

Identification and functional analysis of a prokaryotic-type aspartate aminotransferase: implications for plant amino acid metabolism

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Received 29 September 2005; revised 2 December 2005; accepted 15 December 2005.

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Summary

In this paper, we report the identification of genes from pine (*PpAAT*), *Arabidopsis* (*AtAAT*) and rice (*OsAAT*) encoding a novel class of aspartate aminotransferase (AAT, EC 2.6.1.1) in plants. The enzyme is unrelated to other eukaryotic AATs from plants and animals but similar to bacterial enzymes. Phylogenetic analysis indicates that this prokaryotic-type AAT is closely related to cyanobacterial enzymes, suggesting it might have an endosymbiotic origin. Interestingly, most of the essential residues involved in the interaction with the substrate and the attachment of pyridoxal phosphate cofactor in the active site of the enzyme were conserved in the deduced polypeptide. The polypeptide is processed *in planta* to a mature subunit of 45 kDa that is immunologically distinct from the cytosolic, mitochondrial and chloroplastic isoforms of AAT previously characterized in plants. Functional expression of *PpAAT* sequences in *Escherichia coli* showed that the processed precursor is assembled into a catalytically active homodimeric holoenzyme that is strictly specific for aspartate. These atypical genes are predominantly expressed in green tissues of pine, *Arabidopsis* and rice, suggesting a key role of this AAT in nitrogen metabolism associated with photosynthetic activity. Moreover, immunological analyses revealed that the plant prokaryotic-type AAT is a nuclear-encoded chloroplast protein. This implies that two plastidic AAT co-exist in plants: a eukaryotic type previously characterized and the prokaryotic type described here. The respective roles of these two enzymes in plant amino acid metabolism are discussed.

Keywords: aspartate aminotransferase, prokaryotic-type enzyme, amino acid metabolism, chloroplast.

Introduction

Aspartate aminotransferase (AAT, EC 2.6.1.1) catalyses the reversible reaction of transamination between aspartate and 2-oxoglutarate to generate glutamate and oxaloacetate using pyridoxal 5'-phosphate (PLP) as essential cofactor. The enzyme plays a key role in the metabolic regulation of carbon and nitrogen metabolism in all organisms. In prokaryotes, AAT is a central enzyme in amino acid metabolism. In eukaryotes, AAT has been shown to be involved in the transfer of reducing equivalents between subcellular compartments via the malate–aspartate shuttle (Givan, 1980). Through the intracellular metabolic shuttle system, the enzyme plays a critical role in the interchanges of carbon and nitrogen pools between subcellular compartments of eukaryotic cells.

Aspartate aminotransferases are classified into the group I of the aminotransferase superfamily (Jensen and Gu, 1996). Although the three-dimensional structure is well conserved in all AAT enzymes, significant differences have been found in the primary structure. Thus, the AATs characterized from many organisms have been classified into two subgroups, Ia and Ib, according to their identities in amino acid sequence. About only 15% of identity is observed when the amino acid sequences of subgroups Ia and Ib members are compared (Okamoto *et al.*, 1996). Subgroup Ia includes the AATs from eubacteria and eukaryotes, whereas subgroup Ib comprises the enzymes from some eubacteria including cyanobacteria and archaeobacteria. The enzymes included in the Ia subgroup have been extensively studied and most of our

knowledge on AAT structure and function comes from the research work carried out on its members. However, much less is known about AATs from subgroup Ib.

In higher plants, various AAT isoenzymes have been identified, localized in specific subcellular compartments such as the cytosol, mitochondria, peroxisome and plastid (Ireland and Joy, 1985). In animals, cytosolic and mitochondrial isoforms of AAT have also been characterized. All these isoforms are encoded by a gene family of several members, all belonging to subgroup Ia of the aminotransferase superfamily.

The aspartate aminotransferases best characterized in *Arabidopsis thaliana*. Five genes have been identified encoding AAT polypeptides that are targeted to different subcellular compartments and assembled into functional AAT isoenzymes: ASP1 (mitochondrial), ASP2 and ASP4 (cytosolic), ASP3 (peroxisomal), ASP5 (plastidic) (Coruzzi, 2003; Schultz and Coruzzi, 1995; Wilkie and Warren, 1998; Wilkie *et al.*, 1995).

In this paper, a novel class of AAT has been identified in higher plants. The enzyme is unrelated to the eukaryotic AATs (from plants and animals) included in subgroup Ia of the aminotransferase superfamily but similar to the cyanobacterial AAT and other bacterial enzymes of subgroup Ib. The primary structure of the polypeptide contains a presequence for targeting to the plastid. As the protein is predominant in green tissues, our results suggest a key role of this AAT in nitrogen metabolism associated with photosynthetic activity.

Results

Identification of a prokaryotic-type (PT) AAT in higher plants

Large-scale expressed sequence tag (EST) sequencing projects have been initiated for loblolly (*Pinus taeda*; Allona *et al.*, 1998) and maritime pine (*Pinus pinaster*; Cantón *et al.*, 2003). ESTs databases from different woody tissues have recently been established, and the information is accessible on the internet (<http://pinetree.ccgb.umn.edu>; <http://cbi.labri.fr/outils/SPAM/index.php>). A search in the pine databases identified a total number of 170 EST clones putatively encoding aspartate aminotransferase with high similarity to the Arabidopsis genes for mitochondrial, cytosolic and chloroplastic isoenzymes (data not shown). Interestingly, in addition to these findings, a significant number of ESTs (14) showed a low level of similarity to the Arabidopsis genes but were close in sequence to the AATs of *Synechocystis* and other prokaryotes (data not shown). Five *P. pinaster* clones containing these prokaryotic-like AAT sequences (PP077H03, PP050D02, PP043D11, PP034D09, PP34D08) were selected and the cDNA inserts analysed by agarose gel electrophoresis (data not shown). One of these ESTs harbouring the largest insert, PP034D09, was completely

sequenced and shown to contain a nearly full-length cDNA designated *PpAAT* (EMBL nucleotide sequence database accession number AJ628016). The *PpAAT* sequence contained a 5' untranslated region of 142 nucleotides, an open reading frame encoding a polypeptide of 491 amino acid residues and a 3' non-coding region of 375 nucleotides ending in a short poly(A) tail. The size for the deduced polypeptide was 52.8 kDa and the isoelectric point 7.5.

