·农业生物技术·

家蚕色氨酸羟化酶 (TRH) 基因的克隆及表达特性分析

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摘 要: 5-羟色胺 (5-hydroxytryptamine, 5-HT) 是生物界广泛分布的信号分子, 涉及动物的重要行为。5-HT 是色氨酸羟化酶 (Tryptophan hydroxylase, TRH) 将 L-色氨酸羟化为 5-羟-L-色氨酸, 5-羟-L-色氨酸随即被多巴脱羧酶 (Aromatic L-amino acid decarboxylase, DDC) 脱羧而成。TRH 作为 5-HT 合成的限速酶, 在无脊椎动物神经调控中具有重要地位。鳞翅目昆虫中 TRH 的功能研究并不多。在家蚕中克隆了家蚕 *TRH* (*Bombyx mori TRH*, *BmTRH*) 的 cDNA 序列 1 667 bp, 其中包含 1 632 bp 的开放读码框 (Open reading frame, ORF)。人类 TPH 或者果蝇 TRH (*Drosophila* TRH, DmTRH) 与 BmTRH 有高度相似性, 尤其 BmTRH 和 DmTRH 之间大多数氨基酸保守说明它们在系统发育上的密切关系并可能有相似功能。基因表达分析显示 BmTRH 主要表达于头部和中枢神经组织, 免疫组织化学和 Western blotting 结果显示 BmTRH 只存在于神经组织中, 即 BmTRH 可能仅参与家蚕的神经活动。此外, 家蚕 *DDC* (*B. mori decarboxylase*, *BmDDC*) 和蛋白具有 TRH 活性的苯丙氨酸羟化酶基因 (*Phenylalanine hydroxylase*, *BmPAH*) 也在中枢神经系统中有表达, 暗示家蚕神经系统 5-HT 的合成与果蝇中不同, 可能有两种不同的调控机制。

关键词: 5-羟色胺, 色氨酸羟化酶, 分布, 家蚕

Cloning and expression characteristics of tryptophan hydroxylase (TRH) from silkworm, *Bombyx mori*

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Abstract: The biogenic monoamine 5-hydroxytryptamine (5-HT) is an ancient intracellular signaling molecule widely distributed in all animals with nervous systems, and has been implicated in principal behaviors. Tryptophan hydroxylase

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(TRH) induces a highly specific catalytic reaction that converts L-tryptophan (tryptophan) to 5-hydroxy-L-tryptophan (5-HTP) that is subsequently used as a substrate by aromatic L-amino acid decarboxylase (DDC) to form 5-HT. Five-HT is an ancient intracellular signaling molecule that is widely distributed in the animal kingdom and has been implicated in regulating the behaviors of animals with nervous systems. However, the role of TRH in Lepidoptera is not well understood. In this study, we cloned 1 667 bp cDNAs of *Bombyx mori TRH* (*BmTRH*), which contains a 1 632 bp open reading frame (ORF). Homology analysis revealed that BmTRH shared high amino acid identity with *Homo sapiens* TPH and *Drosophila* TRH (DmTRH). The high homology (70%) of BmTRH with DmTRH suggested that BmTRH could have a function similar to DmTRH. Gene expression analysis revealed that *BmTRH* was mainly expressed in head and central nervous (CNS). Moreover, immunohistochemistry and Western blotting analyses showed that BmTRH was detected only in larval nervous tissues. Taken together, our results indicate that *BmTRH* could likely function in the regulation of neural activities in *B. mori*. The transcripts of *B. mori decarboxylase* (*BmDDC*) and *B. mori phenylalanine hydroxylase* (*BmPAH*) whose proteins had TRH activity, were also expressed in the CNS tissues, indicating that unlike in *Drosophila*, two distinct mechanisms likely regulate 5-HT synthesis in silkworm.

Keywords: 5-HT, Tryptophan hydroxylase, distribution, silkworm

Introduction

The biogenic monoamine 5-hydroxytryptamine (5-HT or serotonin) is an ancient intracellular signaling molecule widely distributed in all animals with nervous systems, and has been implicated in various physiological functions and principal behaviors as a neurotransmitter modulator or a neurohormone. Five-HT was first discovered as a biogenic amine during gastrulation in the developing central nervous system (CNS) of mammals[1-2]. It was found to modulate various behaviors such as feeding, sleep, sexual behavior, body temperature, learning and memory[3-5]. In insects, 5-HT plays a crucial role in the regulation of salivary gland secretion, heart and oviduct contractions, circadian rhythms and diuresis[6-9]. In *Drosophila*, 5-HT was shown to play a vital role in early embryonic development[10-11], and in *Manduca sexta*, it was found to be indispensable for the development of olfactory glomeruli[12-14]. Previous studies in *Bombyx mori* have reported the role of 5-HT in the macroglomerular complex (MGC) and ordinary glomeruli in modulating the response of neuronal populations in the antennal lobe (AL)[15-16]. In summary, 5-HT is an essential compound during both behavioral and developmental processes[17].

Five-HT results from a cascade of reactions initiated by tryptophan hydroxylase (abbreviated as TRH in invertebrates and TPH in vertebrates), which is a rate-limiting enzyme that converts L-tryptophan (tryptophan) to 5-hydroxy-L-tryptophan (5-HTP). TRH is a member of the pterin-dependent aromatic L-amino acid hydroxylase family (AAAHs), which includes phenylalanine hydroxylase (PAH, EC 1.14.16.1) and tyrosine hydroxylases (TH, EC 1.14.16.2). Five-HTP is subsequently decarboxylated to generate 5-HT (a final product) by aromatic L-amino acid decarboxylase (DDC, EC. 4.1.1.28). TRH, as the rate-limiting enzyme, is known to regulate the concentrations of serotonin *in vivo* and has been reported to be more stable than 5-HT in insect neurocytes[18]. Therefore, TRH represents a specific property of 5-HTergic neurons, and evolutionary analyses have revealed that it is likely to have a broader role in the animal kingdom[19].

