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Eun-Jeong Lee
Department of Biological Sciences, University of Calgary, Calgary, Alberta, T2N 1N4, Canada
Tel. +1-403-220-5238
E-mail: leej@ucalgary.ca

Peter Facchini
Department of Biological Sciences, University of Calgary, Calgary, Alberta, T2N 1N4, Canada
Tel. +1-403-220-7651
E-mail: pfacchin@ucalgary.ca
Tyrosine aminotransferase contributes to benzylisoquinoline alkaloid biosynthesis in opium poppy

Eun-Jeong Lee and Peter Facchini*

Department of Biological Sciences, University of Calgary, Calgary, Alberta, T2N 1N4, Canada
Footnotes

1 This work was supported through funding from Genome Canada and Genome Alberta, and by a Natural Sciences and Engineering Research Council of Canada Research Tools and Infrastructure Grant. P.J.F. holds the Canada Research Chair in Plant Metabolic Processes Biotechnology.

* Corresponding author; e-mail pfacchin@ucalgary.ca.

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[1] The online version of this article contains Web-only data.
ABSTRACT
Tyrosine aminotransferase (TyrAT) catalyzes the transamination of L-tyrosine and α-ketoglutarate yielding 4-hydroxyphenylpyruvic acid (4-HPP) and L-glutamate. The decarboxylation product of 4-HPP, 4-hydroxyphenylacetaldehyde (4-HPAA), is a precursor to a large and diverse group of natural products known collectively as benzylisoquinoline alkaloids (BIAs). We have isolated and characterized a TyrAT cDNA from opium poppy (Papaver somniferum), which remains the only commercial source for several pharmaceutical BIAs including codeine, morphine, and noscapine. TyrAT belongs to group I pyridoxal 5’-phosphate (PLP)-dependent enzymes whereby Schiff base formation occurs between PLP and a specific lysine residue. The amino acid sequence of TyrAT showed considerable homology to other putative plant tyrosine aminotransferases, although few of these have been functionally characterized. Purified, recombinant TyrAT displayed a molecular weight of approximately 46 kDa and a substrate preference for L-tyrosine and α-ketoglutarate with the apparent $K_m$ values of 1.82 and 0.35 mM, respectively. No specific requirement for PLP was detected in vitro. Liquid chromatography tandem mass spectrometry confirmed the conversion of L-tyrosine to 4-hydroxyphenylpyruvate. TyrAT gene transcripts were most abundant in roots and stems of mature opium poppy plants. Virus-induced gene silencing was used to evaluate the contribution of TyrAT to BIA metabolism in opium poppy. TyrAT transcript levels were reduced by at least 80% in silenced plants compared with controls, and showed a moderate reduction in total alkaloid content. The modest correlation between transcript levels and BIA accumulation in opium poppy supports a role for TyrAT in the generation of alkaloid precursors, but also suggests the occurrence of other sources for 4-HPAA.
INTRODUCTION

Although many downstream biosynthetic enzymes involved in the biosynthesis of natural products, including the narcotic analgesics codeine and morphine, the cough-suppressant and potential anticancer agent noscapine, and the vasodilator papaverine have been isolated from opium poppy and related plants, enzymes catalyzing the early steps of benzylisoquinoline alkaloids (BIAs) biosynthesis are not well characterized. BIA biosynthesis has been purported to begin with a lattice of decarboxylations, meta-hydroxylations and transaminations that convert L-tyrosine to dopamine and 4-hydroxyphenylacetaldehyde (4-HPPA) (Figure 1; Desgagné-Penix and Facchini, 2011). 4-HPAA is produced by the decarboxylation of 4-hydroxyphenylpyruvate (4-HPP), which is the organic acid of L-tyrosine derived through transamination. L-Dihydroxyphenylalanine (L-DOPA) and dopamine are derived via the corresponding 3-hydroxylation of L-tyrosine and tyramine, whereas tyramine and dopamine result from the decarboxylation of tyrosine and DOPA, respectively (Facchini and De Luca, 1994). Norcoclaurine synthase (NCS) catalyzes the stereoselective condensation of dopamine and 4-HPPA as the first committed step in BIA metabolism.

Transamination reactions occur by a “ping-pong” mechanism whereby two half-reactions are required to complete one catalytic cycle and consequently transfer of the α-amino group from an amino acid to a α-keto acid (Prabhu and Hudson, 2010). This reaction is readily reversible; thus, both amino acids and α-keto acids are substrates for transaminases. Pyridoxal-5’-phosphate (PLP) functions as a coenzyme forming a Schiff base with the amino acid, which is required for activity and involves a conserved lysine residue at the catalytic core of all aminotransferases (Hayashi, 1995). PLP-dependent aminotransferase are divided into four subgroups based on sequence identity. Subgroup I includes aspartate aminotransferase, alanine aminotransferase, histidinol phosphate aminotrasferase, and aromatic amino acid (including tyrosine) aminotransferases. Subgroup II includes acetyl-ornithine aminotransferase, ornithine aminotranferase, and lysine aminotransferase. Subgroup III and IV include serine aminotransferase, phosphoserine aminotransf erase, D-amino acid aminotransferases, and branched-chain amino acid aminotransferases (Hayashi, 1995).

Characterized aromatic amino acid aminotransferases have either specific or a broad range of amino acid and α-keto acid substrates that are used as amino group donors and acceptors, respectively. Although tyrosine, phenylalanine and tryptophan are primarily involved
in protein synthesis a vast array of secondary metabolites are also derived from these aromatic amino acids (Tzin and Galili, 2010). Although the biochemical and structural characterization of tyrosine aminotransferase (TyrAT) in mammals and fungi is well established (Blankenfeldt et al., 1999; Sobrado et al., 2003; Mehere et al., 2010; Schneider et al., 2008) considerably less is known about these enzyme in plants. TyrAT is regulated by coronatine, wounding and methyl jasmonate, and has been implicated as the initial enzyme in tocopherol biosynthesis in *Arabidopsis thaliana* plants (Lopukhina et al., 2001; Holländer-Czyko et al., 2005), and *Amaranthus caudatus* and *Chenopodium quinoa* cell cultures (Antognoni et al., 2009). TyrAT activity was reported in rosmarinic acid-producing cell cultures of *Anchusa officinalis* and *Coleus blumei*, and MeJA-treated hairy root cultures of *Salvia miltiorrhiza* (De-Eknamkul and Ellis, 1987a; Xiao et al., 2009). In plants, tocopherols and rosmarinic acid function as free radical scavengers and confer protection against a variety of biotic and abiotic environmental stress factors (Sattler et al., 2004; Liu et al., 1992; Xiao et al., 2009). These natural products are also associated with potential benefits to human health.

In this study, we report the isolation and characterization of TyrAT involved in the generation of precursors required for the production of BIAs, such as morphine and codeine, in opium poppy. Although much work have been done to identify the genes responsible for downstream BIA metabolism, the enzymes involved in the supply of precursors are still poorly defined. Our work further demonstrates how the availability of high-throughout sequencing technologies, such as 454 pyrosequencing, and the emergence of functional genomics tools in opium poppy (Facchini and De Luca, 2008; Hagel and Facchini, 2010) provide new opportunities to characterize novel biosynthetic genes.

**RESULTS**

**Identification of a TyrAT cDNA from opium poppy**

A deep transcriptome database was generated by 454 GS-FIX Titanium pyrosequencing using a cDNA library prepared from opium poppy cell cultures (Desgagné-Penix et al., 2010)
and several plant cultivars. The assembled and annotated database was initially screened for proteins related to PLP-dependent enzymes and sequences annotated as aminotransferases. Seven full-length cDNAs belonging to the PLP-dependent aspartate aminotransferase superfamily (AAT-like proteins) were identified (Supplemental Figure 1 and Supplemental Table 1). One cDNA with substantial yet differential amino acid identity to putative and functionally validated tyrosine aminotransferases was selected for further characterization. The cDNA contained a 1,257-bp open reading frame and encoding a predicted translation product of 418 amino acids with a molecular weight of 46.3 kDa.

