Planta (2007) 225:1205–1219
DOI 10.1007/s00425-006-0431-9

ORIGINAL ARTICLE

Coordination of *PsAS1* and *PsASPG* expression controls timing of re-allocated N utilization in hypocotyls of pine seedlings

Rafael A. Cañas · Fernando de la Torre · Francisco M. Cánovas · Francisco R. Cantón

Received: 20 July 2006 / Accepted: 13 October 2006 / Published online: 23 November 2006

Abstract During pine seed germination, a large amount of N mobilized from the storage proteins is re-allocated in the hypocotyl as free asparagine, as a result of the high levels of asparagine synthetase (AS) encoded by the *PsAS1* gene. To determine the role of this re-allocated N reserve, a full-length cDNA encoding L-asparaginase (ASPG) has been cloned from Scots pine (*Pinus sylvestris* L.) seedlings and characterized. Like other N-terminal nucleophile hydrolases, pine ASPG requires a post-translational processing to exhibit enzymatic activity. However, in contrast to previous reports on other plant ASPGs, purified recombinant pine ASPG does not undergo autoproteolytic cleavage in vitro. Our results suggest that the processing requires accessory proteins to assist in the proteolysis or in the proper folding before autocleavage in a divalent cation-dependent manner. Sequence comparison analysis revealed that the pine protein is included in the K^+-dependent subfamily of plant ASPGs. The expression of the ASPG-encoding gene (*PsASPG*) was higher in organs with extensive secondary development of the vascular system. The increase in transcript abundance observed at advanced stages of hypocotyl development was concomitant with a decrease of *PsAS1* transcript abundance and a remarkable increase in the number of xylem elements and highly lignified cell walls. These results, together with the precise localization of *PsASPG* transcripts in cells of the cambial region, suggest that the expression of *PsAS1* and *PsASPG* is temporally coordinated, to control the re-allocation of N from seed storage proteins toward the hypocotyl to be later used during early development of secondary vascular system.

Keywords Asparagine metabolism · L-asparaginase · Nitrogen re-allocation · Vascular system development · *Pinus sylvestris*

Abbreviations

EST	Expressed sequence tag
ASPG	L-Asparaginase
AS	Asparagine synthetase
N	Nitrogen
C	Carbon
Ntn	N-terminal nucleophile
UTR	Untranslated region
TIGR	The institute for Genomic Research
RT-PCR	Reverse transcription polymerase chain reaction

Introduction

In plants, asparagine is a key metabolite for N transport (Joy 1988; Sieciechowicz et al. 1988; Lea et al. 1990). Several chemical properties make asparagine a convenient vehicle for N transport, it is more soluble than ureides and more stable than glutamine and it is also a more efficient N carrier than glutamine because it has a higher N/C ratio (Urquhart and Joy 1981). As an N transport molecule, asparagine appears to be targeted toward sink tissues where large amounts of N is

R. A. Cañas · F. de la Torre · F. M. Cánovas · F. R. Cantón (✉)
Departamento Biología Molecular y Bioquímica, Universidad de Málaga, Campus de Teatinos s/n, 29071 Málaga, Spain
e-mail: frcanton@uma.es

demanded (Sieciechowicz et al. 1988), and different studies have shown that glutamine-dependent AS activity (EC 6.3.5.4) is the main source of asparagine for N transport in plants (Azevedo et al. 2006). In particular, during seed germination in angiosperms AS plays a major role in mobilization of N resources, since storage proteins are converted to asparagine for transport to growing apices (Lea and Fowden 1975; Rognes 1975, 1980; Kern and Chrispeels 1978). It has also been proposed that asparagine may play an important role in N storage; however, the studies supporting this role are limited.

Two routes have been described for mobilization of N contained in asparagine. The first one is the reaction catalyzed by ASPG (EC 3.5.1.1) for the hydrolysis of asparagine to aspartate and ammonium. A second route involves the removal of the amino group of asparagine by transamination to an oxo-acid acceptor (Streeter 1977). The transaminase activity (EC 2.6.1.14) has been detected in leaves of soybean (Streeter 1977), pea (Ireland and Joy 1981) and lupin (Atkins et al. 1983). Several studies support the view that ASPG is a key enzyme involved in mobilizing N from asparagine in tissues demanding high amounts of N, such as developing seeds and young leaves (Sodek et al. 1980; Ireland and Joy 1981; Grant and Bevan 1994), whereas asparagine aminotransferase seems to have minor importance in these tissues (Ireland and Joy 1981; Atkins et al. 1983).

Based on amino acid sequences and biochemical properties, enzymes with ASPG activity have been classified in three families: bacterial-type ASPG, plant-type ASPGs, and enzymes similar to *Rhizobium etli* ASPG (Borek and Jaskólski 2001). Plant ASPGs belong to the second family, which also includes enzymes with aspartylglucosaminidase activity (EC 3.5.1.26) that catalyze the hydrolysis of glycosidic bonds between sugar chains and the L-asparagine side chain. Aspartylglucosaminidases and plant ASPGs belong to the group of Ntn-hydrolases (Guan et al. 1998; Guo et al. 1998; Borek et al. 2004; Bruneau et al. 2006; Michalska et al. 2006). Both types of enzyme are heterotetramers, formed by two types of subunits (α and β) created by autoproteolytic cleavage of two identical precursor polypeptides. The cleavage of the precursor creates also the N-terminal nucleophilic residue. Enzymes related to plant ASPGs are also found in other organisms. In particular, three enzymes with ASPG activity have been characterized in *Escherichia coli*: cytosolic EcAI and periplasmic EcAII are bacterial-type ASPGs, whereas EcAIII is a plant-type ASPG catalytic and structurally related to plant ASPGs (Borek et al. 2004).

The biochemical characterization of recombinant ASPGs obtained by expression in *E. coli* of two cDNA from different genes of *Arabidopsis* have revealed that they correspond to the K^+-dependent and K^+-independent subfamilies of plant ASPGs (Bruneau et al. 2006). Sequence comparison and phylogenetic analysis suggest the co-occurrence of both subfamilies in different plant species. This finding explains the detection at the biochemical level of both types of activities in early works (Sodek et al. 1980; Chang and Farnden 1981; Lea et al. 1984). Both types of enzyme show other catalytic difference, the *Arabidopsis* K^+-independent enzyme exhibits both L-asparaginase and isoaspartyl dipeptidase activities, whereas the K^+-dependent enzyme is strictly specific for L-asparagine (Bruneau et al. 2006).

Pine seedlings accumulate high amounts of free asparagine during germination (King and Gifford 1997, Cañas et al. 2006). In early steps of pine seed germination, the megagametophyte provides the embryo with C and N to support development and the differentiation of seedling structures (Todd and Gifford 2002). In particular, the mobilization of storage proteins in the megagametophyte causes a large increase in the levels of free arginine, and a flow of this amino acid toward the embryo, where it is hydrolyzed to ornithine and urea in the reaction catalyzed by arginase (Todd et al. 2001). The urea molecule could provide ammonium to the embryo, through the hydrolysis reaction catalyzed by urease (Todd and Gifford 2002). Finally, glutamine synthetase catalyzes the recycling of ammonium into glutamine, to be used in the synthesis of all N-containing compounds of the seedling (Avila et al. 2001). The fate of N contained in the ornithine molecule derived from arginine hydrolysis is still unknown. Later in germination, and coinciding with the elongation of the radicle and the expansion and development of cotyledons, accumulation of large amount of free asparagine occurs mainly in the hypocotyl (Cañas et al. 2006). This drastic change in the free amino acid profile is a result of the induction of a gene encoding glutamine-dependent AS, which is expressed at high levels in the hypocotyl. The spatial location of arginase, glutamine synthetase and AS in early developing pine seedlings suggests that asparagine is not synthesized as a result of C economy or as a vehicle to transport N from cotyledons to hypocotyl, but rather as a specific N reserve (Cañas et al. 2006).

Genes encoding AS and ASPG have been separately studied in the context of germination and seedling development of angiosperms, and the information about the coordination of asparagine synthesis and hydrolysis is limited. In this paper, we

report the molecular cloning of a cDNA for a *Pinus sylvestris* gene (*PsASPG*) encoding a plant-type ASPG. Precursor processing and enzyme activity have been assayed with recombinant pine ASPG. Phylogenetic relationships of the pine protein and the expression patterns of *PsAS1* and *PsASPG* genes during germination and seedling development are also described.

Materials and methods

Plant material and growth conditions

Scots pine seeds (*P. sylvestris* L.) were obtained from Ministerio de Medio Ambiente (Madrid, Spain). Seeds were immersed in deionized water for 24 h, under continuous aeration and germinated in soil. Seedlings were grown in a controlled culture chamber (Ibercex H-900-B, ASL, SA, Madrid, Spain) at 24°C, with 16 h light/8 h dark cycles. White light was provided by fluorescent lamps (Sylvania F-48T12/CW/WHO; Koxka, Pamplona, Spain) at a fluence rate of 150 μmol m^{-2} s^{-1}. Plants were watered regularly but no N fertilizer was added. Samples were always harvested at the same time in the light/dark cycle.

