated carbon and respiratory metabolism.* Dordrecht: Kluwer Academic Publishers.

Forde BG. 1997. Project Lotus: investigating the regulation of the nitrate assimilatory pathway in a higher plant through genetic manipulation and mutagenesis. In: Hoeveler A, Cresti M, eds. *Biotechnology (1992–1994)*. Luxembourg: European Commission, 347–350.

Forde BG, Clarkson DT. 1999. Nitrate and ammonium nutrition of plants: physiological and molecular perspectives. *Advances in Botanical Research* **30,** 1–90.

Glaab J, Kaiser WM. 1993. Rapid modulation of nitrate reductase in pea roots. *Planta* **191,** 173–179.

Gojon A, Plassard C, Bussi C. 1994. Root/shoot distribution of NO_3^- assimilation in herbaceous and woody species. In: Roy J, Garnier E, eds. *A whole plant perspective on carbon–nitrogen interaction*. The Hague: SPB Academic Publishing, 131–147.

Gordon AJ, Lea PJ, Rosenberg C, Trinchant JC. 2001. Nodule formation and function. In: Lea PJ, Morot-Gaudry J-F, eds. *Plant nitrogen*. Berlin: Springer-Verlag, 101–146.

Handberg K, Stougaard J. 1992. *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. *The Plant Journal* **2,** 487–496.

Harrison J, Hirel B, Limami M. 2004. Variation in nitrate uptake and assimilation between two ecotypes of *Lotus japonicus* and their recombinant inbred lines. *Physiologia Plantarum* **120,** 124–131.

Harrison J, Pou de Crescenzo M-A, Sené O, Hirel B. 2003. Does lowering glutamine synthetase activity in nodules moify nitrogen metabolism and growth of *Lotus japonicus*? *Plant Physiology* **133,** 253–262.

Hoshida H, Tanaka Y, Hibino T, Hayashi Y, Tanaka A, Takabe T, Takabe T. 2000. Enhanced tolerance to salt stress in transgenic rice that overexpresses chloroplast glutamine synthetase. *Plant Molecular Biology* **43,** 103–111.

Kato K, Okamura Y, Kanahama K, Kanayama Y. 2003. Nitrate-independent expression of plant nitrate reductase in *Lotus japonicus* root nodules. *Journal of Experimental Botany* **54,** 1685–1690.

Lawlor DW, Gastal F, Lemaire G. 2001. Nitrogen, plant growth and crop yield. In: Lea PJ, Morot-Gaudry J-F, eds. *Plant nitrogen*. Berlin: Springer-Verlag, 343–367.

Lea PJ. 1999. Nitrogen metabolism. In: Lea PJ, Leegood RC, eds. *Plant biochemistry and molecular biology*. Chichester: Wiley, 163–191.

Lea PJ, Forde BG. 1994. The use of mutants and transgenic plants to study amino acid metabolism. *Plant, Cell and Environment* **17,** 541–556.

Lea PJ, Morot-Gaudry. 2001. *Plant nitrogen*. Berlin: Springer-Verlag.

Limami A, Phillipson B, Ameziane R, *et al*. 1999. Does root glutamine synthetase control plant biomass production in *Lotus japonicus* L.? *Planta* **209,** 495–502.

Márquez AJ. 2005. Lotus japonicus *handbook*. Dordrecht: Springer (in press).

Márquez AJ, Arcondeguy T, Betti M, Díaz P, García-Calderón M, Orea A. 2002. Studies of glutamine synthetase mutants in legume plants. *Nitrate assimilation: molecular and genetic aspects (Namga) book of abstracts*. University of Córdoba, Spain, 81–82.

Márquez AJ, Betti M, García-Calderón M, Estivill G, Credali A, Pajuelo P, Orea A, Pajuelo E, Galván F. 2005. Nitrate and ammonium assimilatory enzymes. In: Márquez AJ, ed. Lotus japonicus *handbook.* Dordrecht: Springer (in press).

Márquez AJ, Orea A, Pajuelo P, *et al*. 2004. Nitrogen assimilation in roots of the model legume *Lotus japonicus*. *Biología* **59,** 69–76.

McIntyre GI. 1997. The role of nitrate in the osmotic and nutritional control of plant development. *Australian Journal of Plant Physiology* **24,** 103–118.

Melo PM, Lima LM, Santos IM, Carvalho HG, Cullimore JV. 2003. Expression of the plastid-located glutamine synthetase of *Medicago truncatula*. Accumulation of the precursor in root nodules reveals an *in vivo* control at the level of protein import into plastids. *Plant Physiology* **132,** 390–399.

Meyer C, Caboche M. 1998. Manipulation of nitrogen metabolism. In: Lindsey K, ed. *Transgenic plant research*. Australia: Harwood Academic Publishers, 125–133.

