Plastidic aspartate aminotransferases and the biosynthesis of essential amino acids in plants

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Abstract

In the chloroplasts and in non-green plastids of plants, aspartate is the precursor for the biosynthesis of different amino acids and derived metabolites that play distinct and important roles in plant growth, reproduction, development or defence. Aspartate biosynthesis is mediated by the enzyme aspartate aminotransferase (EC 2.6.1.1), which catalyses the reversible transamination between glutamate and oxaloacetate to generate aspartate and 2-oxoglutarate. Plastids contain two aspartate aminotransferases: a eukaryotic-type and a prokaryotic-type bifunctional enzyme displaying aspartate and prephenate aminotransferase activities. A general overview of the biochemistry, regulation, functional significance, and phylogenetic origin of both enzymes is presented. The roles of these plastidic aminotransferases in the biosynthesis of essential amino acids are discussed.

Key words: Arogenate, aromatic amino acids, aspartate, aspartate metabolic pathway, plastid, prephenate.

Introduction

Nitrogen (N) is incorporated into carbon skeletons for the biosynthesis of the primary amino acids, glutamine and glutamate, which serve as N donors for the biosynthesis of major N compounds in plants, including other amino acids, nucleic acid bases, polyamines, and chlorophylls. For instance, N can be channelled through transamination for the biosynthesis of aspartate in different subcellular compartments and asparagine in the cytosol (Cánovas et al., 2007; Lea and Azevedo, 2007). Control of aspartate levels in the interior of plastids is particularly important for its role as a starting point in multiple amino acid biosynthesis pathways. In turn, these amino acids are precursors of numerous plant-derived metabolites playing distinct roles in plant growth, reproduction, development or defence. Figure 1 shows a schematic representation of the metabolic fates of aspartate within the plastids. Thus, aspartate lies at the head of the so-called aspartate metabolic pathway resulting in the biosynthesis of methionine, threonine, lysine, and isoleucine localized in the chloroplasts or in the plastids of non-photosynthetic cells (Azevedo et al., 2006; Galili, 2011). Aspartate within the plastids can also be combined with prephenate to generate arogenate, the direct precursor for the biosynthesis of the aromatic amino acids phenylalanine and tyrosine (Maeda et al., 2011; Maeda and Dudareva, 2012). Humans and many animals are unable to synthesize methionine, threonine, lysine, isoleucine, phenylalanine, and tyrosine and therefore these so-called essential amino acids must be supplied in sufficient amounts in the diet to meet their metabolic needs (Mathews et al., 2012). The metabolic pathways of essential amino acid biosynthesis are not only of central importance to plant growth and development, but attract interest from a nutritional and pharmaceutical perspective as sources of natural molecules. Aspartate can also participate in a range of metabolic reactions in the plastids such as the synthesis of argininosuccinate, the...
precursor for the biosynthesis of arginine (Slocum, 2005), the biosynthesis of carbamoyl-aspartate, a precursor of pyrimidine nucleotides also used for the biosynthesis of arginine (Zrenner et al., 2006) and the biosynthesis of pyridine nucleotides (Katoh et al., 2006).

Aspartate biosynthesis is mediated by the enzyme aspartate aminotransferase (AAT; aspartate:2-oxoglutarate aminotransferase; EC 2.6.1.1) which catalyses the reversible transamination between glutamate and oxaloacetate to generate aspartate and 2-oxoglutarate via a ping-pong bi-bi kinetic mechanism. The enzyme plays a key role in the metabolic regulation of C and N metabolism in all organisms. Most of our current knowledge on AAT enzymes comes from studies in micro-organisms. AAT enzymes have been included in the aminotransferase family I and then classified into subfamilies according to their amino acid sequence identities (Jensen and Gu, 1996). Subfamily Iα includes AAT from eubacteria and eukaryotes, while subfamily Iβ mainly includes enzymes from bacteria and archaea. The amino acid sequence identities between members of subfamilies Iα and Iβ is quite low (about 15% of identity). However, despite the observed divergence in primary structures, X-ray crystallographic investigations have demonstrated that members of both AAT subfamilies have very similar 3D structures (Okamoto et al., 1994; Nakai et al., 1998).

