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Molecular cloning and characterisation of asparagine synthetase from *Lotus japonicus*: Dynamics of asparagine synthesis in N-sufficient conditions

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Abstract

Two cDNA clones, LJAS1 and LJAS2, encoding different asparagine synthetases (AS) have been identified and sequenced and their expression in *Lotus japonicus* characterised. Analysis of predicted amino acid sequences indicated a high level of identity with other plant AS sequences. No other AS genes were detected in the *L. japonicus* genome. LJAS1 gene expression was found to be root-enhanced and lower levels of transcript were also identified in photosynthetic tissues. In contrast, LJAS2 gene expression was root-specific. These patterns of AS gene expression are different from those seen in pea. AS gene expression was monitored throughout a 16 h light/8 h dark day, under nitrate-sufficient conditions. Neither transcript showed the dark-enhanced accumulation patterns previously reported for other plant AS genes. To evaluate AS activity, the molecular dynamics of asparagine synthesis were examined *in vivo* using ^{15}N-ammonium labelling. A constant rate of asparagine synthesis in the roots was observed. Asparagine was the most predominant amino-component of the xylem sap and became labelled at a slightly slower rate than the asparagine in the roots, indicating that most root asparagine was located in a cytoplasmic 'transport' pool rather than in a vacuolar 'storage' pool. The steady-state mRNA levels and the ^{15}N-labelling data suggest that light regulation of AS gene expression is not a factor controlling N-assimilation in *L. japonicus* roots during stable growth in N-sufficient conditions.

Introduction

In temperate legumes, in contrast to many other plant species, asparagine, rather than glutamine, is the principal molecule used to transport reduced nitrogen within the plant [1, 22]. This is the case in *Lotus japonicus* [7] where asparagine can account for 86% of the nitrogen transported from

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X89409 (LJAS1) and X89410 (LJAS2).

root to shoot, if the plant receives a nitrate supply compatible with its growth rate [12]. In such circumstances, the primary site of asparagine synthesis is the root and it follows that leaves receive asparagine as the principal N-source for amino acid and protein synthesis [20].

Asparagine provides a more economic means for transporting nitrogen than does glutamine, due to its lower N:C atomic ratio (2:4 for asparagine and 2:5 for glutamine), and it has been proposed that this may make it a preferred nitrogen transport and/or storage compound under conditions where the carbon supply may be limited [12, 24, 29], for example, in the dark [11, 27, 28]. In the non-legume *Arabidopsis thaliana*, it has been proposed that asparagine synthetase (AS) may directly reflect physiological conditions (light and dark) [11] and, therefore, may serve as an important switching enzyme, sensing changes in carbon availability and controlling the metabolic flow of nitrogen. However, severe physiological treatments were used to evoke the observed changes in AS expression, such as growth in prolonged periods of continuous darkness (2–5 d) (i.e. dark-adapted plants) or growth on relatively high exogenous sucrose concentrations (3–5%); thus it could not be concluded whether this phenomenon would occur in a more natural diurnal cycle [11]. A dark-enhanced expression of the AS1 gene has been demonstrated to occur during a normal light/dark cycle in pea leaves [28] but AS expression in the roots of these plants was not described. In asparagus [4], experiments were conducted under natural environmental field conditions and asparagine content and AS mRNA abundance were found to increase in harvested spears, in a manner which was insensitive to light but which paralleled the decline in sucrose content of the spears, supporting the view that AS is regulated by the N:C ratio.

In general, a positive regulation of AS genes by darkness, with expression being specifically repressed during illumination, seems an illogical way for plants such as *L. japonicus*, which utilise asparagine as their major N-transport molecule, to undertake N assimilation, since nitrate reduction would be maximal in the light when AS activity would be at a minimum. The properties of AS have proved very difficult to study because it has been found to be highly unstable in tissue extracts [8]. Co-isolation of AS inhibitors [10] and catabolism of asparagine by asparaginase [25] also hamper AS characterisation. To circumvent these difficulties, we report an *in vivo*-labelling technique, using ^{15}N-ammonium, which gives unequivocal evidence about the actual rate of asparagine synthesis and which must, therefore, reflect the actual diurnal activity of AS. The results obtained are compared with the abundance of mRNAs from the transcription of the two AS genes. We describe a model system for the integrated molecular physiological study of N assimilation and transport during stable growth in *L. japonicus*.

Materials and methods

Plant material and growth conditions

Seeds of *L. japonicus* cv. Gifu (GIFU B-129-S9) [7] were obtained from Dr J. Stougaard, Aarhus University, Denmark. Initial plant material used as source for the construction of a cDNA library was grown hydroponically. Seeds were surface-sterilised and germinated on a fine mesh supported over the surface of a large tank holding 30 l of a dilute nutrient solution, in which nitrogen was supplied as nitrate (10 mM) and which was supplemented with minor elements [13]. The nutrient solution was replaced at weekly intervals and aerated throughout plant growth. Plants were grown hydroponically in growth chambers under a 16 h/8 h light/dark regime and 23 °C light and 17 °C dark temperatures.

Plants used for the characterisation of AS expression and asparagine synthesis were grown in an aeroponic mist system, established in an air-cooled greenhouse (23 °C day and 18 °C night with supplementary lighting between 05.00 and 21.00). Short stem cuttings comprising an apical bud and 3–5 cm of stem were taken from established plants. The lower leaves were stripped off and the basal end of each cutting was inserted into sterilised sand wetted with culture solution

and contained in standard 50 cm × 30 cm PVC seed trays. The use of sterilised sand for rooting prevented nodulation of *L. japonicus* roots. The trays were kept in a growth chamber at 20 °C light and 18 °C dark and were covered with transparent hoods to maintain humidity. A high proportion of the cuttings developed roots within 2–3 weeks. Rooted cuttings were carefully removed from the sand, their roots were rinsed in deionised water and the plantlets were inserted individually into 10 cm × 10 cm white opaque polycarbonate plastic holders and held in place with a small collar of autoclaved polyurethane foam. The holders were then positioned on the perforated lid of a 500 l mist tank. The mist tank contained ca. 42 l of culture solution and two spinning disc humidifiers. The solution was held at ca. 5 °C lower than ambient temperature by pumping cold glycol through two stainless steel coils immersed in the solution. Within two weeks of transfer, the cuttings arrived at a stable shoot/root biomass ratio, at which point they were judged to be suitable for sampling and labelling studies.

Preparation of L. japonicus *cDNA library*

Lotus japonicus roots (25 g) were harvested (10.00) from the hydroponic system (rinsed, blotted dry on tissue paper) and rapidly frozen in liquid nitrogen before being stored at −80 °C. The root material was ground under liquid nitrogen and RNA which was essentially free of contaminating DNA was prepared using an acid SDS-phenol extraction [2]. Ethanol precipitated RNA was resuspended in 4 ml TE buffer [21] and poly(A)$^+$ mRNA was recovered using oligo (dT) cellulose as described by the manufacturer (Boehringer). Purified mRNA was quantified spectrophotometrically.