The predicted opium poppy TyrAT polypeptide contains a catalytic lysine residue found in all AAT-like proteins and ten conserved domains that putatively bind a single PLP molecule as the enzymatic co-factor (Supplemental Figure 2). The NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) structure prediction tool also suggests that the TyrAT candidate possesses several homodimer interfaces. The ClustalW2 program was used to compare the amino acid sequence of the predicted protein with known and putative tyrosine aminotransferases. The primary structures of all selected proteins were similar with respect to overall length and the position of conserved domains (Supplemental Figure 2). An unrooted neighbour-joining tree showing the phylogenetic relationships between the opium poppy TyrAT candidate and related plant enzymes is shown in Figure 2. The Papaver somniferum (Ps TyrAT) showed the highest identity (59-62%) with Ricinus communis Rc TyrAT (Genbank accession number XP_002517869), Populus trichocarpa Pt TyrAT (XP_002328046), Solanum pennelli Sp TyrAT (ADZ24702), and Oryza sativa japonica group Os TyrAT (BAF95202). The Ps TyrAT protein also exhibited considerable sequence identity (55-56%) with Salvia miltiorrhiza Sm TyrAT (ABC60050), Solenostemon scutellaridoides Ss TyrAT (CAD30341), Medicago truncatula Mt TyrAT (AAAY5183), and Glycine max Gm TyrAT (AAAY21813). However, it is important to note that none of these purported TyrAT candidates has been demonstrated to accept tyrosine as a substrate transamination. In contrast, Ps TyrAT showed relatively lower amino acid identity with Arabidopsis thaliana At TyrAT-1 (AAN15626), At TyrAT-2 (NP_180058), and At TyrAT-3 (AAG37062), which have been shown to function as tyrosine aminotransferases (Lopukhina et al., 2001; Holländer-Czytko et al. 2005). Recently, Cucumis melo Cm TyrAT (ADC45389) and A. thaliana At TyrAT-4 (NM_124776) were
characterized as tyrosine aminotransferases (Prabhu and Hudson, 2010; Gonda et al., 2010) and showed considerable sequence identity (55 and 58%, respectively) to Ps TyrAT.

**Purification and functional characterization of opium poppy TyrAT**

The full-length cDNA was cloned into the expression vector pQE30 with a translational fusion to an N-terminal His6-tag, and expressed in *Escherichia coli*. Recombinant Ps TyrAT exhibited a molecular weight of 46 kDa and was purified by cobalt-affinity chromatography and to a high degree of purity (Figure 3). To screen for the transamination of L-tyrosine yielding 4-HPP, enzyme assays containing purified, recombinant Ps TyrAT were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Figure 4). To confirm TyrAT enzyme activity, the native enzyme was compared with heat-inactivated enzyme as a negative control. Reactions were monitored by LC-MS/MS in multiple-reaction monitoring (MRM) mode and collision-induced dissociation (CID) mass spectra were generated for selected compounds. Using authentic standards, product ion spectra were used to determine compound-specific MRM transitions of 180.1 $\rightarrow$ 163.1, 180.1 $\rightarrow$ 119.2 for L-tyrosine and 179.1 $\rightarrow$ 157.1, 179.1 $\rightarrow$ 107.1 for 4-HPP. The MRM transitions and the CID spectrum of the native TyrAT reaction product eluting at 0.54 min were identical to those of the authentic 4-HPP standard (Figure 4). The CID mass spectrum of 4-HPP at $m/z$ 179.1 contained a major fragment at $m/z$ 107.1 $[M-H]^-$. In contrast, the L-tyrosine spectrum showed deprotonated molecular ions at $m/z$ 163.1 and 119.2. Neither the $m/z$ 179.1 precursor nor the $m/z$ 107.1 fragment ions corresponding to 4-HPP were detected in reactions using heat-inactivated enzyme (Figure 4). However, the $m/z$ 179.8 $[M-H]^-$ precursor and $m/z$ 119.2 and 163.1 fragment ions corresponding to L-tyrosine were present.

TyrAT activity was also assayed using $[^{14}C-(U)]$-L-tyrosine as a the amino group donor, and detecting the formation of $[^{14}C-(U)]$-4-HPP by TLC (Supplemental Figure 3). The identity of the reaction product was determined by comparison of the $R_f$ value with that of an authentic $[^{14}C-(U)]$-4-HPP standard. The intensity of the reaction product was directly proportional to the amount of recombinant enzyme used in the assay up to 7 μg of purified protein (Supplemental Figure 3B), and was directly proportional to the amount of $[^{14}C-(U)]$-L-tyrosine up to at least 11 μmol (Supplemental Figure 3C). $[^{14}C-(U)]$-4-HPP was not detected in assays using heat-inactivated enzyme. These data provided an empirical basis for the optimal amount of recombinant Ps TyrAT and L-tyrosine used in the standard enzyme assay.
Kinetic Parameters of Opium Poppy TyrAT

The effect of pH on TyrAT activity was determined between pH 6.0 and 10.0 in HEPES buffer. Optimal activity of Ps TyrAT was measured at pH 8.5, which was about 4 times higher than the activity at pH 7 (Figure 5). The substrate specificity of recombinant Ps TyrAT was tested using the L-amino acids tyrosine, tryptophan and phenylalanine, and the α-keto acids α-ketoglutarate, pyruvate and oxaloacetate as possible amino group donors and acceptors, respectively. Using α-ketoglutarate as the acceptor, L-tyrosine was the preferred donor. L-Tryptophan and L-phenylalanine were also accepted as substrates with lower efficiency than L-tyrosine (Supplemental Figure 4). Using L-tyrosine as the donor, α-ketoglutarate was the preferred acceptor, followed by pyruvate and oxaloacetate (Supplemental Figure 4). Kinetic parameters were determined based on the Michaelis-Menten equation using a nonlinear least-squares approach. The apparent \( K_m \) values for L-tyrosine, L-tryptophan, and L-phenylalanine at a saturating concentration (0.5 mM) of α-ketoglutarate were 1.82 mM, 7.83 mM, and 6.33 mM, respectively (Table 1, Supplemental Figure 5). \( K_m \) values for α-ketoglutarate, pyruvate, and oxaloacetate at a saturating concentration (3 mM) of L-tyrosine were 0.35 mM, 2.45 mM, and 56.13 mM, respectively (Table 1, Supplemental Figure 5). No substantial substrate or product inhibition was detected. The enzyme efficiency \( (k_{cat}/K_m) \) for L-tyrosine was 2.6- and 13-fold greater than those of L-tryptophan and L-phenylalanine, respectively. The \( k_{cat}/K_m \) for α-ketoglutarate was 4.5- and 63-fold greater than those of pyruvate and oxaloacetate, respectively. Recombinant Ps TyrAT activity did not increase in response to the addition of PLP to reaction mixture. The purified enzyme was stable for several days in 100 mM HEPES buffer at -80°C, but its activity gradually decreased after longer-term storage.