In eukaryotic cells, the presence of isoforms in different subcellular compartments plays a critical role in the interchanges of C and N pools. Various AAT isoenzymes have been identified in plants, localized in specific subcellular compartments: cytosol, mitochondria, peroxisome, and plastid (Ireland and Joy, 1985). In A. thaliana, five genes encode distinct AAT isoforms: Asp1 (mitochondrial), Asp2 and Asp4 (cytosolic), Asp3 (peroxisomal), and Asp5 (plastidic) (Schultz and Coruzzi, 1995; Wilkie and Warren, 1998). All these AAT isoenzymes, independent of localization, belong to subfamily Iα. A novel form of plastid AAT exhibiting the characteristics of subfamily Iβ enzymes in prokaryotes was reported by de la Torre et al. (2006). The enzyme is unrelated to the eukaryotic AAT from plants and animals but is similar to bacterial enzymes and present in all characterized plant genomes.

This report presents an overview of the biochemistry, regulation, functional significance, and phylogenetic origin of the plastidic eukaryotic-type (ET-pAAT) and prokaryotic-type (PT-pAAT) aspartate aminotransferases. The functional roles of these two enzymes in the biosynthesis of essential amino acids are also analysed.

**Molecular and kinetic characteristics of plastid aspartate aminotransferases**

Plastidic enzymes are composed of two identical monomers with a molecular size of about 45 kDa, as are nearly all of the AAT enzymes that have been studied (Wilkie et al., 1996;
Each folded monomer comprises a large domain, a small domain, and an extended N-terminal arm, the end of which interacts with the other monomer. Both eukaryotic and prokaryotic-type AAT monomers contain a putative N-terminal signal peptide for plastid targeting (Wilkie et al., 1996; de la Torre et al., 2009), 15 (ET-pAAT) or 16 (PT-pAAT) α-helices, and 11 β-strands. When dimerized, both ET-pAAT and PT-pAAT contain two equivalent active sites, each formed from residues of both subunits (Wilkie et al., 1996; de la Torre et al., 2009). Despite the high structural similarities between the two holoenzymes, the identities in their amino acid sequences are low, as is the case for any pairwise comparison between subfamilies Iα and Iβ. This strongly suggests that the enzymes’ tridimensional structures define their catalytic function. Indeed, there are major differences between subfamilies Iα and Iβ with respect to the recognition, stabilization, and optimal orientation of dicarboxylate substrates in the AAT active centre. For instance, the substrate’s α-carboxylate group is recognized and stabilized by an arginine residue in all AAT that have been described to date. Enzymes from the Iα subfamily such as ET-pAAT have a second conserved AAT arginine residue that interacts with the distal carboxyl group of the substrate. However, this residue is not conserved in the Iβ subfamily. Interestingly, the mutation of this arginine residue in the E. coli AAT substantially improved activity of the enzyme with aromatic substrates (Rothamn et al., 2006). In subfamily Iβ AAT from micro-organisms and plants (PT-pAAT), this role is performed instead by a lysine residue (Nobe et al., 1998) with the assistance of additional residues in the active centre (de la Torre et al., 2009). Site-directed mutagenesis experiments suggest that the presence of this residue in the active site of the enzyme may increase its flexibility and thus its ability to interact with acidic and hydrophobic substrates (Ura et al., 2001). Interestingly, the active site of plant L-l-diaminopimelate aminotransferase (a prokaryotic-type enzyme involved in lysine biosynthesis) contains a similar lysine residue that stabilizes the side-chain carboxylate groups of L-glutamate and L-l-diaminopimelate (Watanabe and James, 2011). It is not known whether this plant enzyme can interact with aromatic substrates but it has recently been reported that a related bacterial N-succinyl diaminopimelate aminotransferase is able to do it (Graindorge et al., 2014). In addition, A. thaliana has two tyrosine aminotransferases (At4g23600 and At4g23590) that contain this lysine residue. However, no studies on their substrate specificity have been reported as yet. Overall, these findings suggest that the presence of a lysine residue in the active centre is a major determinant for substrate recognition in aminotransferases with dual acidic and hydrophobic specificity.

