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ORIGINAL ARTICLE

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High levels of asparagine synthetase in hypocotyls of pine seedlings suggest a role of the enzyme in re-allocation of seed-stored nitrogen

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Abstract A pine asparagine synthetase gene expressed in developing seedlings has been identified by cloning its cDNA (*PsAS1*) from Scots pine (*Pinus sylvestris* L.). Genomic DNA analysis with *PsAS1* probes and a sequence-based phylogenetic tree are consistent with the possibility of more than one gene encoding asparagine synthetase in pine. However, the parallel patterns of free asparagine content and *PsAS1* products indicate that the protein encoded by this gene is mainly responsible for the accumulation of this amino acid during germination and early seedling development. The temporal and spatial patterns of *PsAS1* expression together with the spatial distribution of asparagine content suggest that, early after germination, part of the nitrogen mobilized from the megagametophyte is diverted toward the hypocotyl to produce high levels of asparagine as a reservoir of nitrogen to meet later specific demands of development. Furthermore, the transcript and protein analyses in seedlings germinated and growth for extended periods under continuous light or dark suggest that the spatial expression pattern of *PsAS1* is largely determined by a developmental program. Therefore, our results suggest that the spatial and temporal control of *PsAS1* expression determines the re-allocation of an important amount of seed-stored nitrogen during pine germination.

Keywords Asparagine metabolism · Germination · Nitrogen re-allocation · *Pinus*

Abbreviations AS: Asparagine synthetase · C: Carbon · GS: Glutamine synthetase · N: Nitrogen

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Introduction

Although pine embryos contain some storage material, most of the reserves within the seed are located in the megagametophyte, a maternally derived tissue very rich in lipids and proteins as reservoirs of carbon (C) and nitrogen (N; Groome et al. 1991). Arginine accounts for nearly half of the N in the megagametophyte storage proteins of *Pinus taeda* (King and Gifford 1997). After the breakdown of these proteins during germination, arginine is hydrolyzed to produce ornithine, ammonium and carbon dioxide by the sequential action of the arginase and urease enzymes (Todd and Gifford 2002). Arginase transcript, protein and activity are localized in the expanding cotyledons, structures closely in contact with the megagametophyte. The ammonium released from arginine catabolism is reassimilated by glutamine synthetase (GS) to produce glutamine (Suárez et al. 2002). Two different genes encoding cytosolic GS have been identified in young pine seedlings: an atypical cytosolic GS generating amino donors for the biosynthesis of major N compounds in photosynthetic tissues (Cantón et al. 1993, 1999), and a second typical cytosolic GS involved in the generation of glutamine for N transport (Ávila et al. 2001).

Following the emergence of the radicle a pronounced change in the amino acid profile occurs in the seedling (King and Gifford 1997). In particular, as a result of a dramatic increase, asparagine becomes the most abundant free amino acid, accounting for up to 70% of the free amino acid pool. The main reaction for asparagine biosynthesis in plants is catalyzed by the enzyme asparagine synthetase (AS) using glutamine as N donor. Therefore, AS may play an outstanding role in N re-allocation during post-germinative development.

Current knowledge about AS gene structure, expression and function in plants, comes from studies with herbaceous angiosperms. In several legume and non-legume angiosperms a small family of AS genes

has been identified (Davis and King 1993; Waterhouse et al. 1996; Hughes et al. 1997; Lam et al. 1998; Osuna et al. 2001). Major advances in defining the AS gene family and the particular function of each member have been made for *Arabidopsis thaliana*. In this plant species three AS genes were found (Lam et al. 1994, 1998), and a specific non-overlapping functional expression pattern has been defined for the *ASN1* and *ASN2* genes: the levels of their mRNAs are reciprocally regulated by light and by organic C and N (Lam et al. 1998; Wong et al. 2004). From their specific expression patterns and analysis of transgenic *Arabidopsis* overexpressing the *ASN1* gene differential roles for each AS gene have been proposed: the role of *ASN1* may be related to primary N assimilation and transport, whereas that of the *ASN2* gene may be related, directly or indirectly, to the recapture of lost N resources under stress conditions (Lam et al. 2003; Wong et al. 2004).

Trees are distinct from herbaceous plants in some particular aspects of their physiology and development. For instance, during early development a tree seedling faces a massive demand for C and N to feed the synthesis of cellulose and lignin in the developing stem. Here, we report the isolation of a cDNA clone for AS from the conifer gymnosperm *Pinus sylvestris* and the spatial and temporal expression pattern of this gene during seed germination and early seedling development. The results suggest that this AS gene plays an important role in re-allocation of N released from seed storage material during early stages of conifer development.

Material and methods

Plant material and growth conditions

Scots pine seeds (*P. sylvestris* L.) were obtained from Servicio de Material Genético, Ministerio de Medio Ambiente (Madrid, Spain). Seeds were imbibed in deionized water for 24 h under continuous aeration and germinated in a plastic pot with vermiculite (Eurover, Europerlita, SA, Almería, Spain) as support. Seedlings were grown in a controled culture chamber (Ibercex H-900-B, ASL, SA, Madrid, Spain) at 24°C in cycles of 16 h light/ 8 h dark, except otherwise indicated. Illumination was provided by fluorescent lamps (Sylvania F-48T12/CW/WHO;Koxka, Pamplona, Spain) at a flux density of 150 μmol m^{-2} s^{-1}. Plants were watered regularly but no N fertilization was added. Under these conditions of growth the megagametophyte of conifers supplies N and other nutrients to the developing seedling for a period of 2–3 weeks until seed reserves begin to be exhausted (Flaig and Mohr 1992; Cánovas et al. 1998). When seedlings were grown under photoperiod, samples were always harvested at the same time in the light/dark cycle.

Isolation of pine AS cDNA clones

By comparing the available AS sequences from plant species, degenerated oligonucleotide primers were designed from a highly conserved region: ASN5, 5′·TAY YTDTAYTTCCAYAARGC·3′ and ASN3, 5′·RTANCCAACHCCRTCACT·3′. cDNA was synthesized with the 1st Strand cDNA Synthesis Kit for RT-PCR, AMV (Roche Diagnostics). PCR was performed with Pfu polymerase (Stratagene) and the following conditions: after an initial denaturation step at 95°C three cycles were performed with a denaturation step at 94°C and followed by an annealing step at 42°C and an extension step at 72°C, then 30 additional cycles were carried out with steps at 95, 48 and 72°C. All steps were 30 s in extent. A 10 min final extension was performed. A 323 bp DNA fragment was amplified and cloned in pBluescript SK. After sequencing and BLAST analysis it was confirmed that the PCR product encoded for a fragment of a AS polypeptide.

To clone a full-length cDNA a gene-specific primer was designed from the 323 bp PCR fragment sequence. The primer was used to clone a PCR product by 5′-RACE (Marathon™ cDNA Amplification Kit, BD Biosciences Clontech) containing the 5′end of the AS mRNA. The 5′-end sequence of this PCR product was used to design a primer to amplify a full-length cDNA by 3′-RACE. RACE products were cloned into the pGEM-Teasy vector (Promega). DNA was sequenced using an automatic sequencer ABI 373 XL Stretch and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence was deposited in the EMBL Sequence Database under the accession number AJ496567. Amino acid alignment of several deduced AS polypeptides was carried out with Clustal IV in the DNAstar software package.

