**Biochemical Properties and Subcellular Localization of Tyrosine Aminotransferases in
*Arabidopsis thaliana***

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Abstract

Plants produce various *L*-tyrosine (Tyr)-derived compounds that are of pharmaceutical or nutritional importance to humans. Tyr aminotransferase (TAT) catalyzes the reversible transamination between Tyr and 4-hydroxyphenylpyruvate (HPP), the initial step in the biosynthesis of many Tyr-derived plant natural products. Herein reported is the biochemical characterization and subcellular localization of TAT enzymes from the model plant *Arabidopsis thaliana*. Phylogenetic analysis showed that *Arabidopsis* has at least two homologous *TAT* genes, *At5g53970* (*AtTAT1*) and *At5g36160* (*AtTAT2*). Their recombinant enzymes showed distinct biochemical properties: AtTAT1 had the highest activity towards Tyr, while AtTAT2 exhibited a broad substrate specificity for both amino and keto acid substrates. Also, AtTAT1 favored the direction of Tyr deamination to HPP, whereas AtTAT2 preferred transamination of HPP to Tyr. Subcellular localization analysis using GFP-fusion proteins and confocal microscopy showed that AtTAT1, AtTAT2, and HPP dioxygenase (HPPD), which catalyzes the subsequent step of TAT, are localized in the cytosol, unlike plastid-localized Tyr and tocopherol biosynthetic enzymes. Furthermore, subcellular fractionation indicated that, while HPPD activity is restricted to the cytosol, TAT activity is detected in both cytosolic and plastidic fractions of *Arabidopsis* leaf tissue, suggesting that an unknown aminotransferase(s) having TAT activity is also present in the plastids. Biochemical and cellular analyses of *Arabidopsis* TATs provide a fundamental basis for future *in vivo* studies and metabolic engineering for enhanced production of Tyr-derived phytochemicals in plants.

Keywords

Arabidopsis thaliana; Brassicaceae; Phylogenetic Analysis, Biochemical Characterization and Subcellular Localization; Tyrosine-derived metabolites; Tyrosine Aminotransferase, 4-Hydroxyphenylpyruvate dioxygenase; Tyrosine, 4-Hydroxyphenylpyruvate, Homogentisate.

Chemical Compounds

L-arogenate (PubChem CID: 25244469) (1)

prephenate (PubChem CID: 1028) (2)

L-tyrosine (PubChem CID: 6057) (3)

4-hydroxyphenylpyruvate (PubChem CID: 6971070) (4)

homogentisate (PubChem CID: 780) (5)

1. Introduction

L-Tyrosine (Tyr) (**3**, **Fig. 1**) is required for protein synthesis but also serves as the precursor of several classes of plant metabolites, including alkaloids, prenylquinones, and cyanogenic glycosides (Beaudoin and Facchini, 2014; Block et al., 2014; DellaPenna and Pogson, 2006; Maeda and Dudareva, 2012; Nielsen et al., 2008; Nowicka and Kruk, 2010). Tyr aminotransferase (TAT, EC 2.6.1.5) catalyzes the reversible transamination between Tyr (**3**) and 4-hydroxyphenylpyruvate (HPP) (**4**) and is involved in both synthesis and catabolism of Tyr (**3**) in different organisms (**Fig. 1**). In many microbes (e.g., *E. coli* and yeast), Tyr (**3**) is synthesized from prephenate (**2**), which is oxidatively decarboxylated by prephenate dehydrogenase (PDH/TyrA_p) to HPP (**4**), which is then transaminated to synthesize Tyr (**3**) by TAT (Umbarger, 1978; Urrestarazu et al., 1998). In *E. coli*, TAT activity is derived from three different classes of aminotransferases, TyrB, AspC, and ilvE (Gelfand and Steinberg, 1977). Legumes are the only plant lineages that have a capacity to synthesize Tyr (**3**) via the PDH/TyrA_p pathway, potentially using TAT at the final step of Tyr (**3**) biosynthesis (Rubin and Jensen, 1979; Schenck et al., 2015). In most plants, however, Tyr (**3**) is synthesized via the arogenate dehydrogenase (ADH/TyrA_a) pathway, in which prephenate (**2**) is first transaminated to *L*-arogenate (**1**) and then decarboxylated to Tyr (**3**) (**Fig. 1**, Byng et al., 1981; Connelly and Conn, 1986; Gaines et al., 1982; Rippert and Matringe, 2002; Tzin and Galili, 2010). Thus, TAT activity is likely involved in Tyr (**3**) catabolism by deamination rather than Tyr (**3**) synthesis in most plants.

Deamination of Tyr (**3**) to HPP (**4**), catalyzed by TAT, is the initial step and entry point for biosynthesis of many Tyr-derived compounds. Lineage-specific secondary metabolites such as benzyloquinoline alkaloids in opium poppy (Beaudoin and Facchini, 2014) and rosmarinic acid in *Rosmarinus officinalis* (De-Eknamkul and Ellis, 1987a; Petersen and Simmonds, 2003) are

synthesized via HPP (**Fig. 1**). HPP (**4**) could be further oxidized by HPP dioxygenase (HPPD) to homogentisate, which is further converted to tocochromanols (collectively known as vitamin E) and the photosynthetic electron-carrier, plastoquinone (Fiedler et al., 1982; Norris et al., 1998). Conversion of Tyr (**3**) to homogentisate (**5**) by TAT and HPPD also leads to the degradation pathway of Tyr into the Krebs cycle intermediates (**Fig. 1**, Dixon and Edwards, 2006; Rurand and Zenk, 1974).

