DETERMINATION OF THE PHYSIOLOGICAL IMPACT OF THE MODULATION OF THE PATHWAY OF NITROGEN ASSIMILATION IN PLANTS THROUGH GENETIC MANIPULATION AND BREEDING

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Abstract. The ever-increasing environmental awareness, and increased need to cut costs on fertilisers made the improvement of plant nitrogen use efficiency (NUE: fertiliser taken up by the plants which is assimilated *into* organic N) of high importance for plant breeding industry. To realise potential benefits with respect to sustained agriculture, we need to identify the physiological, biochemical and molecular mechanisms controlling nitrate uptake, nitrate partitioning between roots and shoots, and nitrate reduction and its subsequent assimilation and transfer *into* organic molecules. Furthermore a better understanding of the transition between nitrogen assimilation and nitrogen recycling during leaf senescence will be vital, if improvements in crop nitrogen use efficiency are to reduce fertilisers input and improve or stabilize yield. In this paper, we describe improvement in our understanding of the molecular controls of nitrogen assimilation through the use of transgenic plants and the study of genetic variability in model and crop species. To illustrate this research program, the physiological impact of modified gene expression using either transgenic plants or different genotypes was studied using ^{15}N labelling experiments in order to monitor the influx of nitrate or ammonia and its subsequent incorporation *into* amino acids. Our current knowledge and prospects for future development and application to crop improvement are discussed.

Key words: molecular controls, nitrogen assimilation, transgenic plants, genetic variability.

Introduction

Recent advances both in molecular physiology and genetics allowed for expanding our understanding of the regulatory mechanisms controlling primary steps of inorganic nitrogen absorption, assimilation and relationship with carbon metabolism in relation to growth and development of higher plants (figure 1).

Nitrate is the principal nitrogen source for most wild and crop species. Following its uptake by means of specific transporters located in the root cell membrane [40], nitrate is reduced to nitrite by nitrate reductase that is reduced to ammonium by nitrite reductase. Furthermore ammonium can be generated inside the plant by a variety of metabolic pathways such as photorespiration, phenylpropanoid metabolism, utilization of nitrogen transport compounds and amino acids catabolism, from symbiotically fixed nitrogen or from insect digestion in carnivorous plants [46]. Also, when available in the soil, under particular environments such as rice paddy fields or acidic forest, ammonium can be absorbed by the plants *via* root-specific transporters [33, 45]. In ecosystems characterized by a low rate of mineralisation of organic nitrogenous compounds, plants can directly take up amino acids or through mycorrhizal symbiotic associations [7, 37].

Ammonia, which is the ultimate form of inorganic nitrogen available to the

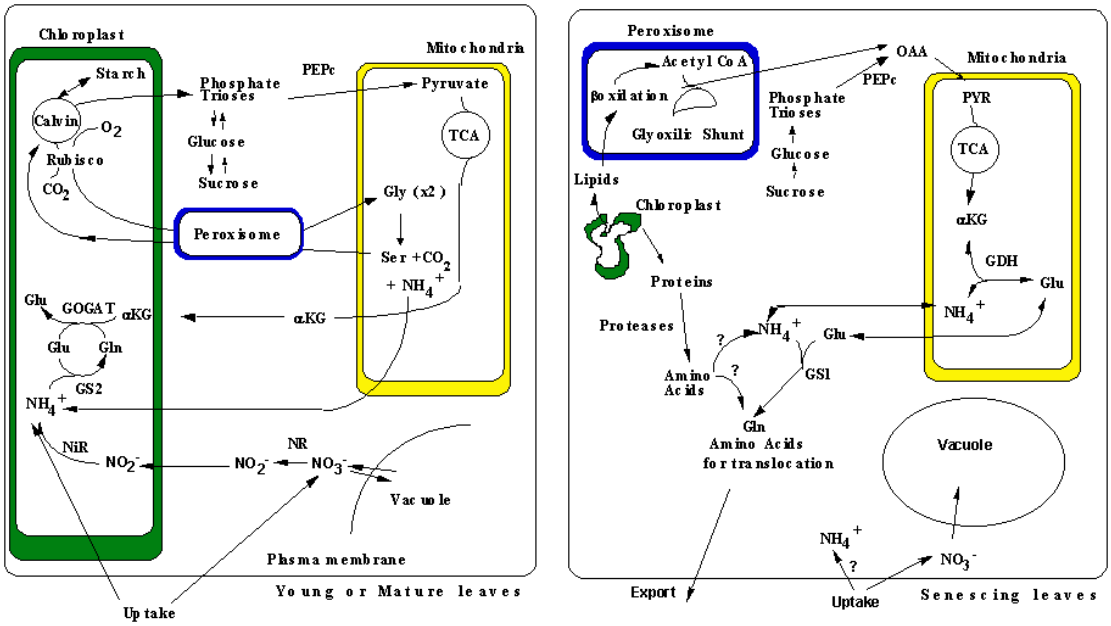

Figure 1. Schematic representation of nitrogen metabolism (uptake and assimilation) and its relationship with carbon metabolism in either young and mature leaves or senescent leaves. NR, nitrate reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase.

plant, is incorporated *into* organic molecules, glutamine and glutamate by the concerted action of two enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT). Both glutamine and glutamate are further used as donors of amino groups to other amino acids utilized for protein synthesis and to nucleotides used as basic molecules for RNA and DNA synthesis [36].

