**Tree Physiology** 27, 1283–1291


# The aspartate aminotransferase family in conifers: biochemical analysis of a prokaryotic-type enzyme from maritime pine

FERNANDO DE LA TORRE,[1] MARÍA FERNANDA SUÁREZ,[1] LAURA DE SANTIS[1] and FRANCISCO M. CÁNOVAS[1,2]

[1] *Departamento de Biología Molecular y Bioquímica, Instituto Andaluz de Biotecnología, Unidad Asociada UMA-CSIC, Campus Universitario de Teatinos, Universidad de Málaga, 29071-Málaga, Spain*

[2] *Corresponding author (canovas@uma.es)*

Received June 22, 2006; accepted January 24, 2007; published online June 1, 2007

**Summary** Plant aspartate aminotransferase (AAT, EC 2.6.1.1) plays a key role in primary nitrogen assimilation, the transfer of reducing equivalents and the interchanges of carbon and nitrogen pools between subcellular compartments. We investigated the AAT family in conifers using maritime pine as the experimental model. Genes for cytosolic, mitochondrial and two plastidic isoenzymes (eukaryotic- and prokaryotic-types) were identified and their deduced amino acid sequences compared. The primary structure of the eukaryotic-type enzymes is quite well conserved, whereas the prokaryotic-type AAT is highly divergent (15% of identity). These molecular data were confirmed by the absence of immunological cross-reactivity between the two types of native AATs. The mature prokaryotic-type polypeptide was overexpressed in *Escherichia coli*, and the native enzyme was purified to apparent homogeneity and its molecular properties determined. The fully active recombinant holoenzyme showed highest catalytic activity at 50–60 °C and was moderately thermostable, retaining about 50% of its activity after incubation at 70 °C for 5–10 min. The presence of pyridoxal 5′-phosphate significantly increased the thermostability of the enzyme. These molecular characteristics were exploited to develop a rapid protocol for the purification of this prokaryotic-type enzyme from pine cotyledons. The results will be useful for studying aspartate and amino acid metabolism in trees.

*Keywords: amino acids, aspartate metabolism, nitrogen assimilation, peptide fingerprinting, recombinant expression.*

## Introduction

Nitrogen (N) availability is often a significant factor limiting plant growth and development. Along with the evolution of land plants, a number of strictly regulated metabolic pathways evolved to guarantee the efficient acquisition, assimilation and use of this essential nutrient. Irrespective of the N source available in soils, ammonium is the form ultimately assimilated into the pool of N-containing biomolecules. This process is catalyzed by the coordinated action of glutamine synthetase and glutamate synthase to generate glutamate, an N donor for the biosynthesis of nitrogenous compounds in plants (Miflin and Lea 1980). Nitrogen from glutamate is channeled to plant metabolism through the reversible reaction of transamination between glutamate and oxaloacetate to generate aspartate and 2-oxoglutarate that is catalyzed by aspartate aminotransferase (AAT, EC 2.6.1.1), with pyridoxal 5′-phosphate (PLP) as the cofactor. Aspartate aminotransferase plays a crucial role in the metabolic regulation of carbon and N metabolism in all organisms. In eukaryotes, AAT is involved in the transfer of reducing equivalents and the interchanges of carbon and N pools between subcellular compartments via the malate aspartate shuttle (Givan 1980).

Aspartate aminotransferases in many organisms are classified in group I of the aminotransferase superfamily (Jensen and Wu 1996) in which two subgroups, Ia and Ib, can be distinguished based on the primary structure of the enzymes. Only about 15% identity is observed between subgroup Ia and Ib members (Okamoto et al. 1996). Despite these differences, most of the active residues essential for catalysis are identical, and tri-dimensional structure is well conserved in all AAT enzymes analyzed to date (Nakai et al. 1999). Subgroup Ia includes the AATs from eubacteria and eukaryotes, whereas subgroup Ib comprises the enzymes from some eubacteria, including cyanobacteria and archaea.

Aspartate aminotransferases exist in higher eukaryotes as distinct isoforms with specific subcellular location and are encoded by a gene family of several members, all belonging to subgroup Ia of the aminotransferase superfamily (Jensen and Wu 1996). In animals, cytosolic and mitochondrial isoforms of AAT have been characterized (Mehta et al. 1989). In higher plants, there are multiple AAT isoenzymes associated with different subcellular compartments such as the cytosol, mitochondria, peroxisome and plastid (Ireland and Joy 1985, Wadsworth 1997). In *Arabidopsis thaliana* L., there are five genes that encode distinct AAT isoenzymes: ASP1 (mitochondrial), ASP2 and ASP4 (cytosolic), ASP3 (peroxisomal) and ASP5 (plastidic) (Schultz and Coruzzi 1995, Wilkie et al. 1995, Wilkie and Warren 1998, Coruzzi 2003). In addition, we

have recently reported the identification of a novel AAT in plants (de la Torre et al. 2006). The enzyme is unrelated to the eukaryotic AATs from plants and animals but is similar to bacterial enzymes, suggesting it belongs to subgroup Ib of the aminotransferase superfamily.

Aspartate is required for asparagine biosynthesis in the reaction catalyzed by asparagine synthetase, and it is the metabolic precursor of several essential amino acids (Azevedo et al. 2006). Aspartate biosynthesis is of particular relevance in the mobilization of N stored in the conifer seed because asparagine is the most abundant amino acid in developing pine seedlings, accounting for about 70% of the total free amino acid pool (King and Gifford 1997). We recently reported the characterization of asparagine synthetase in pine and studied its role in the re-allocation of seed-stored N (Cañas et al. 2006). However, little is known about the genes and enzymes involved in aspartate biosynthesis in pine and other gymnosperms. The objective of this study was to investigate the family of AAT isoenzymes in conifers, using maritime pine (*Pinus pinaster* Ait.) as an experimental model. Genes encoding cytosolic, mitochondrial and two plastidic AAT isoforms (eukaryotic- and prokaryotic-types) were identified. Native gel electrophoresis and immunological analysis were used to resolve and differentiate pine isoenzymes. Recombinant overproduction of substantial amounts of fully active prokaryotic-type AAT allowed biochemical characterization of the enzyme. Knowledge gained from this analysis has been applied to develop a rapid protocol for the purification of the native enzyme from pine cotyledons. The results presented here will facilitate studies on aspartate and amino acid biosynthesis in trees.