Antisera obtention

Both ends of a cDNA encoding the AS of *P. sylvestris* were manipulated by PCR in order to facilitate its in-frame subcloning in the *E. coli* expression vector pET11a (Novagen). For this purpose, an N-terminal primer was designed consisting of a nucleotide sequence from the cDNA and an overhang region containing a unique restriction site for *Ase*I (5′-AGTATTAATGTGTG GAATCCTAGCAG-3′). A C-terminal primer was designed containing a gene-specific cDNA sequence and incorporating a unique restriction site for *Bam*HI downstream from the translation stop codon, (5′-AACGGATCCTCAGCACTTCGCGGGC-3′). Both primers were used to amplify the cDNA by PCR with *Turbo Pfu* (Stratagene) and, after digestion with *Ase*I and *Bam*HI, the cDNA was subcloned in the pET11a vector which had been digested previously with *Nde*I and *Bam*HI. The fidelity of the construction was verified by sequencing.

pET11a containing the in-frame sequence of AS (pET11AS) was transferred to the BL21 DE3 PLyS *E. coli* strain. Cells were grown at 37°C with shaking in LB broth containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) until a cell density (OD 600) of 0.6 was reached. Isopropyl-thio-β-D-galactopyranoside (IPTG) was then added to a concentration of 1 mM and incubation was continued for an additional 3 h. Cells were harvested by centrifugation and resuspended in denaturing buffer (Laemmli 1970). Cell debris was removed by centrifugation. Samples were separated by SDS-PAGE and the band containing the recombinant AS was excised and powdered. The powder was mixed with complete Freund's adjuvant and subdermically injected in two rabbits. Four weeks later, the rabbits were reinjected with protein mixed with incomplete Freund's adjuvant. Ten days later the blood of the rabbits was collected and the serum was satisfactorily assayed against plant and recombinant AS by Western blot. Antibodies were purified against the purified recombinant protein as described by Sakakibara et al. (1991).

Protein extraction, SDS-PAGE, two-dimensional electrophoresis, and Western-blot analysis

All plant material was immediately frozen in liquid nitrogen after harvesting and stored at −80°C until use. Frozen tissues were ground in liquid nitrogen in a mortar, and the powder was transferred into a tube containing extraction buffer (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 0.1% (v/v) 2-mercaptoethanol). The addition of 2% SDS to the extraction buffer prevented the degradation of AS during the extraction procedure observed in the absence of the detergent. The homogenates were centrifuged at 22,000*g* for 20 min at 4°C and the supernatant removed to a new tube. For protein quantification SDS was removed from total protein extract by precipitation with potassium phosphate buffer, following the indications of Zaman and Verwilghen (1979), and the supernatant was used to quantify proteins by the method of Bradford (1976).

SDS-PAGE, electroblotting and immunodetection of AS polypeptides were carried out as described elsewhere (Cantón et al. 1996), except that SDS was not added to the Laemmli buffer. For 2D-gel electrophoresis, proteins were extracted in 10% trichloroacetic acid, 0.07% 2-mercaptoethanol in acetone. The extract was left for 1 h at −20°C and then centrifuged for 15 min at 22,000*g*. The pellet was overlaid with acetone and 0.07% 2-mercaptoethanol and left for 1 h at −20°C. The acetone was removed, the pellet dried and dissolved in UKS buffer [9.5 M urea, 5 mM Na_2CO_3, 1.25% (w/v) SDS, 0.5% (w/v) 1,4-dithio-DL-threitol, 2% (w/v) Pharmalyte™ (Amersham Biosciences), 6% (v/v) Triton X−100]. Proteins were separated by isoelectric focusing (IEF, pH 3–10) and SDS-PAGE, electroblotted onto nitrocellulose, and AS and GS polypeptides revealed by immunobinding to specific antibodies (Cantón et al. 1999)

Analysis of asparagine

Frozen powdered plant material was extracted in 30 mM Tris–HCl, pH 8.0, 1 mM EDTA and 10 mM 2-mercaptoethanol. The extract was centrifuged at 22,000 *g* and the supernatant was recovered and centrifuged again as above. An aliquot of 200 μl was transferred to a fresh tube, 500 μl of methanol were added and mixed for 10 min at 4°C. After centrifugation as above, the supernatant was saved in a fresh tube and the pellet was resuspended in 200 μl of (4:1, v/v) methanol:H_2O mix, stirred for 10 min at 4°C, centrifuged and the supernatant reserved. The last step was repeated twice and all the reserved supernatants combined. The volume was reduced to 200 μl by evaporation at 90°C in an oven. Finally the extract was filtered through a 0.2 μm pore filter.

To determine the asparagine content of the samples, amino acids were separated with no derivatization with a System Gold HPLC BioEssential high-performance liquid chromatograph (HPLC; Beckman-Coulter, Fullerton, CA, USA) using a lithium citrate buffer system and followed by a post-column ninhydrin reaction detection system. For asparagine identification and quantification the corresponding standard was used.

RNA and DNA extraction and analysis

Total RNA was isolated following the procedure described by Chang et al. (1993). Northern-blot analysis was performed as described (Cantón and Quail 1999). ^{32}P-labeled hybridization probes were synthesized with the High Prime System (Roche Diagnostic) using a 240 bp fragment containing only the 3′-UTR derived from the full-length cDNA clone. Prehybridizations and hybridizations were performed according to Church and Gilbert (1984) at 65°C. After hybridization, membranes were washed four times at 65°C in 0.1× SSC/0.1% SDS.

Pinus sylvestris genomic DNA was prepared according to Dellaporta et al. (1983). For Southern-blot analysis, 20 μg of genomic DNA were digested separately with *Hin*dIII, *Bam*HI and *Eco*RI restriction enzymes, DNA samples were run on 0.8% agarose gel, blotted to nylon filters and hybridized to ^{32}P-labeled cDNA probes following the same procedure described above for northern blot. After hybridization membranes were washed at moderate stringency as described elsewhere (Loopstra et al. 1998): three times at 65°C in 2× SSC/0.1% SDS.

Preparation of plant tissue for microscopy and immunohistochemical localization

Seedlings were harvested and immediately fixed in freshly prepared 4.0% (v/v) paraformaldehyde in phosphate buffer saline (PBS) for 4 h at 4°C. Plant material was washed in PBS (3×10 min), dehydrated in a graded

ethanol series, and embedded in Fibrowax (BDH Laboratory, VWR Ltd, Lutterworth, UK). The embedded tissues were sectioned at 10 μm thickness, and affixed to poly-L-lysine-coated glass slides. For light microscopy, the Fibrowax was removed with histoclear (National Diagnostic, Atlanta, GA, USA) and the sections were rehydrated, and then washed in tris-buffered saline (TBS, 3×10 min). To inhibit the endogenous phosphatases, the sections were heated for 5 min in citrate buffer 10 mM (pH 6.0). After TBS washing (3×10 min), sections were blocked for 30 min in TBS containing 5% bovine serum albumin (BSA). To detect *AS* polypeptides, the sections were incubated for 1 h with mono-specific antibodies purified against the purified recombinant protein and then for 2 h with alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich). Alkaline phosphatase was detected colorimetrically, by incubation in the dark in TBS (pH 9.0) containing 50 mM $MgCl_2$ and NBT/BCIP as substrate (Roche Diagnostics). The reaction was stopped by washing with water for 10 min.

Results

Isolation and characterization of a pine AS cDNA

A full-length cDNA encoding AS has been cloned from *P. sylvestris* hypocotyl by a RT-PCR/RACE strategy. We first used RT-PCR to amplify a short fragment, 323 bp in size, using degenerated oligos derived from two conserved regions of the polypeptide in higher plants (residues labeled with asterisks in Fig. 1). The amplified product was sequenced and the deduced amino acid sequence had strong identity with other plant AS proteins (data not shown). The sequence was used to design specific oligonucleotides to amplify the complete 5′ region of the mRNA by 5′-RACE. Finally, a new oligonucleotide was designed from the 5′-end available sequence to amplify a full-length cDNA by 3′-RACE.