In vertebrates, TPH is encoded by two genes, and the two transcripts have tissue-specific patterns; *TPH1* is expressed in the periphery while *TPH2* is expressed in the CNS[20-23]. Studies in *Drosophila melanogaster* have revealed that the gene products of both *PAH* and *TRH* have tryptophan hydroxylase activity *in vivo*, and that *TRH* expressed in the neural tissues has a function similar to *TPH2* in mice[24-27]. PAH, which primarily serves in phenylalanine hydroxylation, is expressed predominantly in the periphery and plays a role similar to *TPH1* in rats[21]. Immunohistochemistry using sheep TPH antibody reveals the wide distribution of TRH protein in the brains of several insect species[25]. However, to our knowledge, there is no additional research on *TRH* in insects, particularly the order Lepidoptera, which includes important pests of agricultural crops. In this study, we cloned and

characterized *TRH* from silkworm (a Lepidoptera model insect) to gain insights into its function in silkworm and other Lepidoptera.

1 Materials and methods

1.1 Silkworm strains

Silkworm DaZao strain was maintained at the Southwest University in China, and fed on mulberry leaves under standard conditions (24–26 °C and 70%–85% RH with a photoperiod of 12:12 LD).

1.2 mRNA isolation and cDNA synthesis

Total RNA was purified using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 3 μg RNA was reverse transcribed by Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) to synthesize cDNA.

1.3 cDNA cloning of BmTRH

cDNA from the heads of 5th instar DaZao larvae was used as templates in PCR for *BmTRH* amplification (the sequence of the primers in Table 1). PCR reaction conditions included 94 °C for 4 min, 30 cycles of 94 °C for 40 s, 55.5 °C for 1 min and 72 °C for 1.5 min and a final extension at 72 °C for 10 min. PCR products were purified and cloned into the pMD19-T simple vector (TaKaRa) and sequenced[28].

1.4 Multiple sequence alignment and phylogenetic analysis

Amino acid sequences used for the sequence alignment were identified in the protein database of the NCBI with the amino acid sequence of *BmTRH* as template. Multiple sequence alignments of the amino acid sequences were performed with DNAMAN and ClustalX. Transmembrane spanning domains were predicted by TMHMM (genome.cbs.dtu.dk/services/TMMM). Phosphorylation sites and N-glycosylation sites were predicted by NetPhos (www.cbs.dtu.dk/services/NetPhos) and NetNGlyc (www.cbs.dtu.dk/services/NetNGlyc) respectively. Values for identity (ID) and similarity (S) were calculated by BioEdit. We utilized MEGA 6.0 to calculate the genetic distances among different species and to construct neighbor-joining (NJ) trees with 1 000-fold bootstrap resampling.

1.5 Semi-quantitative RT-PCR analysis

According to the predicted CDS and EST sequences in the SilkDB, primers for expression analysis were designed for *BmPAH*, *BmTRH* and *BmDDC* (the sequences of the primers in Table 1). Conditions of PCR consisted of 94 °C for 4 min, 25 cycles at 94 °C for 40 s, 53 °C for 40 s and 72 °C for 1 min and a final extension at 72 °C for 10 min. Templates for the reaction were cDNA from eleven larval tissues (head, fat body, silk gland, tracheae, central nervous, hemolymph, testis, ovary, integument, malpighian tubule, midgut) from the 3-day-old 5th molting stage. *BmActin3* was used as an internal control (the sequences of the primers in Table 1).

1.6 Recombinant protein expression and purification

The coding sequence of *BmTRH* was amplified by PCR using head cDNA template (the sequence of the primers in Table 1). After digested with *Bam*HⅠ and *Xho*Ⅰ (TaKaRa), the PCR product was ligated

表 1 本研究所用的引物序列

Table 1 The primer sequences used in this study

		Cloning (5′–3′)	Gene expression profiles (5′–3′)	Protein expression (5′–3′)
BmTRH	F	ATTTGGACGCAATGAGTGGT	ACCCAATACATTCGTCACTCGTC	GGATCCAGTGGTTCGGGAAAAGGTCTTCTA
	R	CGTTTCATGAGCGTAATTTCGATAT	ATGCACAAATCTCCTCGTAACTC	CTCGAGTCACACCCACTGACCAGTATG
BmPAH	F		AGTGTTCCACAGCACCCAGTA	
	R		TTGTCCATAGCGTTTAGCAG	
BmDDC	F		GCTAAAATCACTACAGCCAGAC	
	R		GTTTATACGGCGTAATAGTTCTT	
BmActin3	F		TTCGTACTGGCTCTTCTCGT	
	R		CAAAGTTGATAGCAATTCCCT	

into pET-28a vector. Following with transformation into competent *E. coli* Rosetta (DE3) cells, and induced with 0.6 mmol/L isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 6 h at 37 °C before protein extraction. The recombinant protein containing the 6×His tag was purified by affinity chromatography using a Ni^{2+} column. The purified protein was then quantified using the Bradford method.

1.7 Mass chromatographic analysis and antibody preparation

Purified protein was analyzed by MALDI-TOF-MS as described previously[29]. Data were primarily downloaded from the silkworm genome database (http:silkworm.Swu.edu.cn/silkdb) and data from the NCBI protein database was used as a supplement. Peptide mass fingerprinting was analyzed by GPMAW software. The parameters used were as follows: precision = 0.10%; min. prec = 0.50 Da; min. hits =2; max. overlap = 2. Identification criteria were based on the number and coverage of matched peptides: a minimum of 5 peptides were required to match and the coverage of the matched peptides was about 25%.