## Materials and methods

### *Plant material and conditions of germination*

Maritime pine seeds were aerated in distilled water for 2 days and then germinated on moist vermiculite. Seedlings were grown for 3–4 weeks in a growth chamber at a day/night temperature of 22/18 °C in a 16-h photoperiod.

### *Sequence analysis*

Sequencing data were analyzed by EST database searches with the BLAST program (Altschul et al. 1990). Amino acid sequences of AATs were aligned with the Clustal W program (Thompson et al. 1994).

### *Recombinant expression and purification of PpAAT*

A full-length cDNA clone encoding prokaryotic-type AAT was used to generate gene constructs (de la Torre et al. 2006). The structure of inserts and correct orientation with respect to the T7 promoter was determined by DNA sequencing (data not shown). Transformed *Escherichia coli* BL21-codonPlus-(DE3)-RIL (Stratagene, Cedar Creek, TX) cells were grown at 37 °C in 800 ml of LB medium with 100 μg $ml^{-1}$ ampicillin and 34 μg $ml^{-1}$ chloramphenicol. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM when bacterial growth reached an absorbance of 0.8 at 600 nm. Bacterial cells were further grown for 16–20 hours at 10 °C (growth at a higher temperature substantially reduced enzyme activity), collected by centrifugation and resuspended in a buffer containing 50 mM $Na_2HPO_4$, 300 mM NaCl and 10 mM imidazol at pH 8.0. Cells were lysed by sonication, and soluble recombinant proteins were purified to apparent homogeneity by Ni-NTA affinity chromatography (Qiagen, Crawley, U.K.).

### *Protein extraction and quantification*

Soluble protein extracts corresponding to pine tissues were obtained by breaking up the tissues with a pestle and mortar in the presence of liquid nitrogen. When a fine powder was obtained, it was immediately transferred to a tube containing extraction buffer (100 mM Tris-HCl pH 8.0, 2 mM EDTA, 10% (v/v) glycerol and 0.1% (v/v) 2-mercaptoethanol). Soluble protein extracts were separated from cellular debris by centrifugation at 20,000 *g* at 4 °C for 30 min. Protein concentration was determined by the Bradford procedure (Bradford 1976).

### *Protein electrophoresis and western blot analyses*

Proteins were separated by electrophoresis in native and denaturing gels as described previously (Cánovas et al. 1984, Cánovas et al. 1991). Electroblotting of proteins from SDS gels to nitrocellulose membranes and immunodetection of AAT polypeptides were carried out as described by de la Torre et al. (2002).

### *Peptide fingerprinting and MS analysis*

Coomassie-blue-stained bands from electrophoresis gels were excised for further protein identification. About 50% of the protein-containing band was in-gel trypsin digested with ProteineerDP (Bruker, Bremen, Germany) as described by Schevchenko et al. (1996) with minor modifications. After digestion, each sample was dissolved in 20 ml of electrospray ionization (ESI) Buffer A (0.5% acetic acid in water). Liquid chromatography electrospray ionization tandem mass spectrometry (LC ESI MS/MS) analysis was performed as follows. Samples were loaded in a 100 mm × 100 μm I.D. column packed in-house with Kromasil 5 mM C18 beads (EKA Chemicals, Bohus, Sweden) and fractionated in a Famos-Switchos-Ultimate chromatographic system (LCPackings, The Netherlands) with a linear gradient of 5–30% ESI Buffer B (90% acetonitrile, 0.5% acetic acid in water) for 45 min at a flow rate of 500 nl $min^{-1}$. Peptides eluting from the column were directly analyzed on an Esquire $3000^{Plus}$ ion trap mass spectrometer (Brucker Daltonics, Bremen, Germany). Data dependent MS/MS spectra were acquired by automatic switching between MS and MS/MS mode using dynamic exclusion. Fragmentation spectra were searched against the latest version of MSDB (http://csc-fserve.hh.med.ic.ac.uk/msdb.html) and NCBInr databases using a version of the MASCOT search engine (http://www.matrixscience.com). Each positive hit was individually analyzed to confirm the sequence.

*Determination of enzyme activities*

Activity of AAT was determined for both the forward and reverse reactions. The forward reaction was determined by coupling the production of oxaloacetate from aspartate and 2-oxoglutarate to the oxidation of NADH with malate dehydrogenase (Yagi et al. 1985). Enzyme assays were carried out in a reaction mix containing 50 mM Tris-HCl pH 7.8, 50 mM aspartate, 10 mM 2-oxoglutarate, 0.07 mM pyridoxal 5′-phosphate (PLP), 0.1 mM NADH, 2 units of malate dehydrogenase (Roche Farma S.A., Barcelona, Spain) and 100 µl of the AAT enzyme preparation in a final volume of 700 µl. The reaction was initiated by the addition of 2-oxoglutarate, which was followed by the decrease in NADH absorbance at 340 nm for 120 s. The reverse reaction was measured by coupling AAT activity to glutamate dehydrogenase activity in reaction mixtures containing 15 mM glutamate, 1 mM oxaloacetate (OAA), 0.1 mM NADH, 5 units of beef glutamate dehydrogenase (Roche), 0.07 mM PLP and 3.1 mM $NH_4Cl$ in 60 mM potassium phosphate buffer pH 7.5. The reverse reaction was initiated by the addition of glutamate and followed by the decrease in NADH absorbance at 340 nm.