The full-length cDNA is 2,110 bp in size, including a coding region of 1,782 bp flanked by a 89 bp 5′- and a 239 bp 3′-UTRs. The open reading frame encodes a protein of 593 amino acids. The alignment of the predicted amino acid sequence of pine AS with the sequences of *A. thaliana* and the glutamine-dependent *E. coli* enzyme (ASB) showed a high percentage of identity of the pine protein with plant and prokaryotic asparagine synthetases (Fig. 1). In particular, the pine enzyme maintains all residues previously described as essential for glutamine-dependent enzyme activity (Fig. 1, residues in boxes): those involved in the binding and positioning of glutamine substrate within the binding pocket (Zalkin and Smith 1998; Larsen et al. 1999), binding of aspartate (Boehlein et al. 1997a, b) and AMP anchoring (Larsen et al. 1999). Pine AS includes a 37 residue long carboxy-terminal stretch, absent in the bacterial enzyme. Similar additional extensions are found in all plant AS proteins, which vary greatly in length and amino acid sequence among species (data not shown). In fact, yeast and mammal AS show a higher divergence in the carboxyl end in comparison to plants/*E. coli* enzymes. This hypervariable sequence expands the last 77 residues of the gymnosperm enzyme (Fig. 1, labeled with dots over the amino acids). When this stretch is not considered, the percentage of identity of pine AS with the other plant AS sequences is in the range from 79.4% (*Glycine max* AS1) to 83.1% (*Phaseolus vulgaris* AS2). Like other plant AS, the product of the cloned cDNA showed a remarkable percentage of identity with *E. coli* (54.2%), suggesting a high conservation of the enzyme structure between plants and prokaryotes, whereas the percentage of identity of *E. coli* with the hamster and human AS is lower (32.6 and 32.2%, respectively). It is worth noting that plant asparagine synthetases showed a higher percentage of identity to the bacterial enzyme than the yeast enzymes (48.5 and 49.1% for *Saccharomyces* AS1 and AS2, respectively).

To determine the genomic organization and gene copy number of AS in pine, a Southern blot was performed using either a 323 bp PCR fragment as a probe, which spans a short stretch of the coding region (108 amino acids) that is highly conserved among all asparagine synthetases, or a gene-specific fragment of 240 bp containing only the 3′-UTR from the cDNA (Fig. 2). Several DNA fragments were detected in all three different restriction digests of pine genomic DNA when hybridized with the 323 bp probe. For instance, up to 11 bands can be detected in the *Eco*RI genomic DNA restriction digest. However, a single fragment hybridized to the gene-specific probe in five different restriction digests. The above results suggest the possibility of more than one gene encoding for AS in the pine genome.

The existence of more than one AS gene in the pine genome is further supported by a cladistic parsimony analysis using the amino acid sequences of the different plant AS polypeptides and *E. coli* ASB, together with AS sequences from yeast, human and mouse, and after deletion of the divergent C-terminal amino acids (Fig. 3). The phylogenetic tree obtained was consistent with previous reports (Shi et al. 1997; Osuna et al. 2001; Møller et al. 2003). Bacteria, yeast, plants and animals were separated into four groups. Plants were clustered in two main groups: the sequences grouped to *A. thaliana* AS1 (AS1-like cluster), and those grouped to *A. thaliana* AS2 and AS3 (AS2,3-like cluster). In all the analyses performed so far, available monocot sequences were clustered in the second group, whereas all the available legume sequences are clustered in a unique branch into the AS1-like cluster, suggesting that all known legume AS genes are duplications of an ancestral AS1-like sequence. With the inclusion of the *E. coli* ASB sequence as a outgroup, the separation of the two clusters was supported by 100% of 1,000 bootstrap replicates. Within the AS1-like cluster the sequences form two subbranches: the first branch includes only the pine AS polypeptide, while the remaining AS1-like sequences are clustered in a second branch. These two clusters repre-

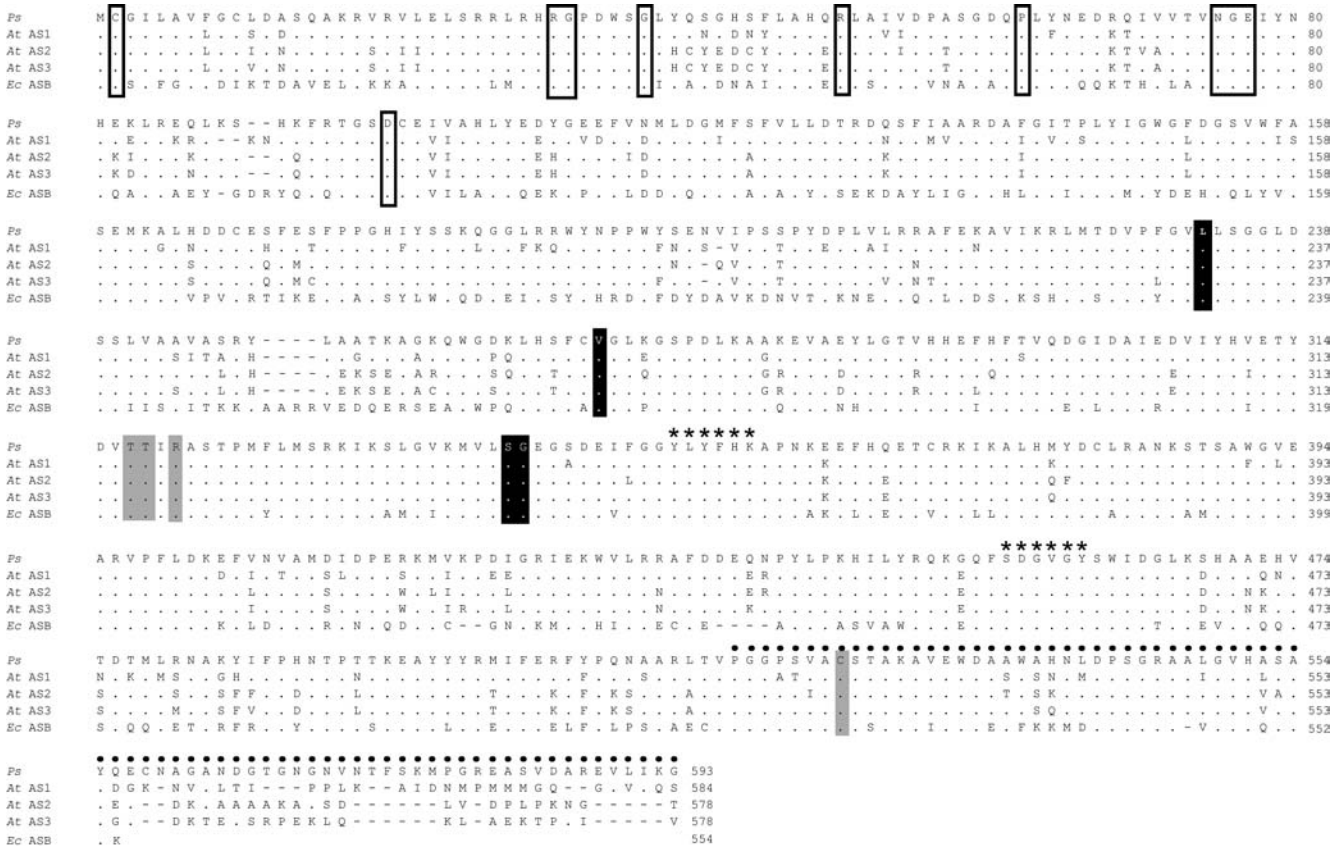


Fig. 1 Amino acid sequence of pine asparagine synthetase polypeptide. The deduced amino acid sequence of *Pinus sylvestris* polypeptide (*Ps*) was aligned with the amino acid sequences of the three *Arabidopsis thaliana* AS and the *Escherichia coli* ASB polypeptide. Accession numbers are indicated in brackets: *At* AS1, *A. thaliana* AS1 (L29083); *At* AS2, *A. thaliana* AS2 (AF095453); *At* AS3, *A. thaliana* AS3 (AF095452); *Ec* ASB, *E. coli* glutamine-dependent ASB (J05554). Identical residues are indicated by *dots*. Gaps introduced to maximize sequence identity are indicated by *dashes*. The position of the last residue in every *line* is indicated by the *number* on the *right*. *Boxes* indicate conserved residues which have been shown to play essential roles in glutamine-dependent AS activity: *b*oxes with *white* background indicate essential residues of the glutamine-binding domain, *black boxes* label residues involved in AMP-anchoring, *gray boxes* label residues involved in aspartate binding. Degenerated oligos used to amplify the first pine AS cDNA fragment were derived from the nucleotide sequences encoding amino acids labeled with *asterisks*. The divergent C-terminal amino acid sequence is shown by *dots* over the residues

sent the gymnosperm/angiosperm separation, and the branching was well supported by 93% of the bootstrap replicas. The position of the pine sequence in the tree suggests that the gene duplication that generated the two main types of AS genes was an early event before the separation between gymnosperm and angiosperms. Therefore, a second type of AS gene should be expected in the conifer genome, as suggested by the Southern blot analysis. Since pine AS is clustered to the *Arabidopsis* AS1-like group, we decided to name this gene *PsAS1*.