The kinetic parameters of purified ET-pAAT and PT-pAAT recombinant proteins have been determined (Wilkie and Warren, 1998; de la Torre et al., 2006). The PT-pAAT enzyme displays a much higher affinity for glutamate ($K_m = 1\, \text{mM}$; de la Torre et al., 2006) than its eukaryotic counterpart ($K_m$ values between 10–30 mM: Taniguchi et al., 1995; Wilkie and Warren, 1998). Given these kinetic properties, both enzymes would be saturated under conditions of high glutamate concentration in the plastid and would, therefore, contribute to aspartate biosynthesis. However, when glutamate is scarce, only PT-pAAT would contribute to the biosynthesis of essential amino acids and other N compounds in the plastids (de la Torre et al., 2009). PT-pAAT is thermostable and this biochemical characteristic permits the separation and rapid purification of the enzyme from plant extracts (de la Torre et al., 2007). Thermostable enzymes with similar biochemical properties have previously been reported (Bonner and Jensen, 1985; Siehl et al., 1986).

It was recently demonstrated that plant PT-pAAT exhibits prephenate aminotransferase activity (PAT; glutamate/aspartate-prephenate aminotransferase; EC 2.6.1.78, EC 2.6.1.79) as well as AAT activity (Graindorge et al., 2010; Maeda et al., 2011). This metabolic reaction represents a key step in the biosynthesis of aromatic amino acids in the plastids because the product of PAT, arogenate, is the immediate precursor for the biosynthesis of phenylalanine and tyrosine using arogenate dehydratase and arogenate dehydrogenase, respectively (Tzin and Galili, 2010; Maeda and Dudareva, 2012). In the presence of glutamate as N donor, PT-pAAT exhibits similar values of the specificity constant ($K_{cat}/K_m$) for oxaloacetate and prephenate, indicating that the enzyme can operate both as a PAT and as a classical AAT (Graindorge et al., 2010). Considering these data, hereafter this bifunctional enzyme will be named PT-pAAT/PAT. The same authors also demonstrated that the ET-pAAT enzyme does not display PAT activity, even in the presence of high levels of prephenate. Consequently, plastids contain two AAT activities for the biosynthesis of aspartate-derived N compounds and PAT activity involved in the biosynthesis of aromatic amino acids.

**Transcriptional regulation**

The *Arabidopsis* AAT gene encoding the ET-pAAT enzyme (*Asp5*) was isolated and the gene structure determined (Wilkie et al., 1996). The regulatory region contained a putative TATA box and multiple copies of two notable sequence motifs: CTCTT, which is associated with nodule-specific gene activity (Fehlberg et al., 2005); and AAGAT, which is a Dof-core site present in genes involved in carbon metabolism (Yanagisawa, 2000). However, the functionality of these cis-elements has not been studied further.

The promoter region of the gene encoding PT-pAAT/PAT has been isolated from maritime pine (Craven-Bartle et al., 2013). *In silico* analysis of a 2064 bp sequence upstream of the translation start codon of the gene revealed a number of putative cis-acting elements including Dof-core sites, GATA and GT1 boxes, and AC elements that were further characterized functionally (Craven-Bartle et al., 2013). AC elements in plant promoters are necessary and sufficient to drive localized xylem expression (Lacombe et al., 2000; Wagner et al., 2012). It was found that the regulatory regions of *PT-pAAT/PAT* and other key genes such as those encoding phenylalanine ammonia lyase and glutamine synthetase contain ACI elements involved in the transcriptional activation mediated by R2R3-Myb factors. A transcription factor, Myb 8, regulates
the co-ordinated expression in vascular cells of the aforementioned genes for prephenate amination and phenylalanine deamination. In phenylalanine metabolism, N recycling is required for the channelling of photosynthetic carbon to lignin biosynthesis during wood formation and is necessary for maintaining N economy in trees (Razal et al., 1996).