## Results and discussion

*Maritime pine aspartate aminotransferase gene family*

Aspartate aminotransferase activity was detected in the cotyledons, stems and roots of developing pine seedlings, and protein extracts from green cotyledons had the highest activities with a specific activity of 9.3 ± 1.3 nkat $mg^{-1}$ protein. Total AAT activity in pine cotyledons was resolved by native gel electrophoresis into several bands corresponding to differentiated AAT isoenzymes (results not shown). Because cytosolic, mitochondrial and plastidic AAT isoenzymes have been characterized in plants (Ireland and Joy 1985), we looked for the occurrence of similar isoforms in pine. In the last few years, several EST sequencing projects have been initiated for conifer species including *Pinus taeda* L. (Allona et al. 1998), *P. pinaster* (Cantón et al. 2003), *P. sylvestris* L., *Picea glauca* (Moench) Voss (http://www.arborea.ulaval.ca), *Picea sitchensis* (Bong.) Carr., *Picea engelmanii* Parry × *P. sitchensis, Cryptomeria japonica* D. Don and *Pseudotsuga menziesii* (Mirb.) Franco (http://www.ncbi.nlm.nih.gov/dbEST). The availability of these genomic resources enabled us to compare the AAT sequences present in the EST conifer databases with those AAT genes identified in the *Arabidopsis* genome. Altogether 223 EST clones were identified with high similarity to genes for cytosolic, mitochondrial and plastidic isoenzymes. In *P. taeda*, the number of EST clones was high enough to establish contigs of sequences showing a high similarity to individual *Arabidopsis* AAT genes and covering the primary structure of individual pine AAT isoforms (Table 1). The corresponding cDNA sequences were named *PtcAAT* (cytosolic), *PtmAAT* (mitochondrial) and *PtpAAT* (plastidic). In addition to these findings, we have recently reported the existence of a gene of plastidic localization and prokaryotic origin (*PpAAT*) encoding a novel class of AAT in plants (de la Torre et al. 2006). A significant number of sequences overlapping the full-length cDNA of the prokaryotic-type AAT from *P. pinaster* were also identified in the EST database of *P. taeda*, although they were insufficient to define an open reading frame encoding the enzyme. The deduced protein sequences for eukaryotic (PtcAAT, PtmAAT and PtpAAT) and prokaryotic (PpAAT) AATs in pine were compared, and the results are presented in Figure 1. The primary structures of PtcAAT, PtmAAT and PtpAAT enzymes are well conserved and quite similar to the mitochondrial, cytosolic and plastidic isoforms previously described in plants (Gantt et al. 1992, Taniguchi et al. 1995, Wilkie et al. 1995, Coruzzi 2003, Silvente et al. 2003). In contrast, the sequence identity of these pine isoenzymes and PpAAT was extremely low (10–20%) (Figure 1). Orthologous single copy genes of PpAAT have been identified in all sequenced plant genomes (de la Torre et al. 2006). The above findings were further supported by phylogenetic analysis of plant AATs. Thus, when a phylogenetic tree was constructed with genes of the AAT family from *Arabidopsis*, rice, poplar and pine, the prokaryotic- and eukaryotic-type enzymes clustered in separate groups (Figure 2). Furthermore, cytosolic (PtcAAT), mitochondrial (PtmAAT) and plastidic (PtpAAT) isoforms from pine clustered with their respective orthologous genes from angiosperms (Figure 2, upper part of the tree). In conclusion, all the above data indicate the existence of two classes of AAT enzymes in conifers: a eukaryotic-type encoded by a gene family of several members as described in angiosperms (Gantt et al. 1992, Taniguchi et al. 1995, Wilkie et al. 1995, Coruzzi 2003, Silvente et al. 2003) and a prokaryotic-type (de la Torre et al. 2006, this study). Because the prokaryotic-type AAT in plants shares a high similarity to cyanobacterial enzymes, a possible endosymbiotic origin during plant evolution has been suggested (de la Torre et al. 2006).

*Biochemical properties of prokaryotic-type AAT in pine*

We examined the biochemical properties of PpAAT to determine the degree of similarity of this atypical enzyme to other AATs previously characterized in plants and cyanobacteria. However, the purification and characterization of enzymes

Table 1. *Pinus taeda* ESTs with similarity to the *Arabidopsis thaliana* aspartate aminotransferases (AATs).

| | AAT isoenzyme | | |
|---|---|---|---|
| | Cytosolic | Mitochondrial | Plastidic |
| EST Accession Nos. | DR687018<br>CO164708<br>DR057726<br>196933<br>164629 | CF663739<br>CO161421<br>AW010831<br>CO162315<br>CO161342<br>DR120588<br>DR101862 | DR109666<br>DR025495<br>DR088419<br>DR023283<br>DR088752 |
| Identity of residues (%) | 77 | 78 | 85 |

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PtcAAT ---------------------------MDGQ------S----DRDGDLSFQPTAGS----- 19
PtmAAT ---------------------------MAMA-----IA----MSRSNLGRGIRLMS----- 20
PtpAAT MATFAAASAKPMAVIDNSQLNLSQKKFDGLRIGHAFA----KRKDSSSLKVKAVSRISMT 56
PpAAT  --MMSASFKCPVSLSGVENICNGDKAIPGINSRTLFTGSSFLQRHHSANKIFFRTCGK-- 56

PtcAAT ---AVSAFQHLEQAPED------PILGVT-----VAYN-------KDPSPVKLNLGVGAYR 59
PtmAAT ---TSGWWKTVEPAPKD------PILGVT-----EAFL-------ADTDESKVNVGVGAYR 60
PtpAAT IATDVSRFEGVTMAPPD------PILGVS-----EAFK-------ADTSDIKLNLGVGAYR 99
PpAAT  -KGSCCLFNIRAMAETLSGNGVPQIDISLSPRVAALKPSKTMAITDLATALKQAGVPVIG 115