The availability of complete genome sequences in plants allowed us to identify single-copy genes in Arabidopsis and rice with similar sequence to the unusual AAT identified in pine, which were named *AtAAT* and *OsAAT* respectively. No similar genes were found in animal genomes, suggesting that this atypical AAT may have a conserved and plant-specific biological role. The amino acid sequences of the *PpAAT*, *AtAAT* and *OsAAT* polypeptides were compared to the primary structure of bacterial and eukaryotic AATs (Figure 1). In spite of the evolutionary distance between gymnosperms and angiosperms, the AAT from *P. pinaster* showed a high degree of identity with the protein from the dicot *A. thaliana* (69.7%) and the monocot *Oryza sativa* (68.5%). Interestingly, the polypeptides deduced from the plant genes *PpAAT*, *AtAAT* and *OsAAT* shared more identity with the AATs from cyanobacteria (40–48%) than with the previously characterized amino acid sequences of cytosolic, mitochondrial and chloroplastic AATs from Arabidopsis (10–20%; Figure 1). Similarly, a low level of identity was found with the cytosolic and mitochondrial isoforms of human AAT and with the *Escherichia coli* enzyme.

As for other PLP-dependent enzymes, aspartate aminotransferases share a common protein scaffold that is related to the positioning of the PLP cofactor (Alexander *et al.*, 1994). Eleven 'anchor' residues are highly conserved and many of them are involved in the interaction with PLP. The deduced amino acid sequences of *PpAAT*, *AtAAT* and *OsAAT* presented most of these conserved residues, suggesting that they encode functional AATs in plants. It is worth noting that one of these residues, G110 in the prototype AAT structure, is conserved in eukaryotic (plant and animal) and *E. coli* AATs but absent in the unusual type of AAT reported here. The above data strongly suggest that *PpAAT*, *AtAAT* and *OsAAT* genes encode a functional PT-AAT in higher plants.

Phylogenetic relationship among AATs from different organisms

To examine the phylogenetic relationship of these new plant genes with AAT genes from different organisms, an unrooted phylogenetic tree was constructed using the neighbour-joining method with 34 full-length AAT amino acid sequences from archeobacteria, bacteria, protozoa, plants and animals that were accessible in the databases. As shown in Figure 2, the phylogenetic tree displays two major classes

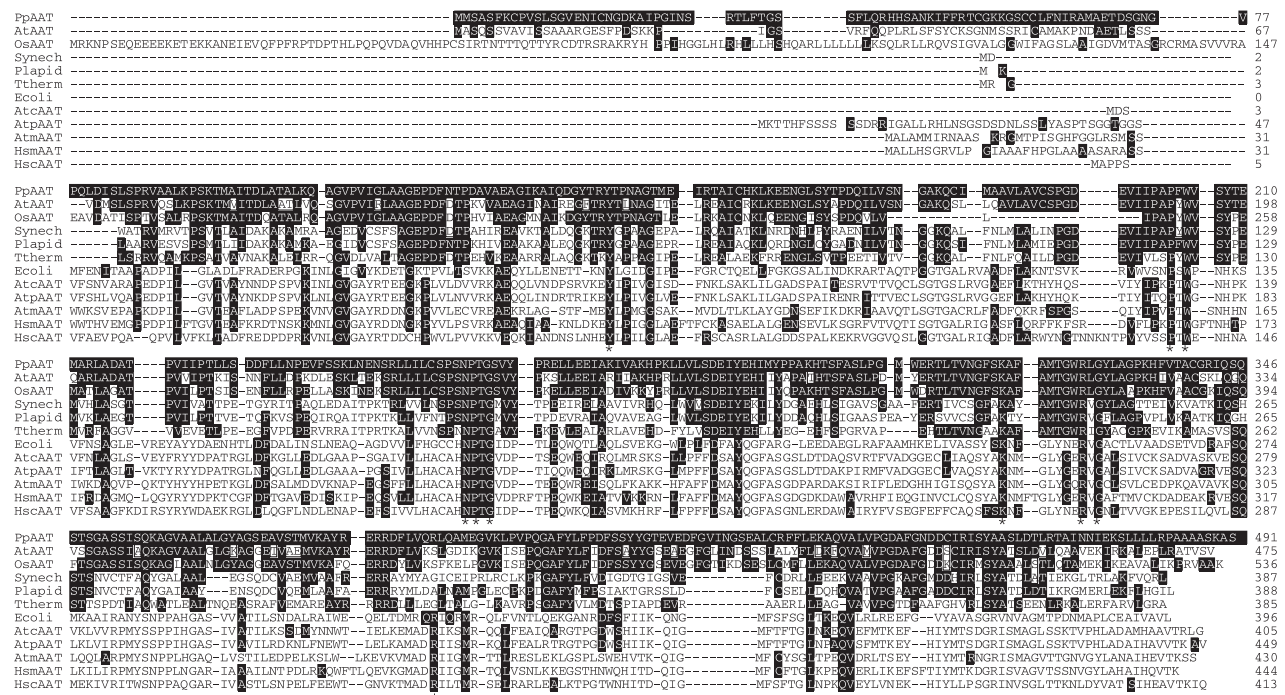


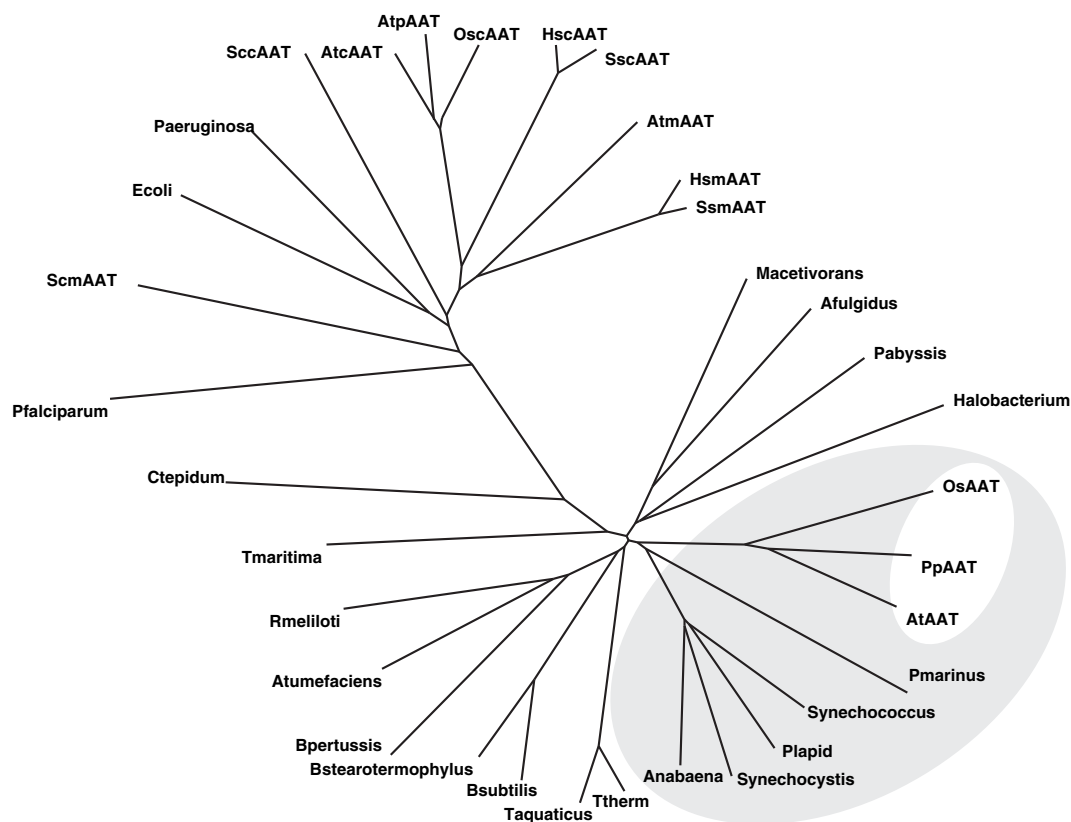
Figure 1. Sequence alignment of AAT from different organisms. Residues identical to PpAAT are indicated in black. Conserved residues involved in the AAT catalytic site are marked with an asterisk. PpAAT, *Pinus pinaster* (Q5F4K8); AtAAT, *Arabidopsis thaliana* (Q9SIE1); OsAAT, *Oryza sativa* (Q943I5); Synech, *Synechococcus* sp strain WH (81081205); PlapId, *Phormidium lapideum* (UJ32B); Therm, *Thermus thermophilus* AAT; EcolI, *Escherichia coli* (P00509); AtcAAT, *Arabidopsis thaliana* cytoplasmic (P46645); AtpAAT, *Arabidopsis thaliana* chloroplastic (P46644); AtmAAT, *Arabidopsis thaliana* mitochondrial (P46643); HsmAAT, *Homo sapiens* mitochondrial (P00505); HscAAT, *Homo sapiens* cytosolic (P17174).