Both GS and GOGAT are represented by numerous isoenzymes harbouring different organ and cell types expression specificities. GS is represented by a cytosolic form located in the cytoplasm and a plastidic form located in chloroplasts of photosynthetic tissues and the plastids of roots and etiolated tissues. The relative proportions of these forms vary within organs of the same plant and between plant species. As such it was proposed that each GS isoform might play a specific role, such as photorespiratory ammonium assimilation, nitrate reduction, nitrogen translocation and recycling [10]. GOGAT is represented by two forms of the enzyme, a Ferredoxin-dependent isoenzyme (Fd-GOGAT) mainly involved, in conjunction with GS2, in the re-assimilation of photorespiratory ammonium and a pyridine-nucleotide-dependent isoenzyme (NADH-GOGAT) involved in the synthesis of glutamate mostly in non-photosynthetic tissues to sustain plant growth and development [51, 52].

GS and GOGAT isoenzymes were shown to play specific roles during the plant life cycle. At least in perennial plants (including most crop species), the plant life cycle can be roughly divided *into* two main phases: (i) the vegetative growth phase, when young developing roots and leaves as sink organs absorb and assimilate efficiently inorganic nitrogen for amino acid and protein synthesis, (ii) the remobilisation phase when senescing tissues as source of both carbon and organic molecules sustain the formation of new developing and/or storage organs. Storage organs are involved in plant survival and are represented by seeds, fruits, tubers, bulbs or trunks [34].

Therefore, during these two phases of plant growth and development, a better understanding of the metabolic and genetic

control of nitrate uptake, nitrate partitioning between roots and shoots, nitrate reduction and its subsequent assimilation and transfer *into* organic molecules is required. This will be of particular importance not only to improve crop quality and productivity but also to avoid excessive use of fertilisers.

In spite of the significant progress that has been made during the last few years on the regulation of inorganic nitrogen metabolism, many aspects of nitrogen assimilation and recycling both at the cellular and whole plant level remain to be discovered and integrated at the whole plant level [49]. This integration is required because in addition to regulating a range of cellular processes including nitrogen assimilation itself through the co-ordination of nitrate or ammonium uptake and use, nitrate and nitrogen metabolites levels in the cell can regulate directly or indirectly a number of closely related metabolic and developmental processes. These processes, which may be regulated through the action of hormones, include the synthesis and accumulation of amino acids and organic acids and the modification of plant development including the extent and form of root growth, the timing of flower induction. All this processes, acting either individually or synergistically, condition nitrogen allocation in newly developing tissues or in storage organs to finally ensure plant vegetative or sexual reproduction [9, 21].

To better understand the regulation of these various processes, several approaches have been undertaken in parallel. They include: (1) the study of the transcriptional and post translational regulation of the proteins and enzymes involved in nitrogen uptake and assimilation and the subsequent incorporation of reduced nitrogen *into* organic molecules. (2) the identification of nitrogen compound(s) which are responsible for the regulation of each specific process under standard or particular environmental conditions such as high CO_2 concentrations, water or salt stress, nitrogen availability and variable light intensities. (3) the development of strategies to alter their concentrations *via* the use of mutants or transgenic plant technology or conventional breeding to increase nitrogen use efficiency. (4) the discovery of how the variations in the relative concentrations of these compounds are sensed and how the information concerning these metabolic changes are channelled through signal transduction pathways to further develop strategies to modify these transduction pathways. (5) the development of integrated research both at the whole plant and crop level in order to evaluate the impact of any genetic or molecular modification of plants in nitrogen use efficiency and associated environmental risks.

In this paper we will focus on the ways in which our understanding of the nature of the molecular controls of nitrogen assimilation has been increased by the use of leguminous and non-leguminous plants with altered capacities for nitrogen assimilation. Conventional biochemical techniques were first used to monitor the changes in carbon and nitrogen metabolites pools to evaluate the physiological impact of inserted genes or genetic variability. However, in a number of cases these techniques only allowed for giving an instant picture of metabolites accumulation, which may be the result of changes occurring, further upstream or downstream in the modified metabolic pathway. To obtain a more dynamic picture of the modifications in the metabolic fluxes, ^{15}N labelling experiments were developed to follow the fate of nitrate of ammonia from their absorption to their incorporation *into* organic molecules.

Modification of nitrate uptake and assimilation

Physiological studies showed that three transport systems do exist in plants for nitrate uptake [for review see 17]. A