Involvement of TyrAT in BIA Metabolism in Opium Poppy

The highest Ps TyrAT transcript levels were detected in the roots and stems of mature opium poppy plants, whereas lower transcript levels were detected in leaves and carpels (Figure 6). The occurrence of relatively high transcript levels in opium poppy stems facilitated the analysis of stem tissue and latex using virus-induced gene silencing (VIGS) to examine the potential role of TyrAT in BIA metabolism. A 492-bp fragment of the Ps TyrAT coding region was inserted into the pTRV2 vector used for tobacco rattle virus (TRV)-based VIGS (Figure 7A).
*Agrobacterium tumefaciens* containing pTRV1 and pTRV2-TyrAT or the pTRV2 empty vector (EV) were infiltrated into the apical meristems of two-week-old opium poppy plants. The pTRV2 vector encodes the tobacco rattle viral coat protein, transcripts of which were detected in stem tissue approximately 10 weeks after infiltration to confirm the presence of the virus (Figure 7B). Relative Ps TyrAT transcript levels were reduced by at least 80% in plants infiltrated with *A. tumefaciens* harboring the pTRV2-TyrAT vector compared with empty-vector controls (Figure 7C). To test the effect of suppressing Ps TyrAT transcript levels on the accumulation of BIAs, latex samples from plants infiltrated with pTRV2-TyrAT or pTRV2-EV and showing the occurrence of TRV2 coat protein transcripts were analyzed by HPLC. Levels of the six major BIAs in opium poppy were reduced in plants infiltrated with *A. tumefaciens* harboring the pTRV2-TyrAT vector compared with empty-vector controls (Figure 7D). The specific alkaloid content showed considerable variation in individual plants, which contributed to the relatively large standard deviation in the mean values. The correlation between relative Ps TyrAT transcript levels and alkaloid accumulation was not fully proportional suggesting that other factors are involved in the supply of precursors for BIA metabolism.

**DISCUSSION**

A full-length cDNA encoding tyrosine aminotransferase was isolated based on its annotation in a deep transcript library generated by 454 pyrosequencing of opium poppy (Desgagné-Penix et al., 2010), and the predicted amino acid sequence was used to reanalyze the transcriptome databases through a BLASTx search. Six full-length cDNAs were revealed (Supplemental Figure 1), five of which annotated as alanine aminotransferase or hypothetical protein and displayed only 16-22% amino acid identity compared with Ps TyrAT (Supplemental Table 1). The other predicted protein (cl.10988) showed 68% amino acid identity with Ps TyrAT, but was not selected for further analysis because corresponding gene transcripts were not detected in elicitor-treated cell cultures (Desgagné-Penix et al., 2010). In contrast, Ps TyrAT transcripts were detected in the 454 databases of elicitor-treated cell cultures and stems, which are both capable of BIA biosynthesis. TyrAT converts L-tyrosine to 4-hydroxyphenylpyruvate.
(HPP) via pyridoxal phosphate-dependent transamination reaction. The proposed early steps in the formation of the central BIA intermediate (S)-norcoclaurine were based on the incorporation of radiolabelled precursors (Figure 1) (Holland et al., 1979; Schumacher et al., 1983; Rueffer and Zenk, 1987). Only $[^{14}C]$-L-tyrosine was equally incorporated into both the “upper” isoquinoline and “lower” benzylic portions of the BIA backbone. In contrast L-DOPA, dopamine, and tyramine were predominantly incorporated into the isoquinoline moiety. Enzyme activities corresponding to the purported transamination, decarboxylation, and hydroxylation reactions have been reported in crude protein extracts (Rueffer and Zenk, 1987; Hara et al., 1994). However, only cDNAs encoding tyrosine/dopa decarboxylase (TYDC; EC 4.1.1.25), which converts L-tyrosine to tyramine and L-DOPA to dopamine, have been isolated from BIA-producing plants (Facchini and De Luca, 1994). Through the isolation and characterization of a cDNA encoding tyrosine aminotransferase from opium poppy, we provide biochemical and physiological support for the involvement of 4-HPP as an intermediate in the formation of the 4-HPAA precursor used in BIA biosynthesis, in support of the proposed pathway (Figure 1).

Previously, TyrAT cDNAs implicated in the biosynthesis of tocopherols or in fruit ripening have been characterized in Arabidopsis and melon, respectively (Lopukhina et al., 2001; Holländ-Czyko et al., 2005; Prabhu and Hudson, 2010; Gonda et al., 2010).

L-Tyrosine aminotransferases are well characterized in mammals, *Escherichia coli*, and *Trypanosoma cruzi*. Several cDNAs encoding TyrAT have been isolated and characterized from rat, mouse, and human (Shinomiya et al., 1984; Müller et al., 1985; Grange et al., 1985; Andersson and Pispa, 1982) and the crystal structures have been solved (Ko et al., 1999; Blankenfeldt et al., 1999; Mehere et al., 2010; Protein Data Bank code 3dyd). Several amino acids have been implicated in catalyzing the transamination reaction based on the structural features of TyrAT from various organisms. Highly conserved catalytic Lys and Asp residues interact with the pyridine nitrogen of PLP (Mehere et al., 2010), which is covalently bound to the □-amino group of the Lys residue via a Schiff base linkage (Hayashi, 1995). Once the amino acid substrate interacts with the active site a new Schiff base is generated. Opium poppy TyrAT shared approximately 30% amino acid identity with TyrATs from rat, mouse, and *T. cruzi*, but less than 10% amino acid identity with TyrAT from *E. coli*. In opium poppy TyrAT, Lys251 and Asp222 are assumed to serve in the same capacity as Lys280 and Asp247 from mouse TyrAT in the binding of PLP (Supplemental Figure 2). Arg417 is the one of the residues responsible for
the interaction of tyrosine with mouse and rat TyrAT (Mehere et al., 2010; Sobrado et al., 2003), and Arg390 of Ps TyrAT might perform the same function. As reported for other investigations of plant aminotransferases, the in vitro activity of Ps TyrAT was not affected by the addition of exogenous PLP. Transamination activity occurred without the addition of PLP in crude protein extracts of bushbean (Forest and Wightman, 1972), tomato (Gibson et al., 1972), mung bean (Truelsen, 1972), peanut (Mazelis and Fowden, 1969), wheat (Cruickshank and Isherwood, 1958), and cauliflower (Eliis and Davis, 1961). Plant aminotransferases appear to function as holoenzymes composed of an apoenzyme tightly bound to the coenzyme moiety, whereas the apoenzyme and coenzyme components of mammalian aminotransferases can be separated (Forest and Wightman, 1972; Wightman and Forest, 1978).

In rat, TyrAT activity is regulated by glucocorticoids, insulin, and glucagon (Rettenmeier et al., 1990). TyrAT deficiency leads to type II tyrosinemia in humans, which is associated with microcephaly, tremor, ataxia, language deficits, and convulsions (Bein and Goldsmith, 1977; Cavelier-Balloy et al., 1985). Fungal TyrATs have been implicated in the biosynthesis of atromentin in Tapinella panuoides (Schneider et al., 2008). Unlike mammalian and microbial enzymes, the physiological functions of plant TyrATs are not well understood. Among the plant enzymes included in the phylogentic analysis (Figure 2) only TyrATs from Arabidopsis and melon have isolated and characterized (Lopukhina et al., 2001; Holländer-Czyko et al., 2005; Prabhu and Hudson, 2010; Gonda et al., 2010). ArAT from Cucumis melo (FJ896816; designated here as Cm TyrAT) displayed aromatic and branched-chain amino acid transaminase activities in flesh and rind tissues during melon fruit ripening (Gonda et al., 2010). The TAT1 (COR13; At4g23600) gene and six related sequences in the Arabidopsis genome, which encode class I aminotransferases were identified by differential display analysis in Arabidopsis plants treated with the phytotoxin coronatine (Lopukhina et al., 2001). Transcript levels for TAT1 (designated At TyrAT-1 herein) increased in response to methyl jasmonate or methyl 12-oxophytodienoic acid (MeOPDA) treatment, and by wounding. The deduced amino acid sequence of TAT1 showed 35% identity with human and rat TyrATs, and shares extensive sequence similarity with nicotianamine aminotransferase involved in the biosynthesis of mugineic acid family phytosiderophores (Takahashi et al., 1999). However, the TyrAT activity of TAT1 remains controversial. In one study, the TAT1 gene was suggested to encode a PLP-dependent cystine lyase (Cys-lyase) and not TyrAT since recombinant TAT1 protein showed higher Cys-lyase than
TyrAT activity. Moreover, TAT1 shares substantial (79%) amino acid identity with the *Brassica oleracea* BOCL3 gene product, which exhibits Cys-lyase activity (Jones et al., 2003). In support of this suggestion, human kynerenine aminotransferase I/glutamine transaminase K (EC 2.6.1.64; KAT-1) was proposed to play a dual function in catalyzing the transamination of several amino acids and also showed Cys S-conjugate β-lyase activity (EC 4.4.1.13) (Cooper, 2004). Alanine and aspartate aminotransferase from procine heart was also able to cleave Cys conjugates (Adcock et al., 1996). Aminotransferases might generally possess multifunctional potential in complex metabolic networks.