in the analysis. One group is formed by *E. coli* and eukaryotic AATs, including enzymes from protozoa, animals and plants. The other group, constituted almost exclusively by AATs from archaeobacteria and bacteria, also includes the plant genes *PpAAT*, *AtAAT* and *OsAAT*. It should be noted that all the animal and plant AATs cluster together, regardless of whether they are located in the cytosol, mitochondria or plastid. This clustering suggests that genes encoding the eukaryotic isoenzymes evolved from a common ancestor after the prokaryotic/eukaryotic split. However, the novel genes identified in this work appear to have evolved independently from a prokaryotic origin.

PT-AAT contains a putative transit peptide for organellar localization

The amino acid sequence deduced from *PpAAT*, *AtAAT* and *OsAAT* has N-terminal extensions compared with other AATs previously characterized in plants. Prediction of sub-cellular localization using Predator (<http://www.inra.fr/predator>) and TARGETP version 1.0 (<http://www.cbs.dtu.dk/services/TargetP>) suggested the existence of a signal peptide for plastid targeting in *PpAAT* and *AtAAT*. In contrast, bioinformatic programs did not predict any targeting pre-sequence in the *OsAAT* N-terminal.

To examine the functional characteristics of this atypical plant AAT enzyme, we decided to overexpress the pine *PpAAT* cDNA in *E. coli* (Figure 3). According to the prediction of a targeting presequence in the polypeptide, two different constructions were made: one contained the complete amino acid sequence (named *PpAAT*) whereas the other contained only the putative processed polypeptide (named p-*PpAAT*) starting in another methionine residue at the N-terminal of the polypeptide. As shown in Figure 3(a), the two forms of AAT were functionally active in *E. coli* protein extracts; however, their respective activities differed considerably. Thus, the specific activity of p-*PpAAT* (16.2 nkat mg⁻¹) was significantly higher than that of *PpAAT* (3.1 nkat mg⁻¹). The enzymes were purified to apparent homogeneity from bacterial extracts by using His-tag affinity chromatography and were shown to be composed of single polypeptides of the expected molecular sizes, i.e. 53 kDa for *PpAAT* and 45 kDa for p-*PpAAT* (Figure 3b). The specific activity and catalytic constant of the homogenous p-*PpAAT* preparation were much higher (over 20-fold) than those of the *PpAAT* holoenzyme (results not shown), a finding that was consistent with the observed values of enzyme activity in bacterial crude extracts (Figure 3a). The purified preparations of the enzymes were then used to determine the functional properties of the overexpressed gene products. In



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Figure 2. Phylogenetic tree of AATs from different organisms.

The phylogenetic tree was constructed with full-length AAT amino acid sequences using the neighbour-joining method of the CLUSTAL W program (Thompson *et al.*, 1994). Abbreviations in addition to those indicated in Figure 1: Macetivorans, *Methanosarcina acetivorans* (Q8TPT6); Afulgidus, *Archaeoglobus fulgidus* (Q28151); Pabyssis, *Pyrococcus abyssi* (Q9UZG3); Halobacterium sp (Q9HMR6); Pmarinus, *Prochlorococcus marinus* (Q7VA14); Synechocystis sp (Q55128); Anabaena sp (Q8YM56); Taquaticus, *Thermus aquaticus* (Q33822); Bsubtilis, *Bacillus subtilis* (Q07597); Bestarotermophilus, *Bacillus stearothermophilus* (93600); Bpertussis, *Bordetella pertussis* (Q7VYW8); Atumefaciens, *Agrobacterium tumefaciens* (AAL43185); Rmeliloti, *Rhizobium meliloti* (P58350); Tmaritima, *Thermotoga maritima* (Q9X0Y2); Ctepidum, *Chlorobium tepidum* (Q8KCN2); Pfciparum, *Plasmodium falciparum* (O96142); ScmAAT, *Saccharomyces cerevisiae* mitochondrial (Q01802); Ecoli, *Escherichia coli* (P00509); Paeruginosa, *Pseudomonas aeruginosa* (P72173); SccAAT, *Saccharomyces cerevisiae* cytosolic (P23542); SsmAAT, *Sus scrofa* mitochondrial (P00506); SscAAT, *Sus scrofa* cytosolic (P00503).

parallel, a purified preparation of PpAAT was used to raise polyclonal antibodies in rabbits according to the method described by Cantón *et al.* (1996).

Molecular and kinetic properties of PT-AAT

In order to determine how PT-AAT polypeptides are assembled into functional holoenzymes, the apparent size of the native enzymes was determined by gel filtration chromatography using protein markers of a known size as standards for column calibration. The PpAAT holoenzyme showed a molecular mass of about 120 kDa, whereas the estimated size of the p-PpAAT enzyme was 94 kDa (Figure 4). These data indicate that the recombinant polypeptides of pine AAT are assembled into homodimeric functional holoenzymes.

To further characterize the molecular properties of this unusual AAT in plants, it was important to determine the substrate specificity of the enzyme. Purified preparations of the functionally expressed holoenzyme in bacteria (p-PpAAT) were used to determine its relative activity towards different amino acid substrates (Table 1). The enzyme was found to be absolutely specific for aspartate as amino donor, with 2-oxoglutarate as amino acceptor. No enzyme activity was detectable in the presence of glutamine, asparagine, alanine, histidine, leucine, methionine, lysine, arginine, tryptophan, tyrosine, phenylalanine or kynureine.