PtcAAT TEEGKPLVLNVVRQAEELLIQDRSRYKEYLPITGLAEFNKLSAKLILGDGSPAIAEKRVA 119
PtmAAT DDHGKPVVLQQVRFAERRIMG--KENMEYLPMGGSVKMVEDSIKLAYGENADVLKDKRVA 118
PtpAAT TEELQPYVLNVVKKTENLMLE-KGDNKEYLPIEGLAAFNKATVELLLGADNNATKQGRVA 158
PpAAT  LAAGEPDFNTPDAVAEAGIKAIQDGYTRYTPNAGTMEIRTAICHKLKEENGLSYTPDQIL 175

PtcAAT TAQCLSGTGSLRVGAEFLAKHYSQRIIYIPVPTWGNHPKIFTLGGLSVKTYRYYDPRTRG 179
PtmAAT AVQALSGTGACRLFADFQKRFRPESQIYIPVPTWANHHNIWRDAHVPQRTFHYYHPETKG 178
PtpAAT TVQGLSGTGSLRLAAAFIQRYFPGAQVLISSPTWGNHKNIFNDAGVPWSEYRYYDEKTVG 218
PpAAT  VSNGAKQC--I--MAAVLAVCSPGDEVIIPAPFWVSYTEMARLADATPVIIPTLLSDDFL 231

PtcAAT LDYQGMLEDLQAASPGAIVLLHACAHNPTGVDPTQDQWVGIRQLIRSK-DLLPFFDSAYQ 238
PtmAAT LDFASLMDDVKNAPNGSFFLLHACAHNPTGVDPTEEQWKEISYQFKVK-NHFPFFDMAYQ 237
PtpAAT LDFEGMIADIKAAPSGSFVLLHGCAHNPTGIDPTPEQWEKIADVIQEK-NLTAFFDVAYQ 277
PpAAT  LNPEVFSSKLNENSR---LLILCSPSNPTGSVYPRELLEEIAKIVAKHPKLIVLSDEIYE 288

PtcAAT GFASGSLDADAYAVRLFVGDGGECFIAQSYAKNMGLYGERVGALSIVCRSATVATRVESQ 298
PtmAAT GFASGDTDRDAKAIRIFLEDGHQIACAQSFAKNMGLYGCRVGCLSVICDDAKQAVAVKSQ 297
PtpAAT GFASGSLDQDASSVRLFVPRGMEVFVAQSYSKNIGLYAERTGAINVVCSSSDAATRVKSQ 337
PpAAT  HIMYPPAKHTSFAS--LPGMWERTLTVNGFSKAFAMTGWRLGYLAGPKHFVTACGRIQSQ 346

PtcAAT LKLVIRPMYSSPPIHG-ALIVATILSDRNLNYNWTVELKNMADRIISMRHQLYDALKARG 357
PtmAAT LQQIARPMYSNPPVHG-ALIVSSILSDPDLKAIWHKEVKVMADRIIGMRKALRGNLEKLG 356
PtpAAT LKRLARPMYSNPPVHG-ARIVANVVGNPTLFKEWKSEMELMSGRIKGVRQRLYDNLSAKD 396
PpAAT  STSGASSISQKAGVAALALGYAGSEAVSTMVKAYRERRDFLVQRLQAMEGVKLPVPQGAF 406

PtcAAT TPG-DWS-HIIKQIGMFTFTGLNKDQVAFMTAEYHIYLTSDGRISMAGLS----SKTVPH 411
PtmAAT SP-LSWN-FVTEQIGMFCYSGMTPEQVDRLTSEFHIYMTRNGRISMAGVT----TGNVEY 410
PtpAAT KSGKDWS-FILRQIGMFSFTGLNKAQSDNMTNKWHVYMTKDGRISIAGLS----LSKCEY 451
PpAAT  YLFPDFSSYYGTEVEDFGVINGSEALCRFFLEKAQVALVPGDAFGNDDCIRISYAASLDT 466

PtcAAT LADAINAAVLRRG                                               424
PtmAAT LANAINEVTKSSEKEMKVGAI                                       431
PtpAAT LADAIFDSYYNVS                                               464
PpAAT  LRTAINNIEKSLLLLRPAAAASKAS                                   491
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Figure 1. Comparison of amino acid sequences for pine aspartate aminotransferase polypeptides. The ESTs with homology to *Arabidopsis thaliana* aspartate aminotransferases (AATs) were compiled to establish the individual primary sequences of *Pinus taeda* cytosolic (PtcAAT), mitochondrial (PtmAAT) and plastidic (PtpAAT) isoforms. PtcAAT, PtmAAT and PtpAAT were compared with the deduced amino acid sequence of PpAAT (de la Torre et al. 2006). The complete sequence of the precursors is shown for the two plastidic AATs, PtpAAT and PpAAT, including the N-terminal peptides for organelle targeting. Residues identical to PtcAAT are boxed in black.

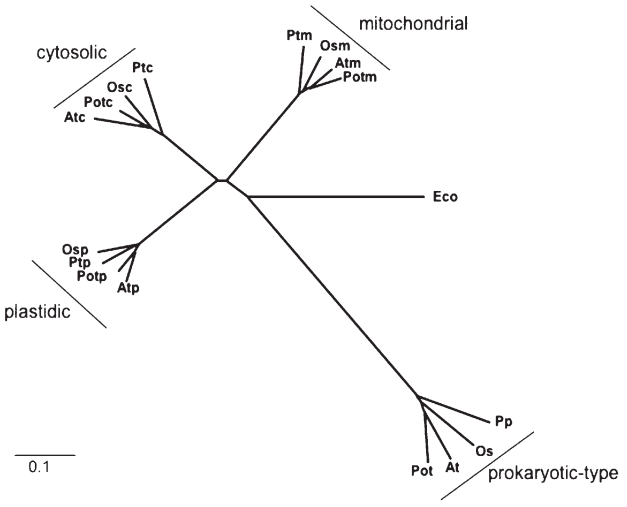



Figure 2. Phylogenetic relationships among plant aspartate aminotransferases (AATs). An unrooted phylogenetic tree was constructed using the neighbor-joining method with AAT amino acid sequences from arabidopsis (At, *Arabidopsis thaliana*), rice (Os, *Oryza sativa*), poplar (Pot, *Populus trichocarpa*) and pine (Pt, *Pinus taeda* and Pp, *Pinus pinaster*). Abbreviations: m, mitochondrial isoform; c, cytosolic isoform; p, plastidic isoform. Transit peptides in the organellar polypeptides were omitted from the analysis. The amino acid sequence of the *Escherichia coli* AAT (Eco), a member of subgroup Ia of the aminotransferase family, is included as a reference.