The TAT3 gene (At2g24850; designated At TyrAT-2 herein) was shown to encode the enzyme catalyzing the first step in tocopherol biosynthesis and was induced by MeJA and MeOPDA, wounding, high light intensity, UV light, and the herbicide oxyfluorfen (Sandorf and Hollander-Czytko, 2002). The rooty/superroot1 gene (At2g20610; designated At TyrAT-3 herein) is a locus on Arabidopsis chromosome 2 encoding a protein suggested to have TyrAT activity (Gopalraj et al., 1996). However, the major role of the rooty/superroot1 gene product is apparently either tryptophan aminotransferase (EC 2.6.1.27) or Cys-lyase, implying a role in modulating IAA levels in tryptophan-derived specialized metabolism such as indole glucosinolate biosynthesis (Gopalraj et al., 1996; Nonhebel et al., 1993; Jones et al., 2003). Recently, another Arabidopsis TyrAT gene (At5g36160; designated here as At TyrAT-4) was reported as an aminotransferase capable of interconverting L-tyrosine and 4-hydroxyphenylpyruvate, and L-phenylalanine and phenylpyruvate (Prabhu and Hudson, 2010). The transcript level of TyrAT in crude protein extracts of *Salvia miltiorrhiza* hairy root cultures increased along with phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-hydroxyphenylpyruvate reductase (HPPR), and 4-hydroxyphenylpyruvate dioxygenase (HPPD) transcript levels in response to MeJA treatment (Xiao et al., 2009). TyrAT with a high substrate specificity for tyrosine and broad specificity toward amino group acceptors was purified from rosmarinic acid-producing cell cultures of *Anchusa officinalis* and *Cocleus blumei* (De-Eknamkul and Ellis, 1987b), and an increase in TyrAT activity in response to MeJA treatment was accompanied by higher α-tocopherol levels in *Amaranthus caudatus* cell cultures (Antognoni et al., 2009). Overall, TyrATs are regulated by stresses factors via a complex signaling network, and are associated with multiple metabolic and other physiological processes. A role for TyrAT in BIA metabolism was proposed based on the detection of enzyme activity in
cell cultures (Rueffer and Zenk, 1987). The isolation and functional characterization of a TyrAT cDNA in opium poppy plants validates the contribution of the enzyme in the provision of 4-HPAA for the formation of (S)-norcoclaurine.

The relatively high pH optimum of pH 8.5 (Figure 5) of Ps TyrAT is consistent with the reported maximal activity at pH 8.2 for TyrATs from other plants (De-Eknamkul and Ellis, 1987a; Prabhu and Hudson, 2010). Opium poppy TyrAT also showed a pronounced preference for L-tyrosine over other aromatic amino acid substrates, but relatively broader specificity toward the amino group acceptors α-ketoglutarate, pyruvate, and oxaloacetate (Supplementary Figure 4). The apparent $K_m$ and $V_{max}$ for L-tyrosine (using α-ketoglutarate as the amino group acceptor) of 1.82 mM and 0.63 μmol min$^{-1}$ mg$^{-1}$, respectively, are within the broad range of values reported for other plant enzymes with TyrAT activity. Three purified TyrAT isoforms from A. officinalis cell cultures displayed $K_m$ values for L-tyrosine between 0.45 and 20 mM (De-Eknamkul and Ellis, 1987a). One isoform showed a relatively strict specificity toward L-tyrosine/α-ketoglutarate, whereas another exhibited higher specificity for L-asparate/α-ketoglutarate compared with L-tyrosine/α-ketoglutarate. In Arabidopsis, an apparent $K_m$ of 0.19 mM and $V_{max}$ of 5 μmol min$^{-1}$ mg$^{-1}$ were reported for a recently isolated enzyme with TyrAT activity (Prabhu and Hudson, 2010). In contrast, a mouse TyrAT displayed a $K_m$ of 1.8 mM for L-tyrosine, 11.4 mM for phenylalanine, and 4.9 mM for glutamate (Mehere et al., 2010). T. cruzi TyrAT showed a broader substrate specificity that extended to L-alanine (Nowicki et al., 2001; Sivaraman and Kirsch, 2006). Interestingly, the affinity of Ps TyrAT for L-tyrosine is lower than that of one reported enzyme from Arabidopsis, similar to that of mouse TyrAT, and higher than that of TyrAT isoforms from A. officinalis cell cultures. TyrATs from mammals (Andersson and Pispa, 1982; Mehere et al., 2010) and T. cruzi (Blankenfeldt et al., 1999) have been shown to function as homodimers, whereas TyrATs from Arabidopsis (Lopukhina et al., 2001) and A. officinalis (De-Eknamkul and Ellis, 1987a) were reported as a homodimer and a homotetramer, respectively. Accordingly, the $k_{cat}$ of Ps TyrAT was calculated assuming that the enzyme functions as a homodimer. The catalytic efficiencies ($k_{cat}/K_m$) toward L-phenylalanine and L-tryptophan were much lower than that of L-tyrosine (with α-ketoglutarate), in support of a predominant role in the interconversion of L-tyrosine and α-ketoglutarate (Table 1).

A high $K_m$ for tyrosine suggests that the cellular pools of this aromatic amino acid are comparatively abundant. Although its absolute levels in opium poppy have not been determined,
tyrosine appears to be generally abundant in plants. The concentration of tyrosine in the phloem sap of barley leaves was reported as high as 1.6 ± 1.3 mM (Winter et al., 1992). In the phloem sap of *Papaver dubium*, tyrosine levels were higher than other amino acids (Wilkinson et al., 2001). Similarly, tyrosine was also more abundant than other amino acids in elicitor-treated opium poppy cell cultures (Zulak et al., 2008).

The highest transcript levels for most biosynthetic genes involved in BIA metabolism occurs in stems and roots (Bird et al., 2003; Samanani et al., 2006). In particular, transcripts encoding TYDC and NCS, the other known enzymes involved in the early step of BIA pathway, were abundant in stems and roots and were found only at low levels in leaves and developing seed capsules (Facchini and De Luca, 1995; Lee and Facchini 2010). The distribution of Ps TyrAT transcripts was consistent with a preeminent role of stems and roots in the biosynthesis of BIAs in opium poppy (Figure 6). NCS (Lee and Facchini, 2010) and other BIA biosynthetic enzymes are also specifically localized, or at least most abundant in sieve element of the phloem in opium poppy (Bird et al., 2003; Samanani et al., 2006). The occurrence of TyrAT in sieve elements would facilitate access to L-tyrosine translocated in the phloem to ensure adequate precursor availability for BIA biosynthesis. The localization of aminotransferase isoforms to different subcellular compartments has previously been reported (Wightman and Forest, 1978).