Isolation of *PsASPG* cDNA and sequence analysis

A set of primers were designed from a maritime pine (*P. pinaster* Ait.) partial cDNA sequence with homology to plant ASPGs, derived from an EST project of developing xylem from maritime pine (ASPG1, 5′-GT GGGTTGTGTTGTTGTTGA-3′ and ASPG2, 5′-CA CAGGCTCTGAACATTCC-3′). These primers were used to amplify a cDNA fragment from *P. sylvestris* by RT-PCR. The RNA sample used for cDNA synthesis was extracted from hypocotyls as described below, and it was evaluated in denaturing agarose-gel stained with ethidium bromide to ensure that they contained intact rRNA and were free of genomic DNA contamination. cDNA was synthesized with AMV reverse transcriptase (Roche Diagnostics). PCR was performed with *Pfu* DNA polymerase (Stratagene). A single DNA fragment of 352 bp was amplified and cloned in the pGEM-Teasy vector (Promega). The cDNA fragment 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). Comparison with sequences in databases confirmed that the PCR product encoded a fragment of an ASPG polypeptide.

To clone a full-length cDNA, a reverse gene-specific primer was designed from the sequence of the 352 bp PCR fragment (ASPG3, 5′-CACCCTTACCATCTTC CAACCTC-3′). The primer was used in a PCR reaction together with the primer complementary to the adaptor sequence AP1 (5′-CCATCCTAATACGAC TCACTATAGGGC-3′) to clone a PCR product by 5′-RACE (Marathon™ cDNA Amplification Kit, Clontech) containing the 5′-end of the mRNA. A nested PCR reaction was performed using the primary product as a template and ASPG3 and the oligonucleotide complementary to the adaptor sequence AP2 (5′-ACTCACTATAGGGCTCGAGCGGC-3′) as primers. The primary product was cloned in the pGEM-Teasy vector (Promega) and completely sequenced to verify the identity of the partial cDNA. To amplify a full-length cDNA containing the complete coding region, the 5′-end sequence of this PCR product was used to design a forward primer (ASPG4, 5′-CTGCTTTCTGCTCTTTATC-3′) that was used together with the AP1 primer in a 3′-RACE reaction. A single product was amplified both in the 5′- and 3′-RACE reactions. To verify the sequence, three full-length cDNAs obtained in independent RT-PCR reactions were completely sequenced. As a control that PCR products were not derived from genomic DNA, PCR reactions were performed with an equivalent amount of RNA, but without performing a previous reverse transcription reaction. No amplification was observed in the control reactions. The nucleotide sequence was deposited in the EMBL sequence database under the accession number AM265537. Amino acid alignment of several deduced ASPG polypeptides was carried out with Clustal IV in the DNAstar software package (DNASTAR Inc.). Phylogenetic analyses were performed with the PAUP software version 3.1.1 (Phylogenetic analysis using parsimony. Swofford DL, Illinois Natural History Survey, Champaign, IL, USA, 1993) using the heuristic option. The unrooted tree was constructed with the TreeView software version 1.6.6.

Recombinant protein production and purification

To facilitate the purification of recombinant pine ASPG, the cDNA encoding *P. sylvestris* ASPG was manipulated by PCR in order to produce an N-terminal polyhistidine-tagged (His-tagged) protein. A forward primer was designed, containing the 5′-end of the coding region of the pine *PsASPG* cDNA preceded by a unique *Nde*I site and a sequence encoding for six histidine residues (5′-AGAACATATGCACCATCATC ATCATCATGGGTGGGCCATAGCTGTGC-3′). A

reverse primer was designed, containing a gene-specific cDNA sequence and incorporating a unique restriction site for *Bam*HI immediately downstream from the translation stop codon, (5′-CAGAGGGTGGATCCT ACTGC-3′) Both primers were used to amplify the cDNA by PCR with *Pfu* DNA polymerase (Stratagene) and, after digestion with *Nde*I and *Bam*HI, the cDNA was subcloned at the same sites of the pET11a polylinker. The construct was verified by sequencing.

BL21-Codon Plus (DE3) Ril *E. coli* cells, were transformed with the construct. Transformants were grown, by shaking in LB broth containing ampicillin (100 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}) at 37°C, until a cell density (OD 600) of 0.6 was reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM and the incubation was continued for an additional 3 h. Purification of soluble recombinant protein under native conditions was performed as recommended (Qiagen). Cells were pelleted by centrifugation at 15,000*g* for 20 min at 4°C, and resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole). Cells were sonicated, and centrifuged at 10,000*g* for 30 min at 4°C. His-tagged recombinant ASPG was purified from the supernatant by affinity with nickel-nitrilotriacetic silica spin columns as recommended (Qiagen). The purified proteins were desalted on a Amicon Ultra Ultracel−30k centrifugal filter (Millipore) and recovered in 50 mM Tris–HCl pH 8.0, 10% (v:v) glycerol. To purify recombinant ASPG under denaturing conditions, cells were harvested by centrifugation and resuspended in 0.1 M NaH_2PO_4, 10 mM Tris–HCl, pH 8.0, 8 M urea, and incubated 1 h at room temperature to lyse the cells. The debris was separated by centrifugation at 8,900*g* for 20 min at 4°C. Recombinant ASPG protein was purified using nickel-nitrilotriacetic acid silica spin column as recommended (Qiagen), and then extensively dialyzed overnight at 4°C in 0.1 M NaH_2PO_4, 10 mM Tris–HCl, pH 8.0, 10% (v:v) glycerol to remove the urea.

Extraction of soluble proteins from radicles of pine seedlings

Radicles were dissected from seedlings and ground in extraction buffer (50 mM Tris–HCl, pH 8.0, 50 mM KCl, 10% glycerol and 0.1% (v:v) 2-mercaptoethanol). The homogenate was centrifuged at 22,000*g* at 4°C for 20 min and the clarified supernatant saved to a new tube. During the entire process, protein was maintained at 4°C.

Precursor processing, determination of ASPG activity and protein quantification

Aliquots (120 μg) of recombinant ASPG, purified under denaturing conditions as described above, in 900 μl of 0.1 M NaH_2PO_4, 10 mM Tris–HCl, pH 8.0, 10% glycerol were incubated at 22°C for 48 h, with the addition of 65 μl of protein extract from radicles containing 3.5 μg of proteins or an equivalent volume of extraction buffer. To determine the ASPG activity recombinant pine, ASPG was incubated for 1 h at 37°C in activity buffer (50 mM Tris–HCl, pH 8.0, 10 mM asparagine). The reaction rate was linear after 2 h of incubation. The reaction was stopped by heating the samples for 1 min, in a bath with boiling water. After stopping the reaction, the samples were centrifuged at 12,400*g* for 2 min. Finally, free ammonia was measured using the phenol–hypochlorite method, as described by Sodek and Lea (1993). The protein concentration was estimated by the method of Bradford (1976).

Antibody production

For antibodies production against pine ASPG, the recombinant protein was overexpressed in *E. coli*, without the His-Tag. The full-length cDNA was cloned into the pET11a vector, following a similar strategy as described above, with the exception that the forward primer used had the sequence 5′-GTTCAGC-CATATGGGGTGGG-3′, whereas the reverse primer was the same as described above. The construct was verified by sequencing. Preparation of recombinant protein, immunization of rabbits and antibody purification were carried out as described previously (Cañas et al. 2006).

Western-blot analysis

SDS-PAGE and electroblotting were carried out as described elsewhere (Cantón et al. 1996). The membranes were blocked for 1 h with 3% (w/v) BSA dissolved in TPBS: 1X PBS (140 mM NaCl, 3 mM KCl, 5 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), 0.05% (v/v) Tween-20, and incubated overnight at room temperature with 0.95 μg of purified anti-ASPG antibodies diluted in TPBS with 0.05% (w/v) BSA. The membranes were washed three times with TPBS. Antigen-antibody complexes were detected with a secondary peroxidase-conjugated anti-rabbit-IgG antiserum. The membranes were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies diluted in TPBS with 0.05% (w/v) BSA. Finally, the

membranes were washed twice with TPBS and then twice with PBS. Immunodetection was visualized with the Immun-Star™ HRP (for horseradish peroxidase) Chemiluminescent Kit (Bio-Rad Laboratories, Inc.). The membranes were incubated with a 1:1 (v:v) mixture of luminol/enhancer and peroxide buffer solutions for 3–5 min. Finally the membranes were exposed to an X-ray film (X-OMAT AR, Kodak) and developed.

RNA and DNA extraction and gel 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 Diagnostics). Prehybridizations and hybridizations were performed at 65°C according to the method of Church and Gilbert (1984). After hybridization, membranes hybridized with the *PsAS1* probe were washed three times at high stringency (0.1X SSC, 0.1% SDS at 65°C). Membranes hybridized with the *PsASPG* probe were washed twice at 65°C in 2X SSC, 0.1% SDS. To avoid unspecific hybridization, the membranes were additionally washed at high stringency (0.1X SSC/0.1% SDS at 65°C).