Functions of TAT enzymes in different Tyr-derived pathways have been investigated in several plant species. TAT activity has been detected and separated in four distinct chromatographic peaks in *Anchusa officinalis* cell culture, producing rosmarinic acid (De-Eknamkul and Ellis, 1987b). Aromatic amino acid aminotransferases have been isolated and characterized from *Cucumis melo* (melon, Gonda et al., 2010), *Papaver somniferum* (opium poppy, Lee and Facchini, 2011), *Petunia hybrida* (Yoo et al., 2013), *Rosa × damascena* (rose, Hirata et al., 2012), and *Ephedra sinica* (Kilpatrick et al., 2016), all of which can deaminate Tyr (**3**) to HPP (**4**) *in vitro*, whereas that of *Atropa belladonna* (Deadly Nightshade) deaminates Phe to phenylpyruvate using HPP (**4**) as the best keto acceptor (thus synthesizing Tyr (**3**), Bedewitz et al., 2014). RNAi suppression of the *P. somniferum*, *P. hybrida* and *A. belladonna* genes led to reduced production of their downstream products, benzenoid volatiles, morphine alkaloids, and tropane alkaloids, respectively (Bedewitz et al., 2014; Lee and Facchini, 2011; Yoo et al., 2013). Two Arabidopsis TAT enzymes, AtTAT1 (At5g53970) and AtTAT2 (At5g36160), have also been characterized previously (Prabhu and Hudson, 2010; Riewe et al., 2012). Recombinant AtTAT1 enzyme efficiently deaminates Tyr (**3**), but not Phe or tryptophan, using α -ketoglutarate as a keto acceptor (Riewe et al., 2012). AtTAT2 also preferred Tyr (**3**) over Phe or glutamate (Glu, Prabhu and Hudson, 2010). However, other amino donors were not tested in these two studies.

Furthermore, *tat1* knockout mutants of Arabidopsis accumulate more Tyr (3) and less tocopherols than wild type, suggesting that AtTAT1 is involved in tocopherol biosynthesis by deaminating Tyr (3) to HPP (4) in Arabidopsis (Riewe et al., 2012). However, it is still unclear what the substrate specificity of AtTAT1 and AtTAT2 is and if they are the only TATs in Arabidopsis.

Tyr (3) is synthesized within the plastids (Jensen, 1986; Maeda and Dudareva, 2012; Rippert et al., 2009; Tzin and Galili, 2010), and the steps downstream of HPPD in tocopherol and plastoquinone biosynthesis also occur in the plastid (Joyard et al., 2009; Soll and Schultz, 1980; Soll et al., 1985, 1980). However, the subcellular localizations of TAT and HPPD, converting Tyr (3) to homogentisate, are more variable among different plants. In soybean, HPPD enzymes are localized in both cytosol and plastids, while maize HPPD is exclusively localized in the plastids (Siehl et al., 2014). On the other hand, HPPD activity was detected in the cytosolic fraction of carrot cell culture (Garcia et al., 1997) and Arabidopsis HPPD protein heterologously expressed in tobacco was detected in the cytosol (Garcia et al., 1999). For TAT enzymes, only petunia Tyr:phenylpyruvate aminotransferase has been shown to localize in the cytosol (Yoo et al., 2013); however, the localization of other plant TATs including Arabidopsis TATs have not been investigated.

To address these knowledge gaps and obtain biochemical and cellular basis of the initial step of Tyr metabolism, here we examined and compared biochemical characteristics and subcellular localization of two TAT enzymes from Arabidopsis. The obtained data showed clear differences in substrate specificity of AtTAT1 and AtTAT2 enzymes, though both were localized in the cytosol, together with HPPD, in Arabidopsis. This study also revealed that, besides cytosolic AtTAT1 and AtTAT2, an additional aminotransferase(s) having TAT activity is also present in the plastids of Arabidopsis.

2. Results

2.1 Phylogenetic analysis of Arabidopsis TATs

To investigate phylogenetic relationships of potential Arabidopsis TAT enzymes, all previously characterized plant TATs (Bedewitz et al., 2014; Gonda et al., 2010; Lee and Facchini, 2011; Prabhu and Hudson, 2010; Riewe et al., 2012; Yoo et al., 2013) were used as queries for BLAST search against the Arabidopsis genome (www.arabidopsis.org). The top ten hits were At5g53970, At5g36160, At2g20610, At4g28410, At2g24850, At4g23590, At4g23600, At4g28420, At1g77670, and At2g22250 (with $e\text{-value} < 10^{-17}$). Maximum likelihood phylogenetic tree was then constructed for TAT homologues from Arabidopsis as well as other representatives of different lineages of plants and algae (**Fig. 2**). The result showed that At5g53970 (AtATAT1) and At5g36160 (AtTAT2) belong to a monophyletic clade containing all previously characterized TATs from flowering plants, *C. melo* (CmArAT1), *P. somniferum* (PsTyrAT), *P. hybrida* (PhPPY-AT), and *A. belladonna* (Ab-ArAT4, Bedewitz et al., 2014; Gonda et al., 2010; Lee and Facchini, 2011; Yoo et al., 2013). AtTAT1 and its close homologues from all the core eudicots included in the analysis, but not from a basal eudicot (*Aquilegia coerulea*), further formed a well-supported subclade (**Fig. 2**, gray box). At4g23590, At2g24850, At4g28410, and At4g28420, fell within the Brassicaceae-specific clade containing previously characterized carbon-sulfur (C-S) lyases, At2g20610 (SUR1) and At4g23600 (CORI3, Jones et al., 2003; Mikkelsen et al., 2004, **Fig. 2**). At4g23600 was first reported as a TAT (Lopukhina et al., 2001) and a later study (Jones et al., 2003) found to be a cystine-lyase with no TAT activity. At1g77670 and At2g22250 were most distantly related to previously characterized plant TATs and C-S lyases, and At2g22250 was shown to encode prephenate aminotransferase (Graindorge et al., 2010; Maeda et al., 2011). Thus,

AtTAT1 and AtTAT2 are the two best TAT candidates in Arabidopsis based on phylogenetic analysis and consistent with previous biochemical studies of At5g53970 (AtTAT1, Riewe et al., 2012), At5g36160 (AtTAT2, Prabhu and Hudson, 2010), At4g23600 (CORI3, Jones et al., 2003) and At2g20610 (SUR1, Mikkelsen et al., 2004).