The application of VIGS as an effective method to specifically silence targeted genes in opium poppy allows a direct investigation of the physiological role of putative biosynthetic enzymes (Hagel and Facchini, 2010; Lee and Facchini, 2010). The VIGS mechanism is based on cosuppression of a transgene and an endogenous gene involving the formation of double-stranded RNA (Robertson, 2004). Close homologs (>90% nucleotide sequence identity) might also be affected, but none of the TyrAT homologs displayed identities in this range (Supplemental Table 1). Transcripts corresponding to cl.10988 were unaffected by the VIGS-mediated reduction in TyrAT transcript levels (Supplemental Figure 6), in support of the specificity of silencing. VIGS facilitated a significant (P < 0.01) suppression of Ps TyrAT transcript levels in opium poppy stem to less than 20% of that found in control plants (Figure 7C). The combined mean abundance of the six major BIAs (i.e. morphine, codeine, oripavine, thebaine, noscapine, and papaverine) was also significantly reduced by almost 50% in plants that showed a reduction in Ps TyrAT transcript levels compared with controls using one-tailed (P<0.07) and two-tailed (P<0.14) t-test analyses (Figure 7D). The statistical analysis showed a...
balanced effect among TyrAT-VIGS plants. The alkaloid levels in several control plants were significantly higher than those in TyrAT-VIGS plants, whereas most of TyrAT-VIGS plants contained lower to similar alkaloid levels compared with controls. A reduction in the accumulation of several individual BIAs was also detected in plants with suppressed Ps TyrAT transcript levels (Figure 7E), but the natural variation in the ratio of some pathway end products and intermediates reduced statistical confidence when metabolites were considered separately. The correlation between transcript levels and total alkaloid accumulation supports a major physiological role for TyrAT in the generation of precursors for BIA metabolism. The modest correlation between transcript and total alkaloid levels was potentially influenced by several factors. The major reason might be that plant aminotransferases generally exhibit broad substrate specificity, which suggests that other gene products can utilize L-tyrosine as an amino donor and contribute to the cellular pool of 4-HPP. Purified plant aromatic aminotransferases possess properties similar to most animal and microbial aminotransferases. Several reports have suggested that plant tryptophan and aspartate aminotransferase have the same substrate multispecificity (Bonner and Jensen, 1985) as mammalian and microbial aspartate aminotransferases (Mavrides and Orr, 1975). Plant aspartate aminotransferases have been shown to transaminate five L-amino acids: aspartate, glutamate, phenylalanine, tyrosine, and tryptophan using α-ketoglutarate or oxaloacetate as the amino group acceptors (Forest and Wightman, 1972). Moreover, tryptophan aminotransferase was able to catalyze the transamination of other aromatic amino acids including L-tyrosine (Truelsen, 1972; Noguchi and Hayashi, 1980; McQueen-Mason and Hamilton, 1989; Koshiba et al., 1993). The contribution of TyrAT homologs (Supplemental Figure 1; Supplemental Table 1) to maintaining balance in the cellular pools of L-tyrosine and 4-HPP cannot be ruled out.

The complex network of aromatic amino acid metabolism provides substrates leading to the biosynthesis of numerous specialized metabolites with diverse physiological functions. In the formation of rosmarinic acid, L-phenylalanine and L-tyrosine are converted to 4-coumaroyl-CoA and 4-hydroxyphenyllactate, respectively. The synthesis of tocopherols, tocotrienols, and plastoquinones share the same aromatic precursor, homogentisic acid, which is synthesized from 4-HPP by 4-hydroxyphenylpyruvate dioxygenase (HPPD). Interestingly, the content of 4-HPP was stable after treatment of S. miltiorrhiza hairy root cultures with MeJA despite an increase in the transcripts levels of HPPD and 4-hydroxyphenylpyruvate reductase (HPPR), which catalyzes
the conversion of 4-HPP to 4-hydroxylphenyllacetic acid (Xiao et al., 2009). In this case, 4-HPP was suggested to not only as a amino group acceptor, but also as a co-substrate for HPPD and HPPR. Additional metabolic pressure on the cellular pool of 4-HPP could account, in part, for the modest correlation between TyrAT transcript levels and BIA accumulation in opium poppy.

Negative feedback mechanisms has been implicated in the regulation of metabolic pathways involving TyrAT. For example, TyrAT activity was inhibited by α-aminooxyacetic acid (AOA) or α-aminooxy-β-phenylpropionic acid (AOPP), which are also inhibitors of PAL and PLP-dependent enzymes in general (De-Eknamkul and Ellis, 1987b). Furthermore, TyrAT activity was also inhibited by dihydroxy-phenyl-lactic acid (DOPL), a tyrosine metabolite and an intermediate in rosmarinic acid biosynthesis (De-Eknamkul and Ellis, 1987a). Clearly, further investigation is required to better understand the regulation of L-tyrosine catabolism in the context of BIA metabolism. Nevertheless, the biochemical characterization of a recombinant TyrAT in vitro coupled with the physiological evaluation of function in the plant supports the role of 4-HPP as an intermediate in the formation of BIA precursors.

MATERIALS AND METHODS

Chemicals

L-Tyrosine and 4-hydroxyphenylpyruvate were purchased from Sigma-Aldrich (St. Louis, MO; www.sigmaaldrich.com). [14C(U)]-L-Tyrosine (74 kBq, specific activity 450 mCi mmol⁻¹) was purchased from American Radiolabeled Chemicals (St. Louis, MO; www.arc-inc.com). Benzylisoquinoline alkaloid standards were obtained or synthesized as described previously (Hagel and Facchini, 2010). [14C(U)]-4-Hydroxyphenylpyruvate was synthesized from [14C(U)]-L-tyrosine by modifying methods described previously (Barta and Böger, 1996; Rueffer and Zenk, 1987). Briefly, a 20 µL portion of [14C(U)]-L-tyrosine was diluted with phosphate buffer (0.1 M, pH 6.5, 130 µL). Then, 5,000 units of bovine liver catalase (activity 2000~5000 U mg⁻¹, Sigma-Aldrich) and 5 mg of crude L-amino acid oxidase from Crotalus adamanteus L. (Sigma-Aldrich) were added. The mixture was incubated in an open vial with shaking at room temperature for 80 min and was then loaded onto a column containing 500 µL of Dowex 50W
X8 (Sigma-Aldrich) resin equilibrated with 1.0 M hydrochloric acid. The radioactive product was eluted with 2 mL of 0.1 M hydrochloric acid, and subsequently purified on a silica gel 60 F<sub>254</sub> TLC plate (EMD Chemicals, Gibbstown, NJ; www.emdchemicals.com) using ethyl acetate:methanol (4:1, v/v) as the mobile phase.

**Isolation and Cloning of Opium Poppy TyrAT**

A full-length cDNA encoding Ps TyrAT was identified by screening an in-house 454 pyrosequencing database (Desgagné-Penix et al., 2010) for unigenes annotated as PLP-dependent aminotransferases. BLASTx analysis of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) was performed to identify TyrAT orthologs. A codon-optimized synthetic gene encoding the Ps TyrAT enzyme was constructed to improve recombinant protein production in *Escherichia coli* (GenScript, Piscataway, NJ; www.genscript.com). The synthetic gene was amplified by PCR using forward (GAGCTCATGGAGAAAGGGGGGAAGA) and reverse primers (AAGCTTTTACTGTTTGGCGTGCC) containing SacI and HindIII restriction sites, respectively. The amplicon was digested with SacI and HindIII, and cloned into the corresponding sites of pQE-30 vectors (Qiagen, Valencia, CA; www.qiagen.com).