A rabbit polyclonal antiserum was prepared against the recombinant BmTRH (Zoonbio, China). Immunizing a healthy male adult rabbits named New Zealand White and the rabbit was immunized every two weeks (three times more). Then collecting blood to gain the antiserum and finally the antibody titer is measured by ELISA (Sigma) and was 1: 6 000.

1.8 Western blotting analysis

The proteins of fat body, silk gland, tracheae, hemolymph, testis, ovary, integument, malpighian tubule and midgut were extracted from 3-day-old 5th Dazao strain. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 12% gels. After determined with the BCA protein assay, approximately 10 mg total protein was loaded per well. Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) with a Mini-Protein Ⅱ blotting system (Bio-Rad) 200 mA, 100 min at 4 °C in a buffer containing 15% methanol. Membranes were blocked with 5% dry milk in Tris-buffered saline containing Tween 20 (TBS-T; 10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.01% Tween 20) for 30 min at room temperature. Membranes were probed with affinity-purified anti-BmTRH antibodies (dilution 1: 10 000 in TBS-T) and antigen-preabsorbed affinity-purified antibodies at the same concentration. Membranes were washed with TBS-T, followed by incubation with a secondary antibody conjugated to horseradish peroxidase (1: 2 000; Beyotime, China). Signals were visualized with an enhanced chemiluminescence detection system (ECL; Thermo, USA) and photographed using a Clinx ChemiScope 3400 Mini (China Scientific, China). Experiments were repeated for three times with independently isolated protein samples.

1.9 Immunohistochemistry assay

The testis, ovaries, integument and nervous of 3-day-old 5th instars from the silkworm Dazao strain were moved, and rapidly fixed with 4% paraformaldehyde for 2–4 h at room temperature. Samples were embedded in paraffin following dehydration (a sequential ethanol series: 30%, 50% and 70%, 100%). All the materials were treated vertical embedding. When cooled, samples were continually sectioned with the aid of paraffin section technique and the thickness of slices was all 5 μm. Paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol solutions, quenched by 10 min immersion in 3% hydrogen peroxide, and treated with 0.01 mol/L citrate buffer (pH 6.0) at 92–98 °C for 15 min. Sections were incubated for 1–2 h in 10% goat serum at room temperature, and then incubated with the BmTRH antibody (dilution 1: 10 000) overnight at 4 °C. After rinsing Tween-20-phosphated-buffered saline (TPBS), sections were incubated for 1–2 h with an HRP-labeled goat anti-rabbit antibody (dilution 1: 2 000) at room temperature. The sections were developed using DAB (3,3-diaminobenzidine) and observed under a microscope. Serum of goat injected with PBS (negative serums) served as a negative control.

2 Results

2.1 *BmTRH* cloning and expression

We identified three potential TRH silkworm homologs by performing homology searches of the

silkworm database (provide URL) using the nucleotide sequences of tryptophan hydroxylase from *H. sapiens* and *D. melanogaster* as query. Among the three, phenylalanine hydroxylase (*BmPAH*)[30] and tyrosine hydroxylase (*BmTH*)[31] have been previously reported. BmTRH, which had the highest homology with *DmTRH* and *TRP*, spans 10.31 kb and is located on nscaf1690 in chromosome 1. We cloned the 1 667 bp cDNAs sequence of *BmTRH* containing the 1 632 bp ORF (KF650639) from the heads of silkworm larvae by using RT-PCR (Fig. 1). *BmTRH* gene contained 10 exons and 9 introns (Fig. 2A), and

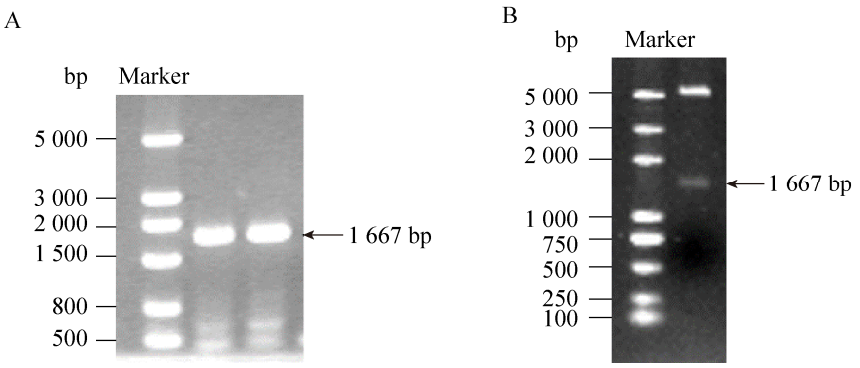

图 1 *Bm TRH* 的克隆和表达鉴定

Fig. 1 The cloning and expression of *BmTRH*. (A) The lane of *BmTRH* is about 1 667 bp for TA cloning. (B) The cloning fragment is inserted into expression plasmid pET-28a.