low capacity, high affinity constitutive system (cHATS) allows plants that have never been exposed to NO_3 to absorb this ion from low external nitrate (< 200 µM). After a first exposure to nitrate a low capacity, high affinity inducible system (iHATS) starts operating beside the former. At high external nitrate it was shown that this ion entered *into* the plant through a high capacity, low affinity system (LATS) [11, 12]. Kinetic of nitrate transport studied by the mean of $^{15}NO_3$ or $^{13}NO_3$ revealed that high affinity nitrate systems are saturable with Km values ranging from 10 to 100 µM while low affinity system showed an intriguing linear concentration response [16, 47, 48]. Two families of genes identified so far as NRT1 and NRT2 have been proposed to encode putative NO_3 transporters [for review see 40]. The first NRT1-family member CHL1 was characterized as a LATS transporter in chlorate resistant mutants of *Arabidopsis thaliana* and named afterwards AtNRT1.1. However when expressed in *Xenopus* oocytes it turned out that AtNRT1.1 is a dual affinity NO_3 transporter showing both low and high affinity nitrate uptake, with a Km of 49 µM in external nitrate concentrations ranging between 0 and 250 µM and a Km of 4 mM for higher external nitrate concentrations up to 30 mM [19, 31]. Other members of this gene family such as AtNRT1.2 were cloned [23], they seemed to rather encode LATS transporters. Members of the family NRT2 seem to encode iHATS transporters. They have been cloned in several plant species, *Nicotiana plumbaginifolia* [41], *Glycine max* [1] on the basis of their sequence homology with a high affinity nitrate transporter gene Crna of *Aspergillus nidulans* [53] and *CrNRT2.1* of *Chlamydomonas reinhardtii* [42]. NRT1 and NRT2 proteins do not share sequence homology but both exhibit the same structure with 12 transmembrane domains constituting two sets of six helices connected by a cytosolic loop.

In order to assign precisely a role to these putative transporters nitrate uptake / influx was measured by the mean of the heavy isotope ($^{15}NO_3$) in transgenic *Nicotiana plumbaginifolia* over-expressing constitutively iHATS gene *NpNRT2.1* either under the control of CaMV 35S or the root specific rolD promoters [13]. When plants were fed nitrate as the only nitrogen source at 10 mM expression of the endogenous *NpNRT2.1* was down regulated by N-metabolites derived from NO_3 reduction. As a result expression of this gene was three to four fold higher in transgenic plants than that in wild type. This expression was positively correlated to $^{15}NO_3$ influx in wild type and transgenic plants, measured at 200 µM external nitrate showing that the protein encoded by *NRT2* is involved in NO_3 uptake being itself the high affinity NO_3 transporter or at least a limiting element of the iHATS. Feeding transgenic plants NH_4NO_3 showed that despite strong constitutive expression of *NpNRT2.1* ammonium still exerted its inhibitory effect on $^{15}NO_3$ influx. These data demonstrated that regulation of iHATS is not confined to the transcriptional level and that either NH_4 or a metabolite derived from its assimilation do exert a post-transcriptional control on this transporter [13].

Control of nitrate ($^{15}NO_3$) uptake and reduction by N-metabolites derived from nitrate reduction was also determined in transgenic tobacco plants either over-expressing or under-expressing nitrate reductase (NR) [18, 28]. $^{15}NO_3$ reduction efficiency calculated as reduced % $^{15}NO_3$ taken up was as expected decreased in NR under-expressers and interestingly increased in NR over-expressers. The latter result moderated the hypothesis supported by several works [43, 55] that in illuminated leaves NO_3 reduction is limited by the substrate NO_3 delivery to the enzyme NR by the xylem or the vacuole and that active NR is present in large excess in the cytosol. It appears then that

metabolic use of NO_3 *in vivo* may be influenced by variations in the level of NRA and that the availability of NO_3 for reduction in the cytosol do not only rely on NO_3 delivery by the xylem, it would be subjected to a competition between the enzyme NR and putative NO_3 transporters on the tonoplast [18]. $^{15}NO_3$ uptake by transgenic plants was also recorded, in the NR over-expressers the increase in nitrate reduction efficiency was coupled to decrease in either $^{15}NO_3$ uptake and NO_3 content in the leaves and a significant increase in free Gln content in the leaves. According to the 'demand' theory the accumulation of Gln in the leaves would mean that the rate of amino acids synthesis in the transgenics exceeded the rate of their incorporation in proteins resulting in a feed back inhibition of NO_3 uptake. Even the mechanistic basis of this inhibition are not yet fully understood, it seems however more likely that inhibition of NO_3 uptake is due to a decrease in NO_3 influx than an increase in NO_3 efflux. Feed back inhibition of NO_3 influx would be exerted by either NO_3 itself or more probably amino acids (Gln), even though the accumulation of these compounds occurred in the leaves it is sensed in the roots *via* the phloem-born amino-acids pool cycling between the shoot and the root [8, 24].