**Heterologous Expression and Purification of Recombinant TyrAT**

The pQE-TyrAT plasmid encoding a translational fusion between Ps TyrAT and an N-terminal His<sub>6</sub>-tag purification tag was expressed in *E. coli* strain SG13009. Transformed bacteria were grown at 37ºC to an OD<sub>600</sub> of 0.4, and were then induced with 0.3 mM isopropyl β-thiogalactopyranoside at room temperature for 4 h. The bacteria were collected by centrifugation at 13,000 g for 10 min, resuspended in 200 mM Tris, 100 mM KCl, 10% (w/v) glycerol, pH 7.5 and lysed by sonication (5 times, 10 seconds each). Debris was collected by centrifugation at 13,000 g for 10 min and the supernatant was used for affinity purification of the recombinant protein over Talon His-Tag Purification Resin (Clontech, Mountain View, CA; www.clontech.com). Proteins eluting between 10-100 µM imidizole were analyzed by SDS-PAGE on a 12% (w/v) acrylamide gel, and visualized using Coomassie Brilliant Blue R-250 stain. Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA; www.bio-rad.com).
Enzyme Assays and Recombinant Protein Characterization

Purified, recombinant TyrAT protein was desalted using a PD-10 column (GE Healthcare, Baie d’Urfé, Canada; www.gehealthcare.com) equilibrated with 100 mM HEPES buffer, pH 8.0. For LC-MS/MS analysis, the reaction mixture contained 2 μg of purified Ps TyrAT protein, 0.1 mM PLP, 0.1 mM EDTA, 0.3 mM α-ketoglutarate, and 3 mM L-tyrosine in HEPES buffer at pH 8.2, to a total volume of 100 μL. As a control, purified TyrAT protein was denatured by boiling for 10 min. For TLC analysis, enzyme assays consisted of 0.1 mM PLP, 0.1 mM EDTA, 0.3 mM α-ketoglutarate, [14C(U)]-L-tyrosine and purified, recombinant TyrAT protein in 100 mM HEPES buffer, pH 8.0, in a total volume of 100 μL. Different concentrations of [14C(U)]-L-tyrosine and amounts of recombinant protein were tested to optimize the enzyme assay.

Reactions were incubated for 1 h at 30°C and terminated by adding 50 μL of 1.0 N HCl. Products were extracted with 1.0 mL of ethyl acetate, which was subsequently evaporated under reduced pressure. The residue was dissolved in 10 μL of methanol and samples were applied to a silica gel 60 F254 TLC plate (EMD Chemicals). Compounds were separated using a mobile phase of ethyl acetate:methanol (4:1, v/v). The reaction product was identified based on its $R_f$ value compared with that of authentic [14C(U)]-4-HPP, and quantified relative to [14C(U)]-L-tyrosine.

Enzyme kinetics were obtained by monitoring the absorbance of various transamination reaction products as described previously (Collier and Kohlhaw, 1972; De-Eknamkul and Ellis, 1987): 4-hydroxyphenylpyruvate at 331 nm ($\varepsilon_{331} = 19,500 \text{ M}^{-1} \text{ cm}^{-1}$), phenylpyruvate at 320 nm ($\varepsilon_{331} = 17,500 \text{ M}^{-1} \text{ cm}^{-1}$), and indole-3-pyruvate at 328 nm ($\varepsilon_{331} = 10,000 \text{ M}^{-1} \text{ cm}^{-1}$) corresponding to the substrates L-tyrosine, L-phenylalanine, and L-tryptophan. The standard assay contained the indicated concentrations of an aromatic amino acid and an organic acid, 2 μg of purified recombinant TyrAT protein, 0.1 mM PLP, 0.1 mM EDTA, and 100 mM HEPES buffer, pH 8.2, in a total volume of 250 μL. The following substrate concentrations were used: 0.1 to 30 mM L-tyrosine, 0.1 to 40 mM L-phenylalanine, 0.1 to 50 mM L-tryptophan, 0.05 to 30 mM α-ketoglutarate, 0.05 to 150 mM pyruvate, and 0.05 to 300 mM oxaloacetate. Reactions were incubated for 1 h at 30°C and terminated by adding a 70 μL of 2.0 N NaOH. $V_{\text{max}}$ and $K_m$ values were calculated according to a nonlinear regression of the Michaelis-Menten equation where $V =$
\[
\frac{V_{\text{max}}S}{(K_m+S)} \quad \text{(Hernández and Ruiz, 1998)}.
\]

The \textit{kcat} value is defined as \(V_{\text{max}}/E_t\) where \(E_t\) is the total enzyme concentration.

**Virus-Induced Gene Silencing**

Virus-induced gene silencing (VIGS) was used to determine the effect of reducing the abundance of the Ps TyrAT transcript on the accumulation of BIAs in opium poppy plants. A 492-bp fragment of the Ps TyrAT coding region was amplified by PCR to introduce \textit{Eco}RI and \textit{SacI} restriction sites using forward (GAATTCGATAGTGCCTGGTTTACGAC) and reverse (GAGCTCCTGTGTTGCGTGTCCTAC) primers. The amplicon was cloned into the corresponding restriction sites of pTRV2 vector to produce the pTRV2-TyrAT construct. 

\textit{Agrobacterium tumefaciens} strain GV3101 harboring pTRV1 and pTRV2 empty vector (EV), or pTRV1 and pTRV2-TyrAT, were cultured at 28°C in 300 mL of LB medium containing 10 mM 2-(\(N\)-morpholino) ethanesulfonic acid (MES), 20 \(\mu\)M acetosyringone, and 50 \(\mu\)g mL\(^{-1}\) kanamycin. Bacteria were pelleted at 3,000 \(g\) for 15 min and resuspended in infiltration buffer (10 mM MES, 200 \(\mu\)M acetosyringone, 10 mM MgCl\(_2\)) to an OD\(_{600}\) of 2.5. Two-week-old opium poppy (\textit{Papaver somniferum} L. cultivar Bea’s Choice) seedlings were infiltrated using a 1-cc syringe with a 1:1 (v/v) mixture of \textit{A. tumefaciens} cultures harboring pTRV1 and either pTRV2-TyrAT or pTRV2-EV. Infiltrated plants were analyzed at maturity (i.e. the emergence of flower buds). Stem sections were excised below the flower bud, young stem tissue was flash frozen in liquid N\(_2\) for RT-qPCR analysis, and 10 \(\mu\)L of exuding latex was collected for HPLC analysis.

**RT-qPCR**

Plant tissue was ground under liquid nitrogen and extracted in 0.8 M guanidinium thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, pH 5.0, 5\% (v/v) glycerol, and 38\% (v/v) Tris-buffered phenol. Subsequently, 200 \(\mu\)L CHCl\(_3\) was added and the mixture emulsified. Samples were centrifuged and 400 \(\mu\)L of the aqueous phase was precipitated with 500 \(\mu\)L of isopropanol. After centrifugation, the supernatant was discarded and the pellet was washed with 70\% (v/v) ethanol. The RNA was reduced to dryness and resuspended in 30 \(\mu\)L of sterile water. First-strand cDNA was synthesized from 100-400 ng of total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA; [www.invitrogen.com](http://www.invitrogen.com)), an oligo(dT)20VN primer (2.5 \(\mu\)M), RT buffer (5X first strand buffer), dNTPs (0.5 mM each), and
DTT (5 mM) in a total reaction volume of 20 μL. The occurrence of cDNAs corresponding to TRV2 coat protein was determined by PCR as described previously (Rotenberg et al., 2006).

RT-qPCR was performed using SYBR Green detection on triplicate technical and triplicate biological replicates for each of 8 plants shown to contain TRV2 coat protein transcripts, and infiltrated with A. tumefaciens harboring pTRV2-EV or pTRV2-TyrAT. Reactions were performed in total volume of 10 μL and contained 5 μL of SYBR Green PCR mix (Applied Biosystems, Carlsbad, CA; www.appliedbiosystems.com), 1 μL of cDNA and 5 μM of PCR primers. PCR conditions were 2 min at 50 ºC and 10 min at 95 ºC, followed by 40 cycles of denaturation at 95ºC for 15 s each and annealing/extension at 72°C for 60 s each. Primers used for the relative quantification of transcripts were qRT-FW1 (GTGAAAACAACACATAAATTC) and qRT-RV1 (CCAACTGCTACGATTGAGCAC) for Ps TyrAT, and qRT-FW2 (CAACAGTAGCAATAATCCGC) and qRT-RV2 (ACTAGCAGATACTTGCAACA) for cl.10988. Threshold (Ct) values of Ps TyrAT and cl.10988 were normalized against the Ct of ubiquitin from opium poppy (Genbank accession number JN402989), which served as the reference transcript. Primers used for the quantification of ubiquitin transcripts were qRT-FW3 (TACCCTCCATTTGGTGCTTC) and qRT-RV3 (CCTCTGCTGATCTGGAGGAA). Fluorescent signal intensities were recorded and analyzed on an Applied Biosystems 7300 Real Time PCR System and SDS software. Dissociation curves for each amplicon were generated to confirm the presence of a single amplification product. The relative gene expression of Ps TyrAT was compared in plants infiltrated with pTRV1/pTRV2-EV and pTRV1/pTRV2-TyrAT using the 2^-ΔΔCt method (Livak and Schmittgen, 2001).