from conifers is not a trivial task. Interfering substances, such as polysaccharides, pigments and phenolics, are especially abundant in lignified tissues of conifers. Consequently, to overcome these difficulties, our initial approach was to overexpress the cDNA in *E. coli* in order to produce large amounts of the active enzyme amenable for biochemical studies (see Figure 3). Because the polypeptide encoded by *PpAAT* contained a presequence in the N-terminus for plastid targeting, two different constructs were made: pre-PpAAT, that contained the complete amino acid sequence of the enzyme precursor, and PpAAT, that included only the mature polypeptide (Residues 67 to 491). The recombinant proteins were purified to apparent homogeneity from bacterial extracts by affinity chromatography, and the final homogeneous enzymes had specific activities and catalytic constants that differed considerably. The PpAAT preparation exhibited a specific activity of 281.3 nkat $mg^{-1}$ protein and a turnover number ($K_{cat}$) of 133.5 $s^{-1}$, whereas the corresponding values for pre-PpAAT were considerably lower at 11.0 nkat $mg^{-1}$ protein and 5.9 $s^{-1}$, respectively. These results indicate that overexpression of the mature polypeptide (PpAAT) yielded a much more efficient enzyme.

Purified pre-PpAAT was used to raise polyclonal antibodies in rabbits, whereas highly active PpAAT preparations were used for further biochemical analysis of the enzyme. Previ-

ously, we used these antibodies to show that pine prokaryotic-type AAT is processed in vivo to a mature polypeptide of 45 kDa that is particularly abundant in photosynthetic green tissues and is located in plastids (de la Torre et al. 2006). This polypeptide is similar in size to the recombinant PpAAT subunit that we assembled into a highly active and catalytically efficient enzyme.

One of our objectives was to differentiate the molecular properties of the prokaryotic-type AAT from the eukaryotic-type AAT. Enzymatic activity of the prokaryotic-type AAT increased with increasing temperature, reaching a maximum between 50 and 60 °C (Figure 4A). Incubation of many plant enzymes at temperatures above the optimum leads to fairly rapid loss of catalytic activity. However, the prokaryotic-type AAT retained about 50% of maximal activity at 75 °C (see Figure 4A). The activation energy, calculated by an Arrhenius plot, was 45 kJ mol$^{-1}$ (Figure 4A, inset), a value that is in the range of reported values for other prokaryotic AATs (Kim et al. 2003). Thermostability studies indicated that activity was equally stable at 30 and 50 °C but decreased at higher temperatures (Figure 4B). For example, after incubation at 70 °C for 5–10 min, the remaining activity was about 50% of the initial activity, and it declined to about 25% after 20 min. A similar behavior has been reported for the enzymes from *Thermus thermophilus* (Okamoto et al. 1996) and *Phormidium lapideum* (Kim et al. 2003). It has been suggested that thermostability of prokaryotic AAT enzymes is associated, at least in part, with the high content of proline residues in the β-turns and the possible existence of bond/bridges in the surface. In support of this suggestion, contents of proline in PpAAT and *P. lapideum* enzymes were significantly higher (6.37 mol % and 6.17 mol %, respectively) than the deduced value for the eukaryotic-type enzyme of plastidic location PtpAAT (4.39 mol %) (Table 2). The number of cysteines was also higher in prokaryotic AATs than in eukaryotic AATs, indicating that some of these residues could be involved in disulfide bonds that stabilize the global protein structure. According to these findings, the initial decline in enzymatic activity might be caused by the detachment of the PLP cofactor or by changes in conformation affecting the active site rather than loss of the compact three-dimensional structure that is required for catalytic activity. To test this hypothesis, enzymatic stability was

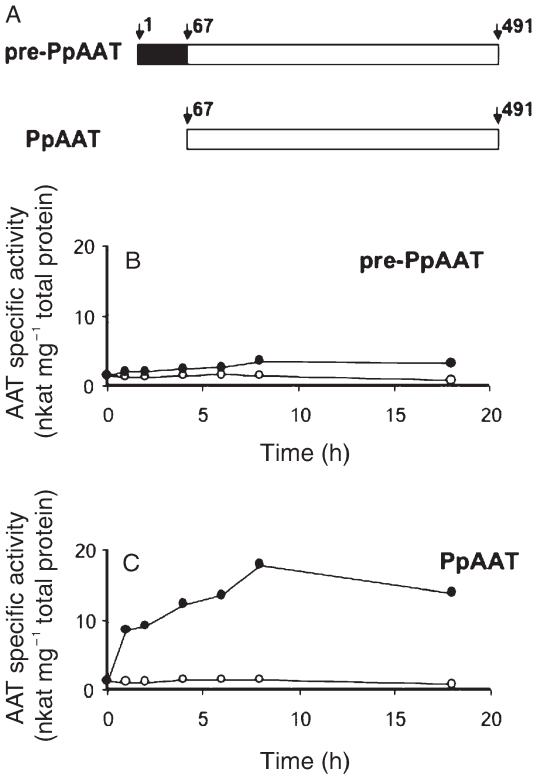



Figure 3. Overexpression in *Escherichia coli* of a prokaryotic-type aspartate aminotransferase (AAT) from pine. (A) Schematic representation of the structures for the complete (pre-PpAAT) and processed (PpAAT) polypeptides. The plastid transit peptide (positions 1–67) is shaded. (B) Time course of development of AAT activity in bacterial cells transformed with pre-PpAAT following culture in the presence (●) or absence (○) of 1 mM IPTG. (C) Time course of development of AAT activity in bacterial cells transformed with PpAAT following culture in the presence (●) or absence (○) of 1 mM IPTG. Bacterial cultures were carried out at 10 °C in a temperature-controlled shaker.