Quantitative genetic approach was recently developed using maize recombinant inbred lines (RILs) by associating metabolic functions and agronomic traits to DNA markers. A significant genotypic variation was observed for various physiological traits related to N metabolism and a positive correlation was observed between nitrate content, GS activity and yield and its components. It was therefore suggested that increased productivity in maize genotypes might be due to their ability to accumulate nitrate in their leaves during vegetative growth and efficiently remobilise this stored N during grain filling [22]. The same pattern was observed in a Lotus RILs population. When Lotus lines were grown under non-limiting N nutrition (10mM NO_3) shoot NO_3 content and biomass production were subjected to genetic variability. Both traits were normally distributed in the progeny, with a positive correlation between them, however these two characters segregated independently. Indeed four classes of plants with respect to the shoot and root biomass / shoot and root NO_3^- concentration relationship were therefore selected: plants with a low biomass and low nitrate content (parental line Gifu and RIL 7), a high biomass and a low nitrate content (RIL 63), a low biomass and a high nitrate content (RIL 11) and a high biomass and high nitrate content (parental line Funakura and RIL 96). Therefore in order to determine the physiological mechanism that underlies differences in the capacity of each line to accumulate NO_3, a ^{15}N-charge/^{14}N-chase labelling experiment was conducted to measure NO_3^- uptake and its components influx and efflux and partitioning of absorbed nitrogen between shoot and root. Plants were fed for 6 charge-hours with a nutrient solution containing 10 mM NO_3 enriched with $^{15}NO_3$ (A % ^{15}N = 2%) then submitted to 48 chase-hours with a nutrient solution containing $^{14}NO_3$ (10 mM). At the end of the charge period the amount of ^{15}N incorporated was 20 % higher both in the shoot and roots of Funakura and high NO_3 type RILs (11 and 96) as compared to Gifu and low NO_3 type RILs (7 and 63) due to a higher NO_3 net uptake (table 1). The partitioning of newly absorbed (^{15}N) nitrogen between shoot and root was similar in all genotypes (table 1), this result is consistent with the amino acids composition of the parental lines and RILs that show an absence of Gln in the roots probably due to a rapid transformation of the latter by transamination *into* Asn that is transported to the shoot.

Monitoring nitrogen ($^{15}NO_3$) influx and efflux showed that the contribution of the influx to net uptake of NO_3 is

predominant in all genotypes. Efflux represented hardly 10 % of the influx in Funakura and high NO_3 type RILs (11 and 96) and almost 5 % of the influx in Gifu and low NO_3 type RILs (7 and 63). This result therefore indicates that NO_3 transport across the plasma membrane of root cells is occurring at a faster rate in the three genotypes accumulating more NO_3 (Funakura and RILs 11 and 96). It can be therefore hypothesized that in these three lines, the increase in NO_3 net uptake / influx could be either due to the presence of more NO_3 transporters on the root plasma membrane or a better activity of the overall NO_3 transport system. However, the presence of higher amounts of mRNA encoding a high affinity NO_3 transporter in Funakura and RILs 11 and 96 is in favour of the former hypothesis. These results confirm that even when plants were grown under a high NO_3 concentration, the level of expression of high affinity NO_3 transporters was correlated with the influx of NO_3 [13, 28].

The capacity of plants to reduce nitrate through the reaction catalysed by the enzyme NR is considered as a potential control point for nitrate accumulation in plant tissue. However when checked out the capacity of Funakura, Gifu and the four RILs to reduce NO_3 *in vivo* (Mg-dependent NR) appeared similar, it cannot therefore explain the observed differences in plant NO_3 contents. We concluded then that in *Lotus japonicus* regardless of the plant biomass accumulation, nitrate uptake is the major controlling point for NO_3 accumulation (figure 2).

Modification of ammonia assimilation

In leaves (for illustration, see figure 2)

Since in a number of higher plants cytosolic GS is not expressed in the leaf mesophyll during the vegetative phase of plant development [5, 26] and is only induced during leaf senescence when ammonia is released as the result of protein hydrolysis, attempts were made to express the enzyme ectopically either in legumes and non legumes. Most of the studies performed using transgenic tobacco [14] or alfalfa [50] revealed that under certain growth conditions the genetic

Table 1. Characterization of nitrate uptake by *Lotus japonicus* parental lines Funakura and Gifu and high (11 and 96) and low (7 and 63) NO_3 type RILs. Plants were submitted to a 6 hours pulse with a nutrient solution containing 10 mM NO_3 enriched with $^{15}NO_3$ (A% ^{15}N = 1.6) followed by 48 hours $^{14}NO_3$ chase. Results are the mean of three replicates, numbers between brackets are SE for $p < 0.05$.

Character	Genotype					
	Funakura	RIL 11	RIL 96	Gifu	RIL 7	RIL 63
	High NO3 type			Low NO3 type		
	µg 15N / g DW / h					
NO3 Net Influx	815.0 (61.8)	720.3 (59.4)	845.0 (65.7)	671.1 (50.6)	615.0 (48.6)	588,3 (43.5)
NO3 Net Efflux	69.5 (5.9)	48.3 (4.5)	59.1 (4.0)	35.7 (3.8)	29.8 (3.5)	24,0 (3.3)
NO3 Net Uptake	745.5 (57.5)	672.0 (50.5)	785.9 (55.4)	635.9 (48.4)	585.2 (45.4)	564.3 (40.2)

