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Isolation and analysis of the triosephosphate isomerase gene, *Hatpi*, from *Helicoverpa armigera*

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Abstract: *Helicoverpa armigera* is an important lepidopteran pest of many crops including cotton. Molecular analysis will promote pest control in the future. The gene that encoding triosephosphate isomerase in *Helicoverpa armigera*, *Hatpi*, was molecularly cloned and analyzed in this study. The cDNA of *Hatpi* (GenBank accession no. AY736358) is 1 149 bp in length and encodes a 248 amino acid protein with the estimated molecular mass of 26.4 kD and isoelectric point of 5.82. $(\beta\alpha)_8$ structure found in the deduced protein structure of HaTPI was similar to that of other triosephosphate isomerase. High identity was also found in the active catalytic sites (Lys12, His94, and Glu165) and peptide motifs (AYEPVWAIGTG and GGASLKPEF). RT-PCR analysis results showed that *Hatpi* was expressed in the embryo, larva, pupa and adult of *H. armigera*, suggesting *Hatpi* may play roles in various developmental stages of *H. armigera*.

Key words: *Helicovepa armigera*; triosephosphate isomerise; gene; RACE; sequence analysis; expression

1 INTRODUCTION

Triosephosphate isomerase (TPI or TIM) is a key enzyme in the glycolytic pathway, which catalyzes the interconversion between dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G-3-P). Triosephosphate isomerase plays an important role in several metabolic pathways and is essential for efficient energy production (Wang *et al.*, 1999). UP to now *tpi* gene has been well characterized from a large number of organisms (Kohl *et al.*, 1994; Aparicio *et al.*, 2003; Gayathri *et al.*, 2007). The active catalytic sites (Lys, His and Glu) and the peptide motifs (AYEPVWSIGT and GGASLKPEF) were conserved in all known TPIs (Wang *et al.*, 1999). α/β barrels with eight parallel β-strands surrounded by a layer of eight α-helices are recently found in subunit of TPI (Gayathri *et al.*, 2007). Instability of the isomerase due to different mutations of the enzyme may underlie the observed reduced catalytic activity. TPI deficiency is usually associated with several genetic disorders such as chronic haemolytic anaemia, cardiomyopathy, susceptibility to infections, severe neurological dysfunction and early death (Schneider, 2000; Olah *et al.*, 2002; Gnerer *et al.*, 2006).

Since DHAP is required by the sn-glycerol-3-phosphate dehydrogenase (α-GPDH)-glycerol phosphate oxidase (GPO) shuttle, the TPI that takes part in the generation of NAD^+ for flight muscle activity becomes more important in insects (Sacktor, 1970). The *tpi* gene has been identified from *Drosophila*, mosquito and *Bombyx mori* (Tittiger *et al.*, 1993; Tyshenko and Walker, 1997; Zhong, 2001). Yet little is known about the *tpi* homologous gene in *H. armigera*. Here, the *H. armigera tpi* gene was cloned and analyzed.

2 MATERIALS AND METHODS

2.1 Test insects

Larvae of *H. armigera* were collected from cotton fields (Xiaoshan, Zhejiang Province, China) in 2005. Cultures were maintained at 25℃ with a 16 h:8 h (light : dark) photoperiod and 40% relative humidity in the laboratory. Larvae were reared on an artificial diet and adults in sealed box with 10% solution of honey in water (Bown *et al.*, 1997).

2.2 Genomic DNA extraction

Genomic DNA was extracted from individuals

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by using a simple organic extraction method outlined by Tobler *et al.* (2005). Approximately 150 mg of adult was placed into 1.5 mL centrifuge tubes and homogenized in 500 μL of insect grinding buffer (10 mmol/L Tris-HCl (pH 8.0), 100 mmol/L EDTA, 0.5% SDS). The homogenate was digested for 3 h at 55℃ with 0.5 mg of proteinase K. The homogenate was then extracted three times, first with equal volumes of buffered phenol, second with equal volumes of buffered phenol/chloroform/isoamyl alcohol (25∶24∶1) and third with equal volumes of chloroform/isoamyl alcohol (24∶1). The DNA was then precipitated with 2 × volume of 95% (v/v) ethanol and 1/10 volume of 3.0 mol/L NaOAC (pH 5.2). Genomic DNA was resuspended in 100 μL 0.1 × TE buffer.