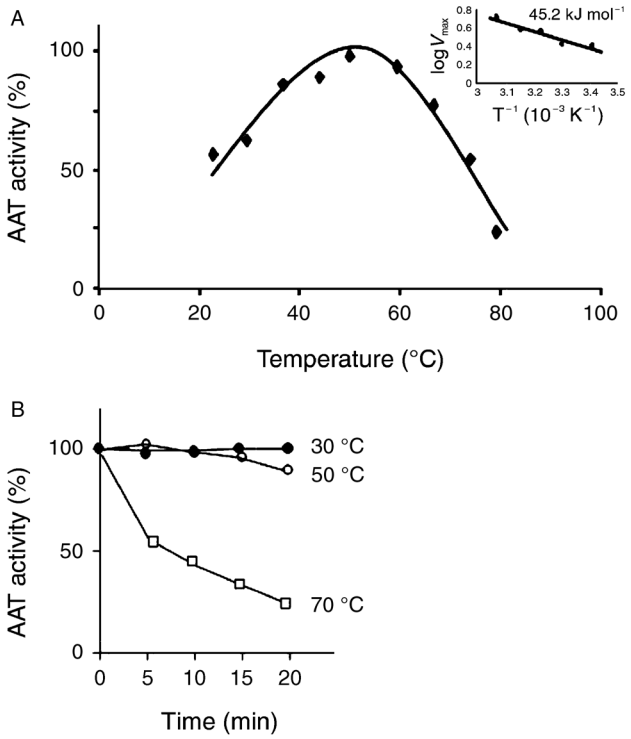



Figure 4. Effects of temperature on the activity and stability of a prokaryotic-type aspartate aminotransferase (AAT) from pine (PpAAT). (A) The catalytic activity of purified PpAAT was assayed at temperatures ranging from 20 to 80 °C. Arrhenius plot is given in the inset (activation energy 45.2 kJ mol$^{-1}$). (B) Purified PpAAT was incubated at temperatures of 30, 50 and 70 °C. At the indicated times (5, 10, 15 and 20 min), aliquots were removed and transferred to 4 °C until enzyme activity was determined.

Table 2. Comparison of the amino acid composition of plant and cyanobacterial aspartate aminotransferases (AATs). The PtpAAT and PpAAT data are derived from cDNA sequences (this study). *Phormidium lapideum* data were determined by analysis of the purified protein (Kim et al. 2003). Abbreviation: no. sub$^{-1}$, number of amino acid residues per subunit of enzyme.

| | PtpAAT | | PpAAT | | *Phormidium lapideum* | |
|---|---|---|---|---|---|---|
| Amino acid | no. sub$^{-1}$ | mol % | no. sub$^{-1}$ | mol % | no. sub$^{-1}$ | mol % |
| Alanine | 38 | 9.27 | 53 | 12.50 | 49 | 12.63 |
| Cysteine | 3 | 0.73 | 7 | 1.65 | 8 | 2.06 |
| Aspartate | 23 | 5.61 | 19 | 4.48 | 19 | 4.90 |
| Glutamate | 20 | 4.88 | 24 | 5.66 | 26 | 6.70 |
| Phenylalanine | 18 | 4.39 | 16 | 3.77 | 15 | 3.87 |
| Glycine | 33 | 8.05 | 29 | 6.84 | 27 | 6.96 |
| Histidine | 5 | 1.22 | 5 | 1.18 | 5 | 1.29 |
| Isoleucine | 19 | 4.63 | 25 | 5.90 | 23 | 5.93 |
| Lysine | 26 | 6.34 | 20 | 4.72 | 21 | 5.41 |
| Leucine | 37 | 9.02 | 43 | 10.14 | 31 | 7.99 |
| Methionine | 11 | 2.68 | 10 | 2.36 | 13 | 3.35 |
| Asparagine | 22 | 5.37 | 14 | 3.30 | 10 | 2.58 |
| Proline | 18 | 4.39 | 27 | 6.37 | 24 | 6.19 |
| Glutamine | 14 | 3.41 | 12 | 2.83 | 15 | 3.87 |
| Arginine | 18 | 4.39 | 17 | 4.01 | 16 | 4.12 |
| Serine | 33 | 8.05 | 35 | 8.25 | 22 | 5.67 |
| Threonine | 24 | 5.66 | 20 | 4.88 | 22 | 5.67 |
| Valine | 30 | 7.32 | 27 | 6.37 | 26 | 6.70 |
| Tryptophan | 6 | 1.46 | 3 | 0.71 | 3 | 0.77 |
| Tyrosine | 16 | 3.90 | 14 | 3.30 | 13 | 3.35 |

studied in the presence of the substrates and the PLP cofactor (Figure 5). Taking as a reference the thermostability behavior in the absence of any ligand (Figure 5, $H_2O$), catalytic activity was completely abolished when the enzyme was incubated for 10 min in the presence of 20 mM aspartate (Figure 5, Asp), and the addition of 20 mM 2-oxoglutarate had no protective effect (Figure 5, 2-OG). However, the addition of 40 mM PLP markedly increased the heat stability of the enzyme and the enzyme remained stable after 10 min at 60 °C and retained about 75% of its activity at 70 °C (Figure 5, PLP). Similar results have been reported for the AATs from *Chlamydomonas reinhardtii* (Lain-Guelbenzu et al. 1990) and *P. lapideum* (Kim et al. 2003).

### *Purification of prokaryotic-type AAT from pine cotyledons*

The development of a protocol for the rapid purification of prokaryotic-type AAT from tree tissues will facilitate characterization of the wild-type enzyme or modified versions of it in mutant or transgenic plants. As the first step in designing a rapid purification procedure, we determined whether the observed thermostability can be employed to enrich the abundance of PpAAT in protein crude extracts from pine.