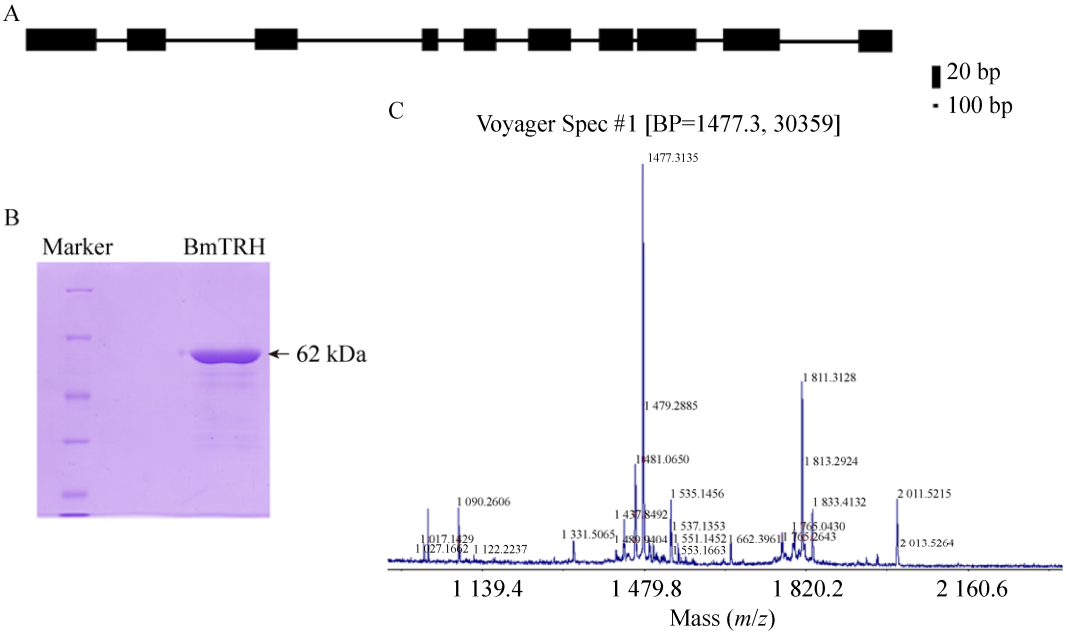

图 2 *BmTRH* 基因结构和 Bm TRH 蛋白体外表达鉴定。(A) *BmTRH* 基因结构。黑框代表外显子，横线代表内含子。(B)考马斯亮蓝染色 12% SDS-PAGE 后在约 62 kDa 大小处出现蛋白条带。(C) BmTRH 蛋白质谱分析图。

Fig. 2 The structure of *BmTRH* and identification of BmTRH expressed *in vitro*. (A) The structure of *BmTRH*. Exons were represented by black boxes and the intron length was represented by lines. (B) 12% SDS-PAGE of proteins stained with Coomassie brilliant blue. The molecular weight of the recombinant protein was about 62 kDa as estimated. (C) Peptide mass fingerprint by MALDI-TOF-MS.

encoded a putative protein containing 543 amino acids (aa) with an expected molecular weight of 62.15 kDa. To investigate of *BmTRH* expression profiles, the complete coding sequence of *BmTRH* was expressed in a prokaryotic expression system. SDS-PAGE analysis showed that the molecular weight of the expressed recombinant protein was about 62 kDa, which was consistent with the predicted weight of BmTRH (Fig. 2B, Fig. 3). Purity of the protein was ⩾ 90% and it was identified as BmTRH by peptide mass fingerprinting (Fig. 2C). The peptide fingerprint masses were analyzed as GPMAW, which showed 20.1% coverage of matched peptides and 8 matched peptides (average difference = 0.49). These results indicated the successful *in vitro* expression of *BmTRH*.

2.2 Sequences analyses

Analysis of the *BmTRH* nucleotide sequence (http://smart.embl-heidelberg.de/) revealed that the predicted BmTRH protein had all the typical features of the AAAH family: an N-terminal regulatory (ACT) domain (spanning 49–113 aa), a catalytic domain (spanning 147–478 aa) and a C-terminal coiled-coil region (spanning 465–476 aa) involved in tetramerization (Fig. 4). Comparison of the BmTRH amino acid sequence with the known sequences of *Homo sapiens* TPH (HsTPH) and *Drosophila* TRH (DmTRH), indicated that the sequences were conserved in the catalytic domain, while the C-terminus containing the regulatory domains diverged among the species (Fig. 4). More importantly, all residues that were identified as important for the structural and functional properties of (HsTPH) and DmTRH[27], were also conserved in BmTRH (Fig. 4). For example, the phosphorylation sites were located in residues S84, 89 and 300; the characteristic VLMYGS, GYLSP, F281, E313, A349 and Y352 were predicted to bind BH4; the residues H291, 312, 317 and E357 were iron binding sites; the conserved Y275, R297, H312, F353, F358 and S377 were tryptophan interaction sites; and the leucine zipper at the N-termini reported to be involved in protein multimerization (Fig. 4). These findings suggested the presence of most typical features of TRH in BmTRH. In addition, the amino acids sequence of *BmTRH* shared 70% identity with DmTRH, 61% with HsTPH1, 57% with HsTPH2, 52% with BmPAH, DmPAH and HsPAH, 46% with BmTH or HsTH, and 43% with DmTH (Table 2).

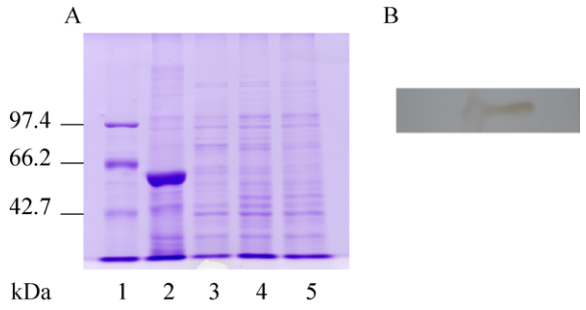

图 3 BmTRH 原核表达及 Western blotting 检测

Fig. 3 The prokaryotic expression and Western blotting analysis of BmTRH. **(**A) The expression of BmTRH in prokaryotic expression system by 0.6 mmol/L isopropyl-β-D-1-thiogalactopyranoside on SDS-PAGE gels. Lane 1: marker. Lane 2: precipitate after induction. Lane 3: supernatant after induction. Lane 4: non-induced control. Lane 5: empty vector induction. (B) Specificity of anti-Bm TRH antibody tested on induced proteins. Immunization with anti- BmTRH antibody (1:10 000) used for Western blotting analysis using 3,3′,5,5′-tetramethylbenzidine assay. A single band of ~62 kDa was detected Western blotting of 10 mg induced proteins.