A cDNA library was prepared with 5 μg mRNA as substrate for the Stratagene Uni-ZAP cDNA synthesis system. The RNA was heated at 68 °C for 5 min and chilled on ice prior to the first strand synthesis reaction to denature possible secondary structures in the mRNA. After packaging (Stratagene, Gigapack II) of 20% of the ligated product, a library of 2 × 10^6 plaque-forming units (pfu) was obtained with *E. coli* XL1 Blue MRF′. The size of the cDNA inserts from phage isolated from a random selection of 10 plaques was evaluated by PCR amplification using the universal forward and reverse primers.

Screening of cDNA library

About 500 000 pfu were plated out for screening. Duplicate plaque lifts using Zetaprobe GT membrane (BioRad) were performed and filter fixation followed standard protocols [20]. Filters were pre-hybridised and hybridised overnight (60 °C) in 6× SSC (sodium chloride-sodium citrate buffer [21]), 5× Denhardt's solution [21] and 0.5% sodium dodecyl sulphate (SDS). The library was screened with a full-length asparagus AS cDNA (pTIP27) [4]. The filters were washed initially in 2× SSC, 1% SDS (2 × 15 min at 50 °C) and then in 0.8× SSC and 0.1% SDS (2 × 15 min at 55 °C). Secondary and tertiary screening of putative positive plaques followed standard procedures [21], under the hybridisation conditions specified above, and the cDNA-containing pBluescript SK + phagemids from the 12 positive phages identified after the tertiary screen were excised according to the manufacturer's instructions. The plasmids were restriction-mapped and two cDNA clones, LJAS1 and LJAS2 (originally identified as pRASP7 and pRASP17 during initial screening procedures), representing the longest of each of two classes of AS insert identified, were selected for DNA sequencing (Pharmacia ^{T7}system). Computer alignment of the deduced amino acid sequences was performed using the Genetics Computer Group (GCG) PILEUP program.

Characterisation of AS genes

Genomic DNA was prepared from *L. japonicus* leaves using CTAB-NaCl purification [17]. Restricted DNA (5 μg) was electrophoresed in agarose gels (0.8%) in a Tris-HCl pH 7.7/acetate

buffer [21] and transferred to Zetaprobe GT membranes. Duplicate filters were screened under low and high stringency hybridisation conditions with either the LJAS1 or LJAS2 ^{32}P-labelled insert. Filters were hybridised overnight [3] in 250 mM Na_2HPO_4 pH 7.2 and 7% SDS at 50 °C. Filters were washed initially in 250 mM Na_2HPO_4 pH 7.2 and 5% SDS (2 × 15 min at 50 °C) and then 2× SSC and 0.5% SDS (1 × 10 min at 50 °C) and for low-stringency conditions, the final wash was in 1× SSC and 0.5% SDS (1 × 15 min at 50 °C). For high-stringency conditions, filters were subsequently washed in 0.1× SSC and 0.1% SDS (2 × 10 min at 65 °C). The sizes of major hybridising bands were calculated from the relative mobilities of ethidium bromide-stained *Hin*dIII-digested Lambda DNA fragments.

Expression of AS genes

RNA was isolated from *L. japonicus* leaves and roots using a modified version of the procedure described by Verwoerd *et al.* [30]. Typically, a ratio of 8 ml extraction buffer/phenol and 4 ml chloroform/isoamyl alcohol (24:1) was used to extract RNA from 2 g ground, frozen tissue. RNA concentrations were estimated spectrophotometrically. RNA (10 μg) for northern blots was denatured using glyoxal and electrophoresed in agarose (0.9%) gels using a Bes buffer [6]. RNA was transferred to Zetaprobe GT membranes by capillary transfer and fixed by baking for 2 h (80 °C). To visualise rRNA and to size hybridising messages, filters were stained with Methylene blue as described by Sambrook *et al.* [21], immediately prior to hybridisation. Confirmation of rRNA and transcript sizes was obtained using standard RNA markers (IBI). Filters were hybridised overnight with ^{32}P-labelled AS cDNA inserts in 250 mM Na_2HPO_4 pH 7.2 and 7% SDS at 65 °C. Both full-length cDNA inserts and the 3′ end of each cDNA were used to probe northern blots, under conditions of high stringency: Southern hybridisations had previously indicated that the full-length cDNA sequences could be used discretely under high-stringency conditions, but as an added precaution for northern hybridisations the 3′ ends of the two AS cDNAs were used as gene-specific probes. Washing was performed at 65 °C for 10 min in each of the following solutions: (a) 250 mM Na_2HPO_4 and 5% SDS; (b) 2× SSC and 1% SDS; (c) 1× SSC and 0.5% SDS and (d) 0.2× SSC and 0.1% SDS. The specificity of the hybridisation conditions used, for the detection of either LJAS1 or LJAS2 transcripts, is demonstrated by the failure of the LJAS2 probe to detect LJAS1 transcripts in leaf RNA preparations. As a control, hybridisations were also performed using *L. japonicus* root-enhanced (cytosolic form) glutamine synthetase (GS1), nitrate reductase (NR) DNA probes (unpublished work) and a soybean ubiquitin cDNA (pUSB6) [5]. Control hybridisations were performed at low stringency (58 °C hybridisation and washing and a final wash in 0.8× SSC and 0.1% SDS). In the case of the GS probe, this permitted the additional detection of leaf GS transcripts, which were not detected at higher stringencies.

Labelling experiments

Since the roots of individual plants in the mist culture did not intermingle it was possible to transfer individuals from the bulk culture to experimental systems with a minimum of physical disturbance. Plants in their holders were placed over individual glass beakers containing ca. 75 ml of the following solution: 450 mM $MgSO_4$; 300 mM KH_2PO_4; 300 mM KCl; 40 mM Fe-EDTA; 450 mM $Ca(NO_3)_2$; Minors [13]; 3000 mM $^{15}NH_4Cl$; 3000 mM MES/Tris pH 6.0.

The solution was gently aerated and kept at 25 °C by immersion in a water bath. During the diurnal cycle, plants were labelled at ambient light and temperature conditions. At night time, all operations were performed under green safelights. After a predetermined period, roots were rinsed for approximately 60 s in unlabelled solution, blotted dry on absorbent paper (30 s) and the fresh weight of shoots and roots determined. The

shoot material was frozen and transferred to a freeze-drier. Root material was treated with 10 vols of ice-cold methanol/chloroform/water (20:5:3) and transferred to −25 °C for ca. 24 h. The extract was allowed to warm up to about 10 °C, vortexed for 60 s and the root residue was washed with solvent. The washings were discarded but the residue was dried at 80 °C overnight and its dry weight, total N and ^{15}N content determined. This procedure gave a measure of the amount of ^{15}N which had entered the pool of insoluble reduced N in the root tissue. Aliquots of extracts (3 ml equivalent to 300 mg fresh weight root) were mixed first with 0.9 ml chloroform and then with 0.6 ml of water which allowed the chloroform and aqueous methanol phases to separate. The aqueous phase was analysed for its amino acid composition by HPLC using norleucine as internal standard. Samples for GC-MS analysis were dried down under vacuum and *N*(*O*)-dimethyl-*tert*-butylsilyl (MTBSTFA) derivatives prepared, as described [14] and mass peaks detected on a spectrometer (Kratos). The total N and ^{15}N of extracts was measured by stable isotope ratio analysis (SIRA) using a mass spectrometer (Europa Scientific, Tracermass) with an automated sample changer and combustion system (Carlo Erba, Strumentazione).