2.3 Isolation of RNA and the RT-PCR analysis

Total RNA was separately from samples by using Trizol reagent according to the manufacture's instruction. For RT experiments, 3 μg of total RNA was denatured at 65℃ for 5 min followed by quick chill on ice in a 14 μL reaction containing 1 μL oligo $(dT)_{18}$ (Sangon Bio.) primer, and 1 μL of 10 mmol/L dNTP mixture (10 mmol/L each dATP, dGTP, dCTP and dTTP at neutral pH). After addition of 2 μL 10 × reaction buffer (KeyGEN, www.keygentec.com.cn), 1 μL AMV (KeyGEN, 10 Units), 1 μL DTT (1 mol/L) and 0.5 μL RNase inhibitor (40 U/μL), the reaction was incubated at 37℃ for 1 h. After terminating, the reaction was heated at 70℃ for 15 min for inactivation. The cDNA fragments were amplified with *Ex Taq* DNA polymerase (TaKaRa) using the *Haactin* (GenBank accession no.: X97614.1) primers Factin (5′-CGCGACCTCACAGACTACCT-3′) and Ractin (5′-GGCCAGACTCATCGTACTCC T-3′) as internal control. Highly conserved fragment of *tpi* was amplified from *H. armigera* with degenerate primers P1 (5′-TYACTGSTGANATNTC VCCMGC-3′) and P2 (5′-AGDCTGVCTCCNCCWA CBAAGAAV-3′). Thermal cycling condition was 95℃ for 5 min followed by 30 cycles of 95℃ 30 s, 58℃ 30 s and 72℃ 1 min. The last cycle was a final extension at 72℃ for 5 min. Amplified products were separated by gel electrophoresis on 1.2% agarose gels at 100 V for approximately 1 h using 1 × TAE buffer (40 mmol/L Tris acetate and 2 mmol/L EDTA in water). After electrophoresis, the gel was stained for 30 min in 0.01% SYBR™ Green I nucleic acid gel stain (FMC Bio.). Following purification of PCR products by Biospin Gel Extraction Kit (BioFlux Bio.), the sequences of purified PCR fragments were TA-cloned into pMD18-T (TaKaRa). The insertions were identified by PCR amplification using M13 forward and reverse primers before sequencing.

2.4 RACE method

The cDNAs were synthesized from 2 μg of total RNA using BD SMART RACE cDNA Amplification Kit (BD Biosciences). The 5′ and 3′ cDNA ends were obtained by touchdown PCR with *LA Taq* DNA polymerase (TaKaRa) using a universal primer mixture (UPM) and the gene-specific primers (GSPs): P3, 5′-TCTCTCCGAAAATTGTTCTC-3′ (5′ end primer) and P4, 5′-GGGCTAATGTTGTAC TCGCT-3′ (3′ end primer) according to manufacture's protocol (http://www.bdbiosciences.com/clontech/). The RT-PCR products were purified directly from bands excised from agarose gels before cloned into pMD18-T (TaKaRa) and sequenced. Full length cDNA and genome primers were P5 (5′-AGCGGTGAAGTGAAGA AGTTTAC-3′) and P6 (5′-GTTTGATTTTTAAGTTTCG ATTG-3′). PCR fragments were cloned and sequenced at least twice per strand.

2.5 Analysis of expression pattern of the *Hatpi* gene

For developmental analysis, total RNA was extracted from various developmental stages including embyro (5th day after hatch), larva (2nd day of the 3rd instar), pupa (3rd day after pupation) and adult (1st day of eclosion) of *H. armigera* respectively. RT-PCR was performed as described above using primers P7 (5′-GTACTGGCA AGACAGCATCTC-3′) and P8 (5′-GTTTGATTTTT AAGTTTCGATTG-3′). *Haactin* were applied as an internal control. Each reaction was repeated at least three times.

