A tyrosine aminotransferase involved in rosmarinic acid biosynthesis in *Prunella vulgaris* L

Mei Ru¹, Kunru Wang¹, Zhenqing Bai⁴, Liang Peng², Shaoxuan He³, Yong Wang¹ & Zongsuo Liang¹,⁴

Rosmarinic acid (RA) and its derivants are medicinal compounds that comprise the active components of several therapeutics. We isolated and characterised a tyrosine aminotransferase of *Prunella vulgaris* (PvTAT). Dduced PvTAT was markedly homologous to other known/putative plant TATs. Cytoplasmic localisation of PvTAT was observed in tobacco protoplasts. Recombinantly expressed and purified PvTAT had substrates preference for L-tyrosine and phenylpyruvate, with apparent **Km** of 0.40 and 0.48 mM, and favoured the conversion of tyrosine to 4-hydroxyphenylpyruvate. In vivo activity was confirmed by functional restoration of the *Escherichia coli* tyrosine auxotrophic mutant DL39. *Agrobacterium rhizogenes*-mediated antisense/sense expression of PvTAT in hairy roots was used to evaluate the contribution of PvTAT to RA synthesis. PvTAT were reduced by 46–95% and RA were decreased by 36–91% with low catalytic activity in antisense transgenic hairy root lines; furthermore, PvTAT were increased 0.77–2.6-fold with increased 1.3–1.8-fold RA and strong catalytic activity in sense transgenic hairy root lines compared with wild-type counterparts. The comprehensive physiological and catalytic evidence fills in the gap in RA-producing plants which didn't provide evidence for TAT expression and catalytic activities in vitro and in vivo. That also highlights RA biosynthesis pathway in *P. vulgaris* and provides useful information to engineer natural products.

The popular medicinal plant, *Prunella vulgaris*, known as ‘heal-all’ or ‘self-heal’, has therapeutical applications in alleviating sore throats, reducing fever, and accelerating wound healing¹. Rosmarinic acid (RA), a phenolic ester, is a prominent secondary metabolite member of the Lamiaceae and Boraginaceae. This compound and its derivates show many notable biological and pharmacological activities, such as anti-colitic², antioxidant³, ⁴, anti-inflammatory⁵, ⁶, anti-leukemic⁷, and anticancer activities⁸, ⁹, as well as neuroprotective activity¹⁰, ¹¹, which has led to their pharmaceutical and analytical development, and examination in clinical studies. In vitro culture systems producing RA, including callus, suspension cell cultures, hairy root cultures, and shoot cultures, have been successfully established from many plant species, such as *Coleus blumei*¹², ¹³, *Anchusa officinalis*¹⁴, *Salvia miltiorrhiza*¹⁵, ¹⁶, *Salvia officinalis*¹⁷, *Agastache rugosa*¹⁸, *Ocimum basilicum*¹⁹, ²⁰, and *Coleus forskohlii*²¹. RA levels in some in vitro culture systems are higher than in the parent plant. Engineered production of RA and its analogues have also been achieved in an *Escherichia coli* culture system²²–²⁴.

Feeding radioactively labeled amino acids to *Mentha* plants and *Coleus* suspension cell cultures have revealed that phenylalanine (Phe) and tyrosine (Tyr) are the only respective amino acid precursors of the caffeoyl moiety and 3,4-dihydroxyphenyllactic acid (DHPL) moiety of RA²⁵–²⁷. Tyrosine aminotransferase (TAT) was confirmed as the entry point enzyme of the tyrosine-derived pathway in RA biosynthesis²⁸. Petersen, *et al.*²⁹ proposed that RA biosynthesis in *C. blumei* involves both the phenylpropanoid pathway (for the caffeic acid moiety) and the tyrosine-derived pathway (for the DHPL moiety) (Fig. 1). Enzymes and genes of RA biosynthesis are well investigated in *C. blumei*, *S. miltiorrhiza*, *Melissa officinalis*, *Lithospermum erythrorhizon*, and other members of the Lamiaceae and Boraginaceae families.³⁰, ³¹ TAT reversibly catalyses Tyr to 4-hydroxyphenylpyruvate (4-HPP), which is the substrate for pathways producing plastoquinone⁶, ³², tocopherols³³, phenolic acid³⁴, ³⁵, and benzylisoquinoline alkaloid (BIA)³⁶ in plants, although tyrosine is synthesised from 4-HPP by TAT in bacteria. These Tyr-derived

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plant metabolites have a noteworthy structural complexity and a variety of pharmacological and biological activities, making them effective nutritional compounds and pharmaceutical drugs. RA is a major component of phenolic acids in *Prunella vulgaris* and controls the quality of this medicinal plant according to National Chinese Pharmacopeia. Kim, *et al.* cloned the genes of enzymes involved in the phenylpropanoid branched-chain of the RA biosynthetic pathway in *P. vulgaris*, however, no biochemical or physiological information has been supported about these enzymes. Moreover, little information about enzymes involved in tyrosine-derived pathway is available in *P. vulgaris*.