We first analyzed whether the relative abundance of PpAAT in extracts increased relative to other AAT holoenzymes (eukaryotic counterparts). Pine AAT isoenzymes were resolved by gel electrophoresis following incubation of crude extracts at 70 °C for 5 min, in the presence of PLP as protectant. As shown in Figure 6A, the fast migrating AAT isoenzymes were highly sensitive to the heat treatment, whereas the slow migrating isoenzymes were more thermostable. Eukaryotic- and prokaryotic-type AATs were clearly identified by Western blot analyses using anti-PpAAT and anti-alfalfa plastidic AAT antisera (Figure 6B). Anti-PpAAT antibodies recognized a single band co-migrating with the recombinant AAT (Figure 6B, panel anti-PpAAT). In contrast, antibodies raised against anti-plastidic AAT from alfalfa (kindly provided by Prof. C.P. Vance, University of Minnesota) recognized both fast- and slow-migrating eukaryotic AATs in pine, but they did not cross-react with PpAAT (Figure 6B, panel anti-alfalfa AAT). These results are consistent with the observed divergence in amino acid sequences found among the different pine AATs (see Figure 1) and clearly indicate that pine prokaryotic and eukaryotic AATs are immunologically unrelated enzymes that differ in thermostability. In addition, the Western blot (Figure 6B, panel anti-alfalfa AAT) showed that the plastidic isoform is associated with the fast-migrating bands, whereas the slow-migrating bands could contain the more thermostable cytosolic isoform (Turano et al. 1991).

We also examined whether the abundance of PpAAT increased relative to the amount of total proteins in the pine extracts. Total soluble proteins from pine cotyledons and the relative abundance of PpAAT protein were analyzed after incubation of crude extracts at high temperature for different periods with PLP as protectant. No significant changes in the profiles of proteins and the abundance of PpAAT polypeptide were found when the incubation was carried out at 60 °C (Figure 7A). However, when the temperature was raised to 70 °C, increased precipitation of soluble proteins was observed in the extracts with increased time of incubation, both in the presence and absence of PLP (Figure 7B). Protein analysis of the

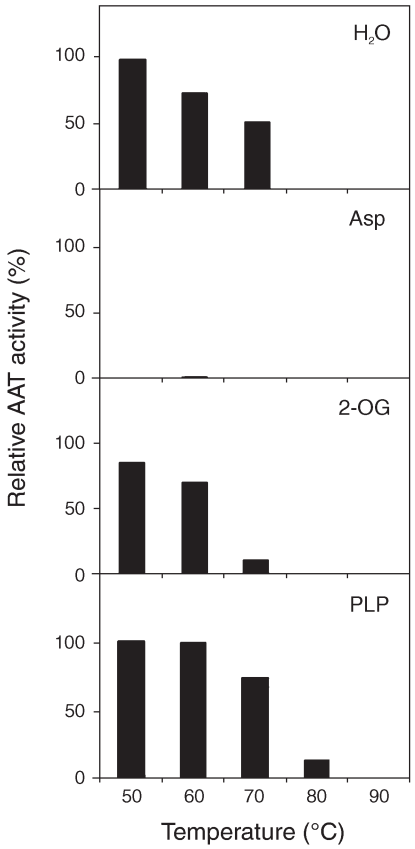



Figure 5. Effect of ligands on the thermal inactivation of recombinant aspartate aminotransferase (AAT). Purified PpAAT was incubated at various temperatures in the absence ($H_2O$) or the presence of 20 mM aspartate (Asp), 20 mM 2-oxoglutarate (2-OG) and 40 mM pyridoxal 5′-phosphate (PLP). The remaining enzyme activity was measured after10 min of incubation.

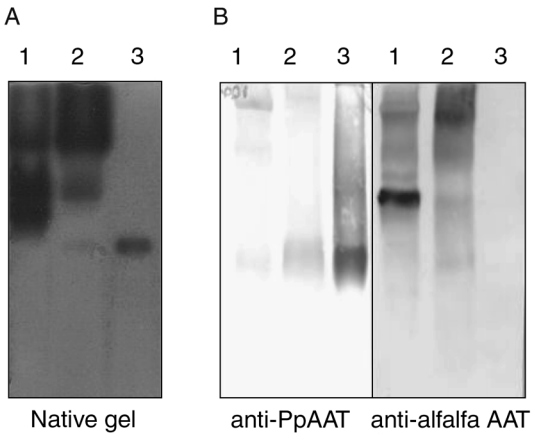



Figure 6. Differential thermostability of native pine aspartate aminotransferase (AAT) isoenzymes. (A) In situ determination of AAT activity in native gels. Lane 1, AAT isoforms in pine cotyledons; Lane 2, AAT isoforms in pine cotyledons after incubation for 5 min at 70 °C in the presence of pyridoxal 5′-phosphate; and Lane 3, recombinant PpAAT. (B) Western blot of the samples resolved in (A) immunorevealed with anti-PpAAT (dilution 1: 2000) and anti-alfalfa AAT (1:1000) antisera.

supernatant of these extracts showed that highly abundant polypeptides, such as the large and small subunit of Rubisco, were absent (cf. upper panels in Figures 7A and 7B). Under these experimental conditions, the relative abundance of PpAAT protein increased dramatically when the incubation was carried out in the presence of PLP, whereas decreasing amounts of the polypeptide were detected with time in the absence of the cofactor (cf. lower panel in Figure 7B).

Based on the above results, we purified PpAAT from pine cotyledons as follows: ammonium sulfate precipitation (30–60%), separation by ion-exchange chromatography on DEAE Sephacel and heat treatment as a basic step. This rapid purification protocol led to a highly purified preparation of native PpAAT containing the polypeptide of 45 kDa of the processed enzyme (Figure 8). The purity and yield of this polypeptide throughout the purification steps were assessed by immunodetection with anti-PpAAT antibodies. The identity of native PpAAT was further confirmed by peptide fingerprinting and mass spectrometry (LC ESI MS/MS) (Figure 8).