Analysis of xylem sap

Samples were analysed as three replicates. Shoots were cut off below the first set of leaves. Glass capillary tubes were placed onto the lower part of the cut stems and sap was collected at intervals. The incorporation of ^{15}N into asparagine in the sap was measured using GC-MS. Sap weights and amino acid pool sizes were determined as above.

Results

Isolation of cDNA clones encoding AS

A cDNA library was constructed in bacteriophage λZAPII using poly(A)$^+$ mRNA isolated from the roots of nitrate-induced *L. japonicus* plants. Following screening of ca. 500000 independent plaques with an asparagus AS cDNA [4], 12 clones were isolated which could be sub-divided by restriction mapping into two classes, of which LJAS1 and LJAS2 were found to contain full-length AS coding sequences representing the longest members of each of these two classes. The open reading frame (ORF) in both LJAS1 and LJAS2 was 1761 nucleotides, whilst the cDNAs had lengths of 2053 and 2187 nucleotides, respectively.

The predicted amino acid sequences of the two *L. japonicus* cDNA clones were aligned with other published plant AS sequences. A high level of identity was detected between the two *L. japonicus* AS peptide sequences (86.5% identity and 92.8% similarity) and with other plant AS sequences (Table 1, Fig. 1), most markedly with those isolated from another legume, pea [27]. In particular, where specific amino acid variations were detected in the *L. japonicus* AS sequences, the alternative amino acids predicted in LJAS2 frequently had the same variant as that reported for the pea AS1 polypeptide (Fig. 1; Table 1). The *purF*-type glutamine-binding domain (a cysteine/histidine/aspartate triad: C, Cys^1; D, Asp^{29}; H, His^{101}) [11, 16] was identified at the N-terminus of both *L. japonicus* AS polypeptides and in addition, the first four amino acids at the N-terminus were also conserved, thus indicating that both LJAS1 and LJAS2 probably encode glutamine-dependent AS enzymes. The C-terminal regions of LJAS1 and LJAS2 (last 31 amino acids) were found to exhibit greater variation; however, again a higher identity was detected between C-terminal AS sequences from *L. japonicus* and pea (Fig. 1),

Table 1. Percentage amino acid identities (similarities) among plant AS sequences.

	LJAS1	LJAS2	asn1_pea	asn2_pea
LJAS1				
LJAS2	86.5 (92.8)			
asn1_pea	86.1 (92.1)	89.4 (95.6)		
asn2_pea	90.1 (94.7)	85.4 (93.1)	85.4 (91.6)	
asn1_aspof	82.8 (90.5)	82.0 (90.4)	80.9 (88.7)	81.1 (88.9)
asn1_arath	83.0 (89.9)	81.8 (90.4)	82.0 (90.1)	81.8 (88.7)

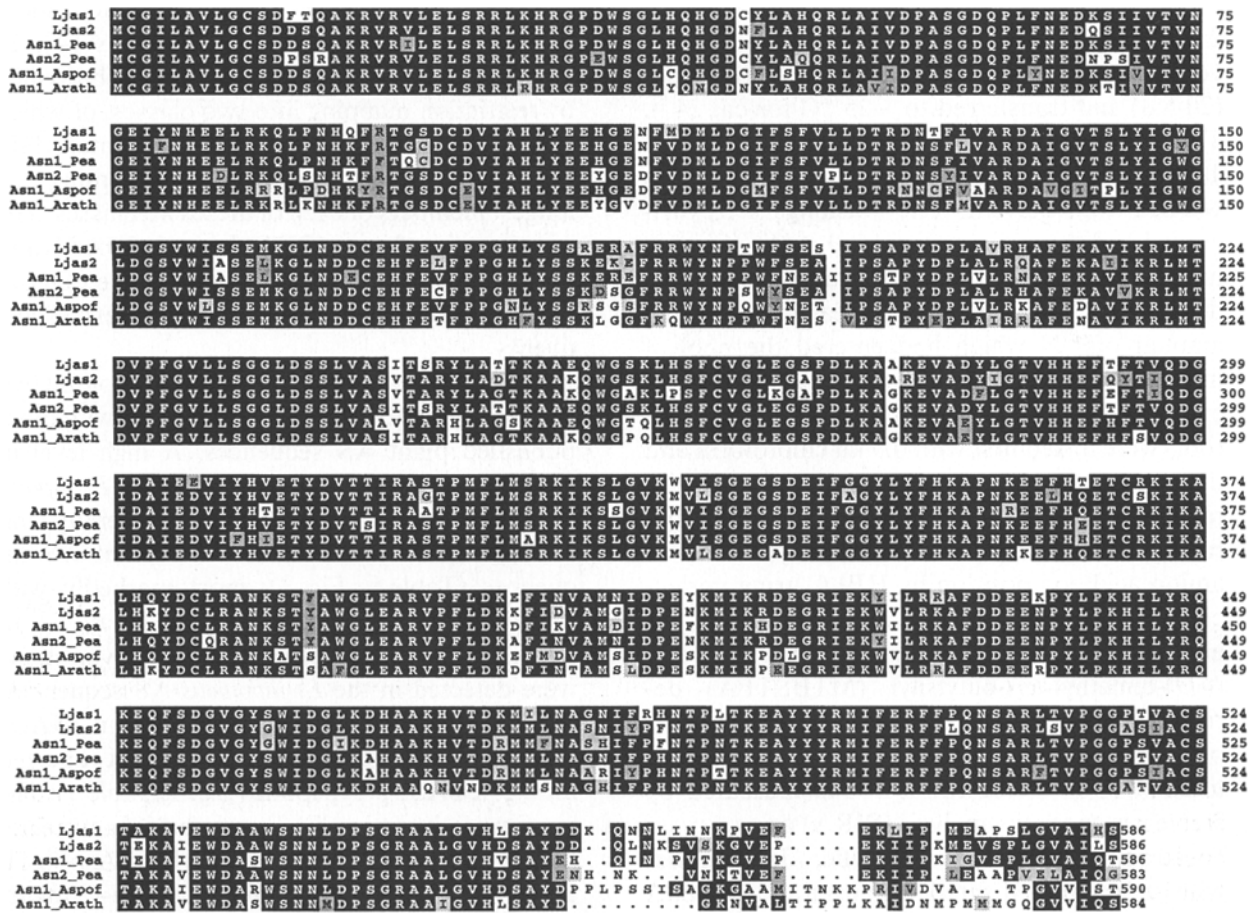

Fig. 1. Comparison of plant AS protein sequences. Amino acid alignment was performed using the Genetics Computer Group (GCG) PILEUP program. Black shading indicates identical amino acids and grey shading indicates conservative substitutions. The sources of AS polypeptide sequences used in this comparison are: LJAS1 *L. japonicus* AS1 (this work); LJAS2, *L. japonicus* AS2 (this work); Asn1-arath, *Arabidopsis* ASN1 (11); Asn1-aspob, asparagus (4); Asn1-pea, pea AS1 (27); Asn2-pea, pea AS2 (28).

than with those from non-legume species (for example, *Arabidopsis* and asparagus).