2.2 Biochemical characterization of recombinant AtTAT1 and AtTAT2

To examine biochemical characteristics of AtTAT1 and AtTAT2 enzymes, their recombinant proteins were individually expressed in *E. coli* as well as an empty vector control, and purified via affinity chromatography (**Fig. S1A**, see **Experimental**). The purified fractions of AtTAT1 and AtTAT2, but not empty vector control, were able to convert Tyr (**3**) into HPP (**4**) using α -ketoglutarate (α -KG) as a keto acceptor (**Fig. S1B**). The TAT activity assay under different pH showed that both AtTAT1 and AtTAT2 exhibit basic optimum pH around 8.5 – 9.0 (**Fig. S1C**), similar to other plant aromatic amino acid aminotransferase activities and enzymes (Bedewitz et al., 2014; De-Eknamkul and Ellis, 1987b; Gadai et al., 1969; Lee and Facchini, 2011; Matheron and Moore, 1973; Prabhu and Hudson, 2010; Yoo et al., 2013). Notably, under the optimum assay condition, TAT1 showed roughly 40 fold higher activity than TAT2 (154.1 vs 3.9 nkat/mg protein, **Fig. S1C**).

Aminotransferase assays using different amino donors (with α -KG as a keto acceptor) further showed that AtTAT1 prefers Tyr (**3**) amino donor but can also use Phe, Trp, His, Met, and Leu (**Fig. 3A**). AtTAT2 exhibited much broader amino donor specificity and took all seven amino donors that AtTAT1 used, but also used additional seven amino donors (Ala, Ser, Cys, Asp, Asn, Gln, and Arg, **Fig. 3A**). Glu, which could not be tested with α -KG as a keto acceptor (**Fig. 3A**), also supported aminotransferase reactions for both AtTAT1 and AtTAT2 with different keto

acceptors (**Table 1**).

Besides α -KG, other common keto acceptors, oxaloacetate, glyoxylate, and pyruvate, could be used by both AtTAT1 and AtTAT2 (**Fig. 3B**). Three aromatic keto acceptors, phenylpyruvate, HPP (**4**), and prephenate (**2**), could be also used by AtTAT2; however, AtTAT1 only used phenylpyruvate and to a much lesser extent HPP (**4**) but not prephenate (**2**) (**Fig. 3B**). Consistent with amino acid specificity, 4-methylthio-2-oxobutanoate (4MTOB) and 4-methyl-2-oxopentanoate (4MOP), keto acids of Met and Leu, respectively, also served as keto acceptors of both AtTAT1 and AtTAT2, whereas 3-methyl-2-oxobutanoate (3MOB) and 3-methyl-2-oxopentanoate (3MOP), keto acids of Val and Ile, respectively, did not (**Fig. 3B**).

Steady state kinetic analyses with different combination of amino donors and keto acceptors further showed that AtTAT1 strongly favors Tyr (**3**) amino donor with apparent K_m of 204 μ M and k_{cat} of 57.3 s^{-1} (with α -KG keto acceptor), and prefers phenylpyruvate as a keto acceptor (K_m of 455 μ M and k_{cat} of 24.5 s^{-1} with Glu amino donor, **Table 1, Figs S2A and S2C**). Notably, apparent K_m of AtTAT1 toward HPP (**4**) was 10.1 mM (with Glu amino donor, **Table 1, Fig. S3A**), suggesting that AtTAT1-catalyzed reaction favors the conversion of Tyr (**3**) to HPP (**4**) in agreement with previous studies (Riewe et al., 2012; Yoo et al., 2013). Although AtTAT2 exhibited a broad substrate specificity (**Fig. 3**), the kinetic analyses showed that AtTAT2 somewhat prefers Met and Tyr (**3**) as amino donors (K_m of 1.7 and 2.9 mM, respectively, with α -KG keto acceptor, **Table 1, Fig. S2A and S2B**) and their corresponding keto acids, 4MTOB and HPP (**4**) (K_m of 807 and 410 μ M, respectively, with Glu amino donor, **Table 1, Fig. S3A and S3B**). Both AtTAT1 and AtTAT2 exhibited poor aspartate aminotransferase activity (Asp to oxaloacetate conversion and *vice versa*) with either very low activity or K_m beyond detection limit (**Table 1, Figs S2D and S2D**). Similar trends were observed for keto acid specificity when

Tyr (**3**) was used as an amino donor (**Table 1, Fig. S4A**).

2.3 Subcellular localization of Arabidopsis TAT1, TAT2 and HPPD

In plants, enzymes involved in Tyr (**3**) biosynthesis are generally localized in the plastids (Dal Cin et al., 2011; Rippert et al., 2009; Siehl et al., 1986). Also, tocopherols and plastoquinone, downstream metabolites derived from HPP (**4**), are synthesized from homogentisate within the plastids (Joyard et al., 2009; Mehrshahi et al., 2014; Schulze-Siebert et al., 1987; Soll and Schultz, 1980; Soll et al., 1985, 1980; Yang et al., 2011). In this study, the subcellular localization of Arabidopsis TAT and HPPD enzymes that catalyze two sequential steps from Tyr (**3**) to homogentisate (**5**) was examined (**Fig. 1**, Fiedler et al., 1982; Löffelhardt and Kindl, 1979; Moran, 2005; Norris et al., 1998; Secor, 1994). Although Arabidopsis HPPD localization was investigated previously (Garcia et al., 1997), the cloned *HPPD* gene was 60 bp shorter than that currently annotated in “The Arabidopsis Information Resource” (TAIR10, www.arabidopsis.org). Thus, in this study, RT-PCR was used to confirm the expression of the full-length *HPPD* gene (including the first 60 bp), which was then cloned for the localization study (**Fig. S5**). GFP was fused to the C-terminal of AtTAT1, AtTAT2, and HPPD, and the fusion proteins were individually expressed in Arabidopsis protoplasts and analyzed by confocal microscopy. The fluorescence signal of GFP fused with AtTAT1, AtTAT2, and HPPD did not overlap with chlorophyll autofluorescence, similar to the free GFP control (**Fig. 4A**). In contrast, the GFP fused with plastidic Arabidopsis arogenate dehydrogenase 2 (ADH2, Rippert et al., 2009) co-localized with chlorophyll autofluorescence (**Fig. 4A**). These results suggest that Arabidopsis TAT1, TAT2, and HPPD are not targeted to the plastids.

