Planta (1997) 201: 424–433

Planta

Overexpression of a soybean gene encoding cytosolic glutamine synthetase in shoots of transgenic *Lotus corniculatus* L. plants triggers changes in ammonium assimilation and plant development

Rémi Vincent[1], Vincent Fraisier[1], Sylvain Chaillou[1], M. Anis Limami[1], Eliane Deleens[2], Belinda Phillipson[1], Corinne Douat[1], Jean-Pierre Boutin[1], Bertrand Hirel[1]

[1]Laboratoire du Métabolisme et de la Nutrition des Plantes, I.N.R.A., Route de St-Cyr, F-78026 Versailles Cedex, France
[2]Laboratoire de Structure et de Métabolisme des Plantes. Institut de Biotechnologie des Plantes. Université de Paris Sud, F-91405 Orsay Cedex, France

Received: 17 July 1996 / Accepted: 16 October 1996

Abstract. A soybean cytosolic glutamine synthetase gene (*GS15*) was fused with the constitutive 35S cauliflower mosaic virus (CaMV) promoter in order to direct overexpression in *Lotus corniculatus* L. plants. Following transformation with *Agrobacterium rhizogenes*, eight independent *Lotus* transformants were obtained which synthesized additional cytosolic glutamine synthetase (GS) in the shoots. To eliminate any interference caused by the T-DNA from the Ri plasmid, three primary transformants were crossed with untransformed plants and progeny devoid of T_L- and T_R-DNA sequences were chosen for further analyses. These plants had a 50–80% increase in total leaf GS activity. Plants were grown under different nitrogen regimes (4 or 12 mM NH_4^+) and aspects of carbon and nitrogen metabolism were examined. In roots, 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. Labelling experiments using $^{15}NH_4^+$ were carried out in order to monitor the influx of ammonium and its subsequent incorporation into amino acids. This experiment showed that both ammonium uptake in the roots and the subsequent translocation of amino acids to the shoots was lower in plants overexpressing GS. It was 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 are grown on an ammonium-rich medium.

Key words: Ammonium ^{15}N labelling – *Lotus* – Senescence – Transgenic plant (development)

Introduction

Recent advances in plant molecular biotechnology combined with more-traditional physiological and biochemical approaches allow more-detailed analyses of the primary steps in inorganic nitrogen assimilation and the subsequent biochemical pathways involved in supplying nitrogen for metabolism in higher plants (Lea and Forde 1994; Temple et al. 1994). Since the discovery that both glutamine synthetase (GS; E.C.6.3.1.2) and glutamate synthase (GOGAT; E.C.1.4.7.1) play a major role in the ammonium assimilation of higher plants (Miflin and Lea 1980), a large number of studies have been made on the mechanisms controlling the expression of the genes encoding these proteins. The reaction catalysed by GS is now considered to be the major route facilitating the incorporation of inorganic nitrogen into organic molecules, in conjunction with GOGAT which recycles glutamate and incorporates carbon skeletons into the cycle for the transfer of amino groups (Joy 1988).

A large number of isoenzymes have been identified for GS. There are two groups of proteins, one group being exclusively located in plastids while the other group of enzymes is restricted to the cytosol. In addition to the dual cellular compartmentation, the relative proportions of the two groups of enzymes have been shown to be different according to the species, the physiological status of the plant, the developmental stage and the organ examined (Hirel et al. 1992a). The synthesis of chloroplastic GS was found to be regulated by light in photosynthetic organs (Lightfoot et al. 1988) while the synthesis of cytosolic GS did not seem to be influenced by external factors, except during plant senescence (Kawakami et al. 1988; Kamachi et al. 1991; Downs et al. 1994) and when ammonium was provided externally to the roots (Hirel et al. 1987).

Abbreviations: A% = atom per cent; CaMV = cauliflower mosaic virus; GS = glutamine synthetase; T_L = left border of the T-DNA; T_R = right border of the T-DNA

Correspondence to: B. Hirel; Fax: 33 (1) 30833096; E-mail: hirel@versailles.inra.fr

More-recent studies have also shown that in higher plants GS is encoded by a multigene family which is expressed in an organ-specific manner. Moreover, a species-specific pattern of gene expression could be observed according to the physiological status or the developmental stage of the plant (see Hirel et al. 1992a, for a review). The specific regulatory mechanisms controlling transcription and translation of particular GS isoforms in different cellular compartments are still not fully understood. Altering ammonium assimilation via genetic manipulation should provide information about the organization and regulation of this complex enzyme.

We have previously isolated several soybean genes encoding cytosolic GS from roots and root nodules (Marsolier et al. 1995). The transcription of one of these genes (*GS15*) was shown to be regulated by multiple and separate promoter sequences controlling organ-specific and ammonium-regulated expression (Marsolier et al. 1993). The coding region of *GS15* was linked to the cauliflower mosaic virus (CaMV) 35S promoter and then introduced into *Lotus corniculatus* plants. Measurements of various plant growth parameters, the concentration of metabolites and ions in combination with results from ^{15}N labelling experiments were used to evaluate the physiological impact of modified GS activity.

Materials and methods

Plant transformation and regeneration. Transgenic *Lotus corniculatus* L. cv Rodeo (Coopérative Ste. Christie, Montestruc, Gers, France) plants were obtained using a *Agrobacterium rhizogenes*-mediated transformation procedure (Petit et al. 1987). Surface-sterilized *L. corniculatus* seeds were germinated and grown on Murashige-Skoog (MS) medium (Murashige and Skoog 1962) without hormones. The hypocotyls of 8-d-old plants were infected with *A. rhizogenes* containing the construct *p35SGS15* (Hirel et al. 1992b). Transformed hairy-roots were selected on MS medium containing kanamycin (25 $mg \cdot ml^{-1}$) and carbenicillin (500 $mg \cdot ml^{-1}$). Regenerated shoots were transferred to MS medium containing 25 $mg \cdot ml^{-1}$ kanamycin. Rooted plants were propagated in acid-washed sand in a growth chamber at 22 °C. New plants were generated from cuttings.

Plant culture. Cuttings of *Lotus* (stems 4–5 cm long) were first grown on sand and watered daily with a complete nutrient solution containing 10 mM NO_3^- plus 2 mM NH_4^+ (Coïc and Lesaint 1971) until 2- to 3-cm long roots developed. After two weeks, 50 plants were placed on a 200-L hydroponic culture unit. This unit was kept in a growth chamber where the day/night aerial temperatures were 22 °C/19 °C with a 16-h day and 8-h night cycle. A photosynthetic photon flux density of 220 $mmol \cdot m^{-2} \cdot s^{-1}$ was provided by metal-halide lamps. During the first week of hydroponic culture the plants received the same nutrient solution as during growth on sand. For the following three weeks, nutrient solution containing 1.25 mM K^+, 0.25 mM Ca^{2+}, 0.25 mM Mg^{2+}, 1.25 mM $H_2PO_4^-$, Fe 21.5 μM (Sequestrene; Ciba-Geigy, Basel, Switzerland), 23 μM B, 9 μM Mn, 0.30 μM Mo, 0.95 μM Cu, and 3.50 μM Zn was used. Nitrogen was supplied as ammonium, either 4 mM (1 mM $NH_4H_2PO_4$ + 1 mM $(NH_4)_2SO_4$ + 1 mM NH_4Cl) or 12 mM (1 mM $NH_4H_2PO_4$ + 2.5 mM $(NH_4)_2SO_4$ + 6 mM NH_4Cl). No nitrate was present in any of these solutions. All the solution was replaced with fresh solution twice a week, and the pH was adjusted daily with 0.2 M KOH to be maintained between 5.0 and 6.0.