In the 3′-untranslated region (UTR) of LJAS2, two *cis*-acting elements which may control message processing and/or stability were identified. A putative polyadenylation sequence, AATAAT, was detected 23 bp upstream of the adenylated tail. A putative upstream accessory element, the TTGTA motif described by Rothnie *et al.* [19], was also identified in LJAS2 located at −53 upstream of the polyadenylated tail. In contrast, in LJAS1, no putative polyadenylation sequence or upstream motifs could be identified in the 3′-UTR.

L. japonicus *AS genes*

Southern hybridisations were performed using full-length cDNA inserts from LJAS1 and LJAS2 as probes under both low and high stringency conditions (Fig. 2). Under low-stringency conditions, both probes gave a complex pattern of overlapping bands, those observed to give a strong hybridisation signal with one probe showing a weak signal with the other probe. The hybridisation patterns observed under low stringency were a composite of the hybridisation patterns obtained for the two probes under high stringency conditions, and were in agreement with restriction

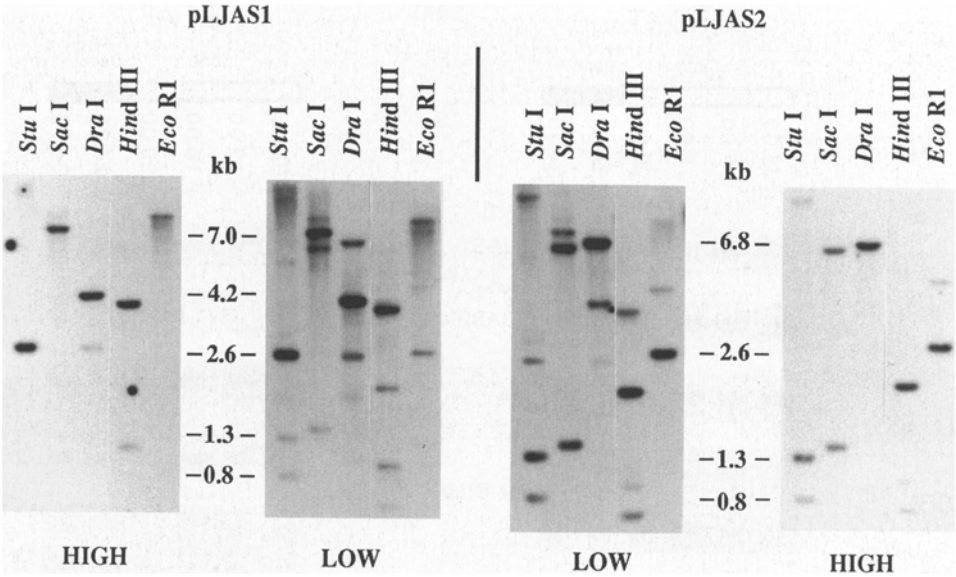

Fig. 2. Genomic Southern blot analysis of *L. japonicus* AS genes. The two *L. japonicus* cDNAs, LJAS1 and LJAS2, were hybridised to restricted *L. japonicus* genomic DNA, under low- or high-stringency conditions (see Materials and methods). The range of restriction enzymes used is indicated at the top of the figure. Hybridised filters were exposed to X-ray film for 16 h (low stringency) and 48 h (high stringency). The sizes of major hybridising DNA bands are shown and were calculated from the relative mobility of a lambda/*Hin*dIII DNA standard.

mapping data of the two clones. Moreover, the fact that all of the AS cDNA clones obtained could be classed in one of these two groups further indicated that LJAS1 and LJAS2 were each encoded by a single gene.

Expression of AS genes in L. japonicus

Northern blot analyses were performed on RNA isolated from *L. japonicus* tissues. Organ-specific differences in the accumulation of both AS gene transcripts (2.2 kb) were detected, with the greatest proportion of both AS mRNAs being located in the roots (Fig. 3A). Transcripts derived from the LJAS1 cDNA were also detected in green photosynthetic tissues, with the abundance of the message being higher in the leaf than in the stem. No transcripts were detected by the LJAS2 probe in either stem or leaf tissues, even after extended exposures of the hybridised filters to film or by using higher loadings of RNA (15 μg). Hybridisation of the LJAS2 cDNA to nodulated root tissue was greater than that observed for the LJAS1 cDNA. As a control, the expression of the AS genes was compared to that of other N-assimilatory genes, for example, nitrate reductase (NR) and glutamine synthetase (cytosolic form GS1). Root-enhanced expression of both of these genes was also detected in *L. japonicus*. The hybridization of the GS1 probe to leaf and stem tissues could only be detected under the low stringency conditions shown here (see Materials and methods) and may represent, in addition to transcripts for the cytosolic GS, the detection of plastidic GS2 transcripts. It should also be noted that since the exposure times shown for the various probes differ considerably (see individual figure legends), this should be considered when comparing the absolute amounts of each transcript.

The expression of the two AS genes was also monitored during normal growth in an air-cooled greenhouse, under a 16 h light/8 h dark photoperiod, with supplementary lighting from 05.00 to 21.00 (Figs. 3B and C). In the roots, both AS genes were strongly expressed throughout the day, although steady state levels of both transcripts appeared higher at 08.00 (3 h into the light period) (Fig. 3B). Small differences in the patterns of