PsAS1 expression in pine seedlings

Pine AS is a highly unstable protein in vitro, similar to other plant AS proteins (Ireland and Lea 1999). In fact, proteins were always extracted in buffer with 2% SDS, otherwise a high degree of degradation was detected in the AS polypeptide. In particular, AS protein was almost undetectable in roots in the absence of SDS. Biochemical studies of AS activity have been hindered by several problems including enzyme instability and rapid physiological turnover, endogenous inhibitors, competing pathways, and asparaginase activity (Romagni and Dayan 2000). Several or all these factors together could determine the lack of success when we tried to measure AS activity in protein extracts from pine seedlings or from *E. coli* overexpressing the pine AS. To show AS activity of the protein encoded by the *PsAS1* gene, complementation of the *E.coli* auxotroph ER, a mutant for both AS genes (Felton et al. 1980), were also attempted. However, the insolubility and instability of the pine AS protein in the *E. coli* mutant in different culture conditions prevented the complementation.

To get insights about the role of the *PsAS1* gene in asparagine synthesis during pine germination the levels of transcript and polypeptide, together with asparagine accumulation, were investigated at different stages of pine seed germination. To detect the pine AS polypeptides,

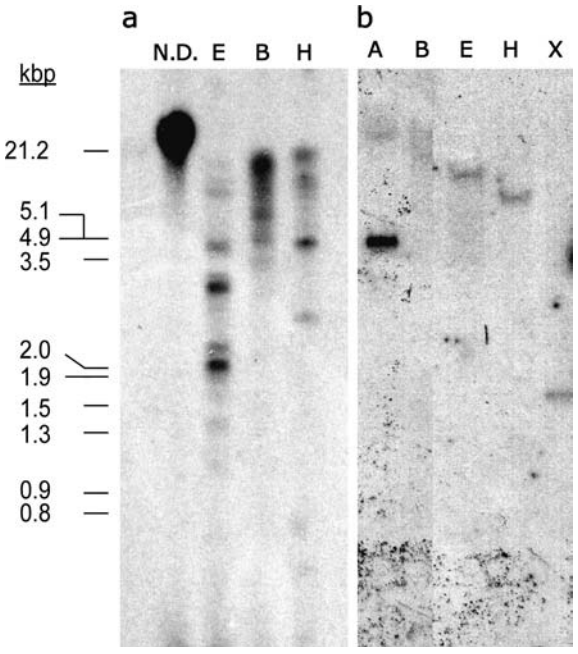


Fig. 2 a, b Genomic Southern blot analysis of *Pinus sylvestris* AS genes. Aliquots of 20 µg of genomic DNA isolated from pine cotyledons were digested with different restriction enzymes, fractioned in an agarose gel and transferred to a nylon membrane. **a** Hybridization with a probe that spans 323 bp of a highly conserved region among AS from different species. **b** Hybridization with a 240 bp fragment containing only the 3′-UTR from the *PsAS1* cDNA. In both cases, after hybridization, membranes were washed at moderate stringency. *Lane N.D.*, undigested pine genomic DNA; *lanes E*, *B*, *H*, *A*, and *X*, 20 µg of pine genomic DNA digested with *Eco*RI, *Bam*HI, *Hin*dIII, *Apa*I *or Xba*I, respectively. *Numbers* on the *left* indicate size in kbp of lambda DNA size markers

an antiserum was obtained in rabbits against the recombinant AS protein expressed in *E. coli*. The antibodies were purified against the recombinant protein and tested by Western blot in parallel with polyclonal antibodies obtained against AS from alfalfa (Twary et al. 1994). A single band was detected in protein extracts from pine hypocotyls, either when probing with antibodies raised against pine or alfalfa AS (Fig. 4a, b; lane 1). The apparent molecular mass calculated for the AS polypeptide was 65.5 kDa, close to the predicted value. Similarly, a single polypeptide was also detected in protein extracts from *E. coli* cultures overexpressing recombinant pine AS (Fig. 4a, b; lane 2). Other faint bands that appeared in the Western probed with the alfalfa antibodies were a result of unspecific recognition of *E. coli* proteins, since they also appeared in the control protein extracts from cells transformed with the pET 11a vector (Fig. 4 b; lane 3). Remarkably, the recombinant polypeptide migrated as a slightly larger molecule than the polypeptide extracted from seedlings. This molecular mass discrepancy could be explained as an electrophoresis artifact or a processing of the native polypeptide in pine cells. However, it is also possible that the observed polypeptide in pine hypocotyls was derived from an alternative pine AS gene.

We proceeded to compare the AS polypeptides and *PsAS1* mRNA accumulation patterns during seed germination and early seedling development. Specific antibodies and a 3′-UTR-derived probe were used to analyze the expression of the *PsAS1* gene in embryos and seedlings (Fig. 5a). In embryos neither protein nor transcript were detected immediately after 24 h of imbibition (Fig. 5a, E). A parallel increase in both, transcript and polypeptide, was observed at the germinated embryo concomitant with radicle emergence and the initiation of seedling elongation, 5 days after seed imbibition (Fig. 5a, G). To determine the *PsAS1* expression in later stages of germination and its spatial pattern, we separately analyzed the levels of *PsAS1* transcript and polypeptide in cotyledons, hypocotyl and radicles from seedlings 8 days after imbibition (Fig 5a, C, H, R). High levels of AS polypeptide were detected in hypocotyls, whereas moderate levels were present in roots and only very low levels in cotyledons. The levels of *PsAS1* transcript were parallel to protein levels in all cases, suggesting that the protein product is mainly, if not completely, the result of *PsAS1* mRNA translation.

We quantified the amount of asparagine in embryos and different organs of the seedling to study the temporal and spatial pattern of accumulation of this amino acid during early stages of germination in *P. sylvestris* and to get insights about the role of *PsAS1* in this accumulation (Fig. 5b). Asparagine levels were very low in the embryo (Fig. 5b, E); they started to increase coinciding with radicle emergence (Fig. 5b, G), and reached high levels at the seedling stage (Fig. 5b, C, H, R). Asparagine was accumulated in seedlings mainly in the hypocotyls, with much lower levels in the radicle and the lowest levels in cotyledons. As shown in Fig. 5, this pattern of asparagine accumulation in the seedlings tightly correlates with the levels of AS polypeptide and *PsAS1* transcript, suggesting that the product of this gene is responsible for asparagine accumulation in developing pine seedlings.