## Conclusions

We demonstrated the existence in conifers of a gene family encoding AAT polypeptides similar to those reported in angiosperms. The pine AATs are similar in primary structure to the isoforms localized in different subcellular compartments such as cytosol, mitochondria and chloroplast. Consequently, they are likely involved in the transfer of reducing equivalents and the interchanges of carbon and N pools between subcellular compartments, a crucial function in eukaryotic cells. Additionally, pine and all studied plants contain another chloro-

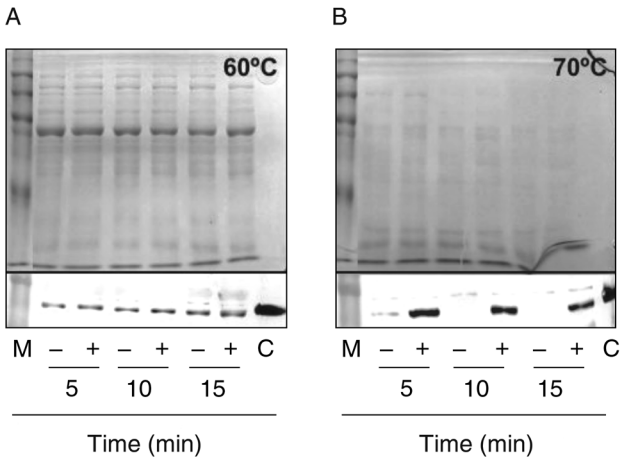
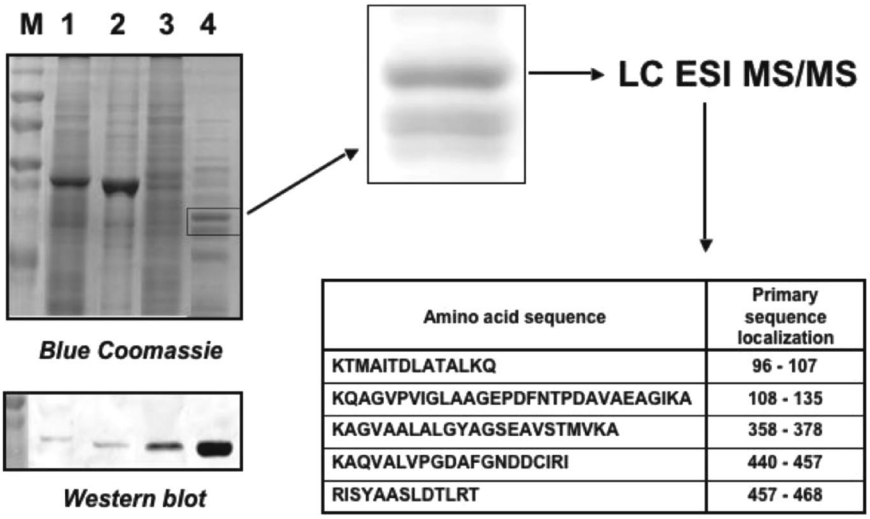



Figure 7. Protection of PpAAT polypeptide by the presence of pyridoxal 5′-phosphate (PLP). Soluble protein extracts from cotyledons of 3-week-old pine seedlings were heated at (A) 60 and (B) 70 °C for increasing periods of time (5, 10 and 15 min) in the presence (+) or absence (–) of PLP. Samples were withdrawn at the indicated times and analyzed by SDS-PAGE and western blotting. The upper panels show protein profiles after staining with Coomassie blue (15 µg of protein was loaded per lane). The lower panels show the corresponding Western blots. Abbreviations: M, molecular size markers; and C, purified PpAAT used as a control.




| Amino acid sequence | Primary sequence localization |
|---|---|
| KTMAITDLATALKQ | 96 - 107 |
| KQAGVPVIGLAAGEPDFNTPDAVAEAGIKA | 108 - 135 |
| KAGVAALALGYAGSEAVSTMVKA | 358 - 378 |
| KAQVALVPGDAFGNDDCIRI | 440 - 457 |
| RISYAASLDTLRT | 457 - 468 |

Figure 8. Electrophoretic and immunochemical analyses of PpAAT purification from pine cotyledons. Thirty µg of soluble proteins from each purification step was separated by SDS-PAGE and PpAAT polypeptides identified by Western blotting. Lane 1, crude extract; Lane 2, ammonium sulphate precipitation (30–60%), Lane 3, DEAE-Sephacel chromatography; Lane 4, heat treatment at 70 °C for 10 min in the presence of 40 mM pyridoxal 5′-phosphate; and M, molecular size markers. The prominent band of protein (45 kDda) was digested with trypsin and subjected to peptide fingerprinting analysis by LC ESI MS/MS. The inset table shows the sequences of PpAAT peptides that were identified.

plast-located and nuclear-encoded AAT that exhibits distinct molecular and functional properties. The amino acid sequence of this novel AAT (PpAAT) is unrelated to the eukaryotic AATs but is quite similar to cyanobacterial enzymes. We suggest that it arose by an endosymbiotic event and could be involved in the biosynthesis of aspartate derived amino acids lysine, threonine and isoleucine, as well as precursors of methionine, all of which are produced exclusively in the plastid (Azevedo et al. 2006). Purified preparations of the fully active recombinant holoenzyme were used to determine the molecular properties of PpAAT and to study the influence of temperature on enzymatic activity and stability. Our results indicate that this conifer enzyme exhibits biochemical characteristics similar to prokaryotic AATs included in subgroup Ib of the aminotransferase superfamily. Knowledge derived from molecular characterization of the recombinant protein has been successfully applied to develop a rapid protocol for the purification of the enzyme from pine cotyledons.

## Acknowledgments

We thank Remedios Crespillo for excellent technical assistance. We are indebted to Prof. Carrol P. Vance, University of Minnesota, for providing antibodies anti-plastidic AAT from alfalfa. Grants (BMC2003-04772 and BIO2006-06216) from the Spanish Ministerio de Educación y Ciencia supported this research. The financial support of Junta de Andalucía (grupo CVI-114) is also acknowledged.

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