Subcellular fractionation of Arabidopsis leaf tissues was further conducted to analyze the

localization of TAT and HPPD activities (**Fig. 4B**). The cytosolic marker, phosphoenolpyruvate carboxylase (PEPC) activity (Meyer et al., 1988), was only detected in the cytosolic fraction, suggesting that the plastidic fraction is free from cytosolic contamination. Similarly, the plastid marker, nitrite reductase (NiR) activity (Miflin, 1974, 1967), was enriched in the plastidic fraction, and only minor activity was detected in the cytosolic fraction (**Fig. 4B**). Like PEPC, HPPD activity was detected only from the cytosolic fraction, consistent with the localization of the GFP-fused HPPD protein (**Fig. 4A**). On the other hand, TAT activity was detected in both the cytosolic and plastidic fractions, indicating that enzymes having TAT activity are present in both cytosol and plastids. Combined with the GFP localization study, the data suggests that TAT1, TAT2 and HPPD are localized in the cytosol, but other plastidic enzymes, besides AtTAT1 and AtTAT2, also contribute to total TAT activity in Arabidopsis.

3. Discussion

3.1 AtTAT1 and AtTAT2 have distinct substrate specificity and preferred reaction direction

Biochemical characterization of recombinant enzymes showed that AtTAT1 has clear substrate preferences towards Tyr (**3**) compared to other amino acids tested (**Fig. 3A, Table 1, Fig. S2**) and phenylpyruvate as a keto acceptor (**Fig. 3B, Table 1, Figs S3 and S4**). Given that AtTAT1 has a much lower K_m towards Tyr (**3**) than HPP (**4**) (**Table 1**) and that the *tat1* mutants accumulate more Tyr (**3**) and less downstream metabolites (i.e., tocopherols, Riewe et al., 2012), AtTAT1 is involved in Tyr (**3**) deamination to HPP (**4**), but not Tyr (**3**) formation, in Arabidopsis. The phenylpyruvate preference further suggests that AtTAT1 may also contribute to the cytosolic formation of Phe, such as for phenylpropanoid biosynthesis, similar to its homolog in petunia (Yoo et al., 2013). Phylogenetic analysis showed that AtTAT1, but not AtTAT2, belongs to a

well-supported subclade containing its close homologues from all the core eudicots used in the analysis, including three previously characterized aromatic amino acid aminotransferases from *C. melo*, *P. hybrida*, and *A. belladonna* (**Fig. 2**). Like AtTAT1, the petunia enzyme also showed amino and keto substrate preference towards Tyr (**3**) and phenylpyruvate, respectively (Yoo et al., 2013), whereas the *A. belladonna* enzyme (Ab-ArAT4) preferred the reverse reaction, the conversion of HPP (**4**) to Tyr (**3**) using Phe amino donor (Bedewitz et al., 2014). Since *A. belladonna* has two other aromatic amino acid aminotransferase genes within the same clade (Bedewitz et al., 2014), it is likely that Ab-ArAT4 became further specialized for the production of tropane alkaloids derived from phenylpyruvate. These results together suggest that AtTAT1 and its close homologs within core eudicots became specialized in the interconversion of Tyr and HPP in exchange of phenylpyruvate and Phe.

As compared to AtTAT1, AtTAT2 has 40 times lower activity towards Tyr (**Fig. 3, Fig. S1C**) and also favors the conversion of HPP (**4**) to Tyr (**3**) (**Table 1**), suggesting that TAT2 has relatively minor involvement in Tyr (**3**) deamination. Instead, it was found that AtTAT2 can take both aromatic and aliphatic amino acids and their corresponding keto substrates (**Fig. 3, Table 1**), suggesting that it is a plant TAT with multi-substrate specificity. Some microbial TATs also have a broad substrate specificity, including TATs from *Klebsiella pneumoniae* (Heilbronn et al., 1999), *Bacillus sp.* (Berger et al., 2003), and *Trypanosoma cruzi* (Nowickia et al., 2001), which deaminate Tyr (**3**) as well as other aromatic and aliphatic amino acids. Although no plant TAT enzyme with a broad substrate specificity has been reported to date, partially purified aminotransferase activities that deaminate both aromatic and aliphatic amino acid substrates have been reported previously from plant tissues (Gamborg, 1965; Matheron and Moore, 1973). Although yet to be identified physiological substrate(s) beyond proteinogenic amino acids may

be present for AtTAT2, previous and these results together suggest that TAT enzymes with a broad substrate specificity (e.g., AtTAT2) are likely maintained in all core eudicots (**Fig. 2**). Interestingly, the most recently-identified aromatic amino acid aminotransferase from gymnosperm (*E. sinica*, EsAroAT1) has amino donor preference toward Tyr (**3**) among other aromatic amino acids, like AtTAT1, but exhibits a relatively broad keto substrate specificity (Kilpatrick et al., 2016), similar to AtTAT2. Notably, the keto substrates of EsAroAT1 include, besides aromatic keto acids, oxaloacetate, pyruvate, and 4MTOB (Kilpatrick et al., 2016), which were also used by AtTAT2 (**Fig. 3B and Table 1**). Given that EsAroAT1 is basal to both AtTAT1 and AtTAT2 and closely related to the homologues of a gymnosperm (*P. abies*) and a basal angiosperm (*A. trichopoda*, **Fig. 2**), the common ancestor of plant TATs likely had capacities to deaminate Tyr (and other aromatic amino acids) using various keto substrates.