Although the biochemical and structural characterisations of TATs in *E. coli*, *Trypanosoma cruzi*, mouse, *Leishmania infantum*, and *Homo sapiens* (Protein Data Bank code, 3dyd) are well established, considerably less information about these enzymes is available in plants. Three activities of TAT have been extensively purified and characterised from *A. officinalis* cell cultures. TAT has been implicated as the initial enzyme in tocopherol biosynthesis in Arabidopsis plants, in aromatic volatiles biosynthesis of *Cucumis melo* fruits, in BIA biosynthesis of *Papaver somniferum*, in an alternate pathway for phenylalanine biosynthesis. Aminotransferase activity has been shown for Arabidopsis TATs, *C. melo* ArAT, *P. somniferum* TAT, rose petunia PhPPY-AT, *Atropa belladonna* ArAT, and *Ephedra sinica* AroAT by activity measurements of the isolated proteins expressed in bacterial systems and/or complementation of the auxotrophic strain.

**Figure 1.** Biosynthetic pathway leading to rosmarinic acid formation in *Coleus blumei*. PAL: phenylalanine ammonia lyase; C4H: cinnamic acid 4-hydroxylase; 4CL: 4-coumaric acid CoA-ligase; TAT: tyrosine aminotransferase; HPPR: hydroxyphenylpyruvate reductase; RAS: rosmarinic acid synthase (4-coumaroyl-CoA:4′-hydroxyphenyllactic acid 4-coumaroyltransferase); 3- and 3′-hydroxylation can be catalyzed by cytochrome P450-monoxygenase.
E. coli strain DL39. Despite enhanced RA accumulation has been demonstrated by overexpressing TAT in S. miltiorrhiza hairy roots and transgenic P. frutescens plants25, 31. However, biochemical confirmation of their enzymatic activity has not been shown in major RA-producing plants. In plants, tocopherols and RA have been defined roles in free radical scavenging and in defence against abiotic and biotic stresses12, 50, and BIA are used by humans as stimulants, narcotics, and therapeutic agents51, 52. These natural products increase plant fitness and are also linked to potential benefits for human health.

Although much work has been done to identify the genes responsible for RA metabolism, the enzymes involved in the supply of precursors are still poorly defined in P. vulgaris. Here, we isolated and characterised tyrosine aminotransferase cDNA (PvTAT) using homology-based cloning, determined its subcellular localisation, characterised the heterologously overexpressed protein, and showed its functional complementation for tyrosine auxotrophic mutant DL39. Moreover, antisense/sense-based expression showed PvTAT is involved in RA biosynthesis in planta.

Results
Identification of a tyrosine aminotransferase as a candidate rosmarinic acid biosynthetic enzyme. A deep transcriptome database was generated by 454 GS–FLX Titanium pyrosequencing using a cDNA library prepared from P. vulgaris root (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) structure prediction tool also suggested that the PvTAT candidate possessed several homodimer interfaces. An unrooted neighbour-joining tree representing the phylogenetic relationships between the putative PvTAT and related enzymes is shown in Fig. 2b. PvTAT showed the highest sequence identity (76–93%) with S. miltiorrhiza SmTAT (AB600500), P. frutescens PPTAT (ADO17550.1), Solenostemon scutellarioides SsTAT (CAD30341), and Scutellaria baicalensis SbTAT (AIY98132.1) from the same Labiatae family. The PvTAT protein also exhibited considerable sequence identity (65–75%) with Solanum pennellii SpTAT (AD24702.1), A. belladonna Ab-ArAT1 (AHN10101.1), A. belladonna Ab-ArAT5 (AHN10105.1), Medicago truncatula MtTAT (AA685183.1), Glycine max GmTAT (AAY21813.1), and Theobroma cacao TcTAT (XP_007021573.1). However, it is important to note that there is no biochemical confirmation of the enzymatic activities of these proteins available. In contrast, PvTAT showed 43–74% sequence identity with P. somniferum PsTAT (ADC33123.1)15, Arabidopsis AtTAT1 (AAK82963.1)42, 43, AtTAT2 (AF301899.1)46, AtTAT5 (NP_198465.3)46, AtTAT7 (NP_200208.1)35, 47, C. melo CmAraAT1 (ADC45389)44, petunia PhPPY-AT (AH6A8287.1)45, and E. sinica AroAT1 (AGK24944.1)45, which have been shown to function as TATs. Additionally, A. belladonna Ab-ArAT4 (AHN10104.1) preferentially catalyses the transamination of phenylalanine to phenylpyruvate and had a sequence identity of 66%56. Well characterised TATs in mammals showed 30% sequence identity, while there was 12–15% sequence identity with bacterial TATs. All identities of the proteins used for phylogenetic analysis are provided in Supplementary Table S1.

Expression analysis of PvTAT. We measured the relative transcript expression level of PvTAT in different organs (roots, stems, leaves, and spikes) of P. vulgaris at complete flowering stage by qRT-PCR. PvTAT involved in RA biosynthesis was expressed in all the tissues but possessed distinct expression patterns. The highest transcript expression analysis of Ar-ArAT5. We measured the relative transcript expression level of Ar-ArAT5 in different organs (roots, stems, leaves, and spikes) of P. vulgaris at complete flowering stage by qRT-PCR. Ar-ArAT5 involved in RA biosynthesis was expressed in all the tissues but possessed distinct expression patterns. The highest transcript level in the roots was 4.6- and 2.8-fold higher than in the stems, while the transcript level in the spikes was set to 1 (Fig. 2c).