**Gene expression analysis**

Total RNA was isolated from opium poppy organs as described above and cDNAs were synthesized as described above. The cDNA samples served as templates for RT-qPCR analysis to determine the relative abundance of Ps TyrAT transcripts in each organ using the primers and conditions described for the analysis of plant subjected to VIGS analysis. PCR products were purified and sequenced to verify the identity of amplified cDNAs.

**High-Performance Liquid Chromatography**
Latex samples were reduced to dryness to determine dry weight and subsequently resuspended in methanol at a concentration 30 mg mL$^{-1}$. Ten microliters of each extract was diluted to a total volume of 100 μL with solvent A (98% (v/v) water: 2% (v/v) acetonitrile: 0.02% (v/v) phosphoric acid). HPLC was performed using a System Gold HPLC (Beckman-Coulter; www.beckmancoulter.com) equipped with a LiChrospher 60 RP Select B column (146 x 4.1 mm, 5 μm; Merck; www.merck.com) and a mobile phase consisting of solvent A [2% (v/v) acetonitrile; 98% (v/v) water] and solvent B [98% (v/v) acetonitrile; 2% (v/v) water] each containing 0.02% (v/v) phosphoric acid. The column was equilibrated in solvent A and alkaloids were eluted at a flow rate of 1.5 mL min$^{-1}$ using the following gradient: 0-1 min to 10% (v/v) solvent B, 1-50 min to 100% (v/v) solvent B, 50-53 min to 2% (v/v) solvent B, 53-60 min hold at 2% (v/v) solvent B. Dextromethorphan was used as a internal standard. Peaks corresponding to morphine, codeine, thebaine, noscapine, papaverine, and dextromethorphan were monitored at 210 nm and identified on the basis of retention times and UV spectra compared with those of authentic standards. BIA levels were expressed as picogram of alkaloid per μg dry weight$^{-1}$ of latex based on standard quantification curves determined using authentic compounds.

**Liquid chromatography-tandem mass spectrometry**

Enzyme assays contained 2 μg of native or heat-inactivated Ps TyrAT protein, 3 mM L-tyrosine, 1 mM PLP, 0.1 mM EDTA, and 0.3 mM α-ketoglutarate in a total volume of 100 μL HEPES buffer, pH 8.2. Reactions were incubated for 1 h at 30 °C and subsequently diluted with 250 μL of acetonitrile:water (55:45; v/v). L-Tyrosine and 4-HPP standards were prepared by diluting the pure compound dissolved in water with acetonitrile:water (55:45; v/v) to yield final concentration of 500 nM. Enzyme assays were analyzed by LC-MS/MS using a 6400 Triple Quadrupole ESI-MS/MS (Agilent Technologies; www.agilent.com). The Zorbax SB-C18 2.1 mm x 50 mm column containing 1.8 μM particles was run at 45°C. The mobile phase was set for elution with gradients from 5 to 95% (v/v) acetonitrile in water, and at a flow rate 0.5 mL/min for 5 min. The mobile phase returned to 5% (v/v) acetonitrile with a 3 min re-equilibration period. Fragment voltages of 90 V and 60 V were used for the analyses of L-tyrosine and 4-HPP, respectively. In negative ESI mode, the voltage was 4,000 kV, the gas flow was 10 L min$^{-1}$, nebulising pressure was 30 psi, and the gas temperature was 350°C. Collision energy was set at 0 eV and/or -10 eV, which showed the most specific and intense fragment ion for each compound.
In full scan mode, nine [M-H]⁻ ions for L-tyrosine (i.e., m/z 180.1, 163.1, 136.8, 119.2, 107.2, 105.5, 93.1, 74.3, 71.8) and four [M-H]⁻ ions for 4-HPP (i.e., m/z 179.1, 151.1, 114.7, 107.1) were detected.

**Phylogenetic Analysis**

BLASTx searches of the NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and in-house opium poppy nucleotide sequence databases were used to identify orthologs of Ps TyrAT. The multiple sequence alignments and neighbor-joining phylogenetic tree were generated using ClustalW2 (http://www.ebi.ac.uk). The bootstrap analysis was performed using TREECON (Van de Peer and De Wachter, 1994). Difference in the transcript and alkaloid levels of pTRV2-TyrAT and TRV2-EV plants were analyzed by one or two-tailed Student’s t-test and regression analysis.

Sequence data from this article can be found in the Genbank/EMBL data libraries under the accession numbers GU370929 (Ps TyrAT); JN402988 (synthetic Ps TyrAT); JN402989 (ubiquitin); JN542549 (cl.10988); JN542550 (cl.1232); JN542551 (cl.1670); JN542552 (cl.8533); JN542553 (cl.615); JN542554 (cl.5707).

**Supplemental data**

**Supplemental Figure 1.** Alignment of six homologs isolated from opium poppy transcriptome databases and encoding putative PLP-dependent aminotransferases.

**Supplemental Figure 2.** Amino acid sequence alignment of tyrosine aminotransferase (Ps TyrAT) from opium poppy with other plant tyrosine aminotransferases. The catalytic site is the lysine residue shown in red and the pyridoxal-5’-phosphate binding sites are indicated in blue. Abbreviations are defined in the legend of Figure 2.

**Supplemental Figure 3.** Detection of TyrAT enzyme activity by thin-layer chromatography. A, Visualization of [¹⁴C(U)]-4-HPP by autoradiography after incubating different quantities of recombinant Ps TyrAT and [¹⁴C(U)]-L-tyrosine at 30°C for 1 h. B, Relationship between the amount of Ps TyrAT protein in the enzyme assay and the formation of [¹⁴C(U)]-4-HPP. C,
Relationship between the amount of $[^{14}\text{C}(\text{U})]$-L-tyrosine in the enzyme assay and the formation of $[^{14}\text{C}(\text{U})]$-4-HPP. Standard assays were performed using quantities of recombinant protein and L-tyrosine in the linear range.

**Supplemental Figure 4.** Relative substrate specificity of TyrAT from opium poppy. Enzyme assays consisted of 2 μg of recombinant Ps TyrAT protein, 0.1 mM PLP, 0.1 mM EDTA, 5 mM of each L-amino acid substrate with 0.5 mM α-ketoglutarate, or 0.5 mM of each α-keto acid with 3 mM L-tyrosine in 100 mM HEPES buffer, pH 8.0. Reactions were incubated at 30ºC for 1 h. Relative substrate specificity was calculated as a percentage of conversion of each substrate compared with the conversion of L-tyrosine or α-ketoglutarate.

**Supplemental Figure 5.** Steady-state enzyme kinetics of purified recombinant Ps TyrAT with various substrates at different concentrations. $V_{\text{max}}$ and $K_m$ values were calculated according to a nonlinear regression of the Michaelis-Menten equation where $V = \frac{(V_{\text{max}}S)}{(K_m+S)}$. Using different concentrations of L-tyrosine (A), L-phenylalanine (B), and L-tryptophan (C) as amino group donors, enzyme activity was determined from the increase in the absorbance of assays monitored at 331, 320, and 328 nm corresponding to 4-hydroxyphenylpyruvate, phenylpyruvate, and indole-3-pyruvate, respectively. Using different concentrations of α-ketoglutarate (D), pyruvate (E), or oxaloacetate (F) as amino group acceptors, enzyme activity was determined by measuring an increase in absorbance at 331 nm resulting from the transamination of L-tyrosine to 4-hydroxyphenylpyruvate. Molar extinction coefficients were used to calculate the quantity of each reaction product. Incubation time and protein concentration were optimized prior to enzyme kinetic analyses. Values represent the mean specific activity ± standard deviation monitored as a function of substrate concentration for three independent replicates.