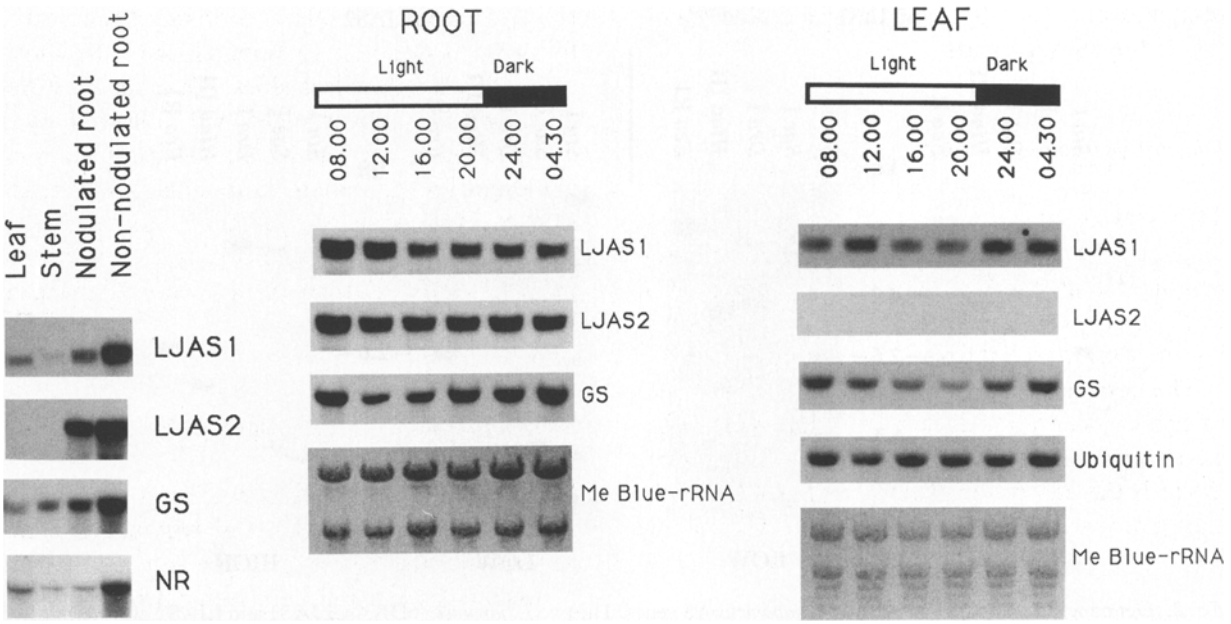

Fig. 3. Northern blot analysis of expression of AS genes in *L. japonicus*.The AS and NR probes were labelled to a high specific activity (2×10^9 cpm/μg) whilst the GS and ubiquitin probes were labelled only to a low specific activity (2×10^8 cpm/μg). GS hybridisation was performed under low stringency (see Materials and methods). Blots were exposed to X-ray film as follows: NR and GS, 4 d; LJAS1 and LJAS2, 30 h. A. mRNA was isolated from 3-week-old aeroponically grown, nodulated *L. japonicus*. Northern blots were loaded with equal amounts (10 μg) of RNA and probed with ^{32}P-labelled DNA probes for different N-assimilation genes. Blots were exposed to X-ray film as follows: NR and GS, 4 d; LJAS1 and LJAS2, 30 h. Confirmation of the uniformity of RNA loading was obtained using Methylene blue staining of the rRNA bands (results not shown). B. Hybridisation of AS and GS probes to root RNA isolated from 2-week-old aeroponically grown, unnodulated, *L. japonicus* harvested during a diurnal cycle. Northern blots were probed under high stringency, with a full-length ^{32}P-labelled cDNA probe representing either LJAS1 and LJAS2 (24 h exposures shown) or under low stringency with a ^{32}P-labelled (low specific activity) genomic DNA probe representing GS (24 h exposure shown). rRNAs were stained before hybridisation with Methylene blue. C. Hybridisation of AS and GS probes to leaf RNA isolated from 2-week-old aeroponically grown, unnodulated, *L. japonicus* harvested during a diurnal cycle. Hybridisation conditions were as described for Fig. 3b; 24 h exposures of filters hybridised with the AS probes, 36 h with the GS and 16 h with ubiquitin are shown. For all northern hybridisations shown the sizes of the different transcripts were calculated from the relative mobilities of the Methylene blue-stained rRNA bands and periodically confirmed using standard markers (IBI). The two AS transcripts were 2.2 kb, the GS transcript 1.4 kb, the ubiquitin transcipt 1.2 kb and the nitrate reductase transcript 3.2 kb long.

steady-state levels of transcripts were detected by LJAS2 compared with those observed for LJAS1 but such differences in AS transcript abundance were minor, particularly when compared to the accumulation pattern of the GS transcript, whose diurnal expression in roots indicated much lower numbers of transcripts during the afternoon (12.00 and 16.00) than at other times of the day. The relative constancy of the Methylene blue-stained rRNA indicated that the difference in the abundance of the GS transcripts in the roots at different times of the day did not arise from an irregular loading of the samples and, therefore, reflected genuine diurnal differences in the steady state root GS mRNA levels.

The accumulation of AS mRNA in leaves (Fig. 3C) was also examined and no hybridisation of LJAS2 to leaf RNA could be detected. The abundance of AS transcripts detected in leaves by LJAS1was much lower than that detected in the root and longer exposures of the filter to film had to be used to obtain similar signal strengths. The steady-state levels of both the LJAS1 and the GS transcripts appeared slightly lower during the late light period (16.00 and 20.00). No irregularities in the leaf signals were detected by either the

ubiquitin probe, or by the Methylene blue staining of the rRNA bands.

Diurnal variation in amino acid pools in roots

Two sets of observations were made during a diurnal cycle using plants exposed to natural day-lengths in a greenhouse. The pool sizes of amino acids in roots were measured at three times during the dark and light periods respectively.

The concentration of asparagine in root tissue varied considerably during the cycle with high values around dawn and a well marked minimum towards the end of the afternoon (Fig. 4A). Both experiments gave the same pattern of values but the two curves are offset, probably because the former was done on 15 June, close to the shortest night with cloudless skies, the other on 9 August, when a longer night was followed by a clouded dawn. By contrast, the pool sizes of glutamine and glutamate, both much smaller than that of asparagine, varied much less, while the small pool of aspartate was greatest during the night (Fig. 4B). Similar results were obtained in both experiments.

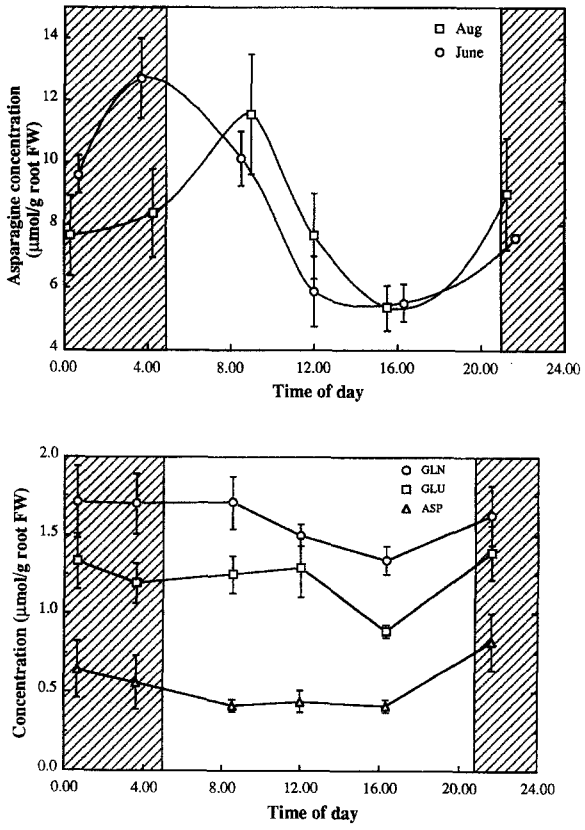

Fig. 4. Diurnal variations in amino acid pools in *L. japonicus* roots. The error bars represent the standard error of the mean (SEM) based on four replicates. A. Accumulation of asparagine during the night in *L. japonicus* roots. B. Fluctations in glutamine, aspartate and glutamate concentrations in *L. japonicus* roots over 24 h.