2.3 Phylogenetic analysis

To explore the phylogenetic relationship between members of AAAHs, we constructed a neighbor joining phylogenetic tree with 51 genes from 13 species including 5 vertebrates [*Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Anolis carolinensis*, *Danio rerio*] and 8 invertebrates [*Tribolium castaneum* (Coleoptera), *Acyrthosiphon pisum* (Hemiptera), *D. melanogaster* (Diptera), *B. mori* (Lepidoptera), *Apis mellifera* (Hymenoptera), *Lepeophtheirus salmonis* (Arthropoda, Crustacea), *Helobdella robusta* (Annelida, Clitellata) and *Caenorhabditis elegans*]. The AAAH of physcomitrella, a lower plant, was used as an out-group; this protein was reported not only to have PAH function but also TH function and/or TRH function. All AAAHs clustered into three distinctclades, suggesting that the members of AAAH which likely derived from an ancestor differed in their structures and/or function[32]. Moreover, the TRH clade was closer to the PAH clade than to the TH

clade (Fig. 5). The first clade was the TH clade, which included two subclades, the invertebrate TH subclade comprised insect TH as well as other invertebrate TH, which served as the out-group while the vertebrate TH subclade consisted of TH1 and TH2 groups. Second was the PAH clade where vertebrate PAH and insect PAH belonged to different categories, and other invertebrate PAH were the out-group. Third was the TRH clade comprised the invertebrate TRH subclade and vertebrate TPH subclade. The invertebrate TRH subclade contained insect TRH groups where silkworm clustered together with *D. melanogaster* and other invertebrate TRH. The vertebrate TPH subclade was divided into TPH1 and TPH2 groups, revealing the likely difference in functions between TPH1 and TPH2. Furthermore, *TPH2* was mainly expressed in the brain stem, while *TPH1* was expressed in the gut, pineal gland, spleen, and thymus in human, mouse and rat[20]. Interestingly, TPH1 in *Danio* genome had two copies corresponding to gene duplication events that occurred specifically in teleost fish[33].