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

Preparation of plant tissue for microscopy and in situ mRNA localization

Seedlings were harvested 30 days after imbibition and the tissues were fixed with freshly prepared FAA solution (5% formaldehyde, 5% glacial acetic acid, 90% ethanol). The samples were then dehydrated in an ethanol series (50, 70, 80, 96, 100%), treated with increasing concentrations of xylene (1:1 ethanol/xylene, xylene, 1:1 paraffin/xylene) and paraffin embedded in an automatic tissue processor TP1020 (Leica Microsystems, Barcelona, Spain). The embedded tissues were sectioned at 10 μm thickness and mounted on poly-L-lysine-coated glass slides (Menzel-Gläser, Braunschweig, Germany). The paraffin was removed with histoclear (National Diagnostic, Atlanta, GA, USA) and the sections were re-hydrated in 100, 90, 70, 50, 30% ethanol in distilled water. Before mRNA hybridization, several treatments were carried out. The tissue sections were incubated in HCl 0.2 M to denature mRNA, and then they were digested with protease K (1 μg ml^{-1}) in TE buffer (100 mM Tris–HCl, pH 8.0, 50 mM EDTA,). Subsequently, the tissue sections were fixed with 4% (w/v) paraformaldehyde in PBS, pH 7.4, and finally acetylated with 0.1% (v/v) acetic acid in TEA [1.3% (v/v) triethanolamine, 0.4% (v/v) HCl 37%]. The full-length *PsASPG* cDNA was subcloned in Bluescript SK-plasmid (Stratagene). Antisense cRNA probe was labeled with digoxigenin(DIG)-11-UTP (Boehringer) in a standard in vitro transcription reaction (Langdale 1993). As a control, a cRNA DIG-labeled sense riboprobe was synthesized. Before hybridization, the slides were air-dried and prehybridized for 2 h, at 37°C, with a solution containing 50% (v/v) formamide, 2X SSC, 1X Denhardt's solution, 10% (w/v) dextran sulphate, 70 mM DTT and 150 μg ml^{-1} yeast tRNA (Roche Diagnostics). Then, the sections were hybridized overnight at 55°C in the same prehybridization solution, but adding 0.5 ng μl^{-1} of labeled riboprobe. After hybridization the sections were washed twice with 1X SSC, 0.1% SDS for 10 min at 55°C. The tissue sections were incubated with alkaline phosphatase-conjugated anti-DIG antibodies for 1 h and alkaline phosphatase activity was developed using Fast Red (Roche Diagnostics) as substrate dissolved in 2 ml of 100 mM Tris–HCl, pH 8.2. Afterward, they were incubated in water for 10 min to stop the alkaline phosphate reaction. An Eclipse E-800 microscope (Nikon, Kingston upon Thames, UK) was used for sample visualization and photography.

In situ lignin detection

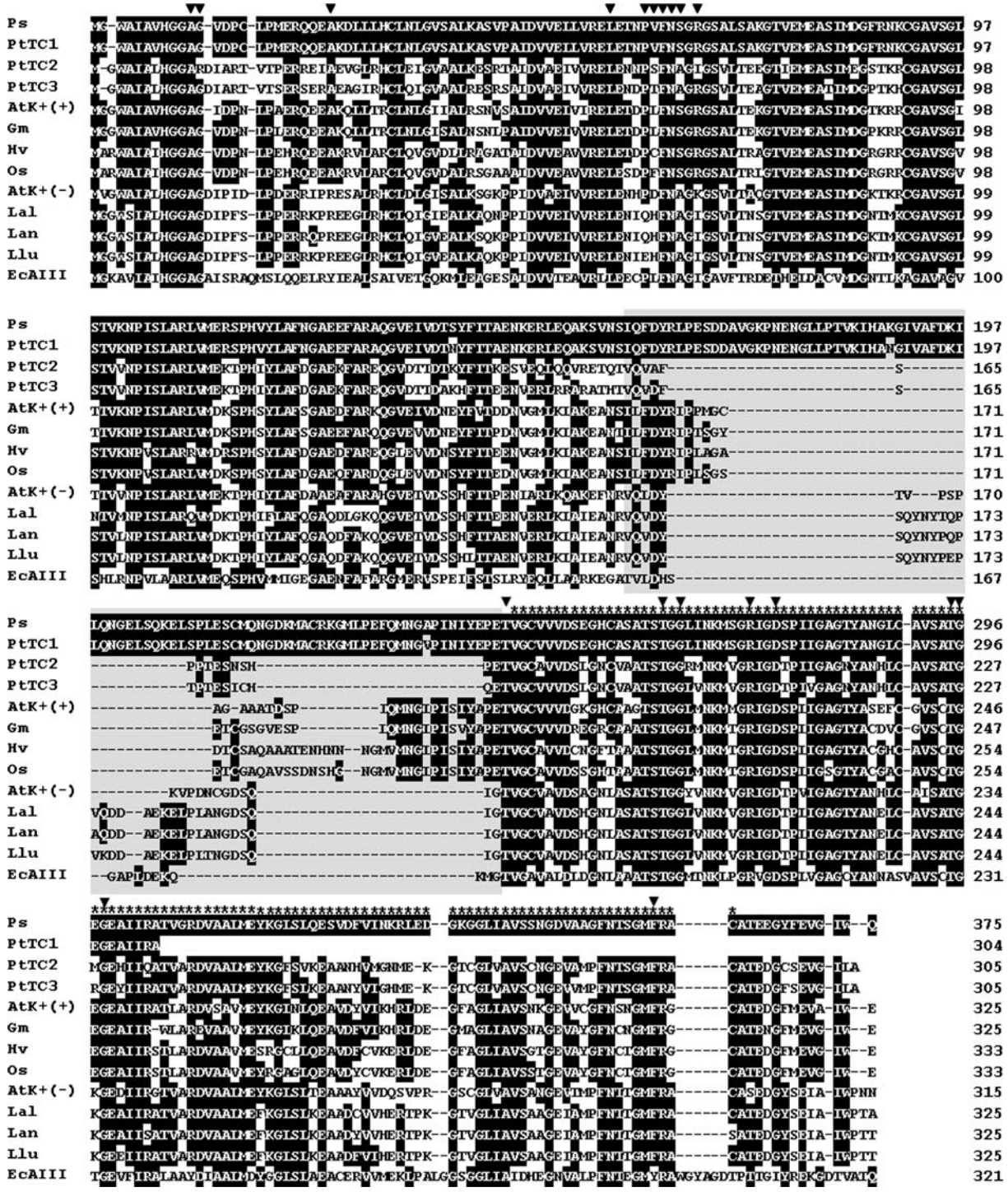
For observation of lignin accumulation, deparaffinized and re-hydrated tissue sections were stained for 3 min in 2.5% (w/v) phloroglucinol–HCl (Sigma-Aldrich), and then rinsed with water. Slides were mounted with glycerol 50%, HCl 50%.

Results

During the development of an EST project of developing xylem from *P pinaster* adult trees, a partial CDNA clone encoding a protein with high similarity to plant-type ASPGs was identified (unpublished data). The full-length cDNA has been cloned from *P. sylvestris*

hypocotyls by a RT-PCR/RACE strategy. We first used RT-PCR to amplify a short fragment (from position 804 to position 1,155 in the full-length cDNA sequence) using primers derived from the *P. pinaster* EST sequence. A single fragment of 352 bp was amplified. The product was sequenced and the deduced amino acid sequence (Fig. 1, residues labeled with asterisks) had a high identity with other plant ASPGs (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 size of the full-length cDNA is 1,360 bp, with a coding region of 1,128 bp, a 5′-UTR of 68 bp and a 3′-UTR of 164 bp, including a poly-A tail with 25 residues.