The kinetic parameters of the purified p-PpAAT holoenzyme were also determined (Table 2). The apparent K_m values for the substrates 2-oxoglutarate and aspartate were in the range for the Arabidopsis isoenzymes overexpressed in bacteria (Wilkie and Warren, 1998) with the exception of

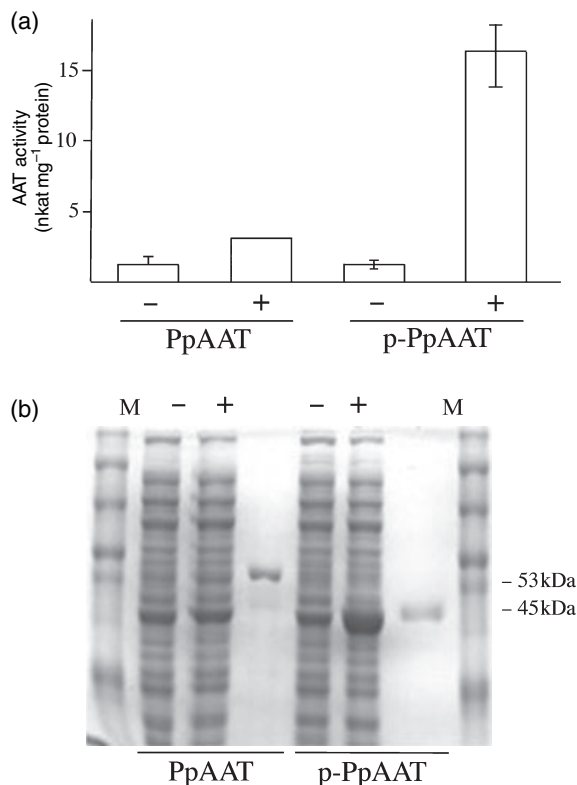


Figure 3. Overexpression of pine AAT enzymes in bacteria.

(a) AAT activity levels in crude extracts from *E. coli* cultures overexpressing the precursor (PpAAT) or the processed (p-PpAAT) polypeptide. Cells harbouring pET11a/(PpAAT) or pET11a/(p-PpAAT) plasmids were grown at 10°C for 14 h either in the presence (+) or the absence (-) of IPTG. Soluble proteins were extracted and AAT activity determined as described in Experimental procedures. The values are given as the means \pm SD of at least three independent determinations assayed twice.

(b) Protein profiles of bacterial extracts (50 μ g) and purified preparations (1 μ g) of PpAAT and p-PpAAT after staining with Coomassie blue. IPTG absence (-) or presence (+). M, protein molecular markers. The molecular sizes of the purified AAT polypeptides are indicated on the right.

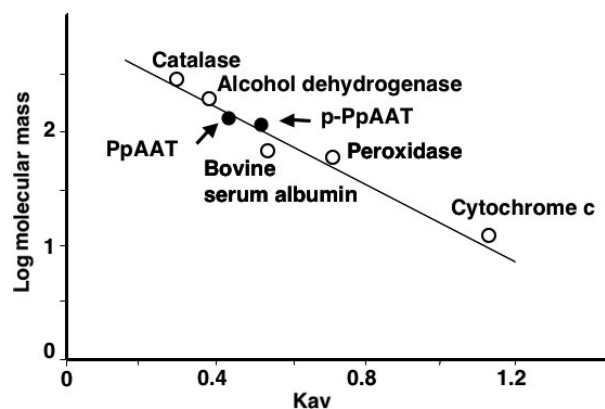


Figure 4. Molecular mass estimation of recombinant AAT enzymes.

The log of molecular mass was plotted versus the partition coefficient ($K_{av} = V_e - V_o / V_t - V_o$) of the proteins eluted through a Sephacryl S-300 column (93 \times 2 cm). V_e , elution volume; V_o , void volume; V_t , total volume.

Table 1 Substrate specificity of pine AAT holoenzyme overexpressed in *Escherichia coli*

	Concentration (mM)	AAT activity (%)
Aspartate	50	100
Asparagine	50	0
Glutamine	50	0
Arginine	50	0
Alanine	50	0
Methionine	50	0
Histidine	25	0
Leucine	25	0
Lysine	25	0
Phenylalanine	50	0
Tryptophan	25	0
Tyrosine	5	0
Kynureine	5	0

the mitochondrial enzyme. In contrast, the kinetic constants for the reverse reaction (oxaloacetate and glutamate) were different from the reported values for mitochondrial, cytosolic and plastidic AATs in Arabidopsis.

PT-AAT is immunologically unrelated to plant AATs and differentially expressed in photosynthetic and non-photosynthetic tissues

Polyclonal antibodies have been used as a molecular tool to further investigate the properties and functional characteristics of the PT-AAT in plants. Samples containing recombinant PpAAT and total protein extracts from pine cotyledons, hypocotyls and roots were analysed by Western blotting (Figure 5a). Anti-PpAAT antiserum specifically recognized decreasing amounts of the PpAAT polypeptide, 53 kDa in size (Figure 5a, lanes 1, 2 and 3), but also a single cross-reacting band in the protein extracts from pine seedlings of approximately 45 kDa (Figure 5a, lanes C, H and R). The size of this later polypeptide corresponds to the predicted value for the processed polypeptide (p-PpAAT) in the cDNA (Figure 3b). It is worth noting that the relative abundance of this AAT in pine extracts was much higher in green (cotyledons and hypocotyls) than non-green organs (roots).

We were interested in determining whether or not PT-AAT had immunological cross-reactivity with the endogenous eukaryotic-type AATs previously characterized in several plant species. Protein extracts from cotyledons were resolved by native gel electrophoresis, stained for AAT activity and immunoprobed with anti-p-PpAAT antiserum. The antibodies exclusively recognized a single band co-migrating with recombinant PpAAT (data not shown). Furthermore, the same protein samples analysed in Figure 5(a) were immunoprobed with AAT antiserum raised against alfalfa plastidic AAT (Gantt *et al.*, 1992), which is presumably able to recognize the polypeptides of cytosolic, plastidic and mitochondrial isoforms (Figures 1 and 2). Figure 5(b) shows

Table 2 Kinetic parameters of eukaryotic and prokaryotic-type AATs in plants. Data reported here for the pine enzyme (*Pinus pinaster*) are compared to those published for recombinant Arabidopsis AATs

	<i>Arabidopsis thaliana</i> ^a			<i>Pinus pinaster</i> ^b
	AAT1	AAT2	AAT5	p-PpAAT
Localization	Mitochondria	Cytosol	Plastid	Plastid (predicted)
Aspartate [K_m (mM)]	3.00	1.38	2.85	2.5
Glutamate [K_m (mM)]	8.15	12.80	11.60	1.0
Oxaloacetate [K_m (μ M)]	38	27	20	150
2-Oxoglutarate [K_m (μ M)]	260	100	90	80

^aKinetic parameters for recombinant AAT isoenzymes from *Arabidopsis thaliana* determined by Wilkie and Warren (1998).