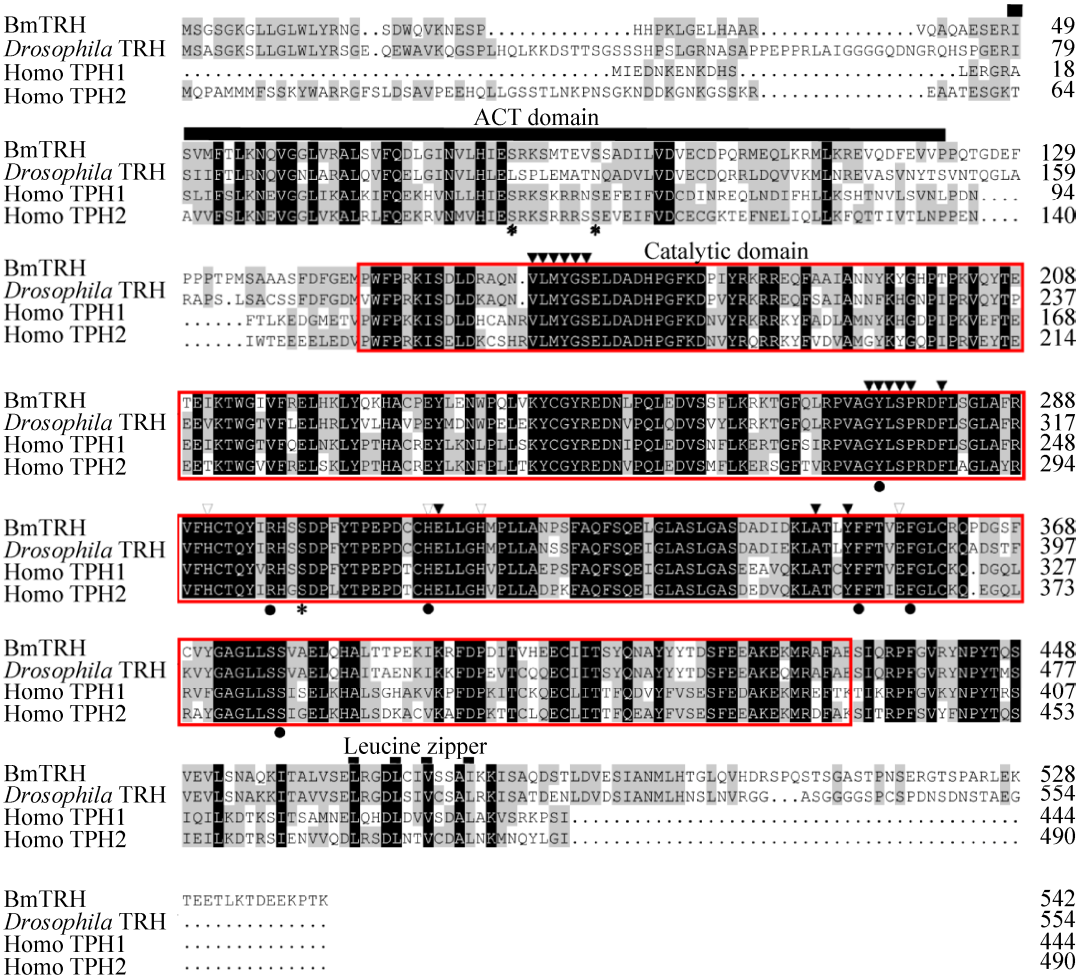

图 4　BmTRH 与人类 TPH1，TPH2 和果蝇 TRH 氨基酸多重序列比对结果

Fig. 4　Comparison of amino acid sequences alignment of BmTRH with *Homo sapiens* TPH1, TPH2 and *Drosophila* TRH. The alignment was generated using ClustalW alignment software. Identical residues were shown as white letters against black, whereas conservatively substituted residues were shaded. Residues implicated in iron binding were marked by a (▽), residues implicated in BH4 binding by a (▼), and residues associated with Leucine zipper by a thinner line. The ACT domains were marked by a bold line, while catalytic domain was marked by a red box. Tryptophan blinding sites were indicated above (●) and phosphorylation sites above (*). The accession numbers of TRH in relevant species in GenBank are listed in Table 3.

表 2 家蚕 TRH 与果蝇 (PAH、TH 和 TRH)、人类 (PAH、TH 和 TPH)之间的氨基酸同源比较分析

Table 2 Amino acid homology scores among *Bombyx* TRH with *Drosophila* (PAH, TH and TRH) and *Homo* (PAH, TH and TPH)

	Bombyx PAH	*Bombyx* TH	*Drosophila* PAH	*Drosophila* TH	*Drosophila* TRH	*Homo* PAH	*Homo* TH	*Homo* TPH1	*Homo* TPH2
Bombyx TRH	52	46	52	43	70	52	46	61	57

The accession numbers of PAH, TH, TRH (TPH) in relevant species in GenBank are listed in Table 3.

表 3 本研究相关物种的 PAH、TH、TRH 蛋白 GeneBank 登录号

Table 3 The accession number of PAH, TH, TRH in relevant species in this study

Genes / Species	PAH	TH	TPH 1	TPH 2
Homo sapiens	NP_000268	NP_954986	NP_004170	NP_775489
Mus musculus	NP_032803	NP_033403	NP_033440	NP_775567
Gallus gallus	NP_001001298	1:NP_990136 2:XP_001235001	NP_990287	NP_001001301
Anolis carolinensis	XP_003220930	1:XP_003214871 2:XP_003220929	XP_003214791.1	XP_00322119
Danio rerio	NP_956845	1:NP_571224 2:NP_001001829	a:NP_840091 b:NP_001001843	NP_999960
Caenorhabditis elegans	NP_495863	NP_871903	NP_495584	
Apis mellifera	XP_623300	NP_001011633	XP_394674	
Drosophila melanogaster	NP_523963	NP_476897	NP_612080	
Tribolium castaneum	XP_967025	NP_001092299	XP_967413	
Bombyx mori	NP_001274766	NP_001138794	NP_001296518	
Acyrthosiphon pisum	XP_001945589	XP_008182999	XP_001952801	
Lepeophtheirus salmonis	EMLSAG00000009260	EMLSAG00000001561	EMLSAG00000001300	
Helobdella robusta	XP_009031440	XP_009022498	XP_009018167	
Physcomitrella patens	AAAH: XP_001774942			

2.4 mRNA expression profile

The TRH activity product, 5-hydroxy-L-tryptophan (5-HTP) is derived from 5-hydroxytryptamine in a reaction that requires aromatic L-amino acid decarboxylase (DDC) that is also involved in the biosynthesis of dopamine[26]. We previously reported that the recombinant BmPAH protein had some TRH activity[30], suggesting that *BmDDC*, *BmPAH* and *BmTRH* could be related to serotonin synthesis. Thus, the tissue-specific expression patterns of *BmTRH*, *BmDDC* and *BmPAH* mRNA in eleven larval tissues (including head, fat body, silk glands, tracheae, central nervous, hemolymph, testis, ovary, integument, malpighian tubule and midgut) were investigated by RT-PCR. We found that the 536 bp *BmTRH* fragment was expressed only in the head and CNS with the PCR product being more intense in CNS than the head. On the other hand, a relatively bright band corresponding to 664 bp *BmDDC* product was detected in the head, fat body, tracheae, CNS, testis, ovary, integument and midgut, while the 567 bp PCR *BmPAH* fragment was observed in head, fat body, CNS, hemolymph, testis, ovary, integument and midgut. Weak PCR amplification was observed for *BmDDC* and *BmPAH* fragments in the hemolymph and malpighian tubule, respectively (Fig. 6 and Fig. 7). These results showed that *BmTRH* mRNA expression was highly restricted in space, and the transcripts of both *BmDDC* and *BmPAH* were expressed widely in most tissues, indicating their functional importance in various physiological contexts.

2.5 BmTRH protein expression profile

BmTRH was detected in larval tissues using anti-BmTRH antibody. Western blotting showed a specific band of ~62 kDa in the head and ventral chain of larvae, and high expression was observed in the ventral chain than the head. BmTRH was not expressed