◀ **Fig. 1** Amino acid sequence alignment of plant ASPG proteins. The following sequences are included (accession numbers in brackets): amino acid sequence deduced from the full-length *PsASPG* cDNA, *Ps*: *Pinus sylvestris* (AM265537); three *P. taeda* sequences derived from tentative consensus of cDNA reported in the TIGR *Pinus* Gene Index database, PtTC1 (*P. taeda*, TC65340), PtTC2 (*P. taeda*, TC68434), PtTC3 (*P. taeda*, TC76010); four ASPG amino acid sequences from angiosperms classified as K^+-dependent (Bruneau et al. 2006), *At*K^+(+) *Arabidopsis thaliana* (GI:9294462), *Gm*: *Glycine max* (GI:29420787), *Hv*: *Hordeum vulgare* (GI:11066973), *Os*: *Oryza sativa* (GI:10241428); four ASPG amino acid sequences from angiosperms classified as K^+-independent, *At*K^+(−) *A. thaliana* (GI:8346547), *Lal*: *Lupinus albus* (GI:496102), *Lan*: *L. angustifolius* (GI:19135), *Llu*: *L. luteus* (GI:4139266); the *Escherichia coli* plant-type ASPG sequence, EcAIII (GI:1787050). Identical residues to *P. sylvestris* sequence are labeled by *black boxes*. 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. The divergent central stretch with variable length is labeled by a *gray background*. The amino acid sequence deduced from the 352 bp RT-PCR product is labeled with *asterisks* over the residues. Positions of residues characterized in the active sites of *L. luteus* and EcAIII ASPGs are labeled with *arrowheads*

The open reading frame encodes a protein of 375 amino acids and a predicted molecular mass of 39.7 kDa. The similarity of the protein deduced from the pine cDNA to plant-type ASPGs was confirmed by analysis of the complete sequence with the GenBank database using the BLAST software. An average identity of 63% was found with ASPG sequences from angiosperms and a value of 38.6% with the *E. coli* EcAIII protein. Overall, the sequence from *P. sylvestris* was more similar to sequences of the K^+-dependent family (identity values range from 64 to 69%) than to sequences of the K^+-independent family (identity values range from 59 to 61%). The alignment in Fig. 1 shows the high percentage of conserved residues between the *P. sylvestris* protein and plant-type ASPGs from other species, including the autoproteolytic cleavage site (Thr–Val–Gly) starting at position 245 of the pine sequence, which determines two potential subunits (α and β)with predicted molecular masses of 26.4 and 13.3 kDa and it also contains the nucleophilic threonine residue (Borek et al. 2004; Bruneau et al. 2006). Residues characterized in *Lupinus luteus* ASPG active site (Michalska et al. 2006) show a high conservation among plant ASPGs and the bacterial EcAIII enzyme. Nevertheless, the percentage of conservation of these residues is variable between the sequence regions corresponding to α and β subunits. All residues of the active site located in the β subunit are absolutely conserved among all plant ASPGs in the alignment and also in EcAIII, with the exception of a phenylalanine residue in plant ASPGs (residue Phe359 in the pine sequence), which is substituted by tyrosine in the bacterial enzyme. However, in the α subunit, only five of ten residues of the active site are conserved in all the sequences.

When pine cDNA sequence was compared to the tentative consensus sequences in the TIGR *Pinus* Gene Index database, the highest scores were obtained for three *P. taeda* sequences annotated as ASPGs (accession number TC65340, TC68434 and TC76010). The tentative consensus TC65340 is a partial cDNA sequence that includes an uncompleted coding region, but the available sequence obtained the highest identity score in the BLAST analysis when compared to the *P. sylvestris* sequence. The alignment in the Fig. 1 shows the high conservation of amino acid residues between the *P. sylvestris* and the *P. taeda* PtTC1 (TC65340) sequence (99% of identity), with only changes in three positions and two of them in the central variable region. At the nucleotide level, both sequences were also very similar (99.2%), with only eight changes. On the contrary, the other two tentative consensus sequences from *P. taeda* were more divergent. In the coding region, the percentages of identity of the *P. sylvestris* cDNA with TC68434 and TC76010 were 57.2 and 58.5, respectively. For the proteins encoded by these *P. taeda* cDNAs, the values of amino acid identity with the *P. sylvestris* protein were 59.7 and 61.0%, respectively. It is worth noticing that the percentage of identity at the amino acid level was only 84.9 between TC68434 and TC76010, and 87.5 when the nucleotide sequences of the coding region were compared. Both tentative consensus sequences were also very divergent in the residues of the UTR regions (data not shown).

The conservation of primary structure in plant ASPGs is interrupted in the central region of the precursor. The function of these amino acids in the structure or activity of the mature enzyme is unknown. In fact, after the initial autolysis, the last 17 residues of the α-subunit are removed from the mature recombinant EcAIII enzyme (Borek et al. 2004). The equivalent region in plant ASPGs shows a high variability in length and composition among different species (Fig. 1, sequence stretch labeled with a gray background). In angiosperms, the composition and length of this stretch is conserved among sequences of the same subfamily (K^+-dependent or -independent), but it diverges between sequences of both subfamilies. With regard to the pine ASPGs, the *P. sylvestris* protein and *P. taeda* PtTC1 (TC65340) include a sequence stretch of 86 residues (from position 159 to 244 in the amino acid sequence), remarkably longer than those found in other plant sequences and very divergent, whereas the equivalent stretch is shorter (16 residues) in PtTC2

(TC68434) and PtTC3 (TC76010), but is also very divergent (Fig. 1). The presence of the longer stretch in the *P. taeda* PtTC1 (TC65340) sequence is supported by several ESTs included in the tentative consensus.

Previous reports have shown that recombinant plant ASPGs purified from *E. coli* were able to catalyze the hydrolysis of the β-amide group from the asparagine after autocatalytic cleavage, even if a poly-histidine tag was present at the N-terminal end of the precursor (Hejazi et al. 2002; Borek et al. 2004). When crude extracts of soluble or total proteins from *E. coli* cells expressing recombinant pine ASPG were analyzed by Western-blot, most of the recombinant protein was observed as a protein with apparent molecular mass of 39 kDa, close to the predicted value of the precursor, including the poly-histidine tag (Fig. 2a, lanes 1 and 2). Other minor bands were also detected, which could be as a result of degradation or displacement in the translational start position. Among those less abundant polypeptides, a fragment was present with apparent molecular mass of 27 kDa, close to the predicted value of 27.2 kDa for the recombinant α subunit including the poly-histidine tag. The polypeptides detected by the antibodies were absent in control extracts of *E. coli* cells that were not induced with IPTG (Fig. 2a, lanes 3 and 4). After affinity purification under native conditions, only the 39 and 27 kDa bands were detected, and the relative amount of both polypeptides was unaltered after 24 and 48 h of incubation at 22°C. Consistently with results from crude extract, most of the recombinant protein was unprocessed. These results suggest that the detected cleavage of the recombinant protein occurred in vivo in *E. coli* cells, and after extraction no further cleavage occurred in vitro. The absence of the β subunit in the purified protein could be explained by the very low levels together with the small size of the protein, which determined that it were not detected in the Western-blot analysis. Another explanation could be that after proteolytic cleavage in *E. coli,* both subunits did not interact and the β subunit was not recovered from the chromatography, since the His-tag was located at the N-terminal end of the α subunit.

To investigate whether plant cellular factors could facilitate or mediate in the processing, we incubated recombinant ASPG protein purified under denaturing conditions with soluble extracts from pine seedlings. To completely remove the urea, the purified protein was extensively dialyzed in urea-free buffer. Recombinant protein purified under denaturing conditions in the

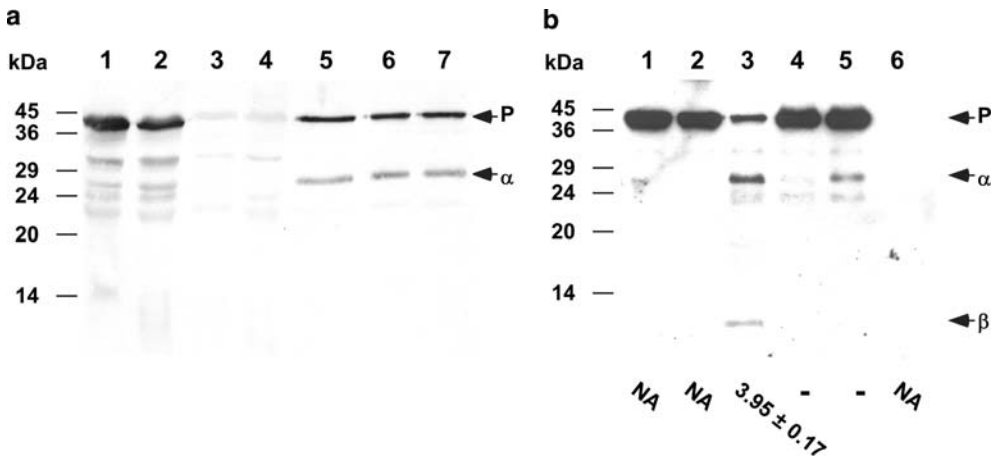


Fig. 2 **a, b** Western-blot analysis and enzymatic activity assay of pine recombinant ASPG. *E. coli* cells transformed with a construct of *PsASPG* cDNA in pET11a were induced to express the recombinant protein with IPTG. **a** Proteins were extracted under native conditions and the recombinant ASPG was purified. *Lane 1* soluble proteins extracted from *E. coli* cells expressing recombinant ASPG; *lane 2* total protein extracted from *E. coli* cells expressing recombinant ASPG; *lane 3* soluble protein control extract from *E. coli* cells that were not induced to express recombinant ASPG; *lane 4* total protein extracted from *E. coli* cells that were not induced to express recombinant ASPG; *lane 5* purified recombinant ASPG; *lane 6* and *7* purified recombinant ASPG incubated at 22°C for 24 and 48 h, respectively. **b** Proteins were extracted in denaturing conditions and the recombinant ASPG was purified. *Lane 1* purified recombinant ASPG; *lane 2* purified recombinant ASPG after incubation at 22°C for 48 h; *lane 3* purified recombinant ASPG incubated at 22°C for 48 h with 3.5 μg of soluble proteins extracted from radicles; *lane 4* purified recombinant ASPG after incubation at 22°C for 48 h with 3.5 μg of soluble proteins extracted from radicles and 10 mM EDTA; *lane 5* purified recombinant ASPG after incubation at 22°C for 48 h with 3.5 μg of heat-denatured soluble proteins extracted from radicles; *lane 6* 2 μg of soluble proteins extracted from radicles. Enzyme activity was tested for different aliquots and results are shown at the bottom of each lane. Enzymatic activity (nkatal mg^{-1}) is the average of three replicates, ±SE. *NA* no activity detected; *dash* non-tested. The molecular masses (kDa) of the protein markers are indicated on the left side in both panels. The complete procedure was repeated three times with similar results