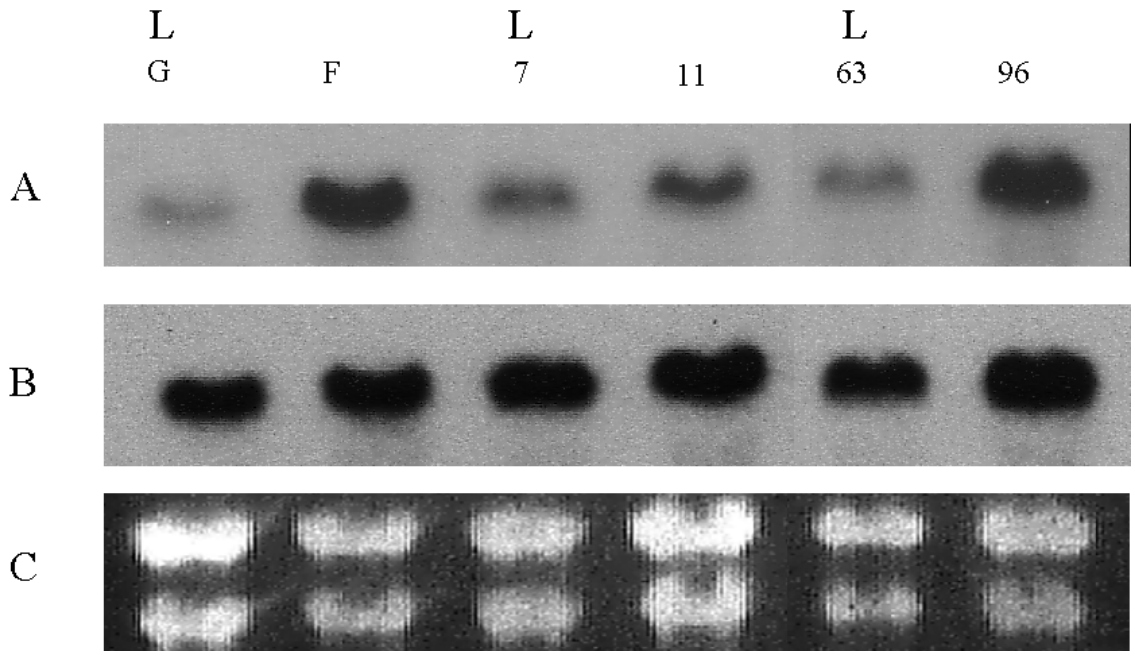

Figure 2. Expression of mRNA encoding high affinity NO_3^- transporter (A) in the roots of two *Lotus japonicus* ecotypes Gifu and Funakura and four RILs of their progeny. H and L indicate respectively high (11 and 96) and low (7 and 63) NO_3 types. (B) corresponds to the expression of Asparagine Synthetase mRNA as a constitutive control. An ethidium bromide stained gel shows that similar amounts of total RNA were loaded in each track (C).

manipulation had an influence on the plant phenotype particularly when nitrogen nutrition was limiting. However, in most of these studies detailed physiological analysis of the impact of the introduced transgene on both nitrogen and carbon assimilation was not thoroughly investigated.

The over-expression of a soybean gene (*GS15*) encoding a cytosolic glutamine synthetase was successfully achieved in *Lotus corniculatus* using the 35S CaMV promoter to allow a strong constitutive expression of the transgene. Several transformants were obtained which synthesized additional cytosolic GS in the shoots representing a 50% increase of the total leaf enzyme activity. Although soybean GS mRNA were highly expressed in roots, surprisingly no enhanced GS activity could be measured in this organ; this was interpreted as root-specific post-transcriptional regulatory mechanism.

Transgenic plants were grown under different nitrogen regimes either on nitrate or ammonia and some aspects of carbon and nitrogen metabolism were examined. Mostly in roots but also in shoots, an increase in free amino acids and ammonium was accompanied by a decrease in soluble carbohydrates in the transgenic plants cultivated with 12 mM NH_4^+ in comparison to the wild type grown under the same conditions. The increase in the ammonium content of the plant was somehow surprising, since an enhanced assimilation of the ion would have been expected as the result of increased GS activity.

Labelling experiments were therefore carried out using $^{15}NH_4^+$ in order to monitor the influx of ammonium and its subsequent incorporation *into* amino acids. This experiment revealed that both ammonium uptake in the roots and the subsequent translocation of amino acids to the shoots was lower in plants over-expressing GS. It was therefore concluded that the build up of ammonium and the increase in amino acid concentration in the roots was the result of shoot protein degradation. Moreover, following three

weeks of hydroponic culture early floral development was observed in the transformed plants. As all these properties are characteristic of senescent plants, these findings suggest that expression of cytosolic GS in the shoots may accelerate plant development leading to early senescence and premature flowering when plants were grown on an ammonium rich medium. This conclusion was in agreement with the current concept concerning the role of the leaf-induced cytosolic GS in the re-assimilation ammonia released during proteolysis [54]. Although the regulatory control mechanisms controlling the induction of GS1 as well as other enzymes involved in nitrogen and carbon recycling are still unknown, this study showed that by modifying the capacity of a plant to assimilate inorganic nitrogen in a different cellular compartment it is possible to influence its developmental cycle. This result also suggests that plant senescence program which is genetically determined may be modified as a result of an