^bKinetic parameters for recombinant p-PpAAT from *Pinus pinaster*. Values given are the average of at least three independent determinations. The differences in each set of data were <10%.

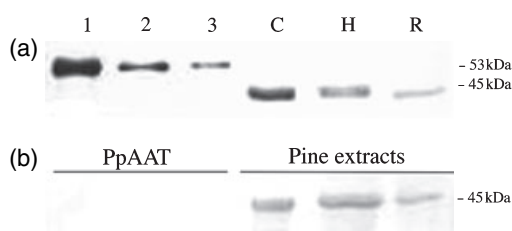


Figure 5. Immunological identification of PT-AAT.

Decreasing amounts of PpAAT (lanes 1, 2 and 3 were loaded with 150, 50 and 20 ng of purified protein, respectively) and soluble protein extracts (30 μ g) from pine cotyledons (lane C), hypocotyls (lane H) and roots (lane R) were subjected to Western blot analyses. (a) Immunoblot probed with anti-PpAAT (dilution 1:2000). (b) Immunoblot probed with alfalfa anti-AAT (dilution 1:1000; Gantt *et al.*, 1992). The molecular sizes of major cross-reacting bands are indicated on the right.

that the antibodies specifically recognized a highly abundant polypeptide of 45 kDa in cotyledons, hypocotyls and roots of pine seedlings. However, the antiserum was absolutely unable to cross-react with the recombinantly expressed PpAAT, indicating that it is a protein immunologically distinct from the eukaryotic AATs. All these data are consistent with the divergence in primary sequence found between both types of enzymes (Figure 2).

Antibodies against PT-AAT from pine were also used as molecular tool to examine the presence of immunologically related proteins in other plant species in which the gene had previously been identified (Figure 1). Proteins were extracted from photosynthetic and non-photosynthetic tissues of pine, Arabidopsis, rice and tomato, and subjected to Western blot analysis with the pine AAT antiserum. As shown in Figure 6(a), the antibodies cross-reacted with single polypeptides of a similar size in all plant species examined. Furthermore, the immunodetected protein band co-migrated with the recombinant p-PpAAT polypeptide included in the gel as a size control, suggesting that the presequences encoded by the corresponding genes are processed *in planta* to mature forms of AAT polypeptides of about 45 kDa. It is also apparent from the Western blot that the steady-state levels of the polypeptide were higher in photo-

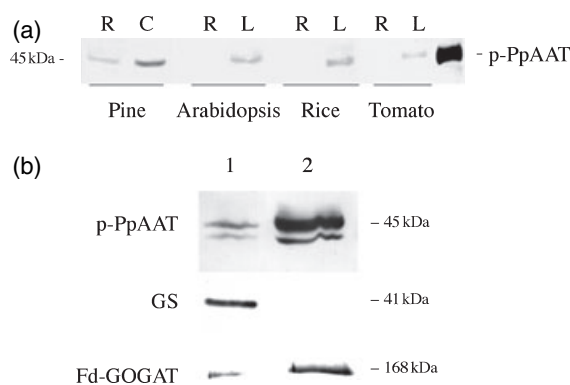


Figure 6. Immunocharacterization of PT-AAT in plants.

(a) Immunodetection of PT-AAT polypeptides in several species. Three-week-old seedlings of pine (*P. pinaster*), Arabidopsis (*A. thaliana*), rice (*O. sativa*) and tomato (*L. esculentum*) were grown under the conditions described in Experimental procedures. Total proteins were extracted from plant tissues and subjected to Western blotting analysis using anti-PpAAT antibodies. Equal amounts of protein (30 μ g) were loaded per lane. R, root, C, cotyledon, L, leaf. Purified p-PpAAT (200 ng) was included as a molecular control.

(b) The PT-AAT is localized in the chloroplasts. Western blot analyses of AAT, GS and Fd-GOGAT in protein extracts from pine cotyledons (lane 1) and chloroplasts (lane 2). AAT (45 kDa) was detected in cotyledon total proteins and in chloroplast proteins. A lower migrating band was also detectable in both extracts, possibly a degradation product of the intact 45 kDa polypeptide. As a control, cytosolic GS (41 kDa) was detected in total proteins but not in chloroplast proteins (Cantón *et al.*, 1996). In contrast Fd-GOGAT (168 kDa) was detected in both total proteins and chloroplast proteins (García-Gutiérrez *et al.*, 1995).

synthetic than in non-photosynthetic tissues in all plant species examined. In fact, at the dilution of the antiserum utilized for detection of AAT in pine, it was almost undetectable in the root extracts from Arabidopsis, rice and tomato.

The PT-AAT of plants is a plastid-located enzyme that accumulates during chloroplast development

We were interested to investigate whether the precursor AAT is processed *in planta* to a 45 kDa polypeptide of plastidic localization. Chloroplasts were isolated from light-grown pine seedlings by differential centrifugation and rapid

purification on Percoll gradients, and soluble proteins from total or chloroplast extracts were analysed by Western blotting. As shown in Figure 6(b), the relative abundance of the processed polypeptide (p-PpAAT) was enriched in the lane containing chloroplast proteins, as also occurred with ferredoxin-dependent glutamate synthase (Fd-GOGAT), a chloroplast-located enzyme (García-Gutiérrez *et al.*, 1995). However, no immunological signal was observed for cytosolic glutamine synthetase (GS) in the chloroplast preparation. We can therefore conclude that the PT-AAT is of plastidic localization.

The biogenesis of chloroplasts is a significant event in plant development that requires enhanced synthesis of a great number of nitrogen compounds. In conifers, the differentiation of the photosynthetic organelles is a light-independent process that is completed during early stages of seedling development (Cánovas *et al.*, 1993; Mariani *et al.*, 1990). We have previously reported the coordinated expression of enzymes for the GS/GOGAT cycle during plastid differentiation in pine seedlings (Cánovas *et al.*, 1991; Palomo *et al.*, 1998; Suárez *et al.*, 2002). To investigate whether the novel AAT is associated with this process, the relative abundance of the protein was determined and compared with that of GS (Figure 7). p-PpAAT and GS were undetectable in the embryo but accumulated upon germination of the seed and during seedling development to reach maximum levels at cotyledon length of 1.5 cm. In *P. pinaster*, the chloroplasts are fully developed when the seedling has this cotyledon size. Interestingly, p-PpAAT was also present at high levels in green needles as observed for GS protein.