**Supplemental Figure 6.** Mean cl.10988 transcript levels in plants infiltrated with pTRV2-TyrAT (black bar) and pTRV2-EV (white bar).

**Supplemental Table 1.** Sequence similarity of six homologs isolated from opium poppy transcriptome databases and encoding putative PLP-dependent aminotransferases compared with Ps TyrAT.
ACKNOWLEDGMENTS

We thank Dr. Shaobo Wu for constructing the pQE30-TyrAT and pTRV2-TyrAT vectors, and Scott Farrow for assistance with the LC-MS/MS analysis.

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Table 1. Kinetic parameters for TyrAT from opium poppy. The data represent the mean of three independent measurements ± standard deviation. The $K_m$ and $V_{max}$ values were calculated from the Michaelis-Menten equation using a least-squares method. The $k_{cat}$ value was calculated by dividing $V_{max}$ by $E_t$ (the number of pmol of enzyme in each assay).

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<th>Co-substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (μmol min$^{-1}$ mg$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
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Figure legends

Figure 1. Pathway leading to the formation of (S)-norcoclaurine, the central intermediate in the biosynthesis of benzylisoquinoline alkaloids (BIAs) in plants, from two molecules of L-tyrosine. Dopamine is derived via decarboxylation and 3-hydroxylation of L-tyrosine, although the prevailing reaction order is not known. Tyrosine/dopa decarboxylase (TYDC) has been shown to accept L-tyrosine and L-dopa as substrates. However, the enzyme (1) responsible for the 3-hydroxylation of L-tyrosine or tyramine has not been identified. 4-Hydroxyphenylacetaldehyde (4-HPAA) is suggested to result from the transamination of L-tyrosine and the subsequent decarboxylation of 4-hydroxyphenylpyruvate (4-HPP). Tyrosine transaminase (2) and 4-HPP decarboxylase (3) activities have been reported in BIA-producing plants, but the corresponding enzymes have not been isolated. The condensation of dopamine and 4-HPPA is catalyzed by (S)-norcoclaurine synthase (NCS).

Figure 2. Unrooted neighbour-joining tree of showing the phylogenetic relationships between opium poppy TyrAT and related plant proteins. Numbers in the tree refer to the bootstrap values for each node over 1,000 iterations. Numbers in parentheses show the percent amino acid identity of each protein compared with TyrAT from opium poppy. The annotations and Genbank accession numbers of each protein are as follows: Ps TyrAT, Papaver somniferum L-tyrosine aminotransferase (GU370929); Os TyrAT, Oryza sativa japonica group putative nicotianamine aminotransferase (BAF95202); Sp TyrAT, Solanum pennelli putative tyrosine aminotransferase (ADZ24702); Rc TyrAT, Ricinus communis putative tyrosine aminotransferase (XP_002517869); Pt TyrAT, Populus trichocarpa aminotransferase family protein (XP_002328046); Ss TyrAT, Solenostemon scutellaridoides putative tyrosine aminotransferase (CAD30341); Sm TyrAT, Salvia miltiorrhiza putative tyrosine aminotransferase (ABC60050); Mt TyrAT, Medicago truncatula putative tyrosine aminotransferase (AAY85183); Gm TyrAT, Glycine max putative tyrosine aminotransferase (AAY21813); Cm TyrAT, Cucumis melo aromatic amino acid transaminase (ADC45389); At TyrAT-1, Arabidopsis thaliana coronatine-regulated tyrosine aminotransferase (TAT1) (AAN15626); At TyrAT-2, Arabidopsis thaliana L-tyrosine:2-oxoglutarate aminotransferase (TAT3) (NP_180058); At TyrAT-3, Arabidopsis thaliana rooty/superroot1 protein (AAG37062); At TyrAT-4, Arabidopsis thaliana tyrosine aminotransferase (NM_124776).
Figure 3. Purification of His6-tagged, recombinant Ps TyrAT from *Escherichia coli* total soluble protein extracts by cobalt-affinity chromatography. Elutions were performed using increasing imidazole concentrations: 10 mM (lane 1), 30 mM (lane 2), 50 mM (lane 3), and 100 mM (lane 4). Lanes contain 20 μL of each fraction, which were separated by SDS-PAGE and visualized using Commassie Brilliant Blue G-250 stain.

Figure 4. Extracted ion chromatograms of *m/z* 179.1 for enzyme assays using native and heat-inactivated Ps TyrAT protein, and the corresponding CID spectrum for the compound eluting at the retention time of 0.54 min. Enzyme assays contained 2 μg of purified, recombinant Ps TyrAT protein incubated with 0.1 mM PLP, 0.1 mM EDTA, 0.3 mM α-ketoglutarate, and 3 mM L-tyrosine for 1 h at 30 ºC. Boiled Ps TyrAT protein was used as the heat-inactivated control. Using the native enzyme, multiple reaction monitoring (MRM) in negative mode showed peaks corresponding to 4-HPP using the fragment ions at *m/z* 151.1 and *m/z* 107.1 for the precursor ion at *m/z* 179.1. No peaks corresponding to 4-HPP were detected using the heat-inactivated enzyme. Collision induced fragment scan (CID) in the range of *m/z* 20-200 confirmed that the extracted ion spectra were derived from 4-HPP by the presence of a fragment ion at *m/z* 107.1 (inset).

Figure 5. Effect of pH on Ps TyrAT activity. Assays were performed for 1 h at 30ºC in HEPES buffer at the pH values indicated, in the presence of 2 μg purified enzyme, 3 mM L-tyrosine, 0.1 mM PLP, 0.1 mM EDTA, and 0.5 mM α-ketoglutarate.

Figure 6. Relative transcript abundance of Ps TyrAT in different opium poppy organs. First-strand cDNAs were synthesized from total RNA and used as a template for RT-qPCR analysis. The transcript abundance of ubiquitin from opium poppy was used as an internal control and relative values were normalized to the Ps TyrAT transcript level in roots. Bars represent the mean ± standard deviation of triplicate experiments.

Figure 7. Effect of reducing Ps TyrAT transcript levels by virus-induced gene silencing (VIGS) in opium poppy plants. A, A 492-bp fragment of the Ps TyrAT coding region was inserted into pTRV2 vector. Two-week-old poppy seedlings were coinfiltrated with pTRV1 and pTRV2
empty vector (EV) or with pTRV1 and pTRV2-TyrAT. After approximately 10 weeks, stem and latex samples were used to determine relative Ps TyrAT transcript abundance and BIA levels, respectively. B, Ethidium bromide-stained agarose gels showing the detection of TRV2 coat protein transcripts in cDNAs synthesized from total stem RNA. C, Mean Ps TyrAT transcript levels in plants infiltrated with pTRV2-TyrAT (black bar) and pTRV2-EV (white bar). D, Relative accumulation of total major BIAs in latex extracted from plants infiltrated with pTRV-TyrAT (black bar) and pTRV2-EV (white bar). E, Relative accumulation of individual BIAs in latex extracted from plants infiltrated with pTRV-TyrAT (black bars) and pTRV2-EV (white bars). Bars represent the mean ± SD of three technical replicates performed on each of three biological replicates for each of eight infiltrated plants.
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Percent identity

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PsTyrAT
A) 492 bp

B) TRV2-TyrAT         TRV2-EV

bp

613

C) Relative Tyr AT transcript level

D) Alkaloid content (pg μg⁻¹ latex [dry weight])

E) Alkaloid content (pg μg⁻¹ latex [dry weight])