2.6 Sequence analysis

The Swiss-Prot and EMBL database (http://cn.expasy.org/tools/blast/) were used to blast the homologs among TPIs. Phylogenetic tree analysis was performed using Clustalx, PHYLIP and TreeView softwares. Multiple sequence alignment was performed by using ClustalW with default parameters set as in the ClustalW web server at EBI (http://www.ebi.ac.uk/clustalw/) and GENEDOC32. TIM barrel structure was analyzed by using 123D Threading (http://123d.ncifcrf.gov/run123D+.html) and the reference result of *Methanocaldococcus jannaschii* TPI (Gayathri *et al.*, 2007).

3 RESULTS

3.1 Isolation and DNA sequence analysis of *tpi* gene of *H. armigera*

A 510 bp DNA fragment of *tpi* cDNA was obtained from *H. armigera* larval by using degenerate primers. After 3′ and 5′ RACE amplification, a 655

bp 3′ end and a 459 bp 5′ end cDNA fragments were obtained respectively. Full-length cDNA and genomic DNA of *H. armigera tpi* were acquired by overlaying the cloned sequences and sequenced with the primer pair (AY736358) for full-length amplification. The cDNA and genome sequence of *H. armigera tpi* are 1 149 bp and 2 239 bp in length, respectively, of which the cDNA was named *Hatpi* and registered in GenBank (GenBank accession no. AY736358). The open reading frame of *Hatpi* cDNA is 747 bp, which encodes a 248 amino acid protein (Fig. 1) with a calculated molecular mass of 26. 4 kD and the isoelectric point of 5.82.

Hatpi gene contained 5 exons and 5 introns. The 1st intron is located in 5′-untranslated region (UTR) (Fig. 2: A) and the other 4 introns fell between Gly75 and Glu76, Lys111 and Val112 and interrupt Val38 and Gln180 (Fig. 2: B).