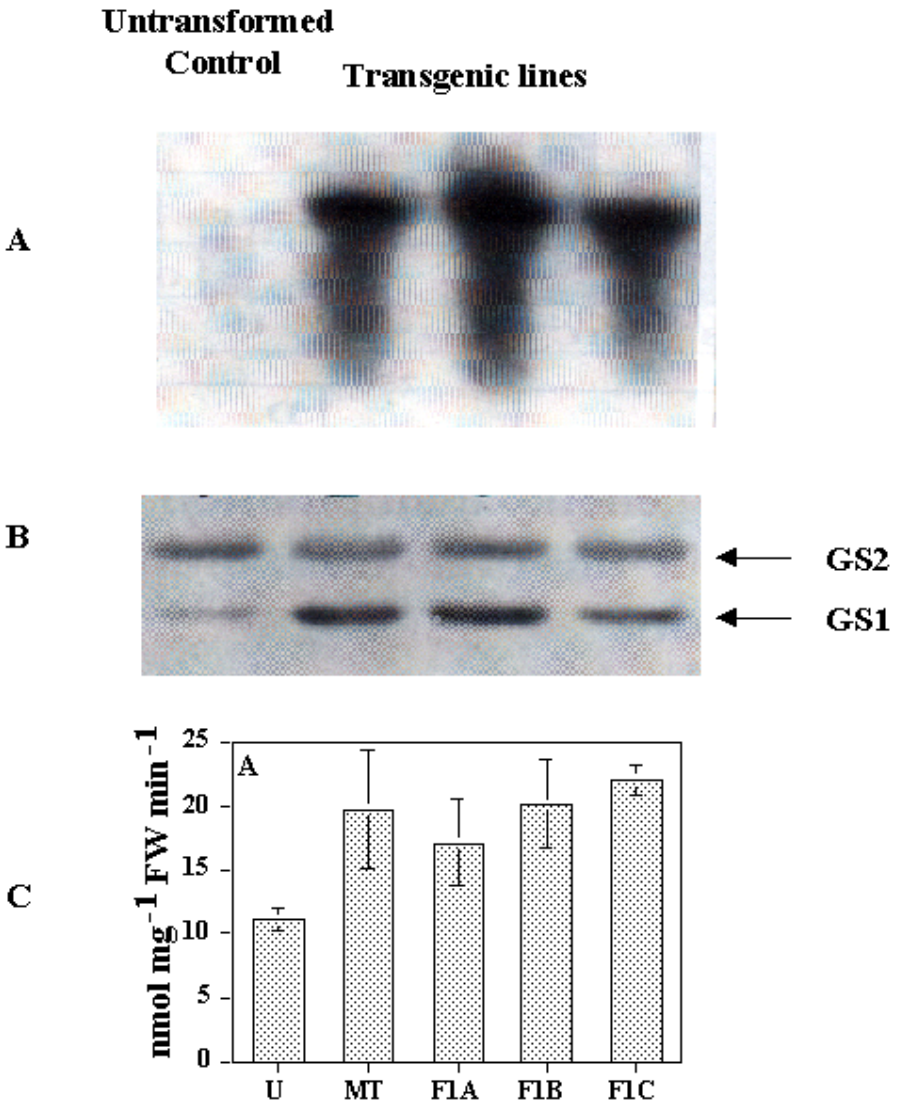

Figure 3. Overexpression of soybean cytosolic GS1 gene in transgenic *Lotus japonicus*. A : Analysis of expression (northern blot) of soybean GS1 gene in control (untransformed) and transgenic plants. B : GS1 and GS2 proteins abundance analysed by western blot in control (untransformed) and transgenic plants. C : GS activity *in vitro* in control (untransformed) and transgenic plants.

imbalance in the leaf metabolism. This imbalance is likely to be similar to that occurring when the plant is subjected to either biotic or abiotic stresses, thus leading to an accelerated development to rapidly produce seeds or fruits to ensure plant survival [2, 41].

In roots and nodules

In a number of reports, it has been suggested that in a number of plant species, legumes in particular, there is a competition between roots and shoots nitrogen assimilation, roots assimilation being rather inefficient compared to its shoots counterpart. It was therefore proposed that shoots nitrogen assimilation could be improved either by down loading it in roots or redirecting it to the shoots [38]. A number of experiments using a specific inhibitor of root cytosdolic GS (tabtoxin-ß-lactam) tented to confirm this hypothesis since shoot biomass production was improved in alfalfa or oat plants treated with this molecule [27].

The use of genetically modified plants for modifying the capacity of the roots to assimilate inorganic nitrogen was another possibility to also ascertain this hypothesis. Since GS in the root cytosol is one of the main enzymes responsible of the incorporation of inorganic nitrogen *into* organic molecules, and was the main target of the inhibitor tabtoxin-ß-lactam, an attempt was made to over express the enzyme in roots using the legume *Lotus* as a model plant.

Transgenic *Lotus* plants over expressing a soybean gene encoding cytosolic GS (GS15) under the control of the *RolD* root-specific promoter were produced. Compared to untransformed control plants, we found that transgenic plants had a decreased plant biomass production. In order to determine if increased root GS activity had modified nitrate uptake in roots and shoots, plants were fed for 6 hours with a nutrient solution containing 10 mM NO_3^- in which 2 % of the total nitrate content was replaced by $^{15}NO_3^-$. Using a pulse chase $^{15}NO_3^-$ labelling experiment, we showed that the decrease in plant biomass production was likely to be due to a lower nitrate uptake accompanied by a redistribution of the newly absorbed nitrogen to the shoots. NO_3^- accumulated in the shoots could not be reduced due to the lack of nitrate reductase activity in this organ [29].

The relationship between plant growth and root GS activity was further assessed using a series of recombinant inbred lines issued from the crossing of two different *Lotus* ecotypes, Gifu and Funakura. We confirmed that a negative relationship exists between root GS expression and plant biomass production in both the two parental lines and their progeny. Statistical analysis allowed us to estimate that at least 13 % of plant growth is dependent upon root GS activity [29].