Cytoplasmic localisation of PvTAT. To experimentally determine the subcellular localisation of the PvTAT in planta, we expressed a PvTAT-GFP fusion protein in tobacco protoplasts. An engineered green-shifted green fluorescent protein (GFP), by itself or fused in frame to the C-terminus of TAT, was driven by the cauliflower mosaic virus (CaMV) 35S promoter. The resulting constructs or GFP alone were transformed into tobacco hairy roots and transgenic P. frutescens plants25, 31. However, biochemical confirmation of their enzymatic activity has not been shown in major RA-producing plants. In plants, tocopherols and RA have been defined roles in free radical scavenging and in defence against abiotic and biotic stresses12, 50, and BIA are used by humans as stimulants, narcotics, and therapeutic agents51, 52. These natural products increase plant fitness and are also linked to potential benefits for human health.

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Cytoplasmic localisation of PvTAT. To experimentally determine the subcellular localisation of the PvTAT in planta, we expressed a PvTAT-GFP fusion protein in tobacco protoplasts. An engineered green-shifted green fluorescent protein (GFP), by itself or fused in frame to the C-terminus of TAT, was driven by the cauliflower mosaic virus (CaMV) 35S promoter. The resulting constructs or GFP alone were transformed into tobacco leaves. As shown in Fig. 2d, when GFP alone was expressed, fluorescence was observed almost throughout the leaf. When PvTAT-GFP was expressed, fluorescence was observed in specific sectors of the leaf, suggesting that PvTAT is involved in RA biosynthesis in planta.
The substrate specificity of purified recombinant PvTAT was assessed using three aromatic amino acids as amino donors, together with several potential oxoacid acceptors as potential co-substrates. The activity of PvTAT indicates that it could use L-Tyr, L-Phe, and L-tryptophan (L-Trp) to varying degrees as amino donors and use α-ketoglutarate, pyruvate, oxaloacetate, and phenylpyruvate to diverse degrees as amino acceptors. With α-ketoglutarate as the amino acceptor, PvTAT shows higher preference for L-Tyr amino donor with an apparent $K_m$ of 0.40 mM than for L-Phe ($K_m$ of 10.2 mM) and L-Trp ($K_m$ of 8.14 mM) (Table 1; Supplemental Fig. S4a–c). The catalytic efficiency ($k_{cat}/K_m$) for L-Tyr was 20- and 23-fold greater than that of L-Phe and L-Trp. With L-Tyr as the amino donor, PvTAT preferentially utilises aromatic oxoacid phenylpyruvate with a $K_m$ of 3.90 mM as the amino acceptor, other than aliphatic oxoacid ($\alpha$-ketoglutarate, pyruvate, and oxaloacetate with a $K_m$ of 10.80, 52.06, and 4.72 mM, respectively) (Table 1; Supplemental Fig. S4d–g). In the reverse direction with L-glutamate (L-Glu) as amino donor, PvTAT favours 4-HPP as oxoacid acceptor with a $K_m$ of 0.48 mM (Table 1; Supplemental Fig. S4h–j). To confirm TAT enzyme activity, the native enzyme was compared to heat-inactivated enzyme as a negative control. We determined that PvTAT preferentially catalyses the transamination of L-Tyr to form 4-HPP, which is used as substrate in RA biosynthesis. Unexpectedly, PvTAT has a preference for phenylpyruvate as the preferred amino acceptor rather than $\alpha$-ketoglutarate. It is likely that PvTAT would catalyse the formation of phenylalanine from tyrosine and phenylpyruvate and that 4-HPP would be produced that in vivo would be channeled toward RA biosynthesis.
cat was calculated by dividing 
curveexpert1.4. Note: For the calculation of cat, a molecular weight of 65 kDa was assumed for the His-tagged 
assay measured the production of 4-hydroxyphenylpyruvate at 331 nm, phenylpyruvate at 320 nm, and indole-
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E.

| Variable substrate (Concentration in assay, mM) | Coubstrate (Concentration in assay, mM) | V_{max} (μmol min^{-1} mg^{-1}) | K_m (mM) | k_{cat} (s^{-1}) | k_{cat}/K_m (mM^{-1}s^{-1}) |
|-------------------------------------------------|----------------------------------------|-------------------------------|----------|----------------|-----------------------------|
| L-Tyr (0–4)                                      | α-ketoglutarate (10)                  | 1.74 ± 0.01                  | 0.40 ± 0.05 | 1.19 ± 0.009 | 3.03 ± 0.35                |
| L-Phe (0–50)                                     | α-ketoglutarate (10)                  | 2.27 ± 0.03                  | 10.2 ± 0.05 | 1.55 ± 0.02   | 0.15 ± 0.04                |
| L-Trp (0–50)                                     | α-ketoglutarate (10)                  | 1.53 ± 0.12                  | 8.14 ± 1.04 | 1.05 ± 0.08   | 0.13 ± 0.008               |
| α-ketoglutarate (0–100)                          | L-Tyr (5.5)                           | 0.92 ± 0.003                 | 10.80 ± 0.19 | 0.63 ± 0.002 | 0.06 ± 0.008               |
| Pyruvate (0–200)                                 | L-Tyr (5.5)                           | 1.30 ± 0.02                  | 52.06 ± 1.38 | 0.89 ± 0.01   | 0.02 ± 0.003               |
| Oxaloacetate (0–300)                             | L-Tyr (5.5)                           | 1.06 ± 0.02                  | 4.72 ± 0.05 | 0.73 ± 0.01   | 0.15 ± 0.005               |
| Phenylpyruvate (0–20)                            | L-Tyr (5.5)                           | 0.43 ± 0.014                 | 3.90 ± 0.067 | 0.30 ± 0.01   | 0.076 ± 0.001              |
| 4-HPP (0–10)                                     | L-Glu (10)                            | 0.18 ± 0.021                 | 0.48 ± 0.004 | 0.121 ± 0.01  | 0.295 ± 0.035              |
| Phenylpyruvate (0–4)                             | L-Glu (10)                            | 0.22 ± 0.009                 | 0.84 ± 0.01 | 0.15 ± 0.006  | 0.18 ± 0.009               |
| Oxaloacetate (0–40)                              | L-Glu (10)                            | 0.16 ± 0.006                 | 1.82 ± 0.03 | 0.110 ± 0.004 | 0.060 ± 0.004              |