The light regulation of AS genes has been extensively studied in *A. thaliana*. In this species three AS encoding genes have been described with differential responses to light, and it has been suggested that they may play different roles in N metabolism. Light represses the expression of the *ASN1* gene, while it induces the levels of *ASN2* transcript (Lam et al. 1998). The phylogenetic analysis suggests that *PsAS1* is an *ASN1*-like gene (Fig. 3), so we decided to investigate whether this conifer gene is also light-repressed. Pine seeds were germinated under continuous light or darkness for 22 days after imbibition. At this point of seedling development the megagametophyte reserves have been completely depleted. The reasons for sampling at this developmental stage were: (1) seedlings at early developmental stage have cotyledons fully or partially covered by the seed, therefore interfering in light perception; (2) at early stages the presence of the megagametophyte can affect light responses of the seedling because storage material maintains a high C status. Previous reports suggested

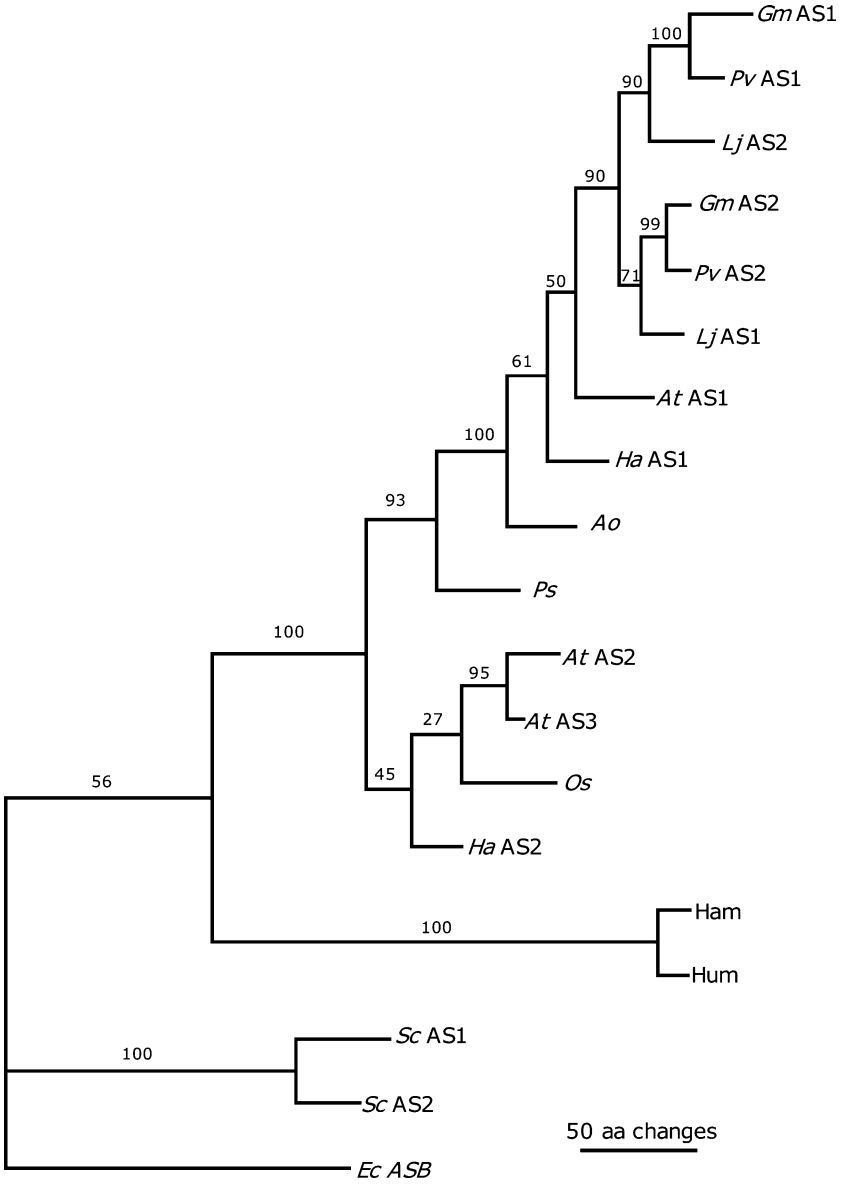


Fig. 3 Phylogenetic tree of AS proteins. Amino acid sequences of 19 asparagine synthetases derived from cDNAs of several plant, yeast, mammals and *E. coli* ASB were aligned with the Clustal IV software after removal of the divergent C-terminal amino acids, corresponding with the last 77 amino acids of the pine sequence. Accession numbers are indicated in brackets: *At* AS1, *Arabidopsis thaliana* AS1 (L29083); *At* AS2, *Arabidopsis thaliana* AS2 (AF095453); *At* AS3, *Arabidopsis thaliana* AS3 (AF095452); *Ao*, *Asparagus officinalis* (X67958); *Gm* AS1, *Glycine max* AS1 (U55874); *Gm* AS2, *Glycine max* AS2 (U77678); *Ha* AS1, *Helianthus annuus* HAS1 (AF190728); *Ha* AS2, *Helianthus annuus* HAS2 (AF190729); *Lj* AS1, *Lotus japonicus* LJAS1 (X89409); *Lj* AS2, *Lotus japonicus* LJAS2 (X89410); *Pv* AS1, *Phaseolus vulgaris* AS1 (AJ133522); *Pv* AS2, *Phaseolus vulgaris* AS2 (AJ009952); *Os*, *Oryza sativa* (D83378); *Sc* AS1, *Saccharomyces cerevisiae* AS1 (Z48675); *Sc* AS2, *S. cerevisiae* AS2 (Z72909); Hum, human AS (M27396); Ham, hamster AS (M27838); *Ec* ASB, *Escherichia coli* glutamine-dependent ASB (J05554). Parsimony analysis was performed using the heuristic search option of the phylogenetic analysis program (PAUP version 3.1.1) and the phylogram was constructed using treeview software version 1.6.6. The length of the branches is proportional to the number of changes along the branch. The value on each branch indicates the percentage of 1,000 bootstrap replicates supporting the branch. The *scale bar* indicates the number of amino acid substitutions required to obtain the branch length

that the light effect on the expression of a cytosolic GS gene of *P. sylvestris* was stronger at later stages of seedling development than at early stages (Cantón et al. 1999). In fact, samples from eight-day-old seedlings germinated under continuous light or dark showed the same *PsAS1* transcript level in both conditions (data not shown). AS and *PsAS1* transcript levels were undetectable in cotyledons of seedlings germinated under continuous light (Fig. 6a; lane 1). However, when the seedlings were germinated under continuous darkness an increase of both, protein and *PsAS1* transcript, was detected (Fig. 6a; lane 2). The parallel increase in *PsAS1* transcript and AS protein supports that the identified polypeptide was the product of the *PsAS1* gene. In

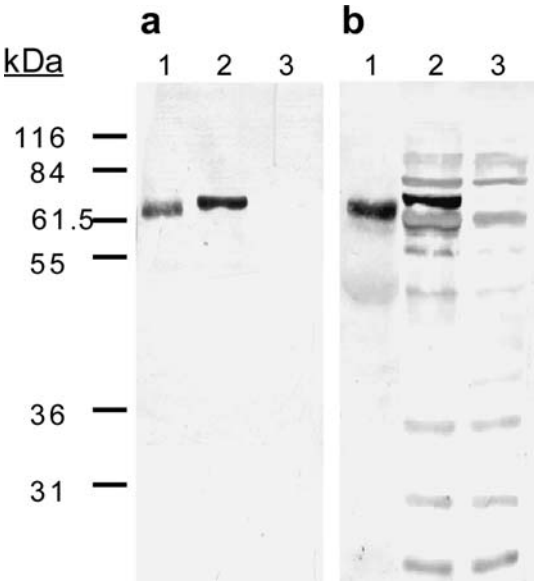
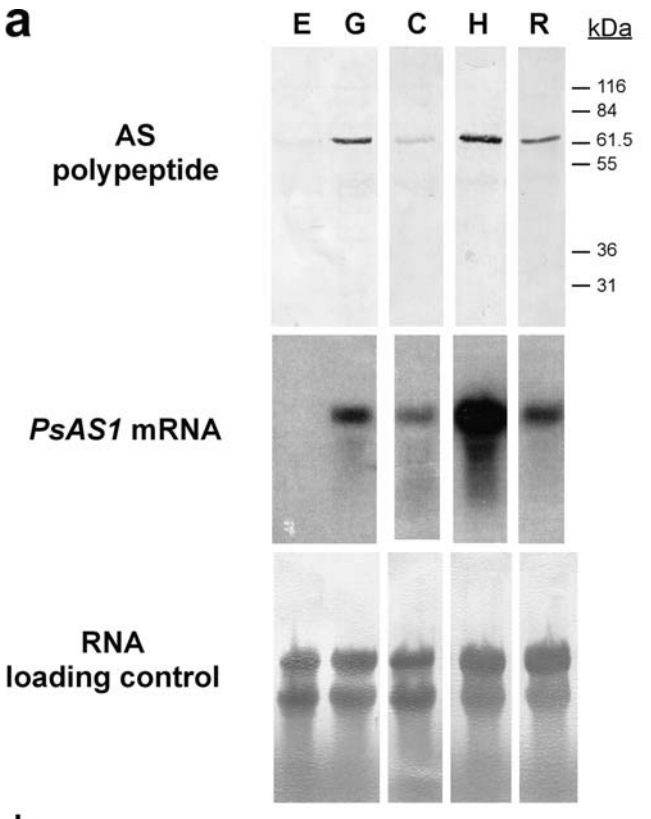