As the others legumes, *Lotus* has the capacity to develop a symbiotic association with a soil bacterium called *Rhizobium* allowing the formation on the roots of specialised organs, the nodules, capable of reducing atmospheric nitrogen *into* ammonia. This symbiotic association allows the plant to grow without any supply of inorganic nitrogen. In the nodules, ammonia is then incorporated *into* amino acids *via* the GS/GOGAT cycle to synthesise, like in the other plant organs, the two basic amino acids glutamine and glutamate.

Lotus plants over expressing GS in the leaf cytosol were grown under nitrogen fixing conditions in order to check if the soybean gene GS15 placed under the control of the constitutive 35SCaMV promoter could be expressed in nodules. Although high levels of GS15 mRNA were expressed in the nodules of plants inoculated with *Rhizobium*, GS activity was 40% lower. Although unexplained this result, was interpreted as a modification in the change of the enzyme turnover and/or stability following the introduction of the foreign soybean GS gene. Even more

surprising was the two-fold increase in plant roots and shoots biomass production and also the higher production of nodules in the transformed plants. Physiological analysis of the transgenic plants was then carried out by monitoring through the rate of ^{15}N dilution, the rate of atmospheric nitrogen fixation and its subsequent translocation in the different part of the plants. Prior inoculation, plants were labelled for a short period with $^{15}NO_3^-$. The dilution of the heavy isotope was then followed up to four weeks after inoculation with *Rhizobium* and allowed to show that the newly fixed nitrogen was first used to form nodules and then for the growth of shoots and roots. On a dry weight basis, the rate of ^{15}N dilution was similar in both control and transformed plants indicating that the rate of atmospheric nitrogen fixation was the same in both types of plants. Since greater quantities of nodules were produced in the transgenic plants it was concluded that the overall levels of nitrogen fixation and assimilation were enhanced which may explained why they produce more biomass [20]. Although this finding was rather unexpected and controversial, it was in agreement with a previous work demonstrating that when root-nodule GS activity is inhibited following the application of the inhibitor tabtoxin-ß-lactam; an increase in biomass production of alfalfa plants is observed [28].

However, further work is still required to confirm this remarkable effect of the enzyme GS on the control of plant growth and development using for example antisense RNA strategy to specifically reduce the activity of the enzyme both in roots and nodules.

In the phloem (for illustration, see table 2)

Among the different cytosolic GS isoenzymes, one was found to be specifically expressed in the phloem companion cells of the vascular tissue. Due to its presence in a tissue specialised in the translocation of assimilates from source to sink organs, it was therefore suggested that the enzyme plays an important role in the synthesis of glutamine used for the export of organic nitrogen [6, 25]. Although logical, this assumption was never demonstrated experimentally.

To identify the physiological role of GS1 in the phloem an antisense strategy was developed using tobacco as a model plant. A fragment of a cDNA (*Gln1-5*) encoding cytosolic was placed in the antisense orientation downstream of the cytosolic Cu/Zn superoxide dismutase promoter of *Nicotiana plumbaginifolia* to direct a specific expression of the antisense in the

Table 2. Concentration and proportion of amino acids in leaves, stems, roots of control and SOD-AS antisense tobacco plants.

Amino acids	Amino acids concentration (nmoles/mg dry weight) and proportions (%) a								
	Leaves			Stems			Roots		
	UT	SOD-AS7	SOD-AS9	UT	SOD-AS7	SOD-AS9	UT	SOD-AS7	SOD-AS9
Asp	1 (0.9)	1 (0.6)	0.7 (0.4)	6.7 (3)b	14 (7.7)b	15.6 (8.2) b	2.6 (4.2)	2.3 (4.6)	2.7 (5.1)
Asn	3.8 (3.4)	4.5 (2.8)	4.6 (2.8)	24.4 (11)	19 (10.4)	22.2 (11.7)	3.7 (6.1)	1.5 (3)	1.5 (2.8)
Glu	1.2 (1.1)	1 (0.6)	1.1 (0.7)	10.9 (4.9)	16.2 (8.9)	13.4 (7)	5.3 (8.6)	4.2 (8.5)	3.6 (6.8)
Gln	8.8 (8)	16.5 (10.5)	20.7 (12.9)	80.5 (36.4)	67.6 (37)	60.2 (31.8)	16 (25.9)	10.3 (20.9)	9.1 (17.3)
Pro	**36.5 (33.4)**	**26 (16.4)b**	**24 (15)b**	**62.5 (28.2)**	**35.1 (19.3)**	**32.3 (17)b**	**4.1 (6.7)b**	**2.1 (4.2)b**	**2.3 (4.3)b**
Ser	23.8 (21.8) b	43.2 (27.5) b	47.6 (29.7) b	13.4 (6.1)	15.1 (8.3)	17.7 (9.3)	4.6 (7.4)	4.5 (9.1)	4.9 (9.3)
Others	34.4 (31.9)	65.6 (41.7)	61.5 (38.4)	22.5 (10.4)	14 (7.7)	27.6 (14.6)	24.6 (41.1)	24.4 (49)	28.4 (54)
Total	109±25 (100)	157±35 (100)	160±28 (100)	221±31 (100)	181±32 (100)	189±14 (100)	61.3±6(100)	49.3±5 (100)	52.5±7 (100)

aAmino acids were separated and quantified in tobacco plants grown for 8 weeks on a complete N12 solution and for an additional two weeks on 8 mM NH_4^+ as sole nitrogen source. Values are the mean of six untransformed control plants (UT) and the two SOD-AS7 and SOD-AS9 transgenic lines for which three individual plants were analyzed (SOD-AS). Relative amino acids proportions are given in brackets. Standard deviation for the individual amino acids was of the same order of magnitude when compared to the total amino acids. bSignificant changes in the amino acid proportions.