Table 1. Kinetic parameters for PvTAT. Data are presented as means ± SD of three replicates. The Keq and 
V_{max} values were calculated from the Michaelis-Menten equation using a non-linear regression method with 
curveexpert1.4. Note: For the calculation of k_{cat}, a molecular weight of 65 kDa was assumed for the His-tagged 
assay measured the production of 4-hydroxyphenylpyruvate at 331 nm, phenylpyruvate at 320 nm, and indole-
leucine, isoleucine and valine, leading to auxotrophy for tyrosine, phenylalanine, aspartate, valine, isoleucine and, leucine. As shown in Fig. 3a, all trans-
formants could grow in M9 medium supplemented with tyrosine, phenylalanine, leucine, isoleucine and valine, whereas only clones transformed with PvTAT were able to grow on M9 medium devoid of tyrosine and phenyla-
lanine. Figure 3b displays the growth curve of the E. coli mutant transformed with the native plasmid or the plas-
mid expressing PvTAT. Bacteria transformed with PvTAT grew continuously, while the bacteria transformed with 
the native plasmid showed little growth over 20 hours. The complementation was only specific to the tyrosine and 
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complements the auxotrophic E. coli mutant and does not participate in the anabolic pathways for aspartate, 
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TAT functionally complements the E. coli tyrosine auxotrophic mutant DL39. To examine 
PvTAT activity in vivo, the recombinant pBAD33-TAT plasmid or pBAD3353 was transformed into the E. coli 
triple mutant DL3946 for an initial functional complementation assay. This strain has mutations in tyrB (tyrosine 
aminotransferase), aspC (aspartate aminotransferase), and ilvE (branched-chain aminotransferase), leading to 
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PvTAT is involved in RA synthesis in P. vulgaris. To determine whether PvTAT is involved in RA biosynthesis in planta, we used an antisense/sense expression-based approach to develop transgenic hairy root 
lines with regulated levels of PvTAT transcription. Wild-type hairy root lines with untransformed A. rhizogenes 
ATCC 15834 and hairy root lines transformed with empty vector pCAMBIA2300 were used as wide-type control 
and vector control, respectively. PCR amplification of rolC/B gene, nptII, and PvTAT confirmed the trans-
formed lines (Supplemental Fig. S5). Despite the relatively low transgenic rate, four antisense and five sense 
PvTAT-expressing hairy root lines were developed that showed 46–95% decreases and 0.77–2.6-fold increases, 
respectively, in transcription levels relative to the wild-type lines (Fig. 4a). To investigate the degree of decrease/increase in total TAT activity due to the antisense/sense expression of PvTAT, we assayed TAT activity in the hairy root lines. Total activity was reduced by 23–67% in antisense-expressing hairy root lines and increased by 43–60% in sense-expressing hairy root lines (Fig. 4b), consistent with the expectation that the antisense/sense expres-
sion of PvTAT leads to loss/gain of TAT activity. Reduced PvTAT expression and activity resulted in a decrease 
in RA levels by 36–92% in all downregulated lines. Increased PvTAT expression and activity led to an increase 
in RA accumulation by 31–83% in all overexpressing lines (Fig. 4c). The positive relationship between PvTAT 
expression, TAT activity, and RA accumulation suggests that PvTAT serves an important role in the production 
of RA in P. vulgaris (Fig. 4d). Together, these results provide in vivo evidence of the major role of PvTAT in RA biosynthesis in P. vulgaris.

Discussion
In plants, many substances are derived from tyrosine, such as RA, homogenitase, tocopherol, and BIA, which 
contribute to human health. RA and its numerous derivatives have many potential nutraceutical and pharma-
ceutical applications51. Since RA was first discovered and isolated in Rosmarinus officinalis52, researchers have 
been studying its biogenesis. Because of its biological and pharmaceutical importance, RA has been studied in 
many medicinal plant species. TAT genes involved in RA biosynthesis have been cloned from C. blumei, S. miltio-
rhiza46, and P. frutescens53. Aminotransferase activity are also implicated in the biosynthesis of aromatic volatiles, 
tocopherol, BIA, and tropane alkaloid and have been characterised in melons, Arabidopsis, P. somniferum, and A.

belladonna43, 42–44, 46, 50, 52, respectively. Here, we cloned the entry-point enzyme TAT from P. vulgaris, a member of the Labiatae. Through the isolation and characterisation of TAT from P. vulgaris, we provide biochemical and 
physiological evidence for the involvement of 4-HPP as an intermediate in RA biosynthesis (Fig. 1).
Based on a phylogenetic tree of sequence similarity, plant, mammalian, and bacterial TATs all differ from each other despite being grouped in the same subdivision within the same subgroup of the class I aminotransferases. Conserved amino acid residues responsible for catalysis and binding PLP are found in all proteins. Lys residue is the catalytic site interacting with the pyridine nitrogen of PLP. Arg in the highly conserved attachment site is one of the residues responsible for the interaction of Tyr with TAT. Although PvTAT shares only 15% amino acid identity with E. coli TAT, it was able to complement the E. coli mutant DL39. This was also the case for Arabidopsis At5g36160, At5g53970, and PhPPY-AT. None of these are involved in the anabolic pathways for leucine, valine, isoleucine, and aspartate.