**Functional role of plastid aspartate aminotransferases**

The function of plastidic ET-pAAT has been investigated using Arabidopsis mutants (Schultz et al., 1998; Miesak and Coruzzi, 2002). Plastid ET-AAT mutants had no visible phenotype and their chloroplasts contained unaltered levels of aspartate and asparagine when compared with wild-type plants. Furthermore, heterologous expression of plastid ET-pAAT from soybean in Arabidopsis did not display an overt phenotype, showing only altered levels in a number of free amino acids such as increased glutamine or asparagine levels, albeit with a great variability between different transgenic lines (Murooka et al., 2002). These data suggest that plastid ET-AAT might have a role in shuttling reducing equivalents, as proposed for the peroxisomal and mitochondrial isoenzymes (Liepman and Olsen, 2004).

The genetic analysis of PT-pAAT function has been less straightforward because its absence is lethal during embryogenesis, as revealed by the study of Arabidopsis transposon mutants defective in female gametogenesis and embryo development (Pagnussat et al., 2005). A lethal phenotype in plants is consistent with the suppression of an essential enzyme for amino acid biosynthesis. Several attempts were made to obtain transgenic Arabidopsis with suppressed PT-pAAT/ PAT gene expression using a reverse genetics approach but all of them proved to be unsuccessful (FM Cánovas, unpublished data). The essential role of the enzyme for plant development is further supported by the absence of homozygous knockout mutants in the Arabidopsis T-DNA database collections (Swarbreck et al., 2008). To overcome this problem, PT-pAAT/PAT expression was locally silenced in petunia petals using an RNAi strategy and the results indicated that the enzyme was implicated in the biosynthesis of phenylalanine (Maeda et al., 2011). Recently, the roles of PT-pAAT and PT-pAAT/PAT in plastids have been investigated using virus-induced gene silencing (VIGS) in N. benthamiana, as an alternative approach to obtain gene silencing in whole plants (de la Torre et al., 2014). The results clearly validated VIGS as an effective method to study the metabolic impact of plastidic AAT suppression in planta given the effective gene silencing, close to 90% of the observed expression levels in control plants. The silencing was further confirmed by analysis of enzyme activity and protein abundance. Phenotypic and metabolic analyses were conducted in silenced plants to investigate the specific roles of these enzymes in the biosynthesis of essential amino acids within the plastid.

The ET-pAAT-silenced plants displayed no overt changes in the phenotype (Fig. 2) in good agreement with the results reported in Arabidopsis (Miesak and Coruzzi, 2002). By contrast, the suppression of PT-pAAT/PAT led to a severe reduction in growth and strong chlorosis symptoms with decreased levels of chlorophyll and lignin (Fig. 2; de la Torre et al., 2014). ET-pAAT-silenced plants exhibited similar levels of free aspartate and glutamate than control plants, but contained decreased levels of asparagine and altered contents of lysine. PT-pAAT/PAT-silenced plants also exhibited similar levels of free aspartate and glutamate, extremely reduced levels of asparagine and were affected in phenylalanine metabolism. The unaltered phenylalanine metabolism in ET-pAAT-silenced plants indicates that the enzyme is not involved in the biosynthesis of aromatic amino acids (de la Torre et al., 2014). A dramatic decrease in free aspartate levels was only observed in co-silenced plants for ET-pAAT and PT-pAAT/PAT, suggesting an overlapping role of the enzymes in the production of this amino acid. Furthermore, the functional analysis of co-silenced plants highlighted the central role of both plastidic aminotransferases in N metabolism (de la Torre et al., 2014).