Discussion

PT-AAT is present in all characterized plant genomes

In this paper, we describe new and atypical AAT genes in pine, *Arabidopsis* and rice named *PpAAT*, *AtAAT* and *OsAAT*

respectively. The deduced amino acid sequence from these genes showed a low level of identity with the nuclear-encoded polypeptides of cytosolic, mitochondrial, peroxisomal and chloroplastic isoforms previously described in plants (Coruzzi, 2003; Gantt *et al.*, 1992; Silvente *et al.*, 2003; Taniguchi *et al.*, 1995; Wadsworth *et al.*, 1993; Wilkie *et al.*, 1995). Furthermore, these novel AAT polypeptides also showed a low degree of similarity with the proteins from vertebrates (e.g. *Homo sapiens*) and certain bacteria (e.g. *E. coli*). However, it is worth noting that the derived polypeptides from *PpAAT*, *AtAAT* and *OsAAT* conserved most of the essential residues involved in the interaction with the substrate and the attachment of PLP cofactor at the active site of the enzyme. Interestingly, the atypical AAT polypeptides showed a high degree of identity with AATs from cyanobacteria (e.g. *Synechococcus p.*), other bacteria and archaeobacteria. The above body of evidence strongly suggests that the *PpAAT*, *AtAAT* and *OsAAT* genes encode a functional PT-AAT in higher plants. Furthermore, the identification of sequences with a similar identity in the recently released poplar genome (<http://genome.jgi-psf.org/Poptr1/Poptr1.home.html>) and the ongoing genome projects of tomato (<http://sgn.cornell.edu>) and maize (<http://www.maizegdb.org>) indicate that this novel form of AAT is widely distributed in the genomes of higher plants, including different plant lineages (monocot and dicot angiosperms and gymnosperms). Taken together, these data indicate the existence of two different classes of AAT enzymes, an eukaryotic-type encoded by a gene family of several members (Coruzzi, 2003; Gantt *et al.*, 1992; Silvente *et al.*, 2003; Taniguchi *et al.*, 1995; Wadsworth *et al.*, 1993; Wilkie *et al.*, 1995) and a prokaryotic-type encoded by a single gene that is described in this paper. The eukaryotic-type AAT in plants belongs to the subgroup Ia, whereas the PT-AAT would be included in the subgroup Ib of the aminotransferase superfamily.

The origin of the PT-AAT is possibly endosymbiotic

To examine explicitly the phylogenetic relationships between the PT-AAT of plants and genes from other organisms, a comparative study was performed with previously described sequences including AATs from animals, plants and prokaryotes. The clustering of *PpAAT*, *AtAAT* and *OsAAT* genes with bacterial AATs further supports the prokaryotic origin of these genes in plants. However, *PpAAT*, *AtAAT* and *OsAAT* are much more closely related to cyanobacterial genes than to other bacterial AAT genes. Therefore, based on the phylogenetic analysis and the low level of similarity with the eukaryotic AATs, we propose that the plant AAT genes described here might have arisen from an endosymbiotic origin. Furthermore, the phylogenetic analysis also indicates that all AATs previously characterized in plants (and other eukaryotic AATs) are closely related to

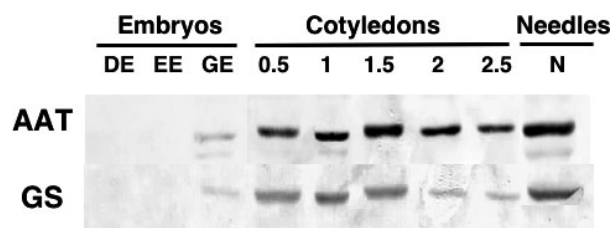


Figure 7. Developmental accumulation of AAT and GS during the initial stages of pine development.

Proteins were extracted from pine tissues (1 g), separated by SDS-PAGE and immunorevealed using anti-PpAAT and anti-GS antibodies. Lanes 1, 2, 3, 4 and 5 correspond in the order given to seedlings with 0.5, 1, 1.5, 2 and 2.5 cm cotyledon length. Similar developmental stages corresponded more closely to cotyledon length than to seedling age. One-month-old seedlings had a cotyledon length of about 2.0–2.5 cm. DE, dry embryo, IE, imbibed embryo, GE germinated embryo, N, needles. Fifty micrograms of protein were loaded per lane.

bacterial genes such as *E. coli*. In conclusion, the results reported here strongly suggest the possibility that the two classes of plant AATs have evolved independently from different prokaryotic genes. Interpreting these data in terms of the possible function of the genes, one may speculate that the prokaryotic-type AAT, which is not found in animals, plays a specific role in photosynthetic organisms such as cyanobacteria and plants (in which it was present at least before the divergence of angiosperms and gymnosperms that occurred >350 million years ago). Members of the well-characterized AAT gene family in plants, which are phylogenetically related to vertebrate AATs, would play other metabolic roles in eukaryotic organisms.

The PT-AAT of plants is a homodimeric holoenzyme strictly specific for aspartate

In order to examine in detail the enzymatic characteristics of this PT-AAT, genetic constructs were made to overexpress the protein in *E. coli*. Our results indicate that the enzyme is catalytically active in bacteria as previously shown for the plant eukaryotic counterparts (Wilkie and Warren, 1998). Protein sequences deduced from *PpAAT* and *AtAAT* cDNAs had a N-terminal region characteristic of plastidic transit peptides. It has been suggested that proteolytic cleavage of the N-terminal targeting signal is required for the correct assembly of the enzymatically active eukaryotic-type isoform located in the chloroplast (Wilkie *et al.*, 1996). Here we report that *PpAAT* subunits assembled into an active dimeric holoenzyme independent of whether or not the N-terminal presequence was present in the overexpressed gene product. These data indicate that the presequence do not block the assembly of subunits and dimerization into an active enzyme. However, the specific activity and catalytic constant of the holoenzyme were much higher when the targeting peptide was removed (p-*PpAAT* holoenzyme). It is clear therefore that removal of the plastid-targeting presequence permits protein assembly in the most appropriate conformation for highly efficient catalytic activity.

The dimeric p-*PpAAT* holoenzyme is a PLP-dependent enzyme as determined by spectroscopic analysis showing a maximum of absorbance at 385 nm (data not shown) corresponding to the bound cofactor (Hayashi *et al.*, 1990). It is well known that many prokaryotic AATs may accept aromatic amino acids as alternative substrates (Jensen and Gu, 1996). In contrast, the p-*PpAAT* holoenzyme showed absolute specificity for aspartate as substrate among a range of tested amino acids, including aromatic amino acids. Taken together, the above results confirm the existence in plants of a new member of the aspartate aminotransferase family of prokaryotic origin that could be plastid-located. Consequently, two plastidic AATs would co-exist in plants: one eukaryotic type and the other prokaryotic type.