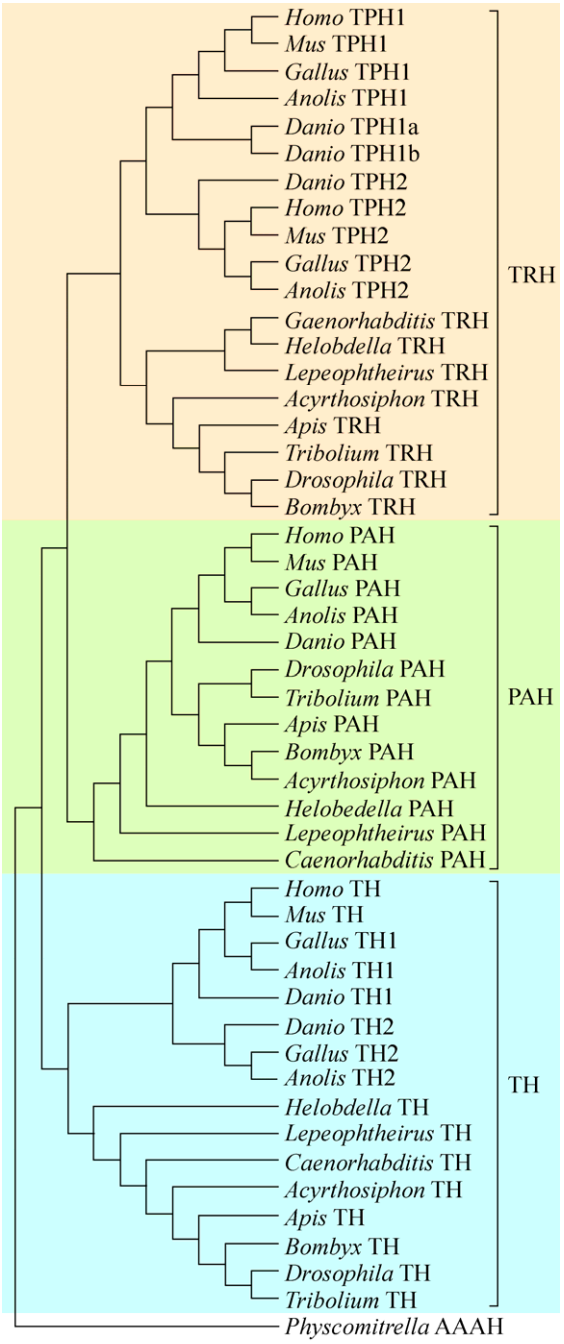

图 5 基于氨基酸序列构建的无脊椎动物与脊椎动物 TRH、TH 和 PAH 系统进化树

Fig. 5 Phylogenetic tree of invertebrates and vertebrates TRH (a narrative convenience, is called vertebrate TPH and invertebrate TRH collectivity in here), TH and PAH. There were three distinct clades, TRH clade, TH clade and PAH clade, for all aromatic amino acid hydroxylases in animal. TRH, PAH and TH was indicated in pink, yellow-green and lake blue respectively. The accession numbers of PAH, TH, TRH in relevant species in GenBank are listed in Table 3.

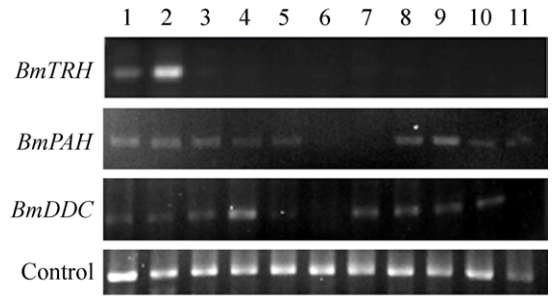

图 6 *BmTRH*、*BmPAH* 和 *BmDDC* 基因在家蚕幼虫各组织中的表达模式

Fig. 6 Expression profiles of *BmTRH*, *BmPAH* and *BmDDC* in larval tissues of silkworm. Lane 1; head; lane 2; central nervous; lane 3; integument; lane 4; fat body; lane 5; hemolymph; lane 6; silk glands; lane 7; midgut; lane 8; testis; lane 9; ovary; lane 10; trachea; lane 11; malpighian tubule; and control; *actin* control. All of the polymerase chain reaction products were approximately 600 bp.

in fat body, silk glands, tracheae, hemolymph, testis, ovary, integument, malpighian tubule and midgut (Fig. 8). These results consistent with the mRNA expression profile of *BmTRH*.

2.6 Localization analysis of *BmTRH* Immunohistochemistry

To further analyze the expression of BmTRH, immunohistochemistry was performed on larval tissues embedded in paraffin sections. Clear positive signal was detected in CNS (ganglion at any region and several nerve cords containing brain), whereas weak signal was found in the integument, testis and ovary (Fig. 9 and Fig. 10). These results suggested that BmTRH expression was restricted to larval nervous tissues.

3 Discussion

In this study, we identified only a single copy of the *TRH* gene in the silkworm genome, which is consistent with reports in other invertebrates. This is the first time of the cloning of its heterologous expression in a Lepidoptera insect. The *BmTRH* gene codes a 61.31 kDa protein consisting of 543 amino acids (aa), similar to *DmTRH*, which encodes a 61 kDa protein with 555 aa. BmTRH also has conserved residues sharing the structural and functional properties of HsTPH or DmTRH. These findings suggest that the *BmTRH* cloned here is a functional *TRH*.

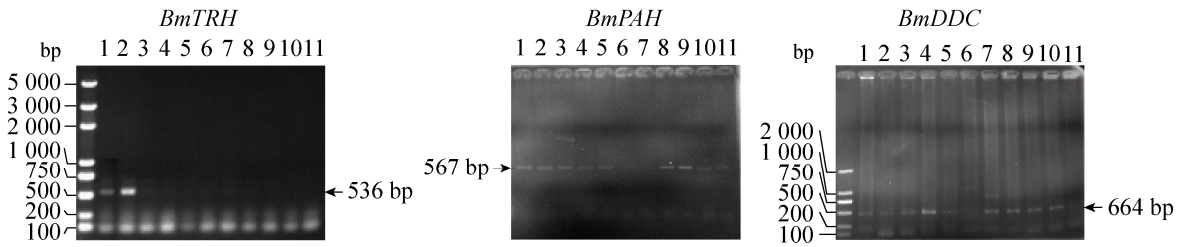

图 7　家蚕幼虫各组织中 *BmTRH*，*BmPAH* and *BmDDC* 基因表达分析 (泳道 1：头；泳道 2：中枢神经；泳道 3：表皮；泳道 4：脂肪体；泳道 5：血淋巴；泳道 6：丝腺；泳道 7：中肠；泳道 8：精巢；泳道 9：卵巢；泳道 10：气管；泳道 11：马氏管。*BmTRH*，*BmPAH* and *BmDDC* 基因 PCR 扩增产物大小分别是：536 bp，664 bp 和 567 bp)