Incorporation of ^{15}N into asparagine

The initial rate of ^{15}N incorporation into asparagine gives a measure of the activity of AS in the root tissue (Fig. 5A). Asparagine contains two N atoms but it is the amido-N which will be labelled first since the substrate for AS will be amido-labelled glutamine. As time passes, an increasing number of glutamine molecules will become labelled at both amino- and amido-N because of the GS/GOGAT cycle and, thus, some of the later synthesised asparagine will also be doubly labelled. Double labelling of asparagine can also result from the reaction of labelled aspartate with amido-labelled glutamine. The relative importance of these two ways of making doubly labelled asparagine will depend on the relative activities of GOGAT and aspartate aminotransferase.

At each of the sampling times indicated in Fig. 4, plants were labelled for 10, 20, 30 or 40 min with a buffered (pH 6) nutrient solution containing 3000 mM NH_4Cl (99.4 atoms% ^{15}N). Asparagine in extracts of these roots was separated into unlabelled, labelled in one N atom (= amido-labelled) and labelled in two N atoms. After 10 min labelling, most of the labelled asparagine had ^{15}N in only one atom. Plotted against time the increase in the proportion of asparagine labelled in two atoms increases (Fig. 5A). A similar pattern of labelling of asparagine was found in the night and during the day, but the proportion labelled varied inversely with the concentration of asparagine in the root tissue with the three samples taken during the day.

These results, on the proportion of labelled

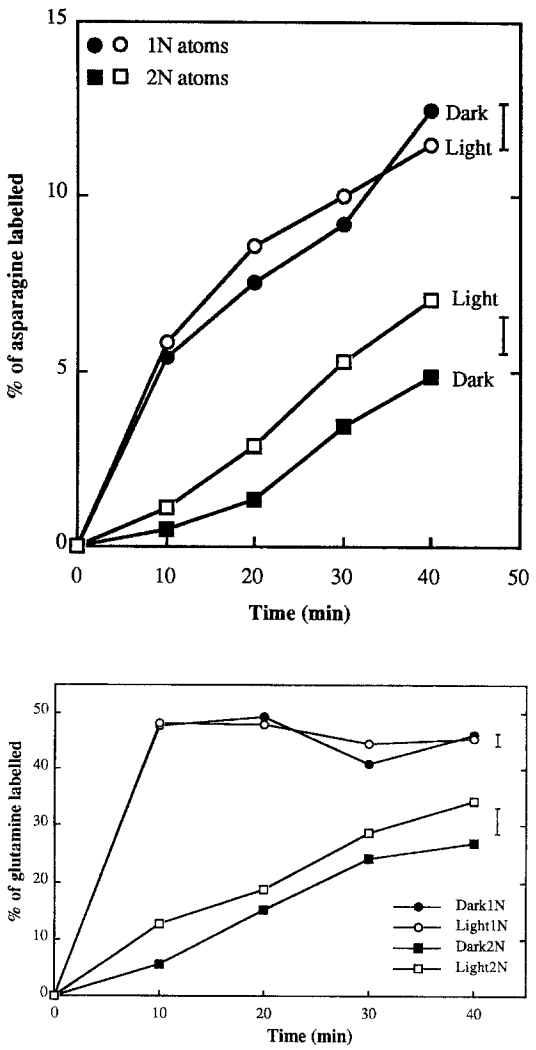

Fig. 5. Kinetics of amino acid labelling by ^{15}N-ammonium supplied to *L. japonicus* roots. The values in the graph are data pooled from 3 samplings in dark and light (June harvest). The bars indicate least significant differences (LSD) of means (22 df) ($p = 0.05$). A. Percentage incorporation of ^{15}N into asparagine in *L. japonicus* roots during dark and light periods. Roots were extracted and the asparagine was analysed by GC-MS in terms of unlabelled, the amido-labelled fraction (labelled at one N atom) and asparagine labelled at both N atoms. B. Percentage incorporation of ^{15}N into glutamine in *L. japonicus* roots during light and dark periods. ^{15}N-labelled glutamine was analysed by GC-MS. Very rapid labelling of the glutamine pool was detected (50% became labelled at one N within 10 min), due to the very small glutamine pool size.

forms of asparagine, can be combined with the quantitative measurements of asparagine-pool sizes to calculate the moles of ^{15}N present in

Table 2. Amounts of ^{15}N found in asparagine from *L. japonicus* root tissue treated during darkness or the day (includes both asparagine labelled in 1 and 2 N atoms).

Period of labelling (min)	Amount of ^{15}N in asparagine (μmol g^{-1} root FW)	
	dark	light
10	0.578	0.593
20	0.891	1.025
30	1.708	1.138
40	2.193	1.703

LSD $p < 0.05$ (22 df) = 0.360.

asparagine. If the ^{15}N found in asparagine in roots is compared in the night and day samples, it appears greater in darkness (Table 2). However, the ^{15}N content of shoot material was appreciable (Table 3); transport to the shoot was evidently greater during the photoperiod when the plants would have been transpiring. It is a reasonable assumption (see below) that the overwhelming majority of the ^{15}N transported to the shoots would have been asparagine. Properly, therefore, this amount should be added to the labelled asparagine measured in the roots. When this was done, there were no longer differences between night and day samples (Table 4).

Incorporation of ^{15}N into other amino compounds

Because the pool of glutamine is so much smaller than that of asparagine, the proportion of it that

Table 3. Labelled N found in *L. japonicus* shoot material in darkness and in light.

Period of labelling (min)	Shoot ^{15}N content (μmol g^{-1} shoot FW)	
	dark	light
10	0.121	0.085
20	0.150	0.389
30	0.247	0.787
40	0.304	1.314

Mean of 3 sampling times in dark and light.
LSD $p < 0.05$ (22 df) = 0.360.

Table 4. Synthesis (*in vivo*) of ^{15}N-asparagine in *L. japonicus* in darkness and in light.

Period of labelling (min)	^{15}N in root and ^{15}N in shoot (μmol per gram FW)	
	dark	light
10	0.650	0.590
20	0.922	1.232
30	1.572	1.640
40	2.002	2.422

Mean results from three samples taken in dark and light. LSD $p < 0.05$ (22 df) = 0.406.

Table 5. Estimates* of rates of synthesis of amino acids in *L. japonicus* during the first 10 min labelling with $^{15}NH_4^+$.