Orea A. 1999. *Nitrate assimilation in the model legume* Lotus japonicus. PhD thesis, University of Seville, Spain.

Orea A, Pajuelo P, Pajuelo E, Márquez AJ, Romero JM. 2001. Characterisation and expression studies of a root cDNA encoding for ferredoxin-nitrite reductase from *Lotus japonicus*. *Physiologia Plantarum* **113,** 193–202.

Orea A, Pajuelo P, Pajuelo E, Quidiello C, Romero JM, Márquez AJ. 2002. Isolation of photorespiratory mutants from *Lotus japonicus* deficient in glutamine synthetase. *Physiologia Plantarum* **115,** 352–361.

Orea A, Pajuelo P, Romero JM, Márquez AJ. 2005*a*. Nitrate assimilation: influence of the nitrogen supply. In: Márquez AJ, ed. Lotus japonicus *handbook*. Dordrecht: Springer (in press).

Orea A, Prosser I, Romero JM, Márquez AJ. 2005*b*. Transgenic plants affected in nitrate assimilation. In: Márquez AJ, ed. Lotus japonicus *handbook*. Dordrecht: Springer (in press).

Ortega JL, Temple SJ, Bagga S, Ghoshroy S, Sengupta-Gopalan C. 2004. Biochemical and molecular characterization of transgenic *Lotus japonicus* plants constitutively over-expressing a cytosolic glutamine synthetase gene. *Planta* **219,** 807–818.

Pajuelo P, Pajuelo E, Orea A, Romero JM, Márquez AJ. 2002. Influence of plant age and growth conditions on nitrate assimilation in roots of *Lotus japonicus* plants. *Functional Plant Biology* **29,** 485–494.

Pal'ove-Balang P, Orea A, Márquez AJ. 2003. Isolation and physiological characterization of chlorate resistant mutants of *Lotus japonicus*. *Abstracts of the 6th International Symposium on Structure and Function of Roots: Plant Root Development and Adaptation to Stresses* held at Stará Lesná, Slovakia (organised by Igor Mistrik, Institute of Botany, Slovak Academy of Sciences, Bratislava, Slovakia), 41.

Papadopoulos Y, Kelman W. 1999. Traditional breeding of *Lotus* species. In: Beuselinck P, ed. *Trefoil: the science and technology of* Lotus. Madison, WI: American Society of Agronomy, 187–198.

Pelsy F, Caboche M. 1992. Molecular genetics of nitrate reductase in higher plants. *Advances in Genetics* **30,** 1–40.

Popova O, Ismailov S, Popova T, Dietz KJ, Golldack D. 2002. Salt-induced expression of NADP-dependent isocitrate

dehydrogenase and ferredoxin dependent glutamate synthase in *Mesembryanthemum crystallinum*. *Planta* **215,** 906–913.

Radutolu S, Madsen LH, Madsen EB, *et al*. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* **425,** 585–592.

Rossens N, Thu T, Iskandar H, Jacobs M. 1998. Isolation of ornithine-δ-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. *Plant Physiology* **117,** 263–271.

Smirnoff N, Stewart GR. 1985. Nitrate assimilation and translocation by higher plants: comparative physiology and ecological consequences. *Physiologia Plantarum* **64,** 133–140.

Stracke S, Kistner C, Yoshida S, *et al*. 2002. A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* **417,** 959–962.

Suarez R, Márquez J, Shishkova S, Hernández G. 2003. Overexpression of alfalfa cystosolic glutamine synthetase in nodules and flowers of transgenic *Lotus japonicus* plants. *Physiologia Plantarum* **117,** 326–336.

Triplett EW. 2000. *Prokaryotic nitrogen fixation*. Wymondham: Horizon Scientific Press.

Verma DPS, Zhang C-S. 1999. Regulation of proline and arginine biosynthesis in plants. In: Singh BK, ed. *Plant amino acids*. New York: Marcel Dekker, 249–265.

Waterhouse RN, Smyth AJ, Massonneau A, Prosser I, Clarkson DT. 1996. Molecular cloning and characterisation of asparagine synthesis from *Lotus japonicus*: dynamics of asparagine synthesis in N-sufficient conditions. *Plant Molecular Biology* **30,** 883–897.

Webb KJ, Robbins M, Wang TL, Parniske M, Márquez AJ. 2005. Mutagenesis. In: Márquez AJ, ed. Lotus japonicus *handbook.* Dordrecht: Springer (in press).

Woodall J, Forde BG. 1996. Glutamine synthetase polypeptides in the roots of 55 legume species in relation to their climatic origin and the partitioning of nitrate assimilation. *Plant, Cell and Environment* **19,** 848–858.

Zhu JK. 2002. Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology* **53,** 247–273.