phloem. Two transgenic lines exhibiting reduced levels of *GS1* mRNA and GS activity in midribs, stems, and roots were obtained. Immunogold labelling experiments allowed us to verify that the GS protein content was severely decreased in the phloem companion cells of transformed plants. A general decrease in proline content in the transgenic plants in comparison to the wild type was observed when plants were forced to assimilate large amounts of ammonium. In contrast, no major changes in the concentration of amino acids used for nitrogen transport were apparent.

A $^{15}NH_4^+$ labelling experiment was then conducted, and the dynamics of primary ammonia assimilation were examined in a pulse (^{15}N) chase (^{14}N) experiment over a 48-hr period to determine whether decreased GS activity in the phloem had also modified the dynamics of proline accumulation. In untransformed tobacco plants, a significant increase in proline content accompanied by a concomitant decrease in the glutamine pool was clearly visible during the 48-hr chase period. In contrast, the pool of glutamate showed a small (but significant) increase. This result suggested that glutamine was used as a precursor for the synthesis of proline *via* glutamate. The latter assumption was strengthened by the simultaneous increase in ^{15}N-proline and decrease in ^{15}N-glutamine, which demonstrates that most of the proline synthesized during the chase period was derived from a pre-existing pool of glutamine. This observation also confirmed the hypothesis that proline may constitute a strong sink for amino-nitrogen in plants when a pulse of ammonia is provided to the plant. Compared to untransformed plants, glutamate, which apparently represents an intermediary pool between glutamine and proline, was increased in transformed plants.

Following two weeks of salt treatment, the transgenic plants had a pronounced stress phenotype, consisting of wilting and bleaching in the older leaves. We conclude that GS in the phloem plays a major role in regulating proline production consistent with the function of proline as a nitrogen source and as a key metabolite synthesized in response to water stress [4].

Genetic variability and nitrogen use efficiency

A few studies have shown that the activities of enzymes involved in nitrogen assimilation may be variable between genotypes, thus suggesting that this variability may have a significant impact on plant nitrogen use efficiency [30, 35]. However, there was no clear demonstration that increased or decreased enzymes activities had a beneficial effect on plant productivity mainly because of the complex interactions between carbon and nitrogen metabolic pathways and their progressive changes during the plant life cycle [35, 49]. Recently, the use of quantitative genetics approaches has allowed starting to dissect the various components of a complex character such as nitrogen use efficiency. Moreover, by the mean of associations with molecular markers it became possible to exploit in a more targeted manner the genetic variability of nitrogen use efficiency in relation to yield and its components [22, 30, 39, 44].

In a limited number of studies, ^{15}N labelling experiments were used to help for the selection of genotypes with greater nitrogen use efficiency. These have demonstrated that, compared to senescent hybrids, "stay green" maize hybrids exhibit prolonged maintenance of green leaf area for photosynthate production during grain fill leading to a prolonged capacity of the plant to take up available soil nitrogen that is further translocated to the grain [32]. It was therefore hypothesised that the nitrate transport systems were active for a longer period as the result of a constant supply of carbon and nitrogen to the roots thus maintaining a greater capacity of the plant to extract nitrogen from the soil [3].

Recently we found that a large genetic variability exists in the shoot NO_3^- concentration of recombinant inbred lines (RILs) of *Lotus japonicus*. To determine the cause of this variability, we have studied some aspects of nitrate uptake and assimilation in the two parental ecotypes (Gifu and Funakura) and four representatives of the RILs population differing both in their shoot biomass and shoot NO_3^-content. Using a $^{15}N/^{14}N$ pulse/ chase experiment, we showed that higher shoot NO_3^-content was mainly due to an increase in the uptake of the ion regardless of the plant biomass production. The positive correlation observed between the shoot NO_3^- content and the steady state level of mRNA encoding high affinity NO_3^- transporters also suggested that the higher NO_3^- influx was due to enhanced expression of the transporters. Although the level of nitrate reductase mRNA was also correlated with the shoot NO_3^-content, the potential enzyme activity *in vivo* in the different lines remains similar and therefore did not seem to have a major impact on the rate of NO_3^-reduction. This indicated that NO_3^- transport in *Lotus* may be one of the main checkpoints controlling shoot NO_3^- accumulation. This type of study therefore demonstrate that ^{15}N labelling experiments can be further applied to identify crops with varied capacity to take up and store nitrogen and select, for example, genotypes exhibiting lower the NO_3^-concentration in the shoots without affecting biomass production. This study also pointed out that the control of NO_3^- uptake and its accumulation in the plant may be subjected to genetic variability regardless of the plant demand, which may help to dissociate nitrogen plant demand and plant growth [15] in future breeding strategies. The concept of exploiting the genetic variability for nitrogen uptake, assimilation and recycling was recently strengthened following a study in which the capacity of different maize genotypes to absorb and utilize nitrogen was tested.

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