In plants, despite L-Phe and L-Tyr are synthesised in plasmids from prephenate, which is derived from chorismate formed through the shikimate pathway. Secondary metabolites, like phenolic acid, flavonoids, volatiles, alkaloids, tocopherols, and cyanogenic glycoside and proteins are derived from the aromatic amino acids L-Phe and L-Tyr. However, plants have retained multiple TATs that likely utilise L-Phe and L-Tyr as substrates and are not plastid localised, but rather cytosol localised. A petunia PhPPY-AT localised in the cytosol was able to directly catalyse the formation of L-Phe from phenylpyruvate using L-Tyr as the amino donor, yielding 4-HPP as a by-product. Moreover, an A. belladonna ArAT4, which is not localised to any organelle, was able to directly catalyse the formation of L-Tyr from 4-HPP using L-Phe as the amino donor, yielding phenylpyruvate as a by-product. The two reactions are mutually inverse and are analogous to the formation of L-Phe and L-Tyr in bacteria, demonstrating the existence of a second, direct, extraplastidic route to L-Phe and L-Tyr biosynthesis in plants and leading to a proposed role for PhPPY-AT and Ab-ArAT4 in modulating aromatic amino acid homeostasis.

Arabidopsis TAT genes (At2g24850, At5g53970) were both shown to encode the enzyme capable of interconverting L-Tyr and 4-HPP, as well as L-Phe and phenylpyruvate, catalysing the first step in tocopherol biosynthesis. Another Arabidopsis TAT gene (At5g36160) was also reported to be capable of interconverting L-Tyr and 4-HPP, as well as L-Phe and phenylpyruvate. More recently, Wang, et al. reported that Arabidopsis At5g53970 favoured the direction of Tyr deamination to 4-HPP but At5g36160 preferred the reverse reaction, both were localised outside of the plastids. The opium poppy TAT was an aminotransferase capable of interconverting L-Tyr and 4-HPP, as well as L-Phe and phenylpyruvate, and contributed to BIA biosynthesis. Additionally, an E. sinica aromatic aminotransferases (EsAroAT1) was shown to function as opium poppy TAT, but is unlikely to contribute to ephedrine alkaloid biosynthesis through the catabolism of L-Phe to phenylpyruvate as a precursor to benzaldehyde. Herein, PvTAT was also confirmed to localise in the cytoplasm and is an aminotransferase capable of interconverting L-Tyr and 4-HPP, interconverting L-Phe and phenylpyruvate.

**Figure 3.** PvTAT complementation of the tyrosine-auxotrophic E. coli mutant DL39. PvTAT was cloned into the expression vector pBAD33 with an arabinose promoter. The recombinant pBAD33-TAT plasmid or empty plasmid was transformed into the E. coli triple mutant DL39. The bacteria were grown to an OD of 1.0 measured at 600 nm, the strains were serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} using 0.85% (w/v) saline and 3μl was replica plated on medium with or without L-tyrosine and L-phenylalanine supplemented with 0.2% (w/v) arabinose. (a) All transformants could grow on solid M9 medium with full supplementation, but only the plasmids expressing PvTAT could grow on medium free of L-tyrosine and L-phenylalanine. (b) Within 20 hours, the growth curve of the DL39 strain transformed with either pBAD33 (triangle) or pBAD33+TAT (circle) in liquid M9 medium lacking L-tyrosine and L-phenylalanine supplemented with 0.2% (w/v) arabinose. (c) The summary of the functional complementation assay of the plant enzyme ability to complement the E. coli triple mutant on M9 medium.

![Diagram](image-url)
Antisense expression of PvTAT led to dramatically decreased RA accumulation (Fig. 4c), confirming the involvement of this enzyme in the formation of RA in *P. vulgaris*. Although we cannot rule out that PvTAT may have amino donor substrates other than L-Tyr, L-Phe, and L-Trp, we demonstrated its strong preference for L-Tyr over the structurally related amino acid L-Phe and L-Trp (Table 1), similar behavior with *A. officinalis* TATs 14, Arabidopsis At5g36160 and At5g53970 45, 46, 48, opium poppy TAT 34, and *E. sinica* AroAT 51. A recombinant TAT from *T. cruzi* was shown to have both tyrosine aminotransferase and alanine aminotransferase activities, displaying an extended substrate enzyme activity 65. Traditionally, TAT enzymes have been characterised with aliphatic keto acid (α-ketoglutarate, oxaloacetate, and pyruvate) as acceptors and α-ketoglutarate or oxaloacetate was considered as the preferred keto acid acceptor 14, 33, 34, 46. However, this perception has been changed since the characterisation of PhPPY-AT and CmArAT1 showed a preference for phenylpyruvate rather than α-ketoglutarate, and interconnected Tyr and Phe metabolism in petunia and melon 45, more and more aminotransferases were found with similar behavior. Ab-ArAT4 has been characterised to prefer the conversion of 4-HPP to Tyr using Phe amino donor, also interconnected Tyr and Phe metabolism in *A. belladonna* 30. What’s more, *Arabidopsis At5g53970* was showed phenylpyruvate preference and may contribute to the cytosolic formation of Phe, like for phenylpropanoid biosynthesis, similar to its homolog in petunia 48. Our work shows that PvTAT shows a substrate preference toward Tyr and phenylpyruvate (Table 1). However, the *Km* (approx. 4 mM) for the best acceptor substrate (phenylpyruvate) is high and there may be more preferred substrates in vivo. Based on the presented data, PvTAT appears to be not an enzyme with high efficiency when compared with *Arabidopsis At5g53970* and Ab-ArAT4 but possesses similar catalytic efficiencies to *Arabidopsis At5g36160* and PhPPY-AT.

