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Biochemical properties and subcellular localization of tyrosine aminotransferases in *Arabidopsis thaliana*

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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**)

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.

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.,

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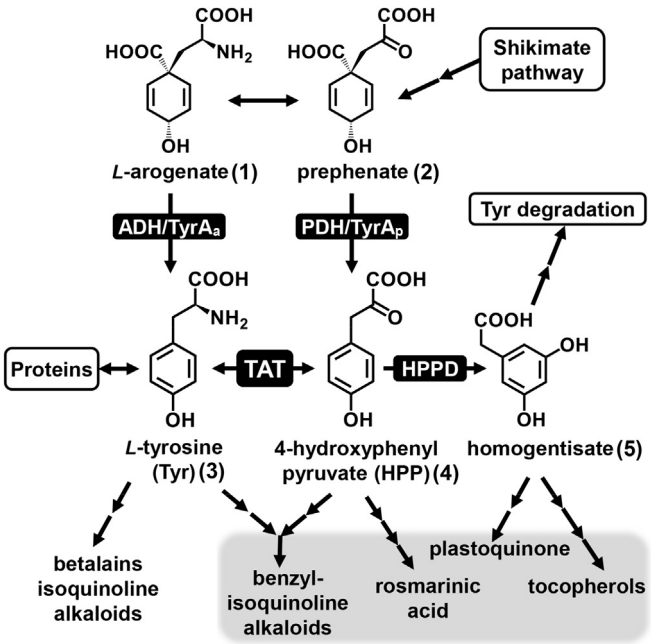

Fig. 1. Biosynthetic and catabolic pathways of tyrosine (3) in plants. In most plants, *L*-tyrosine (Tyr) (**3**) is synthesized via the arogenate dehydrogenase (ADH/TyrA$_a$) pathway and then catabolized into 4-hydroxyphenylpyruvate (HPP) (**4**) by Tyr aminotransferase (TAT). HPP (**4**) serves as 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.

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 benzylisoquinoline 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 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-value < 1^{e-17}). Maximum likelihood phylogenetic tree was then constructed for TAT homologs 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 homologs from all the core eudicots included in the analysis, but not from a