Fig. 4 a, b Western blot analysis of pine AS. **a** Immunoblot performed with affinity purified antibodies obtained against recombinant pine AS protein (1:200 dilution). **b** Immunoblot performed with the antiserum against alfalfa AS protein described by Twary et al. (1994), dilution 1:2000. *Lane 1* total proteins extracted from pine hypocotyls; *lane 2* total proteins extracted from cultures of *E. coli* overexpressing recombinant pine AS; *lane 3* total proteins extracted from cultures of *E. coli* transformed with pET 11a

contrast, the abundance of the *PsAS1* transcript was very similar in hypocotyls under both germination conditions (Fig. 6a; lanes 3 and 4), whereas the levels of AS protein were larger in dark germinated seedlings. This discrepancy between transcript and protein levels could be explained by a post-translational control, a dilution effect in the pool of proteins synthesized in light-grown seedlings or protein levels representing more than one AS polypeptide as a result of induction in darkness of a second AS gene. To investigate the last possibility, we analyzed the AS polypeptides extracted from hypocotyls of dark-grown seedlings by 2D-electrophoresis and Western blotting. GS immunodetection was also included in the experiment as a control of isoelectric focusing resolution. In pine seedlings two cytosolic GS proteins, named GS1a and GS1b, have been identified (Ávila et al. 2001). The percentage of identity in the amino acid sequence is high (82%) between both proteins and they have similar isoelectric point (6.5 and 6.0, respectively). In spite of the similar isoelectric point values, two well-separated spots were resolved for GS, representing GS1a and GS1b polypeptides, whereas a single AS polypeptide was detected (Fig. 6b). The same spot pattern was obtained with



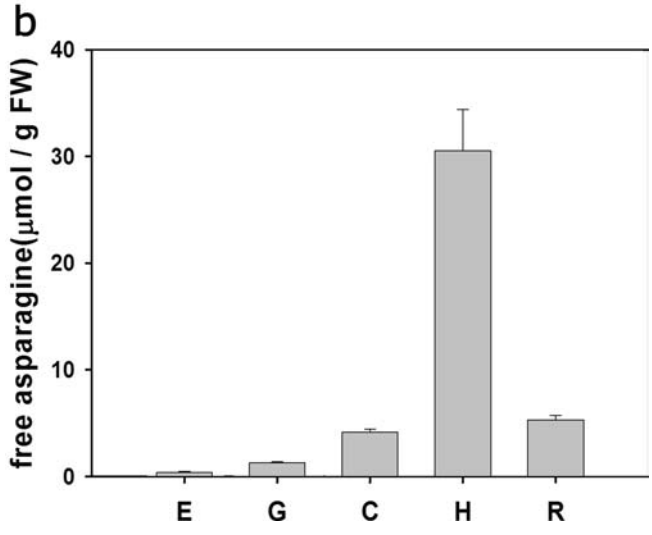


Fig. 5 a, b Expression pattern of *PsAS1* and asparagine levels in embryos and seedlings. **a** Total proteins were prepared from embryos and cotyledons, hypocotyls and radicles of seedlings (*upper panel*). Proteins were separated by SDS-PAGE (15 µg of protein per *lane*), electrotransferred onto nitrocellulose membranes and immunoprobed with affinity purified anti-AS. The molecular mass (kDa) of the protein markers is indicated on the *right*. Total RNA was extracted from embryos and cotyledons, hypocotyls, and radicles of seedlings (*middle panel*); 10 µg from each sample were analyzed by Northern blot using as probe a 240 bp fragment containing the 3′-UTR from the *PsAS1* cDNA. Total RNA transferred onto the nylon membrane was stained with a methylene blue solution to check for equal loading (*lower panel*). Shown data are representative of at least two independent experiments. **b** Embryos, cotyledons, hypocotyls, and radicles from seedlings were analyzed for soluble asparagine content. Each value is the mean ± SE of the results from three separate determinations. *E* embryos harvested immediately after imbibition, *G* embryos harvested 5 days after imbibition, *C* cotyledons, *H* hypocotyls, and *R* radicle from seedlings harvested 8 days after imbibition

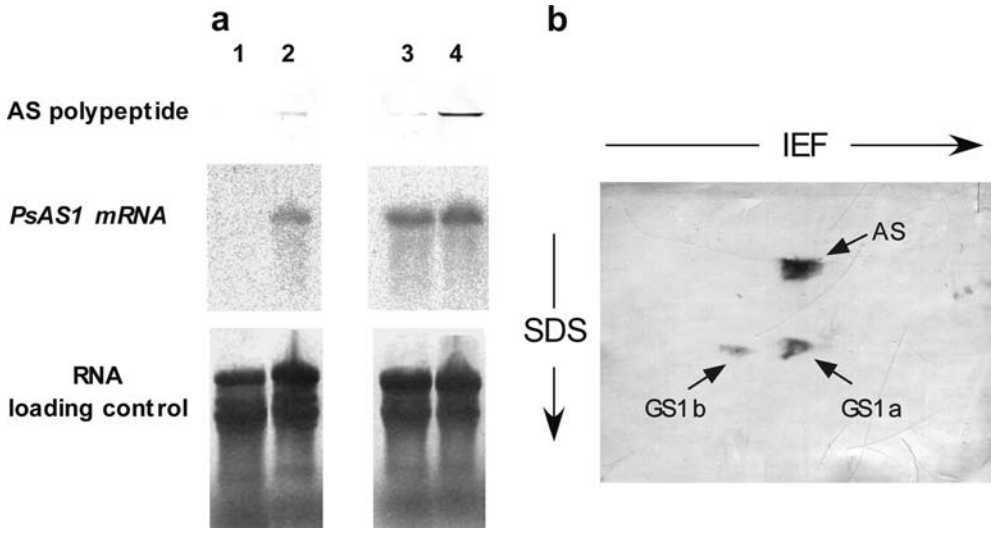


Fig. 6 a, b *PsAS1* expression in cotyledons and hypocotyls of pine seedlings germinated under continuous light or dark. *Pinus sylvestris* seeds were germinated and developed in continuous light or dark for 22 days after imbibition. **a** Proteins (15 μg of total proteins per *lane*) and total RNAs (8 μg of total RNA per *lane*) extracted from cotyledons or hypocotyls were separated by electrophoresis, blotted to filters, and analyzed by Western and Northern blots, respectively. Western blots were immunoprobed with anti-AS antibodies and Northern blots were hybridized with a 240 bp fragment containing the 3′-UTR from the *PsAS1* cDNA. RNA transferred onto the nylon membrane was stained with a methylene blue solution to check for equal loading. *Lane 1* cotyledons from light-germinated seedlings; *lane 2* cotyledons from dark-germinated seedlings; *lane 3* hypocotyl from light-germinated seedlings; *lane 4* hypocotyls from dark-germinated seedlings**. b** 2D-electrophoresis analysis of total proteins extracted from seedlings germinated under continuous dark. After 2D electrophoresis, proteins were blotted to nitrocellulose membranes and immunoprobed with a mixture of antibodies obtained against pine AS and GS. All data are representative of, at least, two independent experiments

protein extracted from hypocotyls of light-grown seedlings (data not shown). These results indicate that a single AS polypeptide accumulates in hypocotyl of pine seedlings.