Many attempts have been made to engineer increased RA production by diverting more primary metabolites toward the production of 4-hydroxyphenylpyruvate or by the regulation of biotic/abiotic factors. Co-expression

**Figure 4.** *PvTAT* gene expression level, TAT activity, RA accumulation in wild-type and transgenic hairy root lines, and the correlation between *PvTAT* gene expression level, TAT activity, and RA accumulation in hairy root lines. (a) Gene expression was determined by qRT-PCR and is shown relative to the expression level of the housekeeping gene β-actin. Transcripts of *PvTAT* in the wild-type hairy root lines were set to 1. (b) TAT enzyme activity was measured as described in the Method. (c) RA accumulation was determined by HPLC. (d) Shows the correlation between *PvTAT* gene expression level, TAT activity, and RA accumulation in hairy root lines. Data are means ± SD of three replicates. *P < 0.05 and **P < 0.01 as determined by Tukey HSD test. WT, the untransformed hairy roots lines (wild-type control); VC, empty vector transformed hairy roots lines (vector control); Anti-TAT, antisense-expressed TAT hairy root lines; Sense-TAT, sense-expressed TAT hairy root lines.
of SmTAT and SmHPPR in S. miltiorrhiza increased RA by 4.3-fold, although overexpression of SmTAT alone did not increase RA content. Overexpression of the PVTAT gene from P. frutescens led to RA levels to be 1.5–2.0 folds higher than controls. By combining the genetic manipulation of the ectopic expression of AtPAP1 and co-suppression of the cinnamoyl-CoA reductase and caffeic acid O-methyltransferase genes in S. miltiorrhiza, a 2.49-fold increase of RA was obtained. Our data showed that overexpression of the PVTAT in hairy roots increased TAT expression, followed by increased enzyme activity, and and 31–83% increases in RA accumulation (Fig. 4c). In contrast, antisense expression of PVTAT successfully suppressed TAT expression, followed by decreased enzyme activity and RA accumulation, especially in antisense line 3 and 4, with 90% and 92% decreases in RA accumulation (Fig. 4c). The positive relationship between PVTAT mRNA levels, total TAT activity, and RA accumulation suggest that PVTAT has an important role in the production of this phenolic acid in P. vulgaris. The biochemical characterisation of a recombinant TAT in vitro, coupled with the physiological evaluation of function in planta, supports the role of 4-HIPP as an intermediate in the formation of RA precursors.

Together, we presented the isolation and characterisation a tyrosine aminotransferases cDNA (PvTAT) from P. vulgaris, commonly known as “heal-all”. We have provided comprehensive information for PVTAT expression and test for the catalytic activities in vitro and in vivo, which fill in the gap in PA-producing plants. What’s more, the physiological evidence that PVTAT important for the synthesis of rosmarinic acid (RA) in P. vulgaris was confirmed by modest correlation between transcript level, TAT activity, and RA accumulation. These results highlight the RA biosynthesis pathway in P. vulgaris, and provide useful information to engineer natural products. These observations open an opportunity to modulate the level of this biotechnologically and pharmaceutically important phenolic acid through the modulation of PVTAT expression.

Materials and Methods

P. vulgaris RNA and DNA isolation and cDNA synthesis. Total RNA from P. vulgaris leaves was isolated using the RNAPrep pure Plant Kit (TIANGEN, Beijing, China) according to the manufacturer’s protocol and reverse transcribed to generate cDNA using the PrimeScript RT reagent kit (Takara, Japan). Genomic DNA was isolated from P. vulgaris leaves using a DNA isolation kit (Omega, USA) following the manufacturer’s protocol.

Amplification of PVTAT cDNA. Homologous cloning was used to obtain the ORF sequence of P. vulgaris. The same Labiatae family members of S. miltiorrhiza (KM575934.1) and C. blumei (AJ458993.1) TAT sequences were used as the query sequence to locally blast against P. vulgaris transcriptome database (Genebank SRX130243) with BioEdit (v7.0.9). Unigenes with more than 80% similarity were chose and assembled using SeqMan of DNASTar. A joint and predicted PVTAT ORF sequence was then obtained. A PCR reaction was carried out using the forward primer (5′-ATGGAGTGTGCAGCTCGGCAGCTGG-3′) and the reverse primer (5′-TTAGGAGCTTGCGCTCGGAAGAC-3′) to amplify PVTAT ORF sequence. Using the same primer pairs, the DNA sequence was amplified using the DAE as a template.

Multiple sequence alignment and phylogenetic analysis. Amino acid alignments were performed using DNAMAN 7.0. An unrooted neighbour-joining phylogenetic tree was constructed using MEGA version 5.05 with the Poisson model and default parameters. A bootstrap test of 1000 replicates was used to value the reliability of the phylogeny. The proteins used for sequence alignments and phylogenetic tree construction are available in Supplemental Table S1.