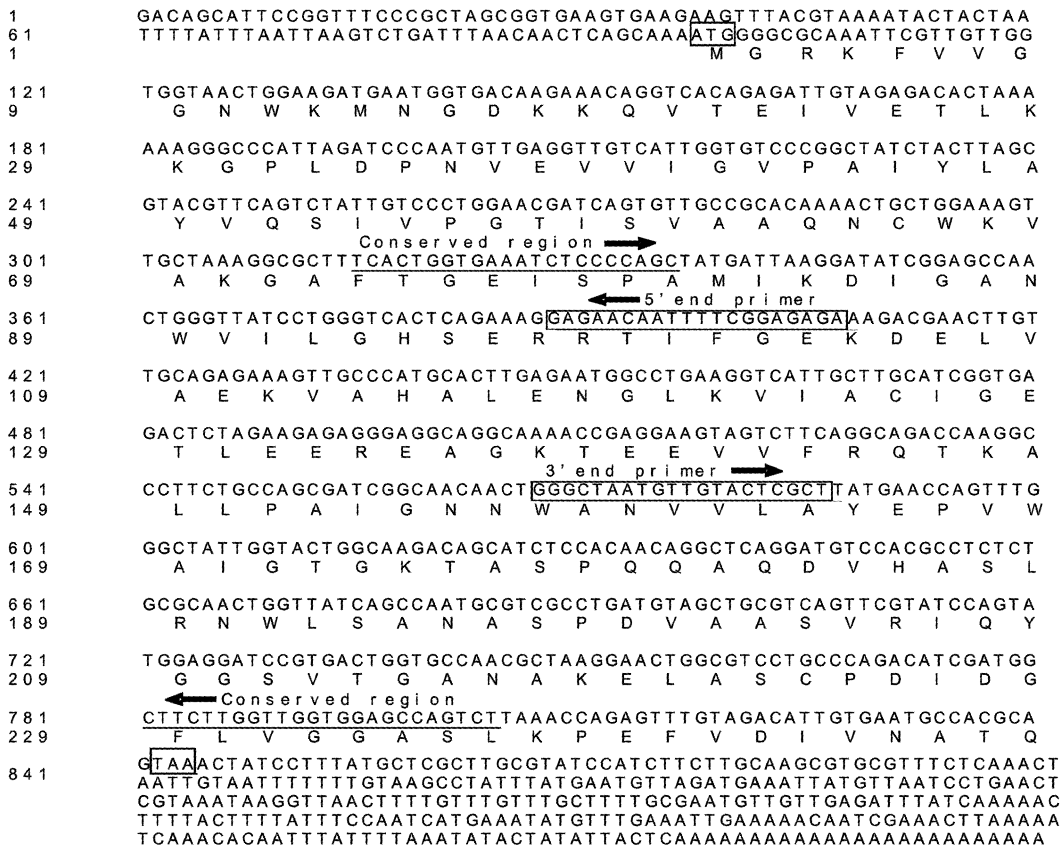

Fig. 1 cDNA and deduced amino acid sequences of *Hatpi* gene

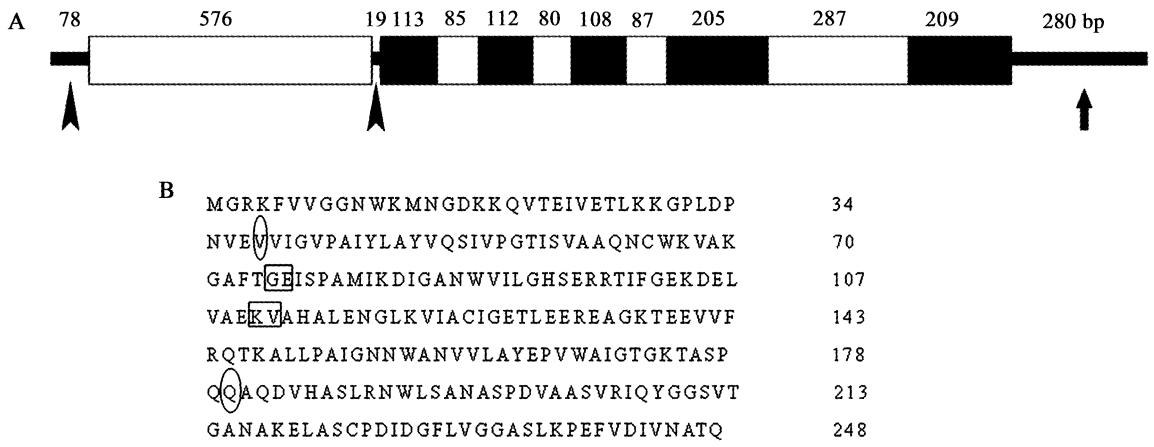

Fig. 2 Exon/intron structure of *Hatpi* gene

A: Characterization of exons and introns in *Hatpi* gene. Black rectangles indicate exons. White rectangles indicate introns, arrowhead indicates the 5′ UTR, and arrow indicates the 3′ UTR. Number indicates the length of exons and introns. B: Intron positions in *Hatpi* gene. Intron positions are showed with boxes where they fall between codons and with ellipses where they interrupt codons in the coding sequence.

Nucleotides are numbered on the left of each line. The deduced amino acid sequence is shown below the nucleotide sequence and numbered from the first methionine. The degenerate primers (P1, P2, marked with conserved region) are shown with underline and arrow. Gene-specific primers (5′ and 3′ end primers) used in RACE were framed and directed by arrow. The initiation and termination codons are shadowed.

3.2 Expression pattern of the *Hatpi* gene

To examine the expression of *Hatpi* gene in various stages, semi-quantitative RT-PCR using total RNA obtained from the embryo, larva, pupa and adult were performed. Results showed that *Hatpi* was expressed in all samples but no significant difference was found in the expression strength (Fig. 3).

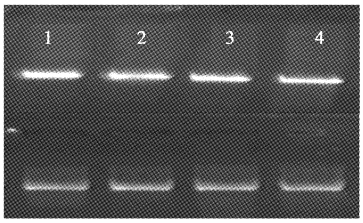

Fig. 3 Expression of *Hatpi* gene in different developmental stages of *Helicoverpa armigera*

1 – 4 stand for the expression level of *Hatpi* gene in embryo, larva, pupa and adult, respectively.

3.3 Phylogenetic analysis of TPI proteins

TPIs from different organism were collected from NCBI to investigate the evolutionary relationships with that of *H. armigera* (Fig. 4). The phylogenetic analysis showed that these enzymes were divided into 6 clades according to their evolutionary relationships. TPIs from mammal were classed into the 1st clade. The 2nd clade contained those from fish, frog and chicken. TPIs from insects including HaTPI in the present paper were grouped to the 3rd clade (arrowhead marked, Fig. 4). TPIs in parasites, such as nematoda, trematoda and cestoda, were classed into the 4th clade. The 5th clade consisted of TPIs from fungi and algae. The 6th clade comprised TPIs from plants. Three chloroplast TPIs were closer to fungi and algal enzymes (asterisk marked), which suggested that these chloroplast TPIs may be from algae or fungi genome. Phylogenetics of TPI reflected the evolutionary of organisms to a great extent, which indicated that *tpi* gene could be an ideal candidate in further evolutionary related research.