Localization of AS polypeptides in pine seedlings

The tissue-specific localization of AS polypeptides was determined in different organs of the seedling using antibodies obtained against the recombinant pine AS protein. Sequence data support that the *PsAS1* gene encodes a glutamine-dependent AS, and it has been suggested that there is a catalytic cycle between AS and the GS1b isoform, spatially associated with vascular tissues (Ávila et al. 2001). Since this metabolic pathway would be involved in the biosynthesis of amides in vascular cells for N transport, we have compared the localization of AS and GS polypeptides to determine whether or not the *PsAS1* polypeptides co-localize with that specific GS isoform.

In developing green cotyledons the accumulation of AS polypeptide was restricted to cells of the vascular bundles (Fig. 7, panel C1). No signals were seen in parenchymatic cells or epidermic cells. In contrast, the GS antibody labeled immunoreactive protein in both the vascular bundles and parenchymatic cells (Fig. 7, panel C2), in good agreement with the accumulation pattern of both GS isoforms (GS1a and GS1b) described previously by immunolocalization (García-Gutiérrez et al. 1998) and in situ hybridization (Ávila et al. 2001).

In the hypocotyl, two different patterns were observed when the upper (UH) and lower (LH) parts were compared (Fig. 7, column 1, rows UH and LH). In the upper section, the stronger signal was mainly located at the pericycle, the Casparian strip, the endodermis, and cells of the inner cortex, although AS polypeptide was also detected in phloem and the pith (Fig. 7, panel UH1). Again, the GS antibodies revealed a much broader localization of polypeptides, as a result of the detection of both GS isoforms (Fig. 7, panel UH2). In the lower hypocotyls, AS was almost restricted to the vascular tissue, mainly phloem cells (Fig. 7, panel LH1), whereas GS showed a broader localization in the vascular cylinder and the cortex (Fig. 7, panel LH2). In all the organs, negative controls did not show any endogenous alkaline phosphatase activity (Fig. 7, panels in column 3).

Discussion

The primary structure of the pine AS polypeptide is similar to the previously reported angiosperm AS proteins (Fig. 1), with an average percentage of identity of 81.2%. Interestingly, pine and angiosperm AS show a high percentage of identity to bacterial glutamine-

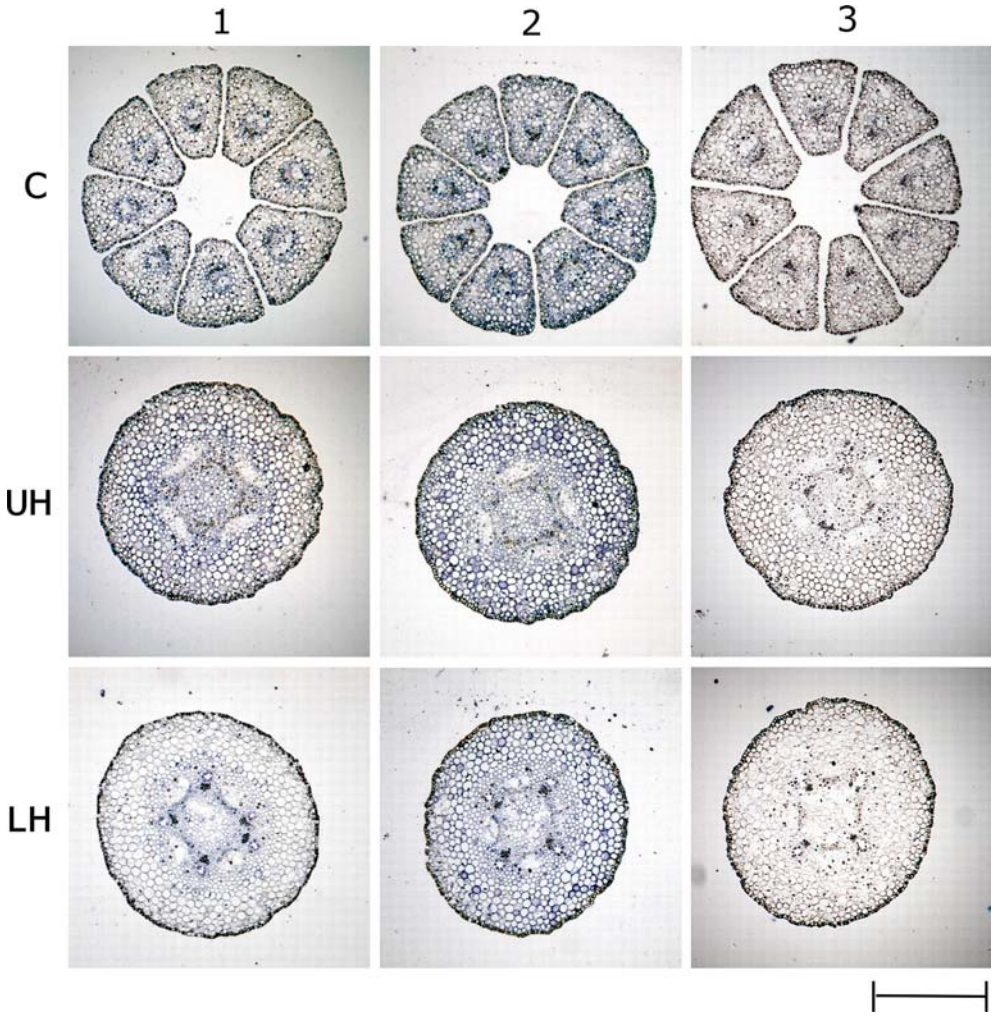


Fig. 7 Histological immunolocalization of AS polypeptides in developing pine seedlings. Sections (10 μm thick) of cotyledons (*C*), upper hypocotyl (*UH*), and lower hypocotyl (*LH*) were probed with purified polyclonal AS IgGs (*column 1*) or purified polyclonal GS IgGs (*column 2*). In the control sections (*column 3*), the whole process was performed but without probing with the AS or GS antibodies. Magnification ×50. *Scale bar* = 500 μm

dependent AS. In addition, all essential residues for the glutamine amidotransferase AS activity (Boehlein et al. 1997a, b; Zalkin and Smith 1998; Larsen et al. 1999) are conserved in the deduced pine polypeptide. Therefore, it can be concluded that this gene, named *PsAS1*, encodes a glutamine-dependent AS. The difference of molecular mass between the pine polypeptide and the recombinant protein observed in Western blot analysis suggests that the AS polypeptide may be processed in pine cells, although we cannot rule out at this point an artifactual effect on migration for unknown reasons. Another explanation for this finding could be that the polyclonal antibodies recognize the product of an alternative pine AS gene. However, the parallel accumulation pattern of the AS polypeptide and *PsAS1* transcript during germination and among seedling organs (Fig. 5), together with the detection of a single AS form as revealed by 2D-electrophoresis (Fig. 6b), strongly suggest that this protein is the product of the *PsAS1* gene.