	Rate of synthesis (μmol ^{15} N per gram root FW h^{-1})			
	glutamine	asparagine	glutamate	aspartate
Dark	10.24	4.15	1.39	0.44
Light	12.72	4.07	1.82	0.32

* Mean results from 3 samples in dark and light.

becomes labelled in a given time is very much greater (Fig. 5B). Within 10 min of labelling, a maximum was reached in the abundance of glutamine labelled only in the amido group. The initial rates of labelling of asparagine, glutamate and aspartate during the first 10 min are compared in Table 5 for samples taken in the night and during the day. Both labelled asparagine and glutamate are derived initially from amido-labelled glutamine and labelled aspartate is derived from glutamate; it is important to note that the flux of ^{15}N into asparagine is 2.98 and 2.24 times greater than that into glutamate in the dark and light, respectively. Evidently, AS competes effectively with GOGAT for recently synthesised glutamine.

Amino composition of xylem sap

Asparagine was the principal amino component of the xylem sap and the flux of asparagine into the sap remained relatively constant over an 80 min period, even though the volume flow decreased markedly (data not shown). The concentration of asparagine varied with water flow but was in the range 7–16 mol/m^3. The kinetics of ^{15}N-labelling of the asparagine in the sap were similar to, but slightly lower than that in the root tissue in the previous experiment (Fig. 6). Aspartate was the next most abundant compound, there being on average about twice as much as glutamine and more than 5 times greater than glutamate (Table 6).

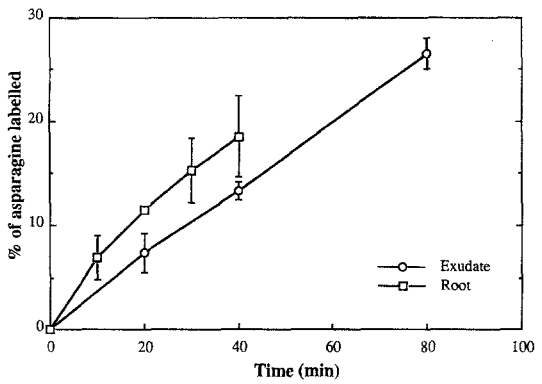

Fig. 6. Comparison of ^{15}N incorporation into asparagine pools in root and xylem sap samples. The error bars represent the standard error of the mean (SEM) based on 4 replicates (June harvest).

Discussion

Lotus japonicus cDNA clones, LJAS1 and LJAS2, share very high DNA and predicted

Table 6. Amino acid compositions and fluxes into xylem sap from excised root systems of *L. japonicus* cuttings.

Component	Concentration, μmol/ml	Flux to xylem μmol per gram root FW per hour
Asparagine	8.692	0.294
Glutamine	0.779	0.0026
Aspartate	1.748	0.059
Glutamate	0.314	0.011
(Water (J))		33.8 mg g^{-1} h^{-1}

Sap collected for 20 min after excision.

amino acid sequence similarities with other plant AS sequences. Both cDNA sequences predict a 585 amino acid polypeptide, and the conservation of their N-termini and the presence of a putative *purF*-type glutamine-binding domain (a cysteine/histidine/aspartate triad: C, Cys^{1}; D, Asp^{29}; H, His^{101}) [11, 16], indicate that both genes encode glutamine-dependent enzymes. In common with other plant AS polypeptides, those of *L. japonicus* have a hypervariable C-terminal end, which is thought to include the aspartate-binding domain [11]. Within this region, amino acid sequence comparisons are now revealing small localised domains of high identity, although these are most marked within the legume family. It is possible that it is these domains, or amino acids contained therein, which represent either regulatory or functional features essential for the specificity of AS activity.

In the LJAS2 sequence, the putative polyadenylation motif (AATAAT) located 23 bp upstream of the adenylated tail and the potential upstream accessory element (TTGTA) [19] at 53 bp from the adenylated tail are likely to direct the termination and processing of this AS transcript. The failure to identify processing determinants in the LJAS1 sequence suggests that the 3′ ends of the two *L. japonicus* AS transcripts are processed by different mechanisms and may be indicative of variations in message termination and/or stability of the two messages. It is tempting to speculate that the divergence of the *L. japonicus* AS 3′-UTR sequences may reflect a difference in the regulation of their expression or the function of each polypeptide. A different functional role for the two AS polypeptides in *L. japonicus* is predicted by their varying tissue specificities, the small differences detected in the roots of the steady-state levels of the two AS transcripts during a day and by the different extents of their response to nodulation. Differences in the level and pattern of expression of the two AS genes in pea have also been reported [27, 28].

Since we wanted to assess asparagine synthesis in the context of physiologically stable growth, we used mature plants, grown in a greenhouse with supplementary lighting, which had attained a stable root:shoot growth balance and in which the roots represent a source of reduced N. A diurnal rhythm in the expression of GS is shown but we also detected distinctive rhythms for the expression of other genes in the roots including NR (data not shown); these provided a good indication of a state of biochemical homeostasis. We were specifically interested in whether a diurnal variation of light influenced AS gene expression during normal N assimilation, since it has been suggested previously that AS gene transcription is up-regulated during dark periods [11, 27, 28]. It is not immediately clear what advantage a plant would gain from utilising such an attribute and it seems strange that plants such as *L. japonicus*, which use asparagine as their major N-transport molecule, would restrict asparagine synthesis to the dark period, and supress it during daylight hours when the energy-requiring nitrate reduction reactions are at a maximum.

Although northern hybridisations indicated that, in *L. japonicus* roots harvested at 08.00, there was a small increase in the abundance of both AS transcripts, there was no evidence supporting a specific dark-stimulated pattern of AS transcript accumulation as has been reported for pea. This suggests that these two members of the legume family, both of which are root N-assimilating species, may operate their N-assimilation in contrasting manners, despite the fact that DNA sequence analysis had indicated a very high level of identity between, in particular, the *L. japonicus* LJAS2 and pea AS1 sequences. Several points of contrast can be highlighted. Firstly, the expression of LJAS1 was root-enhanced whilst that of LJAS2 was root-specific, whereas in pea, transcripts of both genes could be detected in the leaves. Secondly, in *L. japonicus* leaves, the number of transcripts detected by LJAS1 was slightly decreased in only the later stages of the light period (16.00 and 20.00): in contrast, in pea leaves steady state mRNA levels were highest in the latter part of the dark period and were barely detectable after 3 h illumination. Thirdly, in *L. japonicus* roots, both LJAS1 and LJAS2 transcripts were detected at a high level throughout the day whilst in pea roots

although the AS2 transcript was expressed constitutively throughout the day, the AS1 transcript could only be detected during illumination when special precautions were taken to block the small amounts of light which normally penetrate the soil surface [28]. It is possible that our aeroponic mist system excluded light from the roots more efficiently than soil does, since the top of the apparatus is a white opaque polycarbonate sheet placed on the mist tank. However, the system was not designed specifically to be light-proof and the top is kept in place essentially by its own weight. After prolonged periods of darkness (2–3 days), the relative abundance of the AS transcripts in *L. japonicus* roots was seen to decrease (results not shown). Fourthly, in *L. japonicus* only LJAS2 transcipts were detected in significant numbers in nodulated roots whilst in pea both AS genes were expressed at a higher level in nodules than in the control unnodulated roots [27].