Transcription analysis of PVTAT. Plant materials were described in Ru, et al. At the reproductive stage, leaves, stems, roots, and spikes were separately sampled and kept at −80°C before use. Extraction and reverse-transcription of total RNA were carried out as mentioned above. Gene expression was determined by qRT-PCR following our previous method and is shown relative to the expression level of the housekeeping gene β-actin. Relative expression was calculated using the 2−ΔΔCt method. Transcript levels of related genes in the spike were set to 1. Data are means ± SD of three replicates of three independent individuals. Tukey HSD test was used to compare the difference among tissues. Primers were listed in Supplementary Table S2.

Subcellular localisation analysis of PVTAT. The full-length ORF sequence introduced XhoI and Ncol restriction sites was subcloned into the PC0390-pA7-GFP plasmid to produce TAT-GFP fusion proteins. Construction of recombinant plasmid and transient transformation of tobacco were referenced to our previous method. Tobacco protoplasts and leaves were both observed for the determination of PVTAT localisation. Images were acquired using a confocal microscope (Nikon, Japan) and software of NIS-Elements Viewer 4.0. The PC0390-pA7-GFP plasmid was used as a marker. GFP was excited at 488 nm and collected over a 500–550 nm bandpass. Chlorophyll fluorescence was excited at 638 nm and collected over a 662–737 nm bandpass.

Expression and purification of recombinant proteins. The pET32a-TAT plasmid encoding PVTAT was constructed by PCR amplification of the PVTAT ORF sequence that introduced EcoRI and HindIII restriction sites. The PCR fragments were subcloned into the PMD19T Simple cloning vector (Takara) and checked by sequencing. The PMD19T-TAT plasmid and the pET32a (NEB, England) vector were digested by EcoRI-HindIII restriction enzymes to produce the fusion protein with an N-terminal His tag. The resulting plasmid was termed pET32a-TAT and transformed into the E. coli strain Rosetta (DE3).

Escherichia coli strain Rosetta (DE3) cells carrying the pET32a-TAT plasmids or the pET32a empty plasmids were grown at 37°C in 500 ml of Terrific-Broth supplemented with 34 mg l−1 chloramphenicol and 100 mg l−1 carbenicillin. When the culture reached an OD600 of ~0.6, isopropyl-β-D-thiogalactoside (I M) was added to a final concentration of 0.3 mM to induce protein expression and the culture was incubated for 6 h. The cells were harvested by centrifugation at 10,000 g for 10 min at 4°C. The pellet was washed twice with PBS buffer, suspended...
in 200 mM Tris, 100 mM KCl, and 10% glycerol (w/v), pH 8.0, and lysed by high-pressure broken using a cell disrupter (JN-02C, JNBIo, Guangzhou, China). The crude extract was centrifuged at 12,000 g for 20 min at 4 °C to yield a cell-free supernatant. The supernatant was used for affinity purification over His-Tag purification resin (Solaiba, Beijing, China). Proteins eluted with 250 mM imidazole were analysed by SDS-PAGE on a 12% (w/v) acrylamide gel and visualised using Coomassie Brilliant Blue R-250 staining. Protein concentrations were determined using a Bradford Protein Assay Kit (Takara).

Western blotting analysis was determined as Wang, et al.49 described previously, except the protein was transferred to NC membrane (Solarbio). The membrane was incubated with anti-His antibodies at a dilution of 1:3000 in blocking buffer for 1 h, after washing with TBST buffer for 10 min, the membrane was then incubated with goat anti-rabbit IgG alkaline phosphatase-conjugated as secondary antibodies (1:3000 dilution) for 1 h. Antigen-antibody complex was visualised using the BCIP/NBT kit (Betoyime, China).

**Enzyme assay.** To measure TAT activity in *P. vulgaris* hairy root lines, proteins were extracted and determined as Lopukhina, et al.52 described previously with minor modification. Hairy roots (0.2 g fresh weight) were extracted with 3 ml of ice-cold buffer, containing 100 mM potassium phosphate buffer, 0.1 mM EDTA, 8.0 mM α-ketoglutarate, 0.2 mM pyridoxal-5-phosphate (PLP), and 1 mM dithiothreitol, pH 8.0. After centrifugation at 12,000 g at 4 °C for 10 min, the supernatant was incubated on ice and used as a crude extract enzyme. The reaction mixture containing 125 mM KH2PO4/K2HPO4 buffer, 5.5 mM L-Tyr, 0.75 mM EDTA, 0.1 mM PLP, and 10 mM α-ketoglutarate, pH 8.0, was pre-incubated at 30 °C for 30 min. The reaction was initiated by adding 200 μl of crude protein and incubated at 30 °C in a total volume of 3 ml. After 30 min the reaction was terminated with 1 ml of 10 M KOH and extinction of 4-hydroxybenzaldehyde (product derivative) was measured exactly 30 min later at 331 nm using an ultraviolet-visible spectrophotometer (UV-1700, SHIMADZU, Japan). The extinction coefficient used for 4-hydroxybenzaldehyde was 24 900 L mol⁻¹ cm⁻¹.33

To estimate the pH optimum for the recombinant protein, pH values were varied between 6 and 10 and 5 μl (0.2 μg) of purified protein was assayed as described above in a total volume of 1 ml. Enzyme kinetics were obtained by monitoring the absorbance of various transamination reaction products 4-HPP at 331 nm, phenylpyruvate at 320 nm, and indole-3-pyruvate at 328 nm corresponding to the substrates L-Tyr, L-Phe, and L-Trp. The assay contained 125 mM KH2PO4/K2HPO4 buffer, 0.75 mM EDTA, 0.1 mM PLP, and various substrate concentrations, pH 8.0, and incubated at 30 °C in a total volume of 1 ml. When α-ketoglutarate (10 mM) was the amino acceptor, 0–4 mM L-Tyr, 0–50 mM L-Phe, and 0–50 mM L-Trp were used as the amino donor. When L-Tyr (5.5 mM) was the amino donor, 0–100 mM α-ketoglutarate, 0–200 mM pyruvate, 0–300 mM oxaloacetate, and 0–40 mM phenylpyruvate were used as the amino acceptor.