After three weeks, three plants of each line in each solution (4 or 12 mM NH_4^+) were harvested at 4 h into the day period and separated into shoots and roots. The samples were immediately placed in a freezer at −80 °C and later freeze-dried, weighed, ground and then stored in sealed vials until analysis.

Tissue analysis. Total soluble amino acids, free NH_4^+ and soluble carbohydrates were analyzed for the tissue samples of each plant. A 100-mg tissue sample was agitated for 1 h in 5 ml of 80% ethanol at 2 °C. After centrifugation for 10 min at 15 000 · *g* the ethanol fraction was removed and the same process was then repeated with 5 ml water. The ethanol and water fractions were combined and stored at −80 °C. Glucose, fructose and sucrose in the ethanol-water extract were determined using an enzymatic assay from Boehringer (Mannheim, Germany; Bergmeyer 1974). Soluble amino acids were determined on the same extract by a colorimetric reaction with ninhydrin (Rosen 1957). Free NH_4^+ was determined by the phenol hypochlorite assay (Berthelot reaction). A linear reaction as a function of ammonia concentration was obtained in our experimental conditions.

Amino acids were separated by a Biotronik LC5001 ion chromatograph (Maintal, Germany) with lithium citrate buffers and ninhydrin post-column derivatization. They were identified using a standard mixture of amino acids (Benson standard; PANB, Reno, Nev., USA) and quantified using the P.E. Nelson 2100 software (Perkin-Elmer, St-Quentin en Yvelines, France).

^{15}N labelling experiment. Plants were grown as described before, except that after one week of culture on the 12 mM ammonium solution they were removed from the hydroponic unit and placed for 5 min into 0.5 mM $CaSO_4$ to remove ammonium from the root surface and free spaces. The plants were then placed for 6 h in tanks (6 L for ten plants) containing an aerated solution identical to the one in the hydroponic unit except that 1.7% ammonium ions labelled with ^{15}N (supplied as $^{15}NH_4Cl$) were present.

After the labelling period, plants were again rinsed for 5 min in 0.5 mM $CaSO_4$ and they were put back in the hydroponic unit with the 12 mM $^{14}NH_4^+$ solution for a 62-h chase period. The experiment was carried out under the growing conditions described earlier.

Three plants of each line were harvested at the beginning of the labelling period, at the end of the 6-h labelling period, 9, 24, 39 h after the chase period had started, and at the end of the chase period. These plants were separated into shoots and roots, weighed, freeze-dried and reweighed.

Total concentration of N and the ^{15}N enrichment in the total N were determined on samples of dried tissue by an element analyzer (Carlo Erba-Fison Instruments NA 1500; NCS, Arcueil, France) and a mass spectrometer (VG Isotech Optima A07; Micromass France SARL, Villeurbane, France).

In the labelled plant tissue, the measured atom per cent ^{15}N (A% ^{15}N) can be expressed as:

A% ^{15}N = x · (A% ^{15}N natural) + y · (A% ^{15}N exogenous) with A% ^{15}N natural = 0.3655 (measured in unlabelled plants), A% ^{15}N exogenous = 1.6537 (measured in the initial labelled nutrient solution), x being the proportion of natural ^{15}N and y the proportion of exogenous ^{15}N in the plant tissue, x + y = 1.

For each plant tissue sample, one can calculate y, and by multiplying y by the concentration of total N in the plant tissue (per cent dry matter), the concentration of exogenous ^{15}N in the plant can be estimated. This is named ^{15}N input, and is expressed as mg $^{15}N \cdot g^{-1}$ of dry weight.

Isolation of total RNA and Northern blot analysis. Total RNA isolation and Northern blot analysis were carried out as described earlier (Becker et al. 1992). Hybridization was performed with the *Hin*cII-*Kpn*I fragment of the soybean pGS20 cDNA probe (Hirel et al. 1992b). The probe was labelled with [^{32}P]dCTP and hybridization was carried out at 65 °C in 50 mM Tris-HCl (pH 7.6), 2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll (40 000), 0.2% SDS, 0.1% sodium pyrophosphate, 6% NaCl, and

0.1 mg·ml^{-1} denatured calf thymus DNA. Final washes were performed using 0.1 × STE (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; l mM EDTA), 0.1% SDS at 65 °C, and filters were exposed to X-ray film at −80 °C.

Protein extraction, fractionation, enzymatic assay, and Western blotting. Protein extraction and Western blot analysis were carried out as described previously (Hirel et al. 1987) using tobacco GS antibodies (Hirel et al. 1984). Proteins were visualized using peroxidase-conjugated goat anti-rabbit antibodies (Towbin et al. 1979). Glutamine synthetase activity was assayed using the biosynthetic activity as described by O'Neal and Joy (1973) and proteins were quantified using the Bradford method (Bradford 1976). Diethylaminoethyl (DEAE)-Sephacel chromatography was carried out as decribed previously (Hirel et al. 1992b).

Results

Expression of the soybean cytosolic GS15 gene in primary transformants and F1 progeny. A previously made binary vector *p35SGS15* containing the constitutive 35S CaMV promoter upstream of the soybean gene encoding cytosolic GS (*GS15*; Hirel et al. 1992b) was introduced into *Lotus corniculatus* plants, and transgenic plants derived from two independent transformations were analysed. Control plants were either untransformed plants or plants transformed with a similar vector containing the 35S promoter fused to the gene encoding β-glucuronidase (*pBI121*; Jefferson et al. 1987).

The level of GS protein in the transgenic *Lotus* plants was examined by Western blot analysis using specific antibodies recognizing GS (Hirel et al. 1984). This antiserum has been used in several studies and recognizes GS1 (cytosolic GS) and GS2 (plastidic GS) in many different plant species with equal efficiency (Hirel et al. 1987, 1992b). In previous experiments, Western blot analyses were successfully used to identify transformed tobacco plants expressing the soybean cytosolic GS (Hirel et al. 1992b). Therefore, this screening procedure was also used to select primary *Lotus* transformants. Figure 1A shows a Western blot of crude protein extracts from shoots of untransformed or pBI121-transformed plants and eight independent *p35SGS15* transformants immuno-reacted with GS antibodies. It is not possible to distinguish between the endogenous *Lotus* cytosolic GS and the protein encoded by *GS15* since they have a similar molecular mass (40 kDa). In the shoots of control plants most of the GS was chloroplastic (subunit molecular mass of 45 kDa) whereas in transformed plants the cytosolic GS subunit predominated.