In many cases, N assimilation has been investigated in young seedlings. In such cases, the endosperm would be a major source of reduced-N whilst the roots would represent a N sink. In the mature plants we used, the roots represent a major N source and thus our results may also reflect a difference in the physiological background to the analysis. The AS gene expression observed in *L. japonicus* leaves may be a consequence of secondary processes linked to the recovery of ammonia produced during photorespiration rather than to primary N-assimilatory mechanisms. After taking into account the additional information from observations on asparagus [4], where the AS gene was only expressed in spears during the post-harvest period, it becomes clear that AS genes are being expressed in these different plants in response to different biochemical and/or environmental stimuli; thus the gene products are fulfilling different roles.

Unfortunately, interpretation of AS expression has relied heavily on RNA analysis, due to the recalcitrant nature of the AS enzyme which has precluded the use of standard, protein-based approaches to elucidate AS enzyme activities. However, for some N-assimilatory enzymes, for example, NR, a note of caution has been sounded about directly extrapolating from the amounts of mRNA to enzyme activity [20]. We have approached the problem of obtaining a direct analysis of AS activity by observing the ^{15}N labelling of asparagine *in vivo*. This has allowed, for the first time, a direct measure of the *in vivo* rate of asparagine synthesis, by combining GC-MS observations with established procedures which quantify amino acid concentrations in plant tissues. The ^{15}N label was supplied as $^{15}NH_4^+$ which enters the assimilatory process directly; our unpublished observations showed that labelling of asparagine from $^{15}NO_3^-$ was complicated by the mixing of labelled nitrate with unlabelled nitrate from endogenous pools, thus creating uncertainty about the ^{15}N enrichment of the ammonia incorporated into asparagine. It should be pointed out that roots were taken directly from a nitrate-based N supply and treated with ammonium (in the presence of nitrate). Endogenous pools of unlabelled $^{15}NH_4^+$ would have been small and there would have been little opportunity even in the longest exposures of roots to the solution (40 min), for any induction of AS gene expression.

The results obtained showed, in an unequivocal way, that the rate of asparagine synthesis in *L. japonicus* roots, which must reflect the activity of the AS enzyme in the root, remained constant throughout the day, despite the small changes in transcript level. Asparagine does accumulate in *L. japonicus* roots during the dark period but this is not due to an increased rate of synthesis brought about by increased gene expression, since both the rate of asparagine synthesis and of AS transcript abundance were shown to be similar in both light and dark. The accumulation of asparagine in roots during the night is most readily explained by the lower rate of transpiration during the night, which could decrease the export of recently synthesised asparagine from the roots. Consideration of the rate of asparagine formation in darkness (ca. 4 μmol per gram root per hour), with the increase in root asparagine concentration during the night (ca. 7 per gram root FW), showed that the asparagine accumulation was, in fact, only about 17% of the amount which would

have been synthesised. Evidently, the majority of the asparagine synthesised in darkness must have been either exported or further metabolised since a relatively small change in the rate of export would quickly have affected the asparagine concentration.

Our study with the exuding roots showed that asparagine was the predominant amino-component of the xylem sap in *L. japonicus*. Asparagine in the xylem became labelled at a slightly slower rate than the asparagine in the root tissue and suggested that the asparagine in the root was located in a single pool, probably comprising the cytoplasm. Partitioning of the root asparagine into a vacuolar or 'storage' pool and a cytoplasmic or 'transport' pool, would have resulted in a more rapid labelling of the asparagine in the xylem, than in the root as a whole. The latter situation is frequently found in studies on the labelling of nitrate entering the xylem. The present results show that the flux of water across the root, although it follows a different pathway, interacts with the concentration of transportable solutes in the cytoplasm. Thus, the effect was probably to dilute the asparagine in the xylem, thereby increasing its unloading from the symplast. The exuding roots observed in these experiments approximate to the roots of intact plants during the night period, as far as water flow is concerned. The magnitude of the labelled asparagine fluxes was only 20%, at most, of those estimated to have occurred in the intact transpiring plants.

Fluctuations at the micro-molar level in the concentrations of specific metabolites make them ideal candidates for effector molecules [9, 15, 23, 26]. The efficacy of asparagine has not been specifically tested in this study but an increase in the root asparagine pool size was observed around dawn; an increase in number of AS gene transcripts was also detected 3 h into the light period at 08.00. However, this increase in number of transcripts could not be correlated with a subsequent increase in the rate of asparagine synthesis. It is also possible that the differences in AS transcript levels at 08.00 may reflect a changing flow of sucrose *via* the phloem, from the shoot. The overall balance of N:C is thought to be a controlling factor over *Arabidopsis* AS gene expression and growth on an exogenous sucrose-supply caused a decrease in AS transcripts [11]. Much attention is currently focussed on the nature of metabolites which can signal the shoot's nutritional requirements to the root, and sucrose is one such possible molecule. Since *L. japonicus* undertakes N assimilation predominantly in the roots, it provides an excellent model where metabolic control of AS expression under natural lighting conditions can be easily manipulated and investigated, thus removing the requirement for radical treatments which are encountered when experimenting with other, essentially leaf-based expression systems.

It has been suggested recently [11], that the relatively high occurrence of asparagine residues in AS peptides may imply that the enzyme will only be synthesised under times of N sufficiency. The LJAS1 sequence predicts 18 asparagines out of 585 amino acids encoded (3.1%) and the LJAS2 sequence predicts 19 asparagines (3.3%). The only other *L. japonicus* DNA sequences available show a similar ratio of encoded asparagines, for example, NR has 37 asparagines out of 899 encoded amino acids (4.1%) (Lj-nia: accession number X80670) and a cDNA encoding the cytosolic form of GS has 14 asparagines out of 359 amino acids (3.9%) (unpublished work). This relatively high content of asparagine residues of N-assimilatory genes may well indicate that the polypeptides will only be made efficiently during N sufficiency. Conditions of N deprivation (2 d), which are sufficient to deplete the root of its vacuolar reserves of nitrate, are sufficient to silence the expression of NR, GS and AS genes (unpublished work).

The results described herein have demonstrated that *L. japonicus* is a useful model species in which co-ordinate regulation of transport, physiology and inorganic nitrogen assimilation can be studied. Although not exploited in this work, the species nodulates very readily under the conditions we use, thus offering further opportunities for the study of interactions with dinitrogen fixation. Moreover the species is transformable [7] so that direct intervention in the expression of

AS and other enzymes of the transport and assimilatory pathways can be attempted.

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