Putative roles of plastidic AATs in plants

The availability of high amounts of recombinant protein enabled us to raise specific antibodies against p-*PpAAT* that have been used to demonstrate the expression of cross-reacting polypeptides in several plant species. We have found that the enzyme *in planta* is composed of a mature polypeptide of the predicted size (45 kDa). This processed polypeptide is similar in size to the p-*PpAAT* subunit that is assembled into a highly active AAT in bacteria. However, the immunodetected polypeptide was immunologically unrelated to the eukaryotic AAT polypeptides present in the same plant extracts that were specifically revealed using anti-alfalfa AAT antibodies (Gantt *et al.*, 1992). The antibodies were additionally used to demonstrate that orthologous genes to *PpAAT* are also expressed in several plants such as *Arabidopsis*, rice and tomato. Furthermore, *PpAAT* protein was unambiguously located in the chloroplasts, and our results indicate that this plastidic AAT of prokaryotic origin is more abundant in photosynthetic than in non-photosynthetic tissues of plants. These localization and expression data are consistent with molecular data derived from sequence analysis suggesting that this new and undescribed AAT would play a critical and specific role in the nitrogen metabolism of photosynthetic organisms. However, why are two distinct AATs needed in the plastids of plants? It has been proposed, based on evolutionary analyses of plant AAT sequences, that the eukaryotic-type plastidic form was derived from a gene duplication of the cytosolic enzyme (Wadsworth, 1997; Winefield *et al.*, 1995), and that this event occurred at the time that plant and animal evolutionary lines separated. The function of cytosolic and plastidic AATs has been investigated using *Arabidopsis* mutants (Miesak and Coruzzi, 2002; Schultz *et al.*, 1998). Results from these analyses support the conclusion that the cytosolic enzyme (*Asp2*) is involved in aspartate biosynthesis in the light to generate a pool that can be used for asparagine synthesis in darkness. *Asp5* mutants were affected in the plastidic isoenzyme but they had no visible phenotype except that plants contained increased glutamine levels either in light- or dark-growing conditions (Miesak and Coruzzi, 2002; Schultz *et al.*, 1998). These results and recent functional genomic analysis of the aspartate gene family in *Arabidopsis* suggest that the plastidic isoform encoded by *Asp5* has a role in shuttling reducing equivalents as proposed for the peroxisomal and mitochondrial isoenzymes (Liepman and Olsen, 2004). All these functional analyses are in agreement with the evolutionary data (this paper; Wadsworth, 1997; Winefield *et al.*, 1995).

In cyanobacteria, where an incomplete tricarboxylic acid cycle exists, 2-oxoglutarate is channelled through the GS/GOGAT cycle for net glutamate and glutamine biosynthesis. In these photosynthetic organisms, AAT enzyme activity coupled to the GS/GOGAT cycle represents an important

reaction providing a range of amino acids for protein synthesis and many other metabolic pathways such as the biosynthesis of cyanophycin, a polymer exclusively comprised of aspartate and arginine residues that is used as a nitrogen reserve (Kim *et al.*, 2003; Picossi *et al.*, 2004). Our results in plants are also consistent with a function of this unusual AAT associated with the GS/GOGAT cycle. Thus, the expression product of PpAAT accumulated in parallel with the chloroplast development that takes place during the initial stages of pine development as previously described for GS/GOGAT enzymes (Cánovas *et al.*, 1991; García-Gutiérrez *et al.*, 1995; Palomo *et al.*, 1998).

If the plastidic PT-AAT described in this work was derived from an endosymbiont ancestor of cyanobacteria, the physiological role in plants might be related to specific pathways of aspartate metabolism in the plastid. Thus, we propose that this new form of AAT could be involved in biosynthesis of the aspartate-derived amino acids lysine, threonine and isoleucine, as well as precursors of methionine biosynthesis, all of which are produced exclusively in the plastid (Coruzzi and Last, 2000). Interestingly, all these amino acids are absolutely essential for animals where PT-AAT does not exist. Thus, the enzymes reported here represent potential targets for the development of new products acting as herbicides by blocking an important enzyme in plant nitrogen metabolism. These new products would have the advantage of being innocuous to animals as the enzymes are absent in humans and other mammals.

Another question arises from the results reported here: how can the activities of the two plastidic AATs be coordinated in the organelles? Although much research work is needed to answer this question, there are some possible clues. For example, the kinetic properties of PT-AAT for the forward reaction (aspartate and 2-oxoglutarate) are quite similar to those obtained for the native enzymes from *A. thaliana* (Wilkie and Warren, 1998) and *Panicum miliaceum* (Taniguchi *et al.*, 1995), but differ considerably for the reverse reaction. Thus, the affinity of PT-AAT for glutamate (K_m value 1 mM) was much higher than that for the eukaryotic AATs (published K_m values ranging between 10 and 30 mM). According to these findings, PT-AAT activity would ensure the biosynthesis of aspartate-derived amino acids in the chloroplast even at low levels of glutamate. When the amino donor is highly abundant, the alternative AAT would contribute aspartate biosynthesis to dissipate nitrogen excess towards the cytosol. In addition to the kinetic data, there is increased evidence that metabolon formation and metabolic channelling may provide spatial separation of enzyme activities resulting in the biosynthesis of different and specific classes of metabolites (Jorgensen *et al.*, 2005). For example, there is recent evidence for the channelling of intermediates in the phenylpropanoid pathway through independent metabolons that appears to be

differentiated by phenylalanine ammonia lyase isoforms (Achnine *et al.*, 2004; Winkel, 2004).

Summarizing, the results reported here indicate the existence of two forms of AAT in the plastids. Evolutionary and functional analyses support the conclusion that they play different roles in plant nitrogen metabolism. Further research work using single and double knockout mutants and/or transgenic plants with down-regulated plastidic AAT expression is needed to provide further insights on the specific functions of these aspartate aminotransferases in plants.

Experimental procedures

Plant material

Maritime pine (*P. pinaster* Ait) seeds were aerated in distilled water for 2 days and germinated on moist vermiculite. Arabidopsis (*A. thaliana* ecotype Columbia), rice (*O. sativa* cv Puntal) and tomato (*Lycopersicon esculentum* cv Hellfrucht Frühstamm) were germinated on sterile filter paper soaked with water. Seedlings were grown for 3 weeks in culture chamber at 22°C in the light and 18°C in the dark, under a 16 h light/8 h dark photoperiod.

Isolation of pine AAT cDNA clone and sequence analysis

A λ -ZAP composite cDNA library constructed from developing xylem (*P. pinaster*) was used for mass sequencing and EST generation (Cantón *et al.*, 2003). Ten clones containing AAT sequences were identified. Five of them were selected and the cDNA inserts analysed by agarose gel electrophoresis (data not shown). One of these ESTs, named PP034D09, harboured an insert of 2 kb that was sequenced by standard procedures using an ABI automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequence alignments and phylogenetic analyses were performed using the CLUSTAL W program (Thompson *et al.*, 1994).