As the levels of heterologous GS appeared to be very similar in all the transformants three individuals were selected for further studies. The plants were pollinated manually using pollen from untransformed plants and the resulting progeny were analysed. This was done to eliminate any interference caused by either somaclonal variation arising from transformation or by the *Rol* genes (T_L- and T_R- DNA, where L and R indicate the left and right borders of the T-DNA, respectively) of *Agrobacterium rhizogenes* (Webb et al. 1994). Southern blot experiments were carried out where the fragments

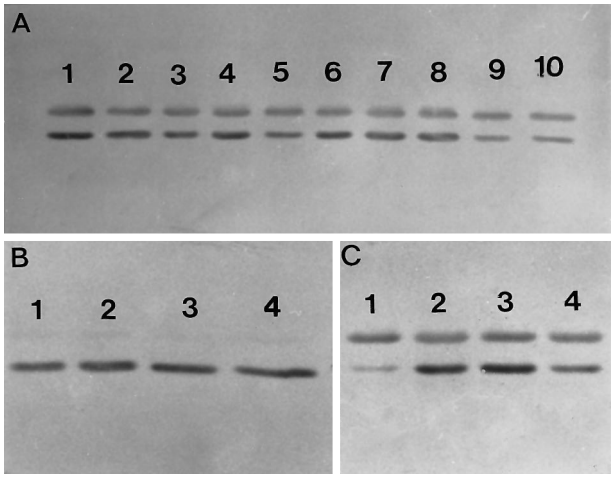

Fig. 1A–C. Western blot analysis of GS subunits in *Lotus* plants transformed with *p35SGS15*. **A** Subunits of GS in leaves of primary transformants. *Lanes 1–8*, plants transformed with *p35SGS15*; *lane 9*, plants transformed with pBI121; *lane 10*, untransformed plants. The upper band (M_r 45 kDa) corresponds to the chloroplastic GS subunits and the lower band (M_r 40 kDa) to the cytosolic GS subunits. **B,C** Subunits of GS in roots (**B**) and leaves (**C**) of the F1 *p35SGS15* plants devoid of T_L- and T_R-DNA (*lanes 2–4*). *Lanes 1* show the GS subunit composition in roots and leaves of an untransformed control plant. Twenty micrograms of soluble protein was loaded in each track

containing the T_L and T_R sequences (Jouanin et al. 1987) were used to probe plant genomic DNA (data not shown). Plants which lacked these sequences were further tested to identify those which still contained the *p35SGS15* construct and which were resistant to kanamycin. One of the plants (from each of the three crosses) which satisfied all these criteria was selected, propagated and analysed with respect to GS isoenzyme content. Southern blot analysis of the three F1 transgenic lines expressing high levels of GS showed random integration of a single copy of the *p35SGS15* construct (data not shown).

In order to avoid genetic heterogeneity in the untransformed plants, two lines of control plants were used in all the experiments. One line was randomly selected from a population of untransformed *Lotus* plants and the other line corresponded to that used for the crossing with the transgenic plants. The results presented are the mean of those obtained with the two untransformed control plants grown under the same conditions.

Figure 1B, C shows results from a Western blot analysis performed using the three F1 transformants. Comparison of the transformed and untransformed plants showed that there was a clear increase in the level of cytosolic GS protein in the shoots of the transgenic F1 plants (Fig. 1C). There appeared to be similar amounts of cytosolic GS protein in the shoots of all the transgenic plants. However, no significant differences could be detected in the levels of cytosolic GS protein found in roots of transformed and untransformed plants (Fig. 1B).

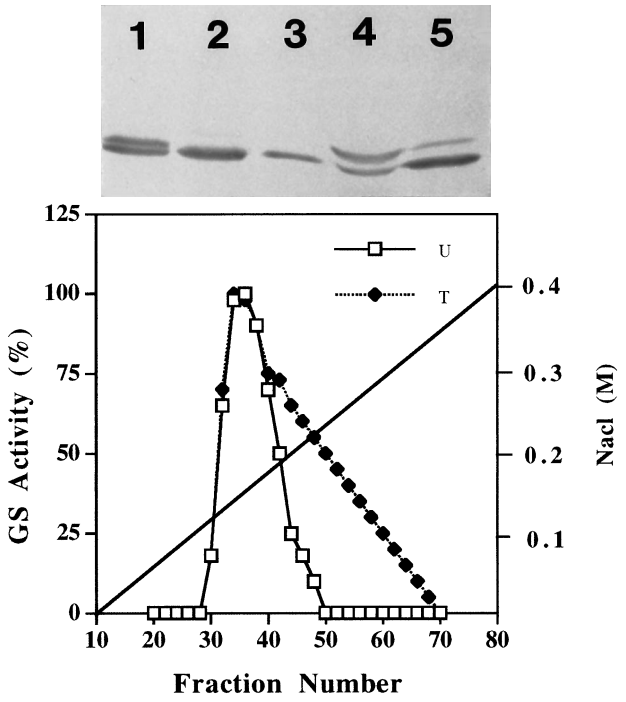

Fig. 2. Diethylaminoethyl-Sephacel chromatography of GS activity in shoot extracts from untransformed and transformed (*p35SGS15*) *Lotus* plants. Extracts from shoots of untransformed plants (*U*) and from shoots of transformed plants (*T*). The top of the figure shows a Western blot with some of the fractions containing GS activity. *Lane 1*, extract from soybean nodule; *lane 2*, additional GS activity in *p35SGS15*-transformed *Lotus* shoots (fractions 50–70); *lane 3*, additional peak of GS activity present in *p35SGS15*-transformed tobacco leaves (Hirel et al. 1992b), *lane 4*, peak of GS activity in untransformed *Lotus* shoots (fractions 30–50); *lane 5*, total GS activity in *p35SGS15*-transformed *Lotus* shoots (fractions 30–70). Twenty micrograms of soluble protein was loaded in each lane

Activity of GS in shoots of transformed *Lotus* plants (Fig. 2) was tested following DEAE-Sephacel chromatography. In shoots of untransformed control plants, a single peak with GS activity eluted at 0.15 M NaCl which corresponded to a mixture of chloroplastic (subunit MW 45 kDa) and cytosolic enzymes (subunit MW 40 kDa; Fig. 2, lane 4). In shoots of transformed plants, a shoulder of GS activity was eluted at 0.25 M NaCl. Western blot analysis of this shoulder of GS activity confirmed the presence of an additional cytosolic GS protein (subunit MW 38 kDa; Fig. 2, lane 2). The molecular weight of the additional cytosolic GS protein was similar to the 38-kDa GS subunit from soybean nodule (Hirel et al. 1987) (Fig. 2, lane 1). In a previous study we have shown that a similar cytosolic GS protein was synthesised in transgenic tobacco plants overexpressing *GS15* in shoots (Hirel et al. 1992b) (Fig. 2, lane 3). Similar results were obtained when the *p35SGS15* plants were grown on nitrate or ammonium.