PT-pAAT/PAT suppression triggered a transcriptional reprogramming in plastid N metabolism involving an up-regulation of genes in the aspartate pathway and a down-regulation of genes involved in the channelling of phenylalanine towards lignin biosynthesis, consistent with the observed reduction in lignin deposition (de la Torre et al., 2014). Unexpectedly, the suppression of PT-pAAT/PAT in petals of petunia (Maeda et al., 2011) and leaves of N. benthamiana (de la Torre et al., 2014) did not result in a dramatic decrease in phenylalanine levels. Recent reports have independently proved that plants are also able to produce phenylalanine by utilizing an alternative microbial-like phenylpyruvate pathway (Yoo et al., 2013; de la Torre et al., 2014), the flux through which is increased when arogenate precursors are limiting. The alternative phenylpyruvate pathway utilizes a cytosolic aminotransferase that links the catabolism of tyrosine to serve as the amino donor, thus interconnecting plastidic and extra-plastidic aromatic amino acid metabolism (Yoo et al., 2013).

Taken together, these results indicate that PT-pAAT/PAT has an overlapping role with ET-pAAT in the biosynthesis of...
Aspartate and a key role in the production of phenylalanine for the biosynthesis of phenylpropanoids. Thus, PT-pAAT/PAT not only plays a role in the biosynthesis of aromatic amino acids but also ensures the biosynthesis of aspartate-derived amino acids and other N compounds in the plastids in stressful conditions (de la Torre et al., 2009; Fig. 3). It is likely that PT-pAAT/PAT is essential for plant growth and development because it represents a safeguard enzyme involved in the biosynthesis of lysine, threonine, isoleucine, methionine, tyrosine, and phenylalanine that are essential amino acids required for protein synthesis and produced exclusively in the plastid (Azevedo et al., 2006; Maeda and Dudareva, 2012). All of these amino acids are essential in the nutrition of animals in which PT-pAAT does not exist (de la Torre et al., 2006).

**Phylogenetic relationships**

Aspartate and tyrosine aminotransferases are homologous proteins with a high degree of sequence similarity and related folding patterns (Mehta et al., 1989). In fact, it has been proposed that these enzymes may have evolved from a common ancestral aminotransferase with a broad specificity for substrates (Jensen and Gu, 1996). Several lines of evidence, reviewed in this work, indicate that aminotransferases with a dual specificity for acidic and hydrophobic substrates have important functions in plants. A tree showing the phylogenetic relationship of tyrosine, aspartate, and bifunctional aspartate/prephenate aminotransferases in plants is shown in Fig. 4. The enzymes of subfamily \( \alpha \) AAT in plants (including ET-pAAT) are similar to vertebrate AAT and closely related to the subfamily \( \alpha \) bacterial enzymes such as those in *E. coli* (Wadsworth, 1997). These early findings are consistent with the molecular and kinetic characteristics of ET-pAAT, a subfamily \( \alpha \) AAT (Taniguchi et al., 1995; Wilkie et al., 1996; Wilkie and Warren, 1998). It has therefore been suggested that ET-pAAT evolved by duplication of an ancestral cytosolic isoform and diverged more recently than the mitochondrial gene (Wadsworth, 1997). By contrast, PT-pAAT/PAT is much more closely related to cyanobacterial genes and it has been proposed that PT-pAAT/PAT arose from an endosymbiont ancestor of cyanobacteria (de la Torre et al., 2006). However, a recent characterization of aminotransferases with PAT activity in micro-organisms has suggested alternative origins for PT-pAAT/PAT in plants (Graindorge et al., 2014).