It was previously found that prephenate can be converted by PDH/TyrA_p enzyme to HPP (**4**), which is then transaminated to form Tyr (**3**) in some plant species (i.e., legumes) but not in Arabidopsis (Rubin and Jensen, 1979; Schenck et al., 2015, **Fig. 1**). Therefore, although AtTAT2 favors conversion of HPP (**4**) to Tyr (**3**) than Tyr (**3**) to HPP (**4**) *in vitro*, it is unlikely that AtTAT2 actively synthesizes Tyr in Arabidopsis. According to Arabidopsis eFP Browser (bar.utoronto.ca/efp), *AtTAT1* is induced during leaf senescence, whereas *AtTAT2* is constitutively expressed throughout development (**Fig. S6**). Also, *AtTAT2* expression is generally higher than *AtTAT1* (**Fig. S6**) likely to partially compensate for its low activity (**Fig. 3, Fig. S1C**). Different isoenzymes of aromatic amino acid pathway enzymes, e.g. arogenate dehydratases and chorismate mutases, are differentially expressed, some of which have house-keeping function and others are likely responsible for synthesis of specialized metabolites (Corea et al., 2012; Mobley et al., 1999). Our working hypothesis here is that AtTAT2 with a broad substrate specificity may

function as a backup enzyme to supplement functions of other enzymes (e.g. AtTAT1) and maintain amino acid homeostasis. Further genetic analysis of their knockout mutants will address the *in vivo* functions of AtTAT1 and AtTAT2.

3.2 AtTAT1 and AtTAT2 connect Tyr synthesis and catabolism in the cytosol

Arabidopsis TAT activities were detected in both cytosol and plastid fractions, but AtTAT1 and AtTAT2, the two Arabidopsis enzymes closely related to previously characterized TATs of flowering plants, are cytosolic, based on confocal microscopy analysis of their GFP-fused proteins (**Fig. 4A**). This result suggests that an enzyme(s) other than AtTAT1 and AtTAT2 capable of converting Tyr (**3**) to HPP (**4**) is present in the plastid. Two previously characterized Arabidopsis C-S lyases are shown as plastidic in the Arabidopsis Subcellular Database (SUBA, Hooper et al., 2014), but they did not exhibit TAT activity (Jones et al., 2003; Mikkelsen et al., 2004). Although other uncharacterized C-S lyase homologs may have some TAT activity, these plastidic TAT activity may be derived from other classes of aminotransferases capable of deaminating Tyr (**3**), which independently evolved Tyr (**3**) specificity or have a broad substrate specificity, like *E. coli* AspC and ilvE (Gelfand and Steinberg, 1977).

Besides AtTATs, our confocal microscopy and subcellular fractionation experiments both showed that the Arabidopsis HPPD enzyme is also cytosolic (**Fig. 4**). Since AtTAT1 is strongly co-expressed with HPPD in Arabidopsis (**Fig. S7**, Aoki et al., 2016), HPPD and AtTAT1 likely work together in the cytosol for Tyr (**3**) deamination to HPP (**4**) and subsequent HPP (**4**) oxidation to homogentisate (**5**). Given that Tyr (**3**) biosynthesis and the steps downstream of HPPD in tocopherols and plastoquinone biosynthesis from homogentisate occur in the plastid (Joyard et al., 2009; Mehrshahi et al., 2014; Schulze-Siebert et al., 1987; Soll and Schultz, 1980; Soll et al., 1985, 1980; Yang et al., 2011), this result suggests that Tyr (**3**) must be first

transported to the cytosol, likely mediated by Arabidopsis homolog of a recently-reported aromatic amino acid transporter from petunia (Widhalm et al., 2015) and that homogentisate (**5**) must be transported back to the plastid via an unknown transporter(s) for tocopherol and plastoquinone biosynthesis. In other plants such as maize or soybean, having HPPD that are localized exclusively in the plastids or both in the cytosol and plastids, respectively (Siehl et al., 2014), the compartmentalization of the Tyr (**3**) deamination step is likely different from Arabidopsis. It is also important to note that homogentisate produced by TAT1 and HPPD may also stay in the cytosol and enter the Tyr (**3**) degradation pathway to form fumarate and acetoacetate (**Fig. 1**), for recycling of the reduced carbon molecule, Tyr (**3**), under various conditions (e.g., senescence).

4. CONCLUSIONS

Comparative biochemical and cellular analysis of Arabidopsis TAT enzymes showed that AtTAT1 and AtTAT2 have similar subcellular localization, but are biochemically distinct, consistent with their phylogenetic divergence within angiosperms. Therefore, although AtTAT1 and AtTAT2 may have some overlapping functions in the cytosol, these two enzymes likely have distinct roles in metabolism and physiology of Arabidopsis plants. For future application to plant biotechnology, upregulation of Tyr-specific AtTAT1 homologs may lead to improved production of HPP-derived plant natural products. On the other hand, downregulation or knockout of TAT1 homologs may redirect carbon flow towards other branches of Tyr (**3**) metabolic pathways (e.g., biosynthesis of betalain pigments and isoquinoline alkaloids, **Fig. 1**). Because of the universal presence of broad substrate TAT2 among angiosperms and of other aminotransferases having TAT activity, the lack of TAT1 may not compromise the overall growth and development of most

plants (Riewe et al., 2012). The study provides essential baseline information for further investigation of their physiological functions and for enhanced production of Tyr-derived phytochemicals with pharmaceutical and nutritional values.