A Northern blot analysis using a ^{32}P-labelled specific DNA fragment from the *GS15* gene (Hirel et al. 1992b) was carried out to measure the levels of mRNA encoding the soybean cytosolic GS in the transgenic *Lotus* plants. Figure 3 shows the amount of soybean GS

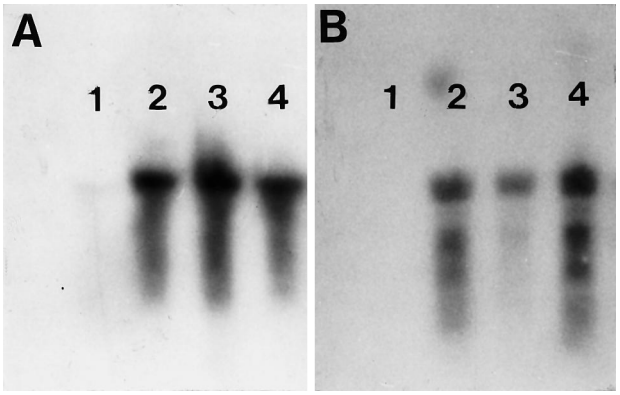

Fig. 3A,B. Expression of mRNA encoding soybean GS in the F1 progeny. **A** root total RNA; **B** leaf total RNA. *Lanes 1*, total RNA from untransformed plants; *lanes 2–4*, RNA from three independent transformants expressing *p35SGS15*

transcripts detected in roots and shoots. The soybean GS probe did not hybridize with the RNA of untransformed plants (Fig. 3, lanes 1) while in the transformed plants the GS transcript was abundant in both roots and shoots (Fig. 3, lanes 2–4)

To determine whether the presence of the GS mRNA in the transgenic *Lotus* plants leads to synthesis of active enzyme, total GS activity was measured in crude extracts from shoots and roots (Table 1). An 80% increase in total GS activity was detected in the shoots of transgenic plants in comparison with the wild type (Table 1), whereas in roots GS activity was not significantly changed (Table 1). Similar results were obtained whether GS activity was expressed on a protein-concentration or fresh-weight basis. Comparable levels of GS activity were obtained in shoots when plants were grown on nitrate plus ammonium, or ammonium (Table 1).

Physiology of the transgenic plants. To determine the physiological impact, in terms of inorganic nitrogen assimilation, of increased levels of GS activity in the cytosol of leaf cells, transformed plants were tested for the ability to assimilate reduced nitrogen when provided as ammonium.

Table 1. Total GS activity in *p35SGS15*-transformed plants. Glutamine synthetase activity was measured in shoot and roots of *Lotus* plants grown for 28 d on a complete nutrient solution containing either 10 mM NO_3^- + 2 mM NH_4^+ or 12 mM NH_4^+. U, untransformed plants; MT, mean of three transgenic lines overexpressing GS. Values are the mean ± SD of three individual plants

	GS activity [nmol·(mg FW)$^{-1}$·min^{-1}]			
	Nitrate + ammonium (10 mM + 2 mM)		Ammonium (12 mM)	
	U	MT	U	MT
Shoot	11.1 ± 0.9	19.8 ± 4.6	8.8 ± 0.9	14.9 ± 0.5
Roots	7.7 ± 0.7	7.1 ± 1.1	3.5 ± 0.6	5.6 ± 1.5

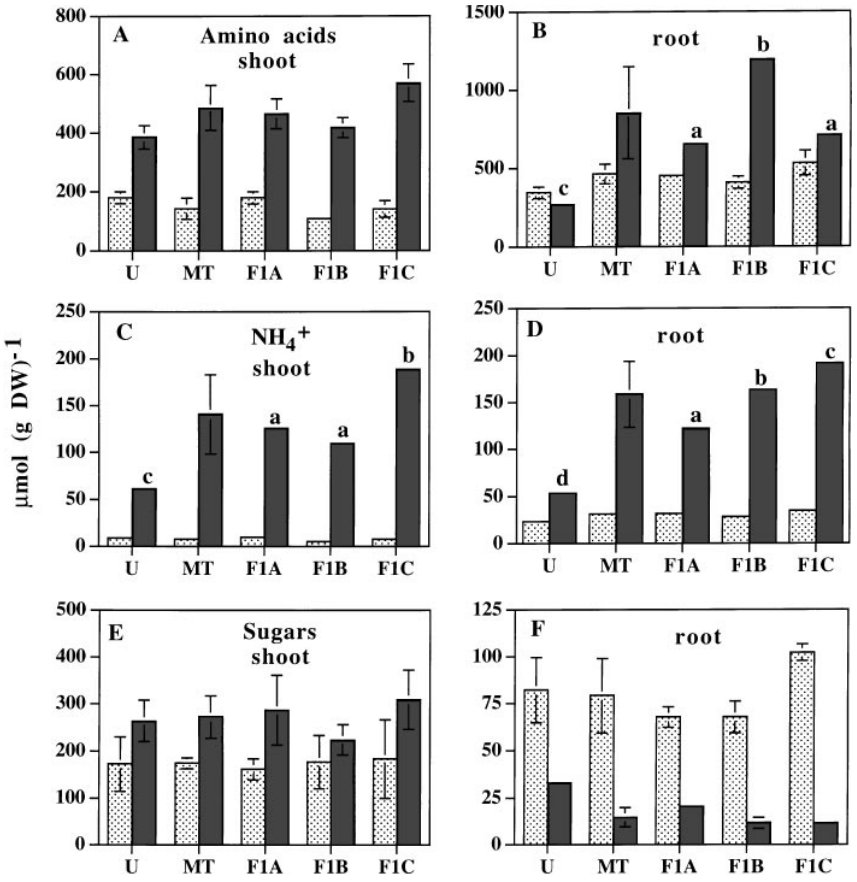

Fig. 4A–F. The physiological characteristics of *p35SGS15*-transformed plants. The concentration of amino acids in shoots (**A**) and roots (**B**), the concentration of free ammonium in shoots (**C**) and roots (**D**), and the concentration of soluble carbohydrates (glucose + fructose + sucrose) in shoots (**E**) and roots (**F**) were measured in *Lotus* plants grown for three weeks on a complete nutrient solution containing 4 mM NH_4^+ (▨) or 12 mM NH_4^+ (■) as the sole nitrogen source. *U*, untransformed plants; *MT*, mean of the three transgenic lines; *F1A, F1B, F1C*, transgenic lines overexpressing GS. Values are the mean ± SD (bars) of three individual plants. Standard deviations are not shown when they are less than the thickness of the line. Values with different letters (*a*,*b*,*c*) are significantly different according to the test of Neuman-Keuls at $P = 0.05$ ($n = 3$)