presence of 8 M urea was enriched in the unprocessed precursor polypeptide, whereas processed fragments were not detected (Fig. 2b; lane 1). The precursor did not exhibit autoproteolytic cleavage, even after 48 h of incubation at 22°C (Fig. 2b; lane 2). However, when the recombinant protein was incubated for 48 h with a small aliquot of soluble protein extracted from pine seedling radicles (3.5 μg of protein), cleavage of the precursor to fragments with apparent molecular masses of 27 and 12 kDa could be observed (Fig. 2b, lane 3). The addition of 10 mM EDTA (Fig. 2b, lane 4) or denaturation of radicle protein extract by boiling for 5 min (Fig. 2b, lane 5) negatively affected cleavage of the precursor. ASPG protein was not detected by Western-blot in the radicle protein extract (Fig. 2b; lane 6). To determine whether the hydrolysis of the pine ASPG precursor could be relevant for enzyme function, ASPG activity was measured for the unprocessed and processed aliquots of the recombinant protein. No activity was detected in purified protein in which the smaller fragments were absent (Fig. 2b, lanes 1 and 2), whereas the detection of ASPG activity was associated to the presence of the 27 and 12 kDa polypeptides (Fig. 2b, lane 4). This result indicates that processing of the precursor is required for enzyme activity as observed in other plant ASPGs and according to their classification as Ntn-hydrolases (Borek et al. 2004).

To detect sequences in the pine genome related to the identified gene, a Southern-blot analysis of *P. sylvestris* genomic DNA was performed with the complete *PsASPG* cDNA as a probe (Fig. 3). When washed at moderate stringency, only a single fragment showed strong hybridization to the probe in each restriction digest. However, additional bands with slight hybridization to the *PsASPG* probe were also detected, suggesting the presence of other related but divergent sequences in the genome. These bands could be fragments derived from the genes homologous to TC68434 and TC76010 in the *P. sylvestris* genome.

To examine the phylogenetic relationships of the *ASPG* enzyme from *P. sylvestris* with the sequences in the TIGR *Pinus* Gene Index and angiosperm ASPGs, an unrooted phylogenetic tree was constructed, using a cladistic parsimony analysis with full-length ASPG amino acid sequences from different organisms accessible in the databases (Fig. 4). Representative sequences from the different phylogenetic groups defined in the analysis of plant-type ASPGs previously reported (Borek and Jaskólski 2001) were used. The human aspartylglucosaminidase (GI:183330) was defined as the outgroup. In accordance with a previous report (Borek and Jaskólski 2001), the maximum parsimony consensus tree shows three main branches: one branch

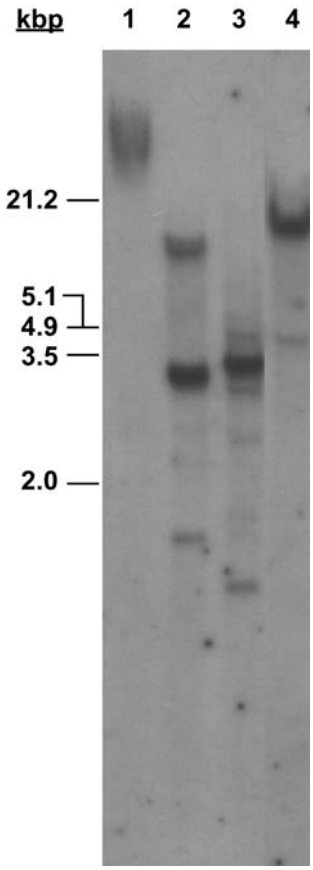


Fig. 3 Southern-blot analysis of *P. sylvestris* genomic DNA. Genomic DNA isolated from pine cotyledons was digested with different restriction enzymes, fractionated in an agarose gel and transferred to a nylon membrane. The membrane was hybridized with a *PsASPG* full-length cDNA and washed at moderate stringency. *Lane 1* 20 μg of undigested pine genomic DNA; *lanes 2*, *3* and *4* 20 μg of pine genomic DNA digested with *Eco*RI, *Hind*III or *Bam*HI respectively. *Numbers* on the left indicate size in kbp of lambda DNA size markers

contains proteins homologous to human threonine aspartase (group I), another branch includes proteins with aspartylglucosaminidase activity (group II) and a third branch includes the plant ASPGs (group III). As defined previously (Bruneau et al. 2006), the plant ASPG family includes two evolutionarily distinct subfamilies: the K^+-dependent subfamily and the K^+-independent subfamily. The bootstrap values show the strong consistency of the different branches. The position of the three pine sequences in the tree indicates that the protein encoded by *PsASPG* is a K^+-dependent ASPG, whereas *P. taeda* PtTC2 (TC68434) and PtTC3 (TC76010) belong to the K^+-independent plant ASPG subfamily. Therefore, the divergence between TC68434 and TC76010 sequences, together with the result of the phylogenetic analysis, suggest that pine genomes contain two genes encoding K^+-independent ASPGs.

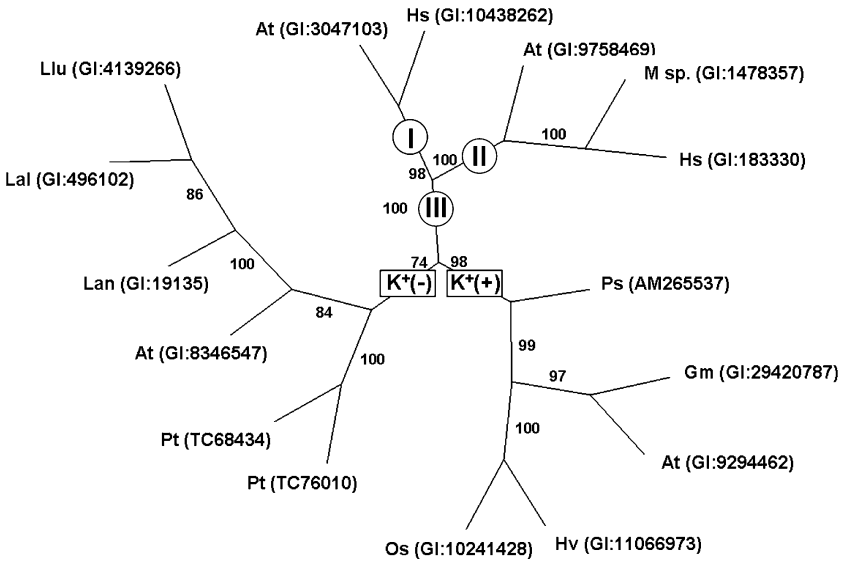


Fig. 4 Maximum parsimony consensus tree of plant ASPGs and several sequence-related proteins. Amino acid sequences of plant ASPGs were aligned with several amino acid sequences representative of ASPG-related proteins. Parsimony analysis was performed using the heuristic search option of the phylogenetic analysis program (PAUP version 3.1.1). The human aspartylglucosaminidase (GI:183330) was used as the outgroup. The main branches are labeled with circles with roman numbers inside. The branches of the K^+-dependent and -independent subfamilies are labeled. 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 numbers in brackets are the sequence accession numbers. *Ps*: *P. sylvestris*; *Gm*: *Glycine max*; *At*: *A. thaliana*; *Hv*: *Hordeum vulgare*; *Os*: *Oryza sativa*; *Pt*: *P. taeda*; *Lan*: *L. angustifolius*; *Lal*: *L. albus*; *Llu*: *L. luteus*; *M sp.*: *Mus sp.*; *Hs*: *Homo sapiens*

To get an insight into the specific function of *PsASPG* during pine germination and early seedling development, the levels of transcripts were analyzed and compared to *PsAS1*, a gene encoding pine AS (Cañas et al. 2006). Since the overall expression of the gene was low, the full-length cDNA was used as a probe, to enhance the signal in the Northern-blot analysis. Washes at high stringency were carried out to avoid cross-hybridization with related sequences. *PsASPG* transcripts were detected in dry embryos (Fig. 5, top panels lane E0) and the levels increased slightly during early stages of germination (Fig. 5, top panels, lanes E1 and G). Developing seedlings were monitored at different times after germination and the levels of transcripts analyzed in dissected cotyledons, hypocotyls and radicles. Cotyledons showed very low levels of transcripts at all analyzed stages, with a slight increase as they developed (Fig. 5, top panels, lanes C1–C4), whereas higher levels were detected in radicles at the different developmental stages analyzed (Fig. 5, top panels, lanes R1–R4). In hypocotyl, the transcript abundance was modulated in a temporal pattern. At early stages of hypocotyl development, the levels were low (Fig. 5, top panels, lanes H1 and H2), but a remarkable increase was detected at later stages (Fig. 5, top panels, lanes H3 and H4). On the contrary, *PsAS1* transcripts were absent in dry and imbibed embryos (Fig. 5, middle panels, lanes E0 and E1), but an increase in transcript abundance was observed during germination, concomitant with radicle emergence and the initiation of seedling elongation (Fig. 5, middle panels, lane G). The expression of the *PsAS1* gene was low during early stages of cotyledon development (Fig. 5, middle panels, lanes C1 and C2) and moderate in radicles (Fig. 5, middle panels, lanes R1 and R2). However, as previously reported (Cañas et al. 2006), high levels of transcripts were detected in hypocotyls at early stages of pine seedlings (Fig. 5, middle panels, lanes H1 and H2). Nevertheless, the levels of *PsAS1* transcripts decreased during subsequent seedling development in all organs until they became undetectable (Fig. 5, middle panels, lanes C3, C4, H3, H4, R3, and R4).