5. Experimental

5.1 Phylogenetic analysis

All the available sequences of previous characterized plant TATs (At5g53970, At5g36160, PsTyrAT ADC33123.1, PhPPY-AT KF511589.1, CmArAT1 MELO3C025613P1, Ab-ArAT4 KC954706.1) were used as queries for BLAST search against representative genomes of different groups of plants and green algae from Phytozome 11 (phytozome.jgi.doe.gov) and Congenie (congenie.org) databases: *Arabidopsis thaliana*, *Brassica rapa*, *Populus trichocarpa*, *Solanum lycopersicum*, *Theobroma cacao*, *Citrus sinensi*, *Vitis vinifera*, *Medicago truncatula*, *Amborella trichopoda*, *Aquilegia coerulea*, *Brachypodium distachyon*, *Sorghum bicolor*, *Selaginella moellendorffii*, *Physicomitrella patens*, *Picea abies* and *Volvox carteri*. TAT homologs showing the majority of their sequences homologous to the queries were included in the analysis. The retrieved sequences had e-value lower than 10^{-17} . The sequence alignment was performed by T-Coffee (**Supplemental Data 2**, Notredame et al., 2000), and used as the input for unrooted phylogenetic tree construction using Mega 6.0 (Tamura et al., 2013) with the maximum likelihood method and 1,000 bootstrap replication with default setting except using partial deletion gaps/missing data mode.

5.2 Heterologous expression and purification of recombinant AtTAT1 and AtTAT2 proteins

Using the primers listed in **Table S1** the full length open reading frame (ORF) of At5g53970.1 (AtTAT1) was amplified from the ORF clone GC105018 obtained from Arabidopsis Biological Resource Center (ABRC), while At5g36160.1 (AtTAT2) was from the pML94-AtTAT2 vector (see below), and introduced into the NdeI and BamHI site of pET28a overexpression vector (Novagen, EMD Millipore, Germany) using Infusion Cloning kit (Clontech, CA). All vectors

were sequenced to confirm that no errors were introduced during cloning. Each vector was introduced into competent Rosetta (DE3) *Escherichia coli* cells (Novagen), which were first inoculated into 10 mL LB medium with 100 $\mu\text{g mL}^{-1}$ kanamycin and grown overnight (37°C, 200 rpm). One milliliter of the culture was then transferred to 25 mL LB medium without antibiotics and incubated for one hour at 37°C, 200 rpm, and then mixed and incubated with 500 mL LB medium with 100 $\mu\text{g mL}^{-1}$ kanamycin at 37°C and 200 rpm until OD₆₀₀ reach 0.2, when the temperature was shifted to 18°C. When OD₆₀₀ reached 0.3, isopropyl β -D-1-thiogalactopyranoside was then added into the medium at 0.4 mM final concentration and further incubated at 18°C and 200 rpm overnight. Cells were harvested by centrifugation, washed with 0.9% sodium chloride, and the pellet was resuspended in 25 mL of lysis buffer containing 50 mM sodium phosphate (pH 8.0), 200 μM pyridoxal-5-phosphate (PLP), 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 $\mu\text{g/mL}$ DNase I (Fisher Scientific, PA) and 1 mg/mL lysozyme (RPI, IL). After disrupting cells by freeze-thaw and sonication, the cell lysate was centrifuged at 4°C, 18,000g for 30 min, and the supernatant was loaded onto HisTrap FF column for purification of the His-tagged recombinant protein using ÄKTAFFPLC system (GE Healthcare Life Sciences, IL) according to manufacturer's protocol. The purified fraction was further desalted by Sephadex G50 column (GE Healthcare) into 50 mM sodium phosphate buffer (pH 8.0) containing 200 μM PLP and 10% glycerol. The purified proteins were loaded on SDS-PAGE gel for determination of their purity using ImageJ (**Supplemental Fig. 1A**).

5.3 Enzyme assays

The aminotransferase reactions were carried out in the reaction mixtures containing 100 mM Tris-HCl (pH 8.5), 200 μM PLP together with an amino donor, a keto acid acceptor, and the

purified recombinant enzymes at final concentrations indicated in each Fig. legend. The reaction was initiated by adding an enzyme. After incubation at 30°C for time periods indicated in each Fig. legend, the reactions were terminated by adding either sodium hydroxide (final 0.4 N) for detecting HPP formation for spectrophotometric assays or methanol (v/v 66% final concentration) for HPLC-based detection. The spectrophotometric HPP (**4**) detection was performed by measuring absorption at 331 nm using NanoDrop 2000 (ThermoFisher Scientific, MA). Various amino acid reaction products were quantified as *o*-phthaldialdehyde (OPA) derivatives using HPLC (Agilent 1260 equipped with Eclipse Plus XDB-C18 column, Maeda et al., 2010). Aspartate, glutamate, and aroenate were separated with a 10-min linear gradient of 10 to 30% MeOH in 0.1% (v/v) NH₄OAc (pH 6.8) at a flow rate of 0.5 mL/min, whereas 0.8 mL/min flow rate with a 30-min linear gradient of 20 to 45% MeOH was used for Tyr (**3**) and Ala. Phe, Gly, Leu, Ile, Val, and Met were separated with a 30-min linear gradient of 10 to 70% MeOH in 0.1% (v/v) NH₄OAc (pH 6.8) at 0.5 mL/min. Standard curves were generated with respective authentic standards for quantification. Kinetic analyses were conducted by hyperbolic regression analysis using Hyper32.exe (<http://homepage.ntlworld.com/john.easterby/hyper32.html>). All enzyme assays were performed under an appropriate enzyme concentration and reaction time, so that velocity was proportional to enzyme concentration and the incubation time, except for the qualitative TAT detection analyses in **Fig. S1B**.