As many plant species differ with respect to ammonium tolerance (Salsac et al. 1987), untransformed *Lotus* plants were initially tested for the ability to grow under different NH_4^+ concentrations. A low concentration (4 mM) and a high concentration (12 mM) of NH_4^+ were selected for this study as both concentrations allowed normal growth and development of plants. In the initial experiments, plants were supplied with either 4 mM or 12 mM ammonium as the sole nitrogen source in the nutrient solution. No major differences were observed with respect to dry weight or total soluble protein content under either of the different nitrogen regimes used (data not shown). When both transformed plants and control plants were grown on 4 mM NH_4^+ for three weeks no marked differences were observed in any of the parameters measured with the exception of a slightly higher accumulation of amino acids in the roots of transformed plants (Fig. 4B). In contrast, after growth on 12 mM NH_4^+ there was a two- to threefold increase in the concentration of both amino acids and free ammonium in the roots of transgenic plants in comparison with the untransformed plants (Fig. 4). The increase in amino acids was caused by a two- to fourfold augmentation in asparagine (Table 2). Other amino acids such as arginine represented about 1% or less of the total. Their relative concentration was not significantly different in untransformed control plants and transformed plants. Similarly, a smaller but significant rise in the concentration of total amino acids (Fig. 4A) and free

Table 2. The concentrations and proportions of amino acids in roots of control and *p35SGS15*-transformed plants. Amino acids were separated and quantified in roots of *Lotus* plants grown for three weeks on a complete nutrient solution with 12 mM NH_4^+. U, untransformed plants; MT, mean of the three transgenic lines; F1A, F1B, F1C, transgenic lines overexpressing GS

Amino acids	Amino acid concentration [µmol (g DW)$^{-1}$] and proportion (%)				
	U	MT	F1A	F1B	F1C
Aspartate	4 (1.9)	6 (1.1)	5 (1.6)	6 (0.7)	5 (1.4)
Asparagine	139 (65.0)	366 (74.0)	251 (74.6)	585 (74.0)	264 (73.7)
Glutamate	3 (1.3)	3 (0.6)	2 (0.6)	4 (0.5)	2 (0.6)
Glutamine	19 (8.8)	42 (8.5)	13 (4.0)	84 (10.6)	30 (8.3)
Others	49 (23.0)	78 (15.8)	65 (19.2)	112 (14.2)	57 (16.0)
Total	214 (100)	495 (100)	336 (100)	791 (100)	358 (100)

Table 3. ^{15}N input at the end of the charge period in control and *p35SGS15*-transformed plants. The ^{15}N input (see *Materials and methods*) was determined in shoots and roots of *Lotus* plants after a 6-h labelling period on 12 mM $^{15}NH_4^+$ solution (A% ^{15}N = 1.7). U, untransformed plants (mean of two untransformed lines); MT, mean of the three transgenic lines; F1A, F1B, F1C, transgenic lines overexpressing GS. Values are the mean ± SD of three individual plants

Plant part	^{15}N input [$\mu g\ ^{15}N \cdot (g\ DW)^{-1}$]				
	U	MT	F1A	F1B	F1C
Shoot	3.91 ± 0.83	1.84 ± 0.60	2.06 ± 0.20	2.22 ± 0.67	1.23 ± 0.35
Roots	58.53 ± 4.62	43.63 ± 4.44	45.25 ± 5.76	44.38 ± 3.62	40.68 ± 3.85

Table 4. Shoot/root ratio of exogenous ^{15}N amount (^{15}N input × dry weight). *Lotus* plants were submitted to a 6 h labelling period on 12 mM $^{15}NH_4^+$ solution (A% ^{15}N = 1.7). *U*, untransformed plants (mean of two untransformed lines); MT, mean of the three transgenic lines; F1A, F1B, F1C, transgenic lines overexpressing GS. Values are the mean ± SD of three individual plants

Line	U	MT	F1A	F1B	F1C
Ratio	0.46 ± 0.09	0.25 ± 0.10	0.24 ± 0.02	0.34 ± 0.13	0.17 ± 0.05

ammonium (Fig. 4C) was also detected in shoot of transgenic plants. Concurrently, the concentration of soluble carbohydrates in the roots of the transformed plants was 50% lower than in roots of untransformed plants (Fig. 4F) but remained not significantly changed in the shoot (Fig. 4E).

There are at least two possible explanations to account for the increase in amino acids and free ammonium in transgenic plants grown on 12 mM NH_4^+. The ammonium uptake and assimilation could be enhanced in *p35SGS15*-transformed plants or there may be an increase in soluble protein hydrolysis.

In order to investigate the two hypotheses, ^{15}N labelling experiments were carried out. These experiments evaluate NH_4^+ uptake, assimilation and subsequent nitrogen translocation by monitoring $^{15}NH_4^+$ input/output and partitioning between roots and shoots. For this purpose, plants were grown for three weeks on a medium containing 12 mM ammonium. Plants were submitted to 6 h of charge with $^{15}NH_4^+$ (A% ^{15}N = 1.7) followed by a 62-h $^{14}NH_4^+$ chase period. At the end of the charge period the level of incorporated ^{15}N was consistently lower both in the shoot and roots of transgenic plants as compared to the untransformed plants. The concentration of ^{15}N in the shoots and roots of transgenic plants was about 50% and 25% lower, respectively, than that in control plants (Table 3). This indicates that ammonium uptake was lower in the roots of transgenic plants. Moreover, at the end of the charge period the shoot-to-root ratio of label was between 0.17 and 0.34 for the *p35SGS15* plants whereas the same ratio was 0.46 in the untransformed plants (Table 4). These results suggest that there is a slower translocation of the newly absorbed nitrogen in *p35SGS15*-transformed plants. This is further supported by results from the previous experiment where the ratios of amino acids in the shoots to the amino acids in the roots were 1.4 and 0.6 in control and transgenic plants, respectively (Fig. 4A,B). Moreover, the ammonium concentration in root exudates was very low and therefore cannot account for the large ammonium accumulation in the leaves (data not shown).