$(\beta\alpha)_8$ barrel and catalytic residues are conserved in reported TPI family, and there is no exception for them in HaTPI, whose conserved catalytic residues (Lys12, His94 and Glu165) and the peptide motifs (AYEPVWAIGTG and GGASLKPEF) are marked in Fig. 5, respectively. Amino acid comparisons revealed 68% – 95% identity between these TPIs.

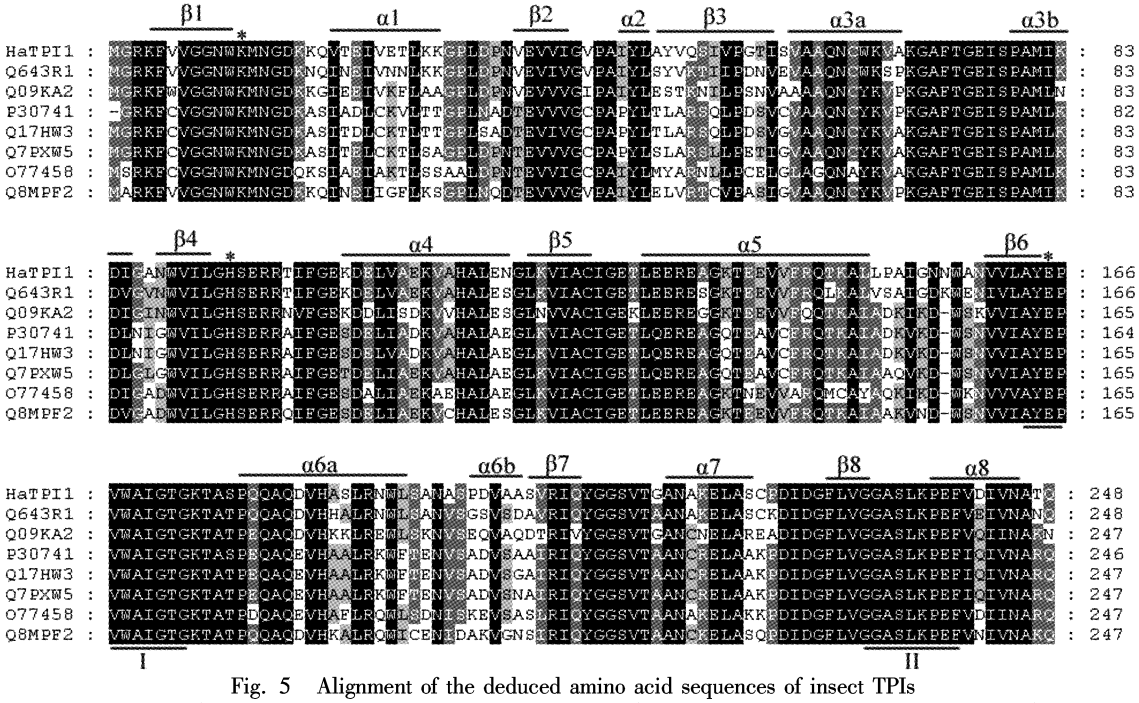

Fig. 5 Alignment of the deduced amino acid sequences of insect TPIs

Q09KA2, *Blattella germanica* (German cockroach); Q8MPF2, *Tenebrio molitor* (yellow mealworm); Q643R1, *Bombyx mori* (silk moth); HaTPI, *Helicoverpa armigera*; P30741, *Culex tarsalis* (encephalitis mosquito); Q17HW3, *Aedes aegypti* (yellowfever mosquito); Q7PXW5, *Anopheles gambiae* str. PEST; O77458, *Drosophila yakuba* (fruit fly). Gray and black shading indicate the conserved and identical residues, respectively. Conserved $(\beta\alpha)_8$ barrel and catalytic residues are shown with underline and asterisk, respectively.