5.4 Expression and localization of Arabidopsis TAT1- , TAT2-, and HPPD-GFP fusion proteins

Full length Arabidopsis *TAT1*, *TAT2* and *HPPD* genes excluding their stop codon were amplified from Arabidopsis leaf cDNA using the primers listed at **Table S1** and cloned into the KpnI and

NotI sites of the pML94 vector (Bionda et al., 2010) using Infusion Cloning method (Clontech). All vectors were sequenced to confirm that the cloned sequences were identical to At5g53970.1 (AtTAT1), At5g36160.1 (AtTAT2), and At1g06570.1 (HPPD) from TAIR. Each plasmid was extracted from the host *E. coli* cells (StellarTM, Clontech) at the concentration around 1 µg/µL. Arabidopsis protoplasts were obtained and transfected with the individual plasmids according to (Wu et al., 2009; Yoo et al., 2007). After 16 hour of transfection, protoplasts were observed and photographed using a Zeiss LSM510 confocal microscope equipped with a LDC-Apochromat 40X/1.1W Korr M27 water immersion objective (Carl Zeiss, Germany). GFP expressions were observed under 488 nm laser excitation and 500-530 nm spectral detection. Chlorophyll auto-fluorescence was visualized under 637-721 nm spectral detection. Images were captured by Zen 2011 software (Carl Zeiss) and processed for display by LSM 5 Series Image Browser (Carl Zeiss).

5.5 Subcellular fractionation and activity assay

Wild-type *Arabidopsis thaliana* (Col-0) was grown under 12/12 hour 100 µE light/dark cycle until four-weeks-old with 85% air humidity in the soil supplied with 1X Hoagland solution. Whole rosette leaves were harvested and processed according to Aryal et al. (2014) with modification not to include the mitochondrial fractions in the assay. Crude, cytosol and plastid fractions were desalted by Sephadex G50 column. Nitrite reductase and phosphoenolpyruvate carboxylase activity assays were performed according to (Meyer et al., 1988; Miflin, 1974, 1967). TAT activity was measured as mentioned above with HPLC, except by detecting the formation of Phe using 2 mM phenylpyruvate in the reaction as keto acceptor. For HPPD assay, the subcellular fractions were first desalted into 50 mM sodium phosphate buffer (pH 7.5). Desalted extracts

were incubated with the final concentrations of 200 μ M HPP (**4**) and 5 mM ascorbate in the total volume of 250 μ L. After 30 min of incubation at 30°C, the reaction was stopped by adding MeOH (500 μ L) containing 100 μ M norvaline as internal standard. The entire mixture (750 μ L) was centrifuged, and the supernatant was dried under vacuum using the CentriVap vacuum concentrator (Labconco, MO), resuspended in pyridine (40 μ L), and derivatized by *n*-tert-butyldimethylsilyl-*n*-methyltrifluoroacetamide [MTBSTFA, (40 μ L) Regis Technologies, IL]. For detection of the formation of homogentisate (**5**), the derivatized sample (1 μ L) was injected into gas chromatography-mass spectrometry (GC-MS, Trace1310-ISQ, ThermoFisher Scientific) with 1 to 10 split ratio, inlet temperature at 260°C, and 1.2 mL/min flow of helium gas. The GC inlet oven program was initially set at 100°C for 1 min, ramped to 300°C by 10°C/min, and held for 5 min. MS electric voltage was set to 70 eV with MS transfer line and source temperature set at 300°C. Homogentisate was detected by SIM mode following ion m/z 453, while norvaline was monitored with ion m/z 186 and 260. Both retention time and mass spectra of homogentisate and norvaline peaks were confirmed by injecting corresponding authentic standards (Sigma-Aldrich, MO).

Acknowledgements

We thank Craig Schenck and Micha Wijesingha Ahchige for critical reading of the manuscript and Sarah Swanson for confocal imaging and analysis training. The confocal imaging was performed at the Newcomb Imaging Center, Department of Botany, University of Wisconsin-Madison. This work was supported by IOS-1354971 from the US National Science Foundation and the start-up funds from the Graduate School, the College of Letters & Science, and the Department of Botany, University of Wisconsin-Madison to H.A.M.

Tables

Table 1. Kinetic parameters of AtTAT1 and AtTAT2 enzymes

variable-substrate	co-substrate ^a	K_m (mM)	V_{max} (nmol s ⁻¹ mg ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	substrate inhibition ^d
<i>AtTAT1</i>						
Tyr (3)	α -KG ^a	0.204 ± 0.01	1192 ± 69	57.3 ± 3.3	281.9 ± 14.4	N.D. ^d
Met	α -KG	24.2 ± 5.8	553 ± 98	26.6 ± 4.7	1.1 ± 0.1	N.D.
Phe	α -KG	6.94 ± 0.56	656 ± 71	31.6 ± 3.4	4.5 ± 0.1	N.D.
Asp	α -KG	^b Activity increased linearly				N.D.
HPP (4)	Glu	10.1 ± 2.9	497.1 ± 86.9	23.9 ± 4.2	2.5 ± 0.2	N.D.
4MTOB	Glu	1.30 ± 0.06	418 ± 11.7	20.1 ± 0.6	15.5 ± 0.3	> 5 mM
PPY	Glu	0.455 ± 0.05	509 ± 48	24.5 ± 2.3	54.2 ± 1.7	> 2 mM
OA	Glu	^c Activity was not detectable				N.A.
α -KG	Tyr (3)	3.33 ± 0.33	998 ± 63	48.0 ± 3.0	15.1 ± 2.4	N.D.
4MTOB	Tyr (3)	3.46 ± 0.44	384 ± 7.1	18.5 ± 0.34	5.5 ± 0.63	N.D.
PPY	Tyr (3)	3.06 ± 0.08	628 ± 18	30.2 ± 0.9	9.9 ± 0.5	N.D.
OA	Tyr (3)	^c Activity was not detectable				N.A.
<i>AtTAT2</i>						
Tyr (3)	α -KG	2.9 ± 0.4	14.2 ± 1.9	0.75 ± 0.1	0.23 ± 0.01	N.D.
Met	α -KG	1.7 ± 0.3	10.9 ± 1.1	0.53 ± 0.05	0.32 ± 0.02	N.D.
Phe	α -KG	6.7 ± 1.0	10.6 ± 1.0	0.51 ± 0.05	0.08 ± 0.01	N.D.
Asp	α -KG	^b Activity increased linearly				N.D.
HPP (4)	Glu	0.410 ± 0.03	11.8 ± 1.7	0.43 ± 0.15	1.38 ± 0.12	> 2 mM
4MTOB	Glu	0.807 ± 0.09	19.1 ± 2.2	0.70 ± 0.24	1.15 ± 0.04	> 5 mM
PPY	Glu	1.28 ± 0.16	12.8 ± 1.3	0.47 ± 0.16	0.49 ± 0.04	> 5 mM
OA	Glu	^b Activity increased linearly				> 10 mM
α -KG	Tyr (3)	7.62 ± 1.08	11.0 ± 0.4	0.53 ± 0.02	0.07 ± 0.01	N.D.
4MTOB	Tyr (3)	0.740 ± 0.06	7.7 ± 0.1	0.37 ± 0.01	0.51 ± 0.04	> 20 mM
PPY	Tyr (3)	3.43 ± 0.47	3.6 ± 0.2	0.13 ± 0.04	0.04 ± 0.00	> 20 mM
OA	Tyr (3)	42.9 ± 15.7	15.3 ± 2.7	0.56 ± 0.21	0.02 ± 0.00	N.D.