Construction of recombinant expression vectors

The full-length cDNA of *P. pinaster* AAT containing the open reading frame sequence (*PpAAT*) and a truncated version containing only the sequence encoding the predicted mature protein (*p-PpAAT*) were subcloned in the expression vector pET11a. For this purpose, 'N-terminal' primers were designed consisting of sequences from the 5' region of the cDNA and an overhang region containing a unique restriction site for *Asel* and encoding a six-histidine tag preceded by a universal ATG start codon (5'-AGTATTAATGCACCATCATCATCATCTTCTGCAAGCTTTAAGTG-3' for *PpAAT* and 5'-AGTATTAATGCACCATCATCATCATCATGCTGAGACGGACAGTGG-3' for *p-PpAAT*). A single 'C-terminal' primer was used for both constructions, including a nucleotide sequence located downstream of the translation stop codon and an overhang region containing a *Bam*HI restriction site (5'-CTTGATCCGCTGGCCACCTTTCAGG-3'). The first and second methionine residues in *PpAAT* were deleted in order to avoid problems in the expression in *E. coli*. For the same reason, methionine 68 was deleted in the *p-PpAAT* construction. PCR reactions were carried out with Turbo Pfu polymerase (Stratagene, Cedar Creek, TX, USA), and the amplified products were digested with *Asel* and *Bam*HI and subcloned in the expression vector.

Expression and purification of the recombinant AAT proteins

The *E. coli* strain BL21-codonPlus-(DE3)-RIL (Stratagene) was transformed with PpAAT and p-PpAAT constructs. Cells were grown at 37°C with shaking in Luria Bertani broth containing 100 µg ml⁻¹ of ampicillin and 34 µg ml⁻¹ of chloramphenicol until a cell density (OD₆₀₀) of 0.6–0.8 was reached. Isopropyl-β-D-thiogalactoside (IPTG) was then added at a final concentration of 1 mM, and incubation was continued for 14 h with shaking at 10°C according to the protein expression protocol described by de la Torre *et al.* (2002). Cells were collected by centrifugation at 8000 *g* and pellets resuspended in a buffer containing 50 mM Na₂HPO₄, 300 mM NaCl and 10 mM imidazole at pH 8.0. The cells were lysed by sonication, and cell debris was removed by centrifugation at 22 000 *g*. Recombinant proteins were purified by affinity chromatography using Ni-NTA columns (Qiagen, Crawley, Sussex, UK) under native conditions.

Antibody production

Antibodies against PpAAT were raised in rabbits by immunization with PpAAT recombinant protein overexpressed in *E. coli* as described by Cantón *et al.* (1996). The serum was titred against the recombinant protein by Western blotting assay. It was determined that a dilution of 1:10 000 was the most appropriate to detect 20–200 ng of purified AAT protein. Pre-immune serum was tested under the same conditions and no immunoreactive signal was detected.

Determination of enzyme activities

AAT activity was determined in crude extracts and recombinant samples by coupling the production of oxaloacetate from aspartate and 2-oxoglutarate to the oxidation of NADH with malate dehydrogenase (Yagi *et al.*, 1985). In the routine assay, the reaction mixture contained 50 mM Tris-HCl pH 7.8, 50 mM L-aspartate, 10 mM 2-oxoglutarate, 0.07 mM PLP, 0.1 mM NADH, 2 U malate dehydrogenase (Roche Farma SA, 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 and followed by the decrease of NADH at 340 nm.

Aminotransferase activities were measured using 2-oxoglutarate as amino acceptor with the pure recombinant PpAAT protein in terms of the transamination of [U-¹⁴C]-2-oxoglutarate. Samples were assayed at 30°C for 15 min in a reaction mixture containing 100 mM Tris-HCl pH 8, the corresponding amino donor, 5 mM of 2-oxoglutarate (mixture of 2-oxoglutarate and [U-¹⁴C]-2-oxoglutarate in a molar ratio of 20:1), 0.07 mM PLP and 5 µg of pure enzyme in a final volume of 1 ml. The reaction was stopped by the addition of 333 µl 1 M acetic acid, and the unreacted 2-oxoglutarate was separated from the reaction product, ¹⁴C-glutamate, by anion-exchange chromatography on Dowex 1X8-400 (Sigma, St. Louis, MO, USA). ¹⁴C-glutamate was eluted with 0.25 M of acetic acid and radioactivity measured with a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA, USA).

Gel filtration chromatography

Purified PpAAT and p-PpAAT holoenzymes were subjected to size-exclusion gel chromatography on a Sephacryl S-300 column (93 × 2 cm). Elution of AAT proteins was carried out at a flow rate of 20 ml h⁻¹, and fractions of 2 ml were collected. The elution peaks of PpAAT and p-PpAAT were determined by enzyme activity measurement in all fractions. Molecular masses were

calculated after calibration of the column with the following standards: catalase (240 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), peroxidase (44 kDa) and cytochrome c (14 kDa).

Protein extraction and chloroplast isolation

Plant tissues were frozen in liquid nitrogen, ground in a mortar and pestle, and the resulting powder immediately transferred into a tube containing extraction buffer (100 mM Tris-HCl pH 8.0, 2 mM EDTA, 10% v/v glycerol, 0.1% v/v 2-mercaptoethanol) to obtain soluble proteins. This buffer was supplemented with 2% w/v SDS when total proteins were extracted. Protein extracts were centrifuged at 22 000 *g* at 4°C for 20 min and the supernatant used for protein analysis and enzyme activity determination. Chloroplasts were isolated from 10 g of 3-week-old light-grown pine cotyledons as described previously (García-Gutiérrez *et al.*, 1995).

Protein determination

Protein concentrations were determined by the Bradford dye-binding method (Bradford, 1976). For protein determination in total protein extracts, SDS was removed following the method described by Zaman and Verwilghen (1979).

Protein electrophoresis and Western blot analysis

Native and denaturing SDS gel electrophoresis and Western blots were carried out as described previously (Cánovas *et al.*, 1984, 1991). Staining for AAT activity in native gels was carried out using Fast Blue BB (Sigma, St. Louis, MO, USA) according to the method of Wendel and Weeden (1989). The following antibodies were used: anti-PpAAT (this paper), anti-plastidic AAT from alfalfa (Gantt *et al.*, 1992), a gift from Professor Carroll P. Vance (University of Minnesota, St. Paul, MN, USA), anti-GS (Cantón *et al.*, 1996) and anti-Fd-GOGAT (García-Gutiérrez *et al.*, 1995).

Acknowledgements

We are grateful to Professor Carroll P. Vance, University of Minnesota, for the provision of the antibody anti-plastidic AAT from alfalfa. L.D.S. is recipient of a studentship from the SOL-Swiss Occidental Leonardo programme, Université de Lausanne, Switzerland. This work was supported by the Ministerio de Educación y Ciencia, Spain (BMC2003-4772).

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EMBL nucleotide sequence database accession number AJ628016.