The increase in *PsASPG* transcript abundance observed at hypocotyl development stages H3 and H4, concomitant with the drastic decrease in *PsAS1* transcript levels, suggests that high levels of ASPG are involved in the mobilization of the asparagine accumulated in the hypocotyl (Cañas et al. 2006). To determine the specific location of *PsASPG* expression and asparagine hydrolysis, we performed in situ hybridization of transversal sections of hypocotyl at the developmental stage H3. *PsASPG* transcripts were detected in the cells located between xylem and

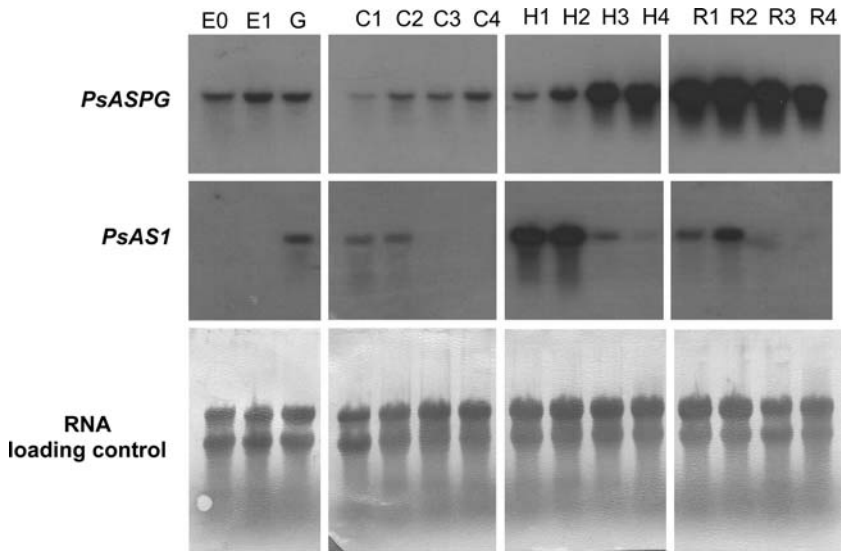


Fig. 5 Expression patterns of *PsASPG* and *PsAS1* during pine seed germination and early development. Total RNA was extracted from embryos and cotyledons, hypocotyls and radicles of seedlings; 10 μg of each sample were analyzed by Northern-blot using a *PsASPG* full-length cDNA and a partial cDNA derived from *PsAS1* cDNA containing the complete 3′-UTR as molecular probes. RNA transferred onto the nylon membrane was stained with a methylene blue solution as a loading control. *Lane E0* dry embryo; *lane E1* wet embryo; *lane G* germinating seed; *lanes C1–C4* cotyledons from seedlings 8, 12, 30 and 90 days after imbibition; *lanes H1–H4* hypocotyls from seedlings 8, 12, 30 and 90 days after imbibition; *lanes R1–R4* radicles from seedlings 8, 12, 30 and 90 days after imbibition

phloem, corresponding to the cambial region (Fig. 6a). Hybridization or background signals were not detected when the sections were hybridized with the sense probe as a control (Fig. 6b). Therefore, this result suggests that the induction of *PsASPG* expression in hypocotyl is specifically located in cells of developing vascular tissues. Lignin content was detected in situ, in transversal sections of H1 hypocotyls and H3 hypocotyls (Fig. 7) as a marker of vascular development. During the elapsed time between H1 and H3 stages, a remarkable development of the vascular system takes place. At the H1 stage, the vascular system is made up of four small bundles containing a few xylem elements, with a limited amount of lignin in the cell walls (Fig. 7a). At the H3 stage, an almost continuous cylinder of vascular elements had been evolved, with a remarkable increase in the number of xylem elements and the lignin content of the cell walls (Fig. 7b).

Discussion

Early after germination, pine seedlings initiate a process of re-allocation of large amounts of seed-stored N, located in the megagametophyte, toward the hypocotyl in the form of free asparagine (Cañas et al. 2006). This soluble N reserve is synthesized by an AS enzyme encoded by the *PsAS1* gene. The strict control of the timing and spatial distribution of *PsAS1* expression during germination suggests that the re-allocation of N reserve in the hypocotyl could play an important role at later developmental stages of pine seedling. To get an insight into the functional role of this N pool accumulated in hypocotyls of pine seedlings, we decided to study the enzymes that could mobilize the N contained in the asparagine.

A cDNA clone encoding a protein from *P. sylvestris* similar to plant ASPGs was amplified by RT-PCR from a RNA sample of hypocotyl. The high similarity with plant ASPGs (Fig. 1), and the conservation of the autoproteolytic cleavage site (Thr–Val–Gly, starting at position 245 of the pine sequence) suggest that this gene encodes an enzyme with ASPG activity. These residues are conserved in plant ASPGs, and they are essential for the enzyme activity, since determines the processing site and contains the threonine residue, that after the cleavage acts as the N-terminal catalytic nucleophile characteristic of Ntn-hydrolases (Borek et al. 2004). Other residues of the β subunit located in the active site of the *L. luteus* enzyme (Michalska et al. 2006), are also conserved in the pine ASPG. The conservation of residues of the active site in the α subunit is lower among plant ASPGs and it could contribute to catalytic differences among them, including the effect of K^+ in the activity.

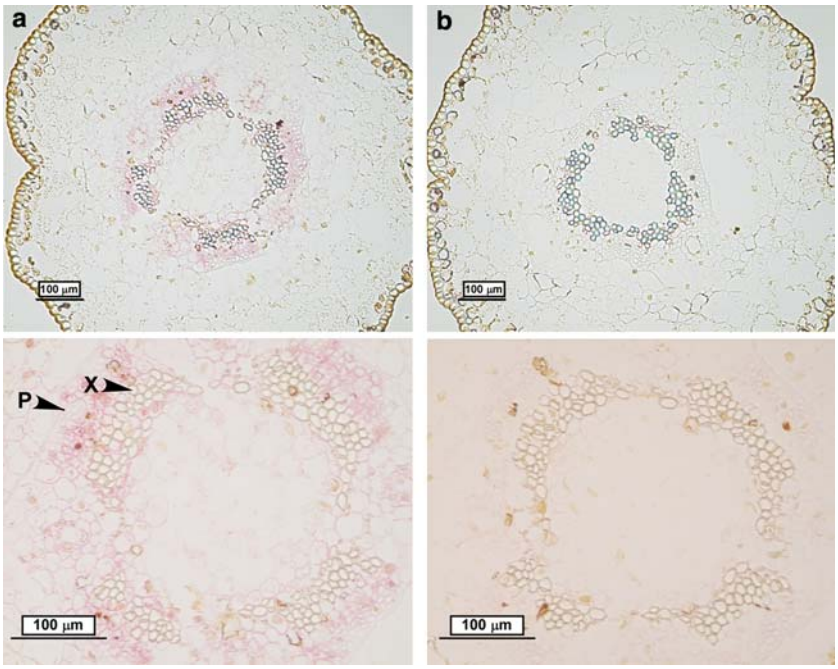


Fig. 6 **a, b** In situ hybridization analysis of *PsASPG* transcripts in hypocotyls of *P. sylvestris* seedlings. **a** Transversal cross-sections through the hypocotyl of H3 developmental stage were used for localization of *PsASPG* mRNAs by in situ hybridization with antisense RNA probes. **b** The specificity of the signals was established by in situ hybridization of control sections with sense probes. The *bottom panels* show a magnification of the vascular system from the picture on top panels. *Arrowheads* point at phloem (*P*) and xylem (*X*). Gene expression signals were observed in *pink color*