For the reverse reaction, the assay was determined as Prabhu and Hudson46 described. The 100 μl assay mixture consisted of 125 mM KH2PO4/K2HPO4 buffer, 0.3 mM NAD, 0.3 mM CoA, 10 mM glutamate, 0.75 mM EDTA, 100 μg 2-ketoglutarate dehydrogenase (0.6 U mg⁻¹ protein) (Sigma), 0.02 μg pure recombinant protein, pH 8.0. After incubated at 30 °C for 30 min, 0–4 mM phenylpyruvate, 0–10 mM 4-hydroxyphenylpyruvate, or 0–40 mM oxaloacetate was added to initiate the reaction. NADH formation was measured at 340 nm using a Multi-Mode Microplate Readers (Spectra Max M 2, Molecular Devices, USA). The extinction coefficient used for NADH was 6220 L mol⁻¹ cm⁻¹.33

\[ Y_{\text{max}} = \frac{(V_{\text{max}}S)}{(K_m + S)} \]

For the calculation of \( k_{\text{cat}} \), a molecular weight of 65 kDa was assumed for the His-tagged TAT protein. All enzyme assays were performed at an appropriate enzyme concentration so that reaction velocity was linear and proportional to enzyme concentration during the incubation time period. Kinetic data were evaluated by curveExpert 1.4. At least triplicate assays were performed for all data points.

**Functional complementation of the *E. coli* DL39 mutant.** The pET32a-TAT and pBAD3357 were both digested by XbaI- HindIII restriction enzymes to produce the pBAD33-TAT constructs. The *E. coli* auxotrophic mutant DL39 (no. 6913)38 with genotype (LAM-, aspC13, fnr-25, rph-1, ilvE12, tyrBS07), obtained from the Coli Genome Stock Center (CGSC) (http://cgsc.biology.yale.edu/), was used for the complementation assays and transformed with either the pBAD33 or pBAD33-TAT constructs. Procedure of complementation assays were performed essentially as Prabhu and Hudson46 previously described.

**Antisense and sense expression of *PvTAT* in *planta*.** To construct pCAMBIA2300-TAT sense and antisense expression vectors, the *PvTAT* ORF, introduced *XbaI* and *KpnI* restriction sites, was inserted into the pCAMBIA2300 vector in sense and antisense orientations. Then, the pCAMBIA2300-TAT and pCAMBIA2300 plasmids were transformed into an *A. rhizogenes* strain ATCC 15834 using the heat shock method. Positive clones were confirmed by PCR and restriction enzymes digestion. Plant transformation was carried out according to our previous method38, except that the transformants were screened with cefotaxim solution and kanamycin together.

**Identification of transgenic hairy roots by PCR.** DNA was isolated from wild-type and transgenic hairy roots using a genomic DNA isolation kit (Omega) according to the manufacturer’s protocol. Four primer pairs, rolB/C, nptII, 35S forward primer 1 (35S F1), and STAT reverse primer (STAT R) were designed for the identification of sense-expressing hairy root lines. 35SF was located at the 35S promoter of the pCAMBIA2300 vector and STATR was from the *PvTAT* gene. 35SF forward primer 2 (35SF2) and ATAT reverse primer (ATATR) were designed for the identification of antisense-expressing hairy root lines. ATATR was also from the *PvTAT* gene. Primers were shown in Supplemental Table S2.
HPLC assay for RA content. RA accumulation in wild-type hairy roots and transgenic hairy root lines was assayed using HPLC according to our previous method\(^4\).

**Analysis for PvTAT gene expression.** qRT-PCR analysis for PvTAT gene expression in wild-type hairy roots and transgenic hairy root lines was determined as mentioned above. Transcripts of PvTAT in the wild-type hairy root lines were set to 1.

**Data analysis.** One-way analysis of variance (ANOVA) was performed to highlight differences in wild-type hairy root lines, hairy root lines transformed with empty vector, and transgenic hairy root lines followed by “Tukey’s HSD” post-hoc multiple comparison tests. Normality and homogeneity of variance of data were tested prior to analysis. All the data analyses were accomplished using the “Statistical Package” for Social Sciences program (SPSS 16.0, SPSS Inc., USA). OriginPro 8.0 (OriginLab Corporation, Northampton, MA) was used for graphical presentation. All the data were expressed as means ± SD with triplicate replicates.

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Author Contributions
R.M. and L.Z.S. conceived the original screening and research plans; L.Z.S. supervised the experiments; R.M., W.K.R., B.Z.Q. performed the experiments; P.L., H.S.X., W.Y. provided technical assistance; R.M. designed the experiments and analysed the data; R.M. and W.K.R. conceived the project and wrote the article with contributions of all the authors; L.Z.S. supervised and complemented the writing.

Additional Information
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