Although it is generally accepted that plastids originated by endosymbiosis of an ancestral cyanobacterium and that genes from the endosymbiont genome were integrated in the host nucleus (Reyes-Prieto et al., 2007), recent large-scale genomic and phylogenomic analyses from prokaryotic and plant genomes are providing new insights into the early events of primary endosymbiosis (Reyes-Prieto et al., 2007). Several lines of evidence support that Chlamydia-like pathogens, which are the second major source of foreign genes in plants, had a significant role during the establishment of endosymbiosis (Fachinelli et al., 2013). The evolutionary origin of PT-pAAT/PAT in plants should, therefore, be re-examined in light of these new findings. Besides PT-pAAT/PAT, other enzymes involved in the biosynthesis of essential amino acids have a prokaryotic origin, possibly associated with early events in plastid formation in plants. For example, LL-DAPAT is another plastid enzyme of potential
Fig. 4. Phylogenetic relationships between aromatic and aspartate aminotransferases in plants. Representative members of the tyrosine (TyrAT), aspartate/prephenate (AAT/PAT), and aspartate aminotransferases (AAT) from the following plants were included in the analysis: Arabidopsis thaliana, Solanum lycopersicum, Populus trichocarpa, and Pinus pinaster. TyrAT from: Arabidopsis thaliana (At4g28420, At2g20610, At2g24850, At4g23590, At4g23600, At5g53970, and At5g36160), Solanum lycopersicum (Soly1g007110, Soly1g088000, Soly1g096240, Soly1g008200, Soly1g053710, and Soly1g053720), Populus trichocarpa (Pt011g115100, Pt017g014200, Pt007g137900, Pt017g014100, and Pt017g014000), and Pinus pinaster (Pp. uni36796, Pp.uni5498, and Pp.uni57779). Bifunctional AAT/PAT from: Arabidopsis thaliana (At2g22250), Solanum lycopersicum (Soly0g054710), Populus trichocarpa (Pt005g072000), and Pinus pinaster (Pp.uni16666), AAT from: Arabidopsis thaliana (AtASP1-At2g30970, AtASP2-at5g19550, AtASP3-At5g1520, AtASP4-At1g62800, and AtASP5-At4g31990), Solanum lycopersicum (Soly0g068330, Soly0g032740, Soly0g041870, Soly0g055210, and Soly1g075170), Populus trichocarpa (Pp006g241600, Pp018g082500, Pp006g241500, Pp006g260200, Pp014g143300, and Pp006g107100), and Pinus pinaster (Pp.uni4800, Pp.uni34766, and Pp.uni17743). The phylogenetic tree was constructed with full-length AAT amino acid sequences using the Neighbour–Joining method of the CLUSTALW program (Thompson et al., 1994). (This figure is available in colour at JXB online.)
cyanobacterial origin responsible for the biosynthesis of lysine in plants and whose activity is essential for plant viability (Hudson et al., 2006). Moreover, LL-DAPAT-like enzymes are also found in Chlamydia supporting an evolutionary relationship between Chlamydia and cyanobacteria in the endosymbiotic ancestry of plastids (McCoy et al., 2006). Another example can be found in the plastid-located shikimate pathway necessary for the biosynthesis of essential aromatic amino acids, given that chorismate is the common precursor for the biosynthesis of phenylalanine, tyrosine, and tryptophan. Phylogenetic analyses have shown that plant shikimate pathway genes are derived from multiple prokaryotic origins including cyanobacteria and eubacteria (Richards et al., 2006), further supporting a tight correlation between the biosynthesis of essential amino acids, the transfer of prokaryotic-type genes, and the primary endosymbiotic event resulting in the plastids. Finally, the phylogeny of genes encoding aspartate metabolic pathway enzymes, such as aspartate kinase-homoserine dehydrogenase, aspartate semialdehyde dehydrogenase, and diaminopimelate epimerase, indicates the existence of additional cyanobacterial-like genes involved in the biosynthesis of essential amino acids (F de la Torre, FM Cánovas, unpublished data).

Overall, the above data suggest a strong connection between the capacity of plants for the synthesis of a set of amino acids and the acquisition of a set of prokaryotic genes responsible for this function. The exact way in which plants acquired the ability to synthesize essential amino acids remains unclear, but phylogenetic and biochemical data suggest that this ability was made possible in an early event, probably related to the primary endosymbiosis of a cyanobacterium and probably facilitated by a concomitant chlamydia infection, which resulted in the plastids (Fachinelli et al., 2013). Further work is necessary to understand fully the origin of this metabolic pathway in the plastids, but the findings presented in this paper would explain the inability of humans and many animals to synthesize essential amino acids and why they should be provided in their foods and feeds.

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