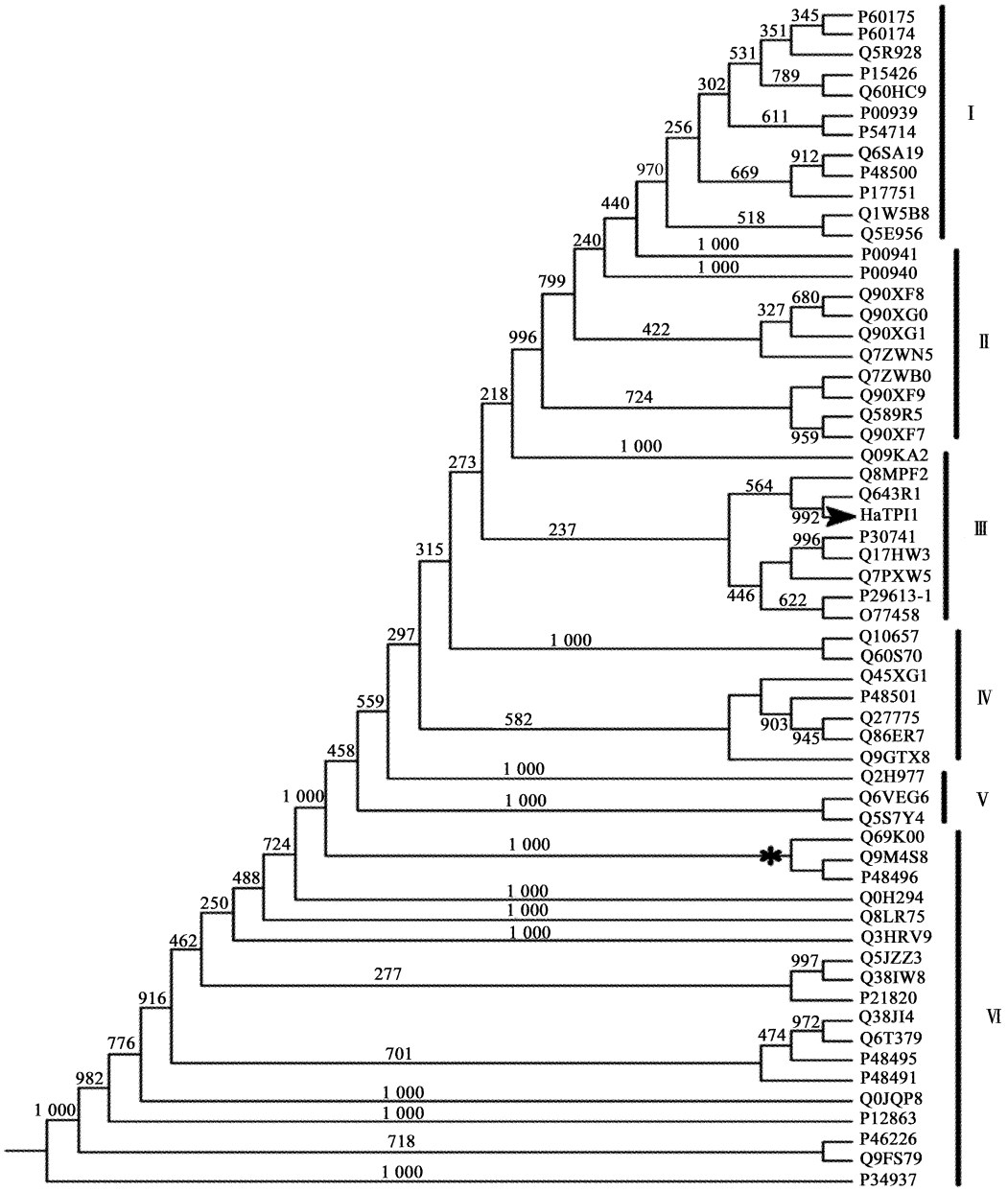

Fig. 4 Phylogenetic tree of the deduced amino acid sequences of 59 TPIs from 52 eukaryotic organisms