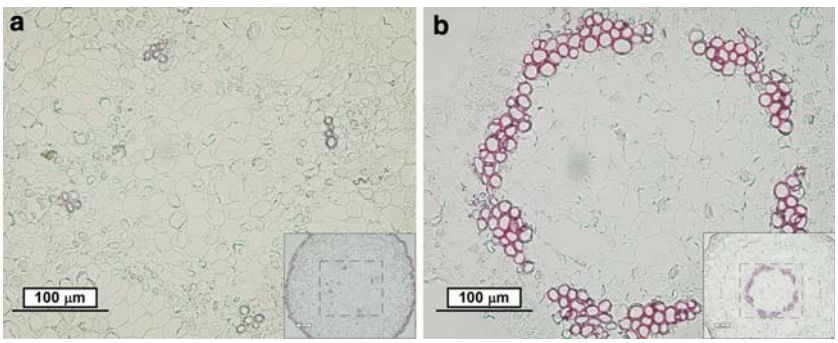


Fig. 7 **a, b** In situ detection of lignin in hypocotyls of *P. sylvestris* seedlings. Transversal cross-sections through the hypocotyl at H1 (**a**) and H3 (**b**) developmental stages were used for localization of lignin accumulation using staining with phloroglucinol. The *small pictures* at the *bottom right side* show the complete section of the hypocotyl

The C-terminal sequence of the predicted α-peptide, assuming that the proteolytic cleavage takes place between the residues Glu244 and Thr245 of the pine precursor, is remarkably longer, compared to other plant-type ASPGs. The primary structure of this region of the precursor shows the highest variability between the two plant ASPG subfamilies and among angiosperms and gymnosperms. In particular, this stretch does not contain any residue conserved in all plant ASPG proteins, even those close to the autoproteolytic cleavage site. However, some residues are conserved between *P. sylvestris* ASPG and PtTC1 (TC65340) and angiosperms K^+-dependent ASPGs, suggesting that they could play a specific role in this subfamily. In the *E. coli* EcAIII precursor this extension is very short and may be absent in the active enzyme, since it has been suggested that after the autoproteolytic cleavage both subunits may undergo further processing shortening the C-terminal end of the α-subunit by 17 residues (Borek et al. 2004) This further processed fragment is shadowed in gray in Fig. 1. Therefore, the comparison of the primary structure of the plant-type ASPGs suggests that this stretch of amino acids is not involved in the basic mechanism of catalysis. Whether these amino acids are required for plant enzyme activity, confer specific properties to both plant ASPG subfamilies or they are also removed in a post-autocleavage processing remain to be determined.

The association of ASPG activity detection with the presence of the 27 and 12 kDa fragments (Fig. 2) suggests that the pine precursor is also processed by cleavage, as reported previously for angiosperm ASPGs (Borek and Jaskólski 2001; Borek et al. 2004). To create the N-terminal catalytic nucleophile residue (Thr245 in the pine sequence), an autocatalytic pocessing is required by all the known Ntn-hydrolases (Oinonen and Rouvinen 2000). However, pine recombinant precursor was unable to undergo an autocatalytic cleavage in vitro, in contrast to the reported autoprocessing ability of *Arabidopsis thaliana* and

L. luteus ASPG precursors (Hejazi et al. 2002; Borek et al. 2004; Bruneau et al. 2006). When recombinant ASPG was purified under native conditions, most of the recombinant protein was unprocessed and only a small amount of apparently processed protein was present (Fig. 2a). Similar proportions of precursor and processed proteins were observed in soluble extracts of *E. coli*, suggesting that the limited processing occurred in vivo, and it did not occur during or after the purification procedure. In fact, the incubation of the purified recombinant protein up to 48 h, did not result in an increase in the amount of processed protein. Purified preparations of recombinant protein under denaturing conditions were enriched in the precursor polypeptide, whereas the processed forms were not detected (Fig. 2b). Probably the enrichment in the amount of precursor was as a result of the solubilization and purification of the insoluble fraction of recombinant protein. It is likely that this fraction, usually precipitated in inclusion bodies, is not capable of undergoing proteolytic processing. The protein, purified under denaturing conditions was also unable to undergo autocatalytic processing in vitro. However, when small amounts of proteins extracted from radicle of pine seedlings were added, the processing of the precursor could be observed. This processing was completely prevented by the addition of 10 mM EDTA and partially by the boiling of the radicle extract. All these results, together, indicate that the product of the gene *PsASPG* does not exhibit autoproteolytic cleavage in vitro. On the contrary, it requires divalent cations and accessory proteins to take place efficiently. Folding and correct assembly of the subunits is suggested to be a triggering step for activation of prokaryotic members of the Ntn-hydrolase, and dimerization of precursors has been suggested to be a prerequisite to trigger autoproteolysis in aspartylglucosaminidase (Riikonen et al. 1996). The long C-terminal end in the α subunit could interfere in the folding of the precursor, requiring accessory proteins to assist in folding in a divalent cation-dependent manner. Another possible mechanism for mediated processing would involve a proteolytic activity requiring divalent cations as cofactor. Further work will be necessary to know the specific mechanism of processing of the pine precursor.

The Southern-blot analysis (Fig. 3) suggests that *PsASPG* is a single copy gene without close-related sequences in the *P. sylvestris* genome. Nevertheless, the detection of additional hybridization bands with very low signal at moderate stringency suggests the presence of related but divergent sequences, probably sequences homologous to the *P. taeda* genes TC68434 and TC76010. Although the amino acid sequence of the *P. sylvestris* ASPG was only a little more similar to K^+-dependent ASPGs than to K^+-independent ASPGs, it clearly clustered with the K^+-dependent subfamily of plant ASPGs in the phylogenetic analysis (Fig. 4). *P. taeda* PtTC2 (TC68434) and PtTC3 (TC76010) sequences were clustered with K^+-independent ASPGs, indicating that those sequences correspond to members of the K^+-independent subfamily in pine. Together with the Southern-blot analysis, these results suggest that the pine genome contains at least one gene encoding K^+-dependent ASPG and two genes encoding K^+-independent ASPGs. Contrary to K^+-independent ASPGs, the K^+-dependent enzyme from *Arabidopsis* showed a strict specificity for L-asparagine and it had no activity toward β-aspartyl dipeptides (Bruneau et al. 2006), suggesting an exclusive functionality as ASPG. Furthermore, the kinetic parameters of the *Arabidopsis* K^+-dependent enzyme suggested that it may metabolize L-asparagine more efficiently under conditions of high metabolic demand for N. Consequently, the identified ASPG may play a relevant role in the mobilization of N in the form of asparagine in pine. The temporal and spatial pattern of expression of this gene is in agreement with this function.

The Northern-blot analysis (Fig. 5) showed a low expression of *PsASPG* in cotyledons, supporting the idea that this organ uses glutamine and glutamate as major N donor compounds (Suárez et al. 2002). The gene is mainly expressed in radicles during seedling development, suggesting that this organ is a major asparagine sink. The expression levels were low in cotyledons at all analyzed stages and the hypocotyls at stages H1 and H2. However, a remarkable increase in mRNA levels was observed at later stages of hypocotyl development (H3 and H4), coinciding with a decrease in *PsAS1* gene expression. These data suggest that once the AS activity decreases, there is an increase in the consumption of asparagine in the hypocotyl. The expression of ASPG genes has often been associated with tissues undergoing active division and protein synthesis (Lough et al. 1992a, b; Grant and Bevan 1994). These tissues use the asparagine provided from sites of synthesis as a primary N source. The apical meristem in the hypocotyl is a site of active division and protein synthesis in developing seedlings. However, in our analysis the apical meristem was detached from hypocotyls. Therefore, the accumulation of *PsASPG* transcripts should be associated with a different type of cells. When the tissue expression pattern was precisely determined by in situ hybridization in hypocotyls of the H3 developmental stage, *PsASPG* transcripts were only detected in cells located between the xylem and

phloem elements (Fig. 6). They are cambial cells and their derivatives undergoing differentiation to xylem and phloem. Therefore, the temporal and spatial expression patterns of *PsASPG* suggest a role in vascular development. In fact, transcripts of glutamine synthetase 1b were found mainly in procambial cells of embryos and seedlings of *P. sylvestris* (Pérez-Rodríguez et al. 2006) suggesting a role of the product of this gene in the re-assimilation of the ammonium released by the ASPG activity. Therefore, the functional association of both enzymes may play a relevant role in utilization of asparagine for vascular development.

The expression of *PsASPG* is high in radicle, an organ that differentiates a lignified vascular system promptly after germination. On the other hand, in the hypocotyl, the transition between stages H2 to H3 coincides with a developmental state when the elongation of this organ stops and structural changes are apparent. The measurement of the hypocotyl length showed that this parameter did not change during the transition from H2 to H3 (average value of 4.2 cm at both stages of development). On the contrary, important changes in composition and structure take place during this developmental period. Vascular structures highly specialized in transporting water and solutes are developed to support growth and development. The lignified xylem is transformed from several separated bundles of xylem elements at H1 developmental stage, to a continuous ring highly lignified at H3 stage (Fig. 7). Developing a secondary cell wall is a process with a high C and N demand. Cellulose is a C sink and although lignin is essentially a C frame structure, monolignols are derived from the amino acid phenylalanine. Thus, during the initial development of the secondary xylem, important amounts of N should be derived to feed the phenylalanine biosynthesis, among other N demanding processes during stem vascular development.