Southern blot analysis with an AS probe from a well-conserved region among all plant AS genes revealed multiple genomic fragments (Fig. 2a). Some of the bands showed a stronger hybridization signal and they are most probably fragments of the *PsAS1* gene. The large genomic fragments with lower hybridization signals could represent other AS genes in the pine genome. In fact, when the hybridization was performed with a *PsAS1* gene-specific probe, only a single genomic fragment was detected in every lane (Fig. 2b). These results suggest that pine genome contains more than one gene encoding AS polypeptides, as described in angiosperms. The other AS genes could be involved in asparagine synthesis in different physiological contexts or developmental stages. Thus, when RNA samples of various tissues from adult pines were hybridized with a *PsAS1* probe no transcript was detected (data not shown), suggesting that other AS gene could be involved in asparagine synthesis in adult pines. The phylogenetic

analysis of AS sequences from different sources correctly separated plant, mammalian, yeast and *E. coli* AS polypeptides (Fig. 3). In the plant cluster, angiosperm species were grouped in two subclasses: (1) AS grouped with the *Arabidopsis* AS1 polypeptide and (2) AS clustered with the *Arabidopsis* AS2 and AS3 polypeptides. The separation of these two groups was well supported by the results of the bootstrap analysis. As previously reported (Osuna et al. 2001), the monocotyledonous AS polypeptide clustered with the AS2/AS3-like group. Pine AS was included in a sub-branch of the AS1-like polypeptides. This classification was also well supported by 93% of the 1,000 bootstrap replicas, and indicates the gymnosperm/angiosperm divergence. The position of the pine polypeptide as a separated branch in the AS1-like group suggest that the AS gene family appeared early in plant evolution. Therefore, since *PsAS1* is an orthologue of the *ASN1* gene of *Arabidopsis*, it could share similar functions in the physiology of the plant.

In *Arabidopsis* the *ASN1* and *ASN2* genes are reciprocally regulated by light and metabolites (Lam et al. 1998). The *ASN1* gene is downregulated by light and sucrose, and amino acids partially relieve the negative effect of the carbohydrates, whereas *ASN2* is induced by light and sucrose and the addition of some amino acids partially repressed carbohydrate induction. It has been suggested that the *ASN1* gene modulates asparagine levels depending on the light conditions or availability of C (Lam et al. 1998). In contrast, a recent report supports the notion that *ASN2* is closely correlated with ammonium metabolism associated with stress (Wong et al. 2004). The higher identity of pine AS primary structure to the *Arabidopsis ASN1* gene product suggests a similar role for the pine enzyme in modulating the levels of asparagine to adjust N assimilation, storage and transport to C availability during germination and seedling development.

PsAS1 mRNA and polypeptide are absent in embryos. However, *PsAS1* gene expression increased when embryo elongation started, suggesting that high levels of AS are required only when N storage material has been mobilized and the embryo undergoes active cell proliferation and development of essential seedling organs and structures. This activation of *PsAS1* expression matches the increase of asparagine levels observed during *P. taeda* germination (King and Gifford 1997), where the most pronounced change in the amino acid profile in the seed occurred following radicle emergence. This change was the result of a large increase in the amounts of a few amino acids, and asparagine became the most abundant. Afterward, during seedling elongation and establishment, *PsAS1* is expressed at high levels in hypocotyls and at moderate levels in radicles, whereas very low amounts of transcript and polypeptide were detected in cotyledons (Fig. 5a). In *P. sylvestris* the abundance of asparagine in the embryo is also very low, but after emergence of the radicle and elongation of the hypocotyls the levels of this amino acid are triggered in the seedling, coinciding with the induction of the *PsAS1* gene (Fig. 5b). Furthermore, our dissection of 8-day-old seedlings showed that the increase in asparagine content is due mainly to the accumulation of this particular amino acid in hypocotyls. The correlation between amino acid levels and *PsAS1* expression during germination and the spatial distribution in the seedling strongly suggest that the asparagine accumulation is a result of the *PsAS1* gene product activity.

The asparagine increase in later stages of pine seedling growth, contrasting with the relatively low concentration in the non-vascularized megagametophyte, was first reported by King and Gifford (1997). In pine seeds 75% of lipid and 80% of proteins stored within the seed are located in the megagametophyte (Groome et al. 1991; Stone and Gifford 1999). Following germination, these reserves are broken down into sugars and amino acids, which are rapidly moved to the seedling where they support seedling growth. Therefore, during early development the large megagametophyte tissue provides the seedling with large amounts of C and N, which may be in excess of their metabolic demands. In fact, much of the sucrose synthesized from lipid reserves in *Pinus edulis* (Murphy and Hammer 1994) and *P. taeda* (Stone and Gifford 1999) is converted to starch and accumulated in cotyledons and hypocotyl, and the embryonic triacylglycerols are not completely exhausted in the seedling during early growth (Stone and Gifford 1999). All the mobilized materials are transported from the megagametophyte to the cotyledons where they are metabolized and distributed to the hypocotyls and the radicle (King and Gifford 1997; Stone and Gifford 1999; Todd and Gifford 2002). According to this scenario, arginase activity, protein and mRNA are predominantly localized in the expanding cotyledons, which remain in contact with the megagametophyte throughout early seedling growth (Todd et al. 2001). Ammonium derived from arginine hydrolysis is assimilated into glutamine by GS, which is also accumulated at high levels in cotyledons during seed germination (Ávila et al. 2001). Our results indicate that during pine seedling development, high levels of asparagine are synthesized in hypocotyl and radicle, with the hypocotyl being the main asparagine production site in the seedling. Therefore glutamine, synthesized from arginine in cotyledons, must be specifically transported to the hypocotyl and the radicle for asparagine synthesis, in a situation in which there is no C limitation. This suggests that asparagine is not synthesized as a result of C economy or as a vehicle to transport the mobilized N from cotyledons to hypocotyl and radicle, but rather as a specific N reserve.

The ability of conifer seeds to synthesize chlorophyll, develop chloroplast-like plastids, and express photosynthesis-related genes in darkness is well known (Bogdanovic 1973; Mariani et al. 1990; Yamamoto et al. 1991, Cánovas et al. 1993). When compared to angiosperms and no-conifer gymnosperms, pine seed germination displays a tightly regulated developmental program with limited environmental interactions (García-Gutiérrez et al. 1998). Although a long period of darkness (22 days) increased the AS content, it

did not affect the relative spatial distribution of the polypeptide. Either in continuous light or darkness AS content was larger in hypocotyls than cotyledons. Moreover, *PsAS1* transcript level in hypocotyl was similar in seedlings germinated under continuous light or darkness. These results suggest that, during pine germination and early seedling growth, there is a developmental control of *PsAS1* expression to ensure a high level of asparagine synthesis in hypocotyls.

The localization of AS protein in cells of the vascular bundles in cotyledons and the lower part of hypocotyls (Fig. 7) suggests that *PsAS1* may have a role in synthesizing asparagine for N transport, probably associated with the GS1b isoenzyme, which is also located in vascular bundles in cotyledons and hypocotils (Ávila et al. 2001). However, the broader tissue localization of AS polypeptides in the upper part of the hypocotyls in other cell types besides the vascular cells, and overlapping with GS1a localization (Ávila et al. 2001), suggest that this part of the seedling could be specialized in synthesizing high amounts of asparagine.

Summarizing, a single AS gene, *PsAS1*, appears to be responsible for asparagine biosynthesis during pine germination and post-germinative growth. *PsAS1* expression is developmentally regulated in pine hypocotyl, where high levels of asparagine are accumulated. Furthermore, our results suggest that an important amount of N mobilized from storage material in megagametophyte to cotyledons is further transferred to synthesize and accumulate asparagine in hypocotyls. The accumulation of N as soluble asparagine may be a metabolic mechanism to preserve the excess of mobilized N that is not immediately used. Thus, the asparagine pool may represent a transitory store of N to be used later to meet specific demands during seedling development. For instance, it might be used as an initial C and N supply during the transformation of the hypocotyl in a highly lignified vascular structure, a crucial process for pine seedling development and survival.

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