Fig. 7　Expression profiles of *BmTRH*, *BmPAH* and *BmDDC* in larval tissues of silkworm. Lane 1: head; lane 2: central nervous; lane 3: integument; lane 4: fat body; lane 5: hemolymph; lane 6: silk glands; lane 7: midgut; lane 8: testis; lane 9: ovary; lane 10: trachea; lane 11: malpighian tubule. The polymerase chain reaction products of *BmTRH*, *BmPAH* and *BmDDC* were 536 bp, 664 bp and 567 bp, respectively.

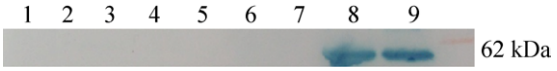

图 8　Bm TRH 在家蚕幼虫各组织的 Western blotting 检测

Fig. 8　Western blotting analysis with the anti-BmTRH antibody in larval tissues of silkworm. Lane 1: tracheal; lane 2: ovary; lane 3: testis; lane 4: silk gland; lane 5: hemolymph; lane 6: fat body; lane 7: integument; lane 8: ventral chain; and lane 9: head. The specific lane was showed in lane 8 and 9.

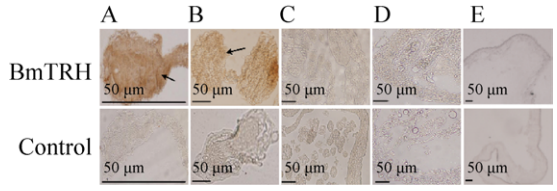

图 9　Bm TRH 在家蚕幼虫中枢神经，表皮，精巢和卵巢中的免疫组织化学分析

Fig. 9　Immunohistochemical analysis of BmTRH in central nervous, integument, testis and ovary of larvae. The arrows indicate positive signals in (A) (ventral ganglion) and (B) (brain). No positive signal was found in (C) (testis), (D) (ovary) or (E) (integument). Incubating with PBS displaced primary antibody was as negative controls.

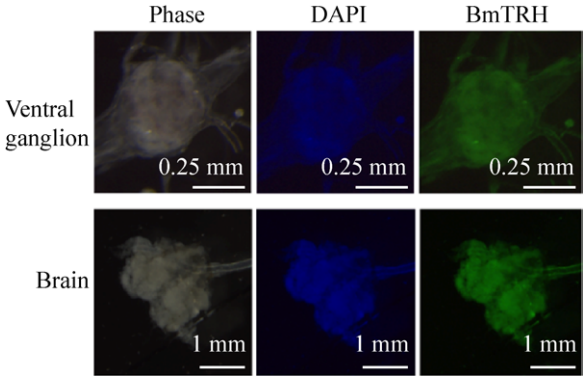

图 10　Bm TRH 蛋白在家蚕脑和腹部神经节的免疫荧光鉴定 (Bm TRH 抗体在脑和腹部神经节中均有表达)

Fig. 10　Immunofluorescence analysis using BmTRH antibodie in ventral ganglion and brain of *B. mori*. BmTRH could be detected in ventral ganalion and brain.

In *D. melanogaster*, DmTRH exhibits high activity with a notable ability to hydroxylate phenylalanine, while DmPAH has strong phenylalanine hydroxylase activity and displays a significant ability to hydroxylate tryptophan. Both DmTRH and DmPAH have been reported, not to have tyrosine hydroxylase activity[24-26]. Our phylogenetic analysis indicates that TRH is closer to PAH than TH, and BmTRH together with DmTRH are clustered to a branch with TRH from other insect. Besides, our previous study also showed that BmPAH expressed *in vitro* was capable of tryptophan and phenylalanine hydroxylation rather than only tyrosine hydroxylation[30]. These results imply that BmTRH, like DmTRH, can hydroxylate both tryptophan and phenylalanine but not tyrosine in silkworm.

The *BmTRH* transcript and BmTRH protein are detected only in the head and CNS with high expression in the CNS. Consistently, immunohistochemistry also shows a strong positive signal only in CNS tissues containing the ventral

ganglion and brain. These suggest that *BmTRH* could likely trigger neural activities in silkworm larvae, and is consistent with previous reports that TRH participates in the neural activity in other animals[34-35].

In *D. melanogaster*, DmPAH and DmTRH are selectively involved in 5-HT synthesis with distinct expression patterns and enzyme activities[36]. However, expression analysis in this study shows that *BmTRH* together with *BmPAH* and *BmDDC* could likely be co-expressed in the head and CNS tissues. These suggest the likelihood of two distinct regulation mechanisms for 5-HT synthesis in CNS, and that BmTRH- and BmPAH-mediated 5-HT biosynthesis pathways may not segregate into neuronal and peripheral tissues in silkworm. A similar phenomenon has been reported in *Gryllus bimaculatus*[37].

4 Conclusion

In this study, we identify and clone a cDNA sequence for *TRH* gene in silkworm, and analyze the phylogenetic relationships to metazoan as a members of aromatic amino acid hydroxylases (AAAHs). By gene and protein expression analysis, we speculate BmTRH could likely function in the regulation of neural activities. This is the first time that the *BmTRH* gene is cloned from silkworm and BmTRH protein is expressed and purified *in vitro* in Lepidoptera.

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