Data are means ± SE (n ≥ 3). Individual Michaelis–Menten saturation curves and reaction conditions are shown in **Figs. S2 to S4**.

^a Concentrations of co-substrate were 20 mM for α -KG and Glu, and 5 mM for Tyr (due to low Tyr solubility).

^b Activity increased linearly

^c Activity was not detectable

^d Activity was inhibited beyond the indicated concentration. N.D., not detected, N.A., not available due to undetectable activity.

OA, oxaloacetate; α -KG, α -ketoglutarate; PPY, phenylpyruvate, 4MTOB, 4-methylthio-2-oxobutanoate; 4MOP, 4-methyl-2-oxopentanoate; 3MOB, 3-methyl-2-oxobutanoate, 3MOP, 3-methyl-2-oxopentanoate, Tyr, tyrosine; Phe, phenylalanine; Met, methionine; Asp, aspartate, Glu, glutamate.

Figures

Figure 1. Biosynthetic and catabolic pathways of tyrosine (3) in plants. In most plants, *L*-tyrosine (Tyr) (3) is synthesized via the arogenate dehydrogenase (ADH/TyrA_a) pathway and then catabolized into 4-hydroxyphenylpyruvate (HPP) (4) by Tyr aminotransferase (TAT). HPP (4) serves as a precursor of many plant natural products (gray highlight). HPP (4) can also be converted by HPP dioxygenase (HPPD) to homogentisate (5) for tocopherol and plastoquinone biosynthesis and degradation of Tyr (3). PDH/TyrA_p, prephenate dehydrogenase.

Figure 2. Maximum likelihood phylogenetic tree of plant TAT homologs. Percentage values of replicate trees in bootstrap test (1000 replicates) is shown next to the branches (value less than 50 are not shown) with branch length indicating the number of substitutions per site. Characterized enzymes are indicated in published names in parentheses and marked with symbol ◇ for prephenate aminotransferase, Δ for C-S lyases, * for Gymnosperm AAA-ATs, and ** for Angiosperm AAA-ATs. The well-supported subclade containing AtTAT1 is highlighted in gray. Symbols of each species: **AT**, *Arabidopsis thaliana*; **Atrichopoda**, *Amborella trichopoda*; **Bradi**, *Brachypodium distachyon*; **Potri**, *Populus trichocarpa*; **Brara**, *Brassica rapa*; **GSVIVG**, *Vitis vinifera*; **MA**, *Picea abies*; **Medtr**, *Medicago truncatula*; **Pp**, *Physcomitrella patens*; **Semoe**, *Selaginella moellendorffii*; **Sobic**, *Sorghum bicolor*; **Solyc**, *Solanum lycopersicum*. **Aquca**, *Aquilegia coerulea*; **Thecc**, *Theobroma cacao*; **orange**, *Citrus sinensis*; **Vocar**, *Volvox carteri*. Arrows denote AtTAT1 and AtTAT2. AAA-AT, aromatic amino acid aminotransferase.

Figure 3. Amino donor and keto acceptor specificities of recombinant AtTAT1 and AtTAT2 enzymes. Aminotransferase activities were analyzed for different amino donors using α -ketoglutarate keto acceptor (A) and for different keto acceptors using glutamate amino donor (B). The reaction mixture containing 5 mM amino and keto substrates and 200 μ M PLP were incubated for 5 min at 30°C together with 1 to 8 μ g/mL or 10 to 80 μ g/mL of purified recombinant AtTAT1 or AtTAT2, respectively. Data are means $\pm$ SE ($n \geq 3$). 4MTOB, 4-methylthio-2-oxobutanoate; 4MOP, 4-methyl-2-oxopentanoate; 3MOB, 3-methyl-2-oxobutanoate; 3MOP, 3-methyl-2-oxopentanoate; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Val, valine.

Figure 4. Subcellular localization of TAT and HPPD in Arabidopsis. (A) GFP-fused Arabidopsis TAT1, TAT2, HPPD, and ADH2 and free GFP were transiently expressed in Arabidopsis protoplasts. Representative images show GFP fluorescence and chlorophyll autofluorescence in green and magenta, respectively. Scale bars, 5 μ m. (B) Specific activities of tyrosine aminotransferase (TAT), 4-hydroxyphenylpyruvate dioxygenase (HPPD), nitrite reductase (NiR) and phosphoenolpyruvate carboxylase (PEPC) detected from the crude, cytosol and plastid fractions of Arabidopsis leaf tissues. TAT activity was measured by detecting the formation of Phe using 2 mM phenylpyruvate as keto acceptor in the reaction. Data are means $\pm$ SE (n = 4). N.D., not detectable.

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