Translocation of the labelled nitrogen was then followed during a 62-h chase period. In the three transgenic lines the concentration of ^{15}N in the shoots remained low and stable during the 62-h chase period (Fig. 5). In contrast, a twofold increase in the concentration of ^{15}N was observed in the shoots of control plants during the first 8 h of the chase (Fig. 5). This result demonstrates that in untransformed plants the newly absorbed nitrogen was more rapidly translocated to the shoots than in transgenic plants. Moreover, in the roots of transgenic plants the decrease in the concentration of ^{15}N during the chase period was more rapid. During the first 8 h of the chase period, this decrease was about 40% in the control plants and about 65% in each of the three different transgenic plants (Fig. 5). The decrease in the concentration of ^{15}N in the roots of the *p35SGS15*-transformed plants was not accompanied by an increase in the concentration of ^{15}N in the shoots. The more rapid decrease in label in the roots of transgenic plants could be caused by an increase in the levels of unlabelled amino acids produced during protein breakdown in the shoots, although an enhanced efflux of $^{15}NH_4^+$ (or other ^{15}N labelled compounds) to the nutrient solution could not be ruled out. In parallel, we have also repeatedly observed that, in spite of insignificant differences in the biomass production, transgenic plants were in full bloom at the time of harvest, whereas untransformed plants were only developing floral buds (Fig. 6). This observation favours the hypothesis of accelerated protein hydrolysis since premature floral development is generally a characteristic of nitrogen remobilization in the early stage of plant senescence.

In order to compare the plants at the same developmental state (full bloom) the untransformed plants were grown on 12 mM NH_4^+ for 38 d and the transgenic plants for 28 d. The concentrations of amino acids (700 $\mu mol \cdot (g\ DW)^{-1}$) and free sugars (5–10 $\mu mol \cdot (g\ DW)^{-1}$) were the same in roots of both untransformed

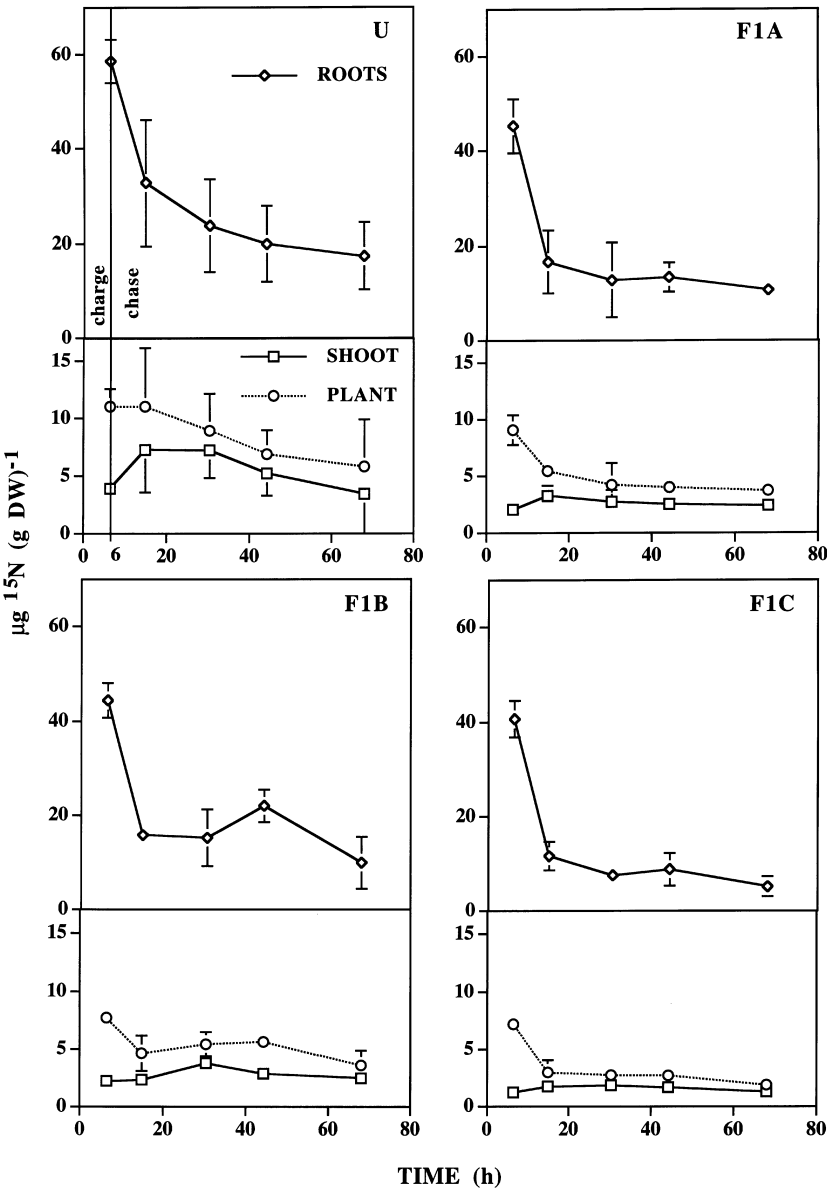

Fig. 5. Changes in the levels of ^{15}N during the 62-h chase period. *Lotus* plants were submitted to a 6-h labelling period on 12 mM $^{15}NH_4^+$ solution (A% ^{15}N = 1.7). This was followed by a 62-h chase period with 12 mM $^{14}NH_4^+$. *U*, untransformed plants (mean of two untransformed lines); *F1A*, *F1B*, *F1C*, transgenic lines overexpressing GS. Values are the mean ± SD of three individual plants

and transgenic plants. However in the shoots of untransformed plants the concentration of amino acids (150 µmol·(g DW)$^{-1}$) and free sugars (110 µmol·(g DW)$^{-1}$) were much lower than in the shoots of transgenic plants (500 and 270 µmoles·(g DW)$^{-1}$, respectively). The concentration of free ammonium was always much lower in the untransformed plants as compared to the transgenic plants at both stages of development (50 v.s. 150 µmol·(g DW)$^{-1}$).

Discussion

In order to understand the regulation of cytosolic GS in higher plants, a soybean cytosolic GS gene was expressed in *Lotus corniculatus* under the control of a strong constitutive (CaMV 35S) promoter. As already observed in a previous study where the same construct was introduced into tobacco plants, the level of expression of the soybean cytosolic GS gene was similar in several independent transformants. This is in contrast to results from some other studies where the level of expression directed by the 35S CaMV promoter was found to be rather variable (Van der Krol et al. 1990; Hobbs et al. 1993; Boerjan et al. 1994). However, although *GS15* was highly transcribed in roots and shoots an increase in GS activity was only detected in shoots. This is in contrast to results previously obtained with *p35SGS15*-transformed tobacco plants where a significant increase in GS activity and protein was also obtained in the roots (Hirel et al. 1992b). It is well established that in tobacco nitrogen reduction occurs mostly in the shoots and therefore tobacco can overcome additional GS activity in the roots (Oaks 1992). However, in temperate legumes the roots are the major sites of primary nitrogen assimilation (Oaks 1992). Therefore, *Lotus,* as a temperate legume which uses asparagine for nitrogen transport (data not shown), may

Fig. 6. Phenotypic characterization of *p35SGS15* transgenic *Lotus* plants. On the left side are F1 transgenic plants after three weeks of culture on a solution containing 12 mM NH_4^+; on the right is an untransformed plant

only be able to tolerate a certain amount of additional GS activity in the roots. This suggests that there is a post-transcriptional mechanism controlling the synthesis, assembly and activity of GS in the roots of temperate legumes. A similar conclusion has been drawn by Temple et al. (1993). These authors postulated that an unknown factor controls the steady-state level of cytosolic GS holoenzyme. When this factor is locked in the assembled form of the enzyme it is no longer available for assembling more GS subunits.