In summary, our results suggest that during the process of development of the hypocotyl to form a highly vascularized and lignified stem, this organ goes through an important metabolic change. At initial developmental stages the hypocotyl is a major site for asparagine production and storage with high levels of *PsAS1* gene expression and free asparagine (Cañas et al. 2006). However, at later stages and coinciding with the transformation of the hypocotyl into a highly vascularized and lignified organ, the levels of *PsAS1* transcripts decrease drastically and coordinately with the increase in *PsASPG* transcript abundance, suggesting that the hypocotyl consumes the previously accumulated asparagine, to provide N and C for this transformation.

Acknowledgments We are grateful to Dr. María Fernanda Suárez for technical assistance with in situ hybridization. This work was supported by a grant from the Spanish Ministry of Science and Education (AGL2003-05191). FRC is supported by a contract from Spanish Ministry of Science and Education (Programa Ramón y Cajal). RAC was supported by a pre-doctoral fellowship from Spanish Ministry of Science and Education.

References

Atkins CA, Pate JS, Peoples MB, Joy KW (1983) Amino acid transport and metabolism in relation to the nitrogen economy of a legume leaf. Plant Physiol 71:841–848

Avila C, Suárez MF, Gómez-Maldonado J, Cánovas FM (2001) Spatial and temporal expression of two cytosolic glutamine synthetase genes in Scots pine: functional implications on nitrogen metabolism during early stages of conifer development. Plant J 25:93–102

Azevedo RA, Lancien M, Lea PJ (2006) The aspartic acid metabolic pathway, an exciting and essential pathway in plants. Amino Acids 30:143–162

Borek D, Jaskólski M (2001) Sequence analysis of enzymes with asparaginase activity. Acta Biochim Pol 48:893–902

Borek D, Michalska K, Brzezinski K, Kisiel A, Podkowinski J, Bonthron DT, Krowarsch D, Otlewski J, Jaskolski M (2004) Expression, purification and catalytic activity of *Lupinus luteus* asparagine β-amidohydrolase and its *Escherichia coli* homolog. Eur J Biochem 271:3215–3226

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

Bruneau L, Chapman R, Marsolais F (2006) Co-occurrence of both L-asparaginase subtypes in *Arabidopsis*: At3g16150 encodes a K+-dependent L-asparaginase. Planta 224:668–679

Cantón FR, García-Gutiérrez A, Crespillo R, Cánovas FM (1996) High-level expression of *Pinus sylvestris* glutamine synthetase in *Escherichia coli*. FEBS Lett 393:205–210

Cantón FR, Quail PH (1999) Both phyA and phyB mediate light-imposed repression of *PHYA* gene expression in *Arabidopsis*. Plant Physiol 121:1207–1215

Cañas RA, de la Torre F, Cánovas FM, Cantón FR (2006) High levels of asparagine synthetase in hypocotyls of pine seedlings reveal an essential role of the enzyme in re-allocation of seed-stored nitrogen. Planta 224: 83–95

Chang KS, Farnden KJ (1981) Purification and properties of asparaginase from *Lupinus arboreus* and *Lupinus angustifolius*. Arch Biochem Biophys 208:49–58

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine tree. Plant Mol Biol Rep 11:113–116

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Dellaporta SL, Wood J, Hicks JB (1983) Isolation of DNA from higher plants. Plant Mol Biol Rep 4:19–21

Grant M, Bevan MW (1994) Asparaginase gene expression is regulated in a complex spatial and temporal pattern in nitrogen-sink tissues. Plant J 5:695–704

Guan C, Liu Y, Shao Y, Cui T, Liao W, Ewel A, Whitaker R, Paulus H (1998) Characterization and functional analysis of the cis-autoproteolysis active center of glycosylasparaginase. J Biol Chem 273:9695–9702

Guo HC, Xu Q, Buckley D, Guan C (1998) Crystal structures of *Flavobacterium* glycosylasparaginase. An N-terminal nucleophile hydrolase activated by intramolecular proteolysis. J Biol Chem 273:20205–20212

Hejazi M, Piotukh K, Mattow J, Deutzmann R, Volkmer-Engert R, Lockau W (2002) Isoaspartyl dipeptidase activity of plant-type asparaginases. Biochem J 364:129–136

Ireland RJ, Joy KW (1981) Two routes for asparagine metabolism in *Pisum sativum* L. Planta 151:289–292

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

Kern R, Chrispeels MJ (1978) Influence of the axis on the enzymes of protein and amide metabolism in the cotyledons of mung bean seedlings. Plant Physiol 62:815–819

King JE, Gifford DJ (1997) Amino acid utilization in seeds of loblolly pine during germination and early seedling growth. Plant Physiol 113:1125–1135

Langdale JA (1993) In situ hybridization. In: Freeling M, Walbot V (eds) The maize handbook. Springer, Berlin Heidelberg New York, pp 165–180

Lea PJ, Fowden L (1975) The purification and properties of glutamine-dependent asparagine synthetase isolated from *Lupinus albus*. Proc R Soc Lond B 192:13–26

Lea PJ, Festenstein GN, Hughes JS, Miflin BJ (1984) An immunological and enzymological survey of asparaginase in seeds of *Lupinus*. Phytochemistry 23:511–514

Lea PJ, Robinson SA, Stewart GR (1990) The enzymology and metabolism of glutamine, glutamate and asparagine. In: Miflin BJ, Lea PJ (eds) The biochemistry of plants, vol 16. Academic, San Diego, pp 147–152

Loopstra CA, Mouradov A, Vivian-Smith A, Glassick TV, Gale BV, Southerton SG, Marshall H, Teasdale RD (1998) Two pine endo-β-1,4-glucanases are associated with rapidly growing reproductive structures. Plant Physiol 116:959–967

Lough TJ, Chang KS, Carne A, Monk BC, Reynolds PHS, Farnden KJF (1992a) L-asparaginase from developing seeds of *Lupinus arboreus*. Phytochemistry 31:1519–1527

Lough TJ, Reddington BD, Grant MR, Hill DF, Reynolds PHS, Farnden KJF (1992b) The isolation and characterization of cDNA clone encoding L-asparaginase from developing seeds of lupin (*Lupinus arboreus*). Plant Mol Biol 19:391–399

Michalska K, Bujacz G, Jaskolski M (2006) Crystal structure of plant asparaginase. J Mol Biol 360:105–116

Oinonen C Rouvinen J (2000) Structural comparison of Ntn-hydrolases. Protein Sci 9:2329–2337

Pérez-Rodríguez MJ, Suárez MF, Heredia R, Ávila C, Breton D, Trontin J-F, Filonova L, Bozhkov P, von Arnold S, Harvengt L, Cánovas FM (2006) Expression patterns of two glutamine synthetase genes in zygotic and somatic pine embryos support specific roles in nitrogen metabolism during embryogenesis. New Phytol 169:35–44

Riikonen A, Rouvinen J, Tikkanen R, Julkunen I, Peltonen L, Jalanko A (1996) Primary folding of aspartylglucosaminidase: significance of disulfide bridges and evidence of early multimerization. J Biol Chem 271:21340–21344

Rognes SE (1975) Glutamine-dependent asparagine synthetase from *Lupinus luteus*. Phytochemistry 14:1975–1982

Rognes SE (1980) Anion regulation of lupin asparagine synthetase: chloride activation of the glutamine-utilizing reactions. Phytochemistry 19:2287–2293

Sieciechowicz KA, Joy KW, Ireland RJ (1988) The metabolism of asparagine in plants. Phytochemistry 27:663–671

Sodek L, Lea PJ, Miflin BJ (1980) Distribution and properties of a potassium-dependent asparaginase isolated from developing seeds of *Pisum sativum* and other plants. Plant Physiol 65:22–26

Sodek L, Lea PJ (1993) Asparaginase from the testa of developing lupin and pea seeds. Phytochemistry 34:51–56

Streeter JG (1977) Asparaginase and asparagine transaminase in soybean leaves and root nodules. Plant Physiol 60:235–239

Suárez MF, Avila C, Gallardo F, Cantón FR, García-Gutiérrez A, Claros MG, Cánovas FM (2002) Molecular and enzymatic analysis of ammonium assimilation in woody plants. J Exp Bot 53:891–904

Todd CD, Cooke JEK, Mullen RT, Gifford DJ (2001) Regulation of loblolly pine (*Pinus taeda* L.) arginase in developing seedling tissue during germination and post-germinative growth. Plant Mol Biol 45:555–565

Todd CD, Gifford DJ (2002) The role of the megagametophyte in maintaining loblolly pine (*Pinus taeda* L.) seedling arginase gene expression in vitro. Planta 215:110–118

Urquhart AA, Joy KW (1981) Use of phloem exudate technique in the study of amino acid transport in pea plants. Plant Physiol 68:750–754