The physiological consequences of the GS overexpression were studied on *Lotus* plants grown under various nitrogen regimes. No major differences were observed with respect to amino acids, free ammonium and sugars when either *p35SGS15*-transformed plants or controls were grown on a nutrient solution containing either 4 mM NH_4^+ or a mixture of 10 mM NO_3^- and 2 mM NH_4^+ (data not shown). From the initial experiments performed to determine which nitrogen regime should be used for growing the plants, it was shown that *Lotus corniculatus* could tolerate up to 12 mM NH_4^+ in the growth medium. This is a rather high concentration in comparison to other plants (Salsac et al. 1987) and probably results from the adaptation of legumes to ammonium nutrition (Oaks 1992). Although high concentrations of ammonium have no major toxic effects on *L. corniculatus*, several changes in the concentration of amino acids, free ammonium and soluble carbohydrates can be observed. When plants were grown on 12 mM ammonium compared to plants grown on 4 mM ammonium, the increase in shoot amino acid concentration and in shoot and root ammonium concentration as well as the decrease in root carbohydrate content was more pronounced in transgenic plants than in untransformed plants. Such symptoms are characteristic of the ammonium syndrome (Mehrer and Mohr 1989; Chaillou et al. 1991). This is interpreted as a greater need for energy and carbon skeletons allowing enhanced ammonium assimilation (Chaillou et al. 1991). Although the transgenic plants grown on 12 mM NH_4^+ displayed a more pronounced ammonium syndrome than the untransformed plants, there were no repercussions on plant growth as measured by the biomass of roots and shoots. The presence of a stronger ammonium syndrome in the transgenic plants grown under high ammonium concentration could be the consequence of at least two different physiological events, one being enhanced ammonium uptake and assimilation and the other being increased soluble protein degradation. Therefore, experiments using labelled $^{15}NH_4^+$ were carried out with plants grown on 12 mM NH_4^+.

The results showed that the transgenic plants had a lower rate of ammonium uptake than the wild type, and in addition the translocation of nitrogen-containing compounds from the roots to the shoots was less efficient in the transgenic plants. This clearly demonstrates that the higher accumulation of amino acids and ammonium in the transgenic plants was not caused by an enhanced ammonium assimilation. Furthermore, the levels of label in these plants decreased more rapidly during the chase, which was probably due to increased protein and amino acid catabolism occurring in the shoots.

As the transgenic plants grown on high concentrations of ammonium developed more rapidly it could be suggested that the changes seen were not caused by the overexpression of GS. However, comparison of untransformed plants and transgenic plants at the same developmental state showed that although some of the differences seen in roots could be explained by the enhanced development there were still dissimilarities between the shoots of these plants. Thus the changes seen were caused by the overexpression of cytosolic GS in the shoots and not by accelerated development. We postulate that the enhanced expression of cytosolic GS in the shoots activated a physiological process leading to premature flowering and senescence. This provided further evidence to suggest that there is increased protein catabolism since nitrogen is remobilized in the cytosol during leaf senescence (Kamachi et al. 1991). It has been well established that accumulation of amides such as glutamine or asparagine is the primary means of coping with high levels of ammonium generated during leaf

senescence. At this time, large amounts of ammonium are produced as the soluble amino acids released by proteolysis are deaminated (Givan 1979). Watanabe et al. (1994) have shown that artificially increasing the glutamine concentration of radish cotyledons grown in the dark induces senescence and also enhances accumulation of cytosolic GS transcripts. Moreover, in a number of other reports a specific increase in the transcription and synthesis of cytosolic GS genes has been observed in the shoots during plant senescence (Kamachi et al. 1991; Bernhard and Matile 1994). This is in contrast with fully developed and photosynthetically active shoots of the same species where only high levels of chloroplastic GS could be detected (Kamachi et al. 1991). These authors postulated that during senescence cytosolic GS was specifically synthesized in order to produce glutamine which was translocated via phloem transport to the developing sink tissues, including the reproductive organs.

However, in the experiments presented here, accelerated development could only be observed when plants were grown with ammonium as the sole nitrogen source. As a relatively high concentration of ammonium was required to produce this effect, it remains to be determined whether ammonium per se and/or changes in the amino acid concentration or carbon/nitrogen balance in the cytosol (Feller and Fisher 1994) triggered these changes. It is more difficult to interpret the decrease in ammonium uptake seen in the transgenic plants grown on 12 mM NH_4^+. It can be suggested that this limited uptake of ammonium may be a consequence of the accelerated development leading to asparagine accumulation during senescence. This hypothesis is in agreement with data published by Lee et al. (1992) who observed that raising the intracellular concentration of glutamine and/or asparagine leads to a supression of net ammonium uptake. As suggested by other authors, it could also be due to an enhanced accumulation of free amino acids and ammonium (Rideout et al. 1994).

Further studies are also being carried out in order to investigate the physiological events resulting from changes in GS activity in different organs and tissues. Recently, in-situ immuno-localizations combined with molecular studies showed that GS is expressed in a number of different cell types, including phloem companion cells (Carvalho et al. 1992) anthers (Marsolier et al. 1993) and nodules. Studies are also being done to direct overexpression of GS in different organs using tissue-specific promoters. The present study was an initial attempt to examine more precisely the physiological consequences of a shift in GS cellular compartmentation. This study has demonstrated that genetic manipulation may help to unravel the fine regulatory mechanisms controlling inorganic nitrogen assimilation and nitrogen use efficiency in plants.

We thank Elisa Carrayol and François Gosse for assistance in tissue and plant culture. This work was funded in part by the European Communities BIOTECH programme, as part of the Project of Technological Priority 1993–1996.

References

Becker TW, Foyer C, Caboche M (1992) Light-regulated expression of the nitrate-reductase and nitrite-reductase genes in tomato and in the phytochrome-deficient *aurea* mutant of tomato. Planta 188: 39–47

Bergmeyer HU (1974) Methods of enzymatic analysis, vol 3. Academic Press, New York

Bernhard WR, Matile P (1994) Differential expression of glutamine synthetase genes during the senescence of *Arabidopsis thaliana* rosette shoots. Plant Sci 98: 7–14

Boerjan W, Bauw G, Van Montagu M, Inzé D (1994) Distinct phenotypes generated by overexpression and supression of S-adenosyl-L-methionine synthetase reveal developmental patterns of silencing in tobacco. Plant Cell 6: 1401–1414

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Carvalho E, Pereira S, Sunkel C, Salema R (1992) Detection of a cytosolic glutamine synthetase in leaves of *Nicotiana tabacum* L. by immunocytochemical methods. Plant Physiol 100: 1591–1594

Chaillou S, Vessey JK, Morot-Gaudry JF, Raper CD Jr, Henry LT, Boutin JP (1991) Expression of characteristics of ammonium nutrition affected by pH of the root medium. J Exp Bot 42: 189–196

Coic Y, Lesaint C (1971) Comment assurer une bonne nutrition en eau et en ions minéraux en horticulture. Hortic Française 8: 11–14

Downs CG, Borst WM, Hurst PL, Stevenson DG (1994) Isoforms of glutamine synthetase in asparagus spears: the cytosolic enzyme increases after harvest. Plant Cell Environ 17: 1045–1052

Feller U, Fisher A (1994) Nitrogen metabolism in senescing leaves. Crit Rev Plant Sci 13: 241–273

Givan CV (1979) Metabolic detoxification of ammonium in higher plants. Phytochemistry 18: 375–382

Hirel B, Weatherley C, Cretin C, Bergounioux D, Gadal P (1984) Multiple subunit composition of chloroplastic glutamine synthetase of *Nicotiana tabacum* L. Plant Physiol 74: 448–450

Hirel B, Bouet C, King B, Layzell D, Jacobs F, Verma DPS (1987) Glutamine synthetase genes are regulated by ammonium provided externally or by symbiotic nitrogen fixation. EMBO J 6: 1167–1171

Hirel B, Miao GH, Verma DPS (1992a) Metabolic and developmental control of glutamine synthetase genes in legume and non-legume plants. In: Verma DPS (ed) Control of plant gene expression. CRC Press, Boca Raton, Fl., pp 443–458

Hirel B, Marsolier MC, Hoarau A, Hoarau J, Branjeon J, Schafer R, Verma DPS (1992b) Forcing expression of a soybean root glutamine synthetase gene in tobacco leaves induces a native gene encoding cytosolic enzyme. Plant Mol Biol 20: 207–218

Hobbs SLA, Warkentin TD, DeLong CMO (1993) Transgene copy number can be positively associated with transgene expression. Plant Mol Biol 21: 17–26

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907

Jouanin L, Guerche P, Pamboukdjian C, Tourneur C, Casse-Delbart F, Tourneur J (1987) Structure of T-DNA in plants regenerated from roots transformed by *Agrobacterium rhizogenes* strain A4. Mol Gen Genet 206: 387–392

Joy KW (1988) Ammonia, glutamine, and asparagine: a carbon-nitrogen interface. Can J Bot 66: 2103–2109

Kamachi K, Yamaya T, Mae T, Ojima K (1991) A role of glutamine synthetase in the remobilisation of leaf nitrogen during natural senescence in rice leaves. Plant Physiol 96: 411–417

Kawakami N, Watanabe A (1988) Senescence-specific increase in cytosolic glutamine synthetase and its mRNA in radish cotyledons. Plant Physiol 88: 1430–1439

Lea PJ, Forde BJ (1994) The use of mutants and transgenic plants to study amino acid metabolism. Plant Cell Environ 17: 541–556

Lee RB, Purves JV, Ratcliffe RG, Saker LR (1992) Nitrogen assimilation and the control of ammonium and nitrate absorption by maize roots. J Exp Bot 43: 1385–1396

Lightfoot DA, Green NK, Cullimore JV (1988) The chloroplast-located glutamine synthetase of *Phaseolus vulgaris* L.: nucleotide sequence, expression in different organs and uptake into isolated chloroplasts. Plant Mol Biol 11: 191–202

Marsolier M, Carrayol E, Hirel B (1993) Multiple functions of promoter sequences involved in organ-specific expression and ammonium regulation of a cytosolic glutamine synthetase gene in transgenic *Lotus corniculatus*. Plant J 3: 405–414

Marsolier MC, Debrosses G, Hirel B (1995) Identification of several soybean cytosolic glutamine synthetase transcripts highly or specifically expressed in nodules: expression studies using one of the corresponding genes in transgenic *Lotus corniculatus*. Plant Mol Biol 27: 1–15

Mehrer I, Mohr H (1989) Ammonium toxicity: description of the syndrome in *Sinapis alba* and the search of its causation. Physiol Plant 77: 545–554

Miflin BJ, Lea PJ (1980) Ammonia assimilation. In: Miflin B J (ed) The biochemistry of plants, vol. 5. Academic Press, New York, pp 169–202

Murashige J, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 115: 473–497

Oaks A (1992) A re-evaluation of nitrogen assimilation in roots. Bioscience 42: 103–110

O'Neal D, Joy KW (1973) Glutamine synthetase in pea leaves. I. Purification, stabilization, and pH optima. Arch Biochem Biophys 159: 113–122

Petit A, Stougaard J, Kühle A, Marcker KA, Tempé J (1987) Transformation and regeneration of the legume *Lotus corniculatus*: a system for molecular studies of symbiotic nitrogen fixation. Mol Gen Genet 207: 245–250

Rideout JW, Chaillou S, Raper CD, Morot-Gaudry JF (1994) Ammonium and nitrate uptake by soybean during recovery from nitrogen deprivation. J Exp Bot 45: 23–33

Rosen H (1957) A modified ninhydrin colorimetric analysis for amino acids. Arch Biochem Biophys 67: 10–15

Salsac L, Chaillou S, Morot-Gaudry JF, Lesaint C, Jolivet E (1987) Nitrate and ammonium nutrition in plants. Plant Physiol Biochem 25: 805–812

Temple SJ, Knight TJ, Unkefer PJ, Sengupta-Gopalan S (1993) Modulation of glutamine synthetase gene expression in tobacco by the introduction of an alfalfa glutamine synthetase gene in sense and antisense orientation: molecular and biochemical analysis. Mol Gen Genet 236: 315–325

Temple SJ, Bagga S, Sengupta-Gopalan S (1994) Can glutamine synthetase activity be modulated in transgenic plants by the use of recombinant DNA technology? Transgenic plants and biochemistry. Biochem Soc Trans 22: 915–920

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc Nat Acad Sci USA 76: 4350–4354

Van der Krol AR, Mur LA, Beld M, Mol JA, Stuije A (1990) Flavonoid gensin *Petunia*: addition of a limited number of gene copies may lead to supression of gene expression. Plant Cell 2: 291–299

Watanabe, Hamada K, Yokoi H, Watanabe A (1994) Biphasic and differential expression of cytosolic glutamine synthetase genes of radish during seed germination and senescence of cotyledons. Plant Mol Biol 26: 1807–1817

Webb KJ, Robbins MP, Mizen S (1994) Expression of GUS in primary transformants and segregation patterns of GUS, T_L- and T_R-DNA in the F1 generation of hairy root transformants of *Lotus corniculatus.* Transgenic Res 3: 232–240