The distance tree was calculated using the MEGA program, which was based on a Clustal alignment of the sequences after phylogenetic analysis. Branch lengths are proportional to percentage sequence difference. Numbers indicate the bootstrap values. I, Mammalia: P60175; *Pantroglodytes* (chimpanzee); P60174, *Homo sapiens*; Q5R928, *Pongo pygmaeus* (orangutan); P15426, *Macaca mulatta* (rhesus macaque); Q60HC9, *Macaca fascicularis* (crab eating macaque) (cynomolgus monkey); P00939, *Oryctolagus cuniculus* (rabbit); P54714, *Canis familiaris* (dog); Q6SA19, *Rattus norvergicus* (rat); P48500, *Rattus norvegicus* (rat); P17751, *Mus musculus* (mouse); Q1W5B8, *Sus scrofa* (pig); Q5E956, *Bos taurus* (bovine). II, Vertebrata, Euteleostomi: P00941, *Latimeria chalumnae* (coelacanth); P00940, *Gallus gallus* (chicken); Q90XF8, *Xiphophorus maculatus* (southern platfish); Q90XG0, *Brachydanio rerio* (zebrafish) (Danio rerio); Q90XG1, *Acipenser brevirostrum* (shortnose sturgeon); Q7ZWN5, *Xenopus laevis* (African clawed frog); Q7ZWB0, *Brachydanio rerio* (zebrafish) (Danio rerio); Q90XF9, *Brachydanio rerio* (zebrafish) (Danio rerio); Q589R5, *Oryzias latipes* (medaka fish) (Japanese ricefish); Q90XF7, *Xiphophorus maculatus* (southern platyfish). III, Arthropoda, Hexapoda, Insecta: Q09KA2, *Blattella germanica* (German cockroach); Q8MPF2, *Tenebrio molitor* (yellow mealworm); Q643R1, *Bombyx mori* (silk moth); HaTPI, *Helicoverpa armigera*; P30741, *Culex tarsalis* (encephalitis mosquito); Q17HW3, *Aedes aegypti* (yellowfever mosquito); Q7PXW5, *Anopheles gambiae* str. PEST; P29613-1, *Drosophila melanogaster* (fruit fly); O77458, *Drosophila yakuba* (fruit fly). IV, Nematoda, Trematoda and Cestoda: Q10657, *Caenorhabditis elegans*; Q60S70, *Caenorhabditis briggsae*; Q45XG1, *Orientobilharzia turkestanicum*; P48501, *Schistosoma mansoni* (blood fluke); Q27775, *Schistosoma japonicum* (blood fluke); Q86ER7, *Schistosoma japonicum* (blood fluke); Q9GTX8, *Taenia solium* (pork tapeworm). V, Fungi, Euglenida: Q2H977, *Chaetomium globosum*; Q6VEG6, *Euglena gracilis*; Q5S7Y4, *Astasia longa* (euglenophycean alga). VI, Plants: Q69K00, chloroplast of *Oryza sativa*; Q9M4S8, chloroplast of *Fragaria ananassa* (strawberry); P48496, chloroplast of *Spinacia oleracea* (spinach); Q0H294, *Pteris vittata* (Chinese ladder brake); Q8LR75, *Oryza sativa*; Q3HRV9, *Solanum tuberosum* (potato); Q5JZZ3, *Phaseolus vulgaris* var. *nanus*; Q38IW8, *Glycine max* (soybean); P21820, *Coptis japonica* (Japanese goldthread); Q38JI4, *Solanum tuberosum* (potato); Q6T379, *Solanum chacoense* (Chaco potato); P48495, *Petunia hybrida* (petunia); P48491, *Arabidopsis thaliana*; Q0JQP8, *Oryza sativa*; P12863, *Zea mays* (maize); P46226, *Secale cereale* (rye); Q9FS79, *Triticum aestivum* (wheat); P34937, *Hordeum vulgare* (barley). Three chloroplast TPI that are closer to fungi and algal enzymes are marked with asterisk.

4 DISCUSSION

The *Hatpi* gene reported in present study contains three catalytic residues Lys12 in loop1, His94 in loop4 and Glu165 in loop6 (Fig. 5), two peptide motifs AYEPVWAIGTG around the glutamic acid residues and GGASLKPEF around the C-terminus, which are consistent with previous study (Schliebs *et al.*, 1996; Wang *et al.*, 1999). These results verified that TPI is a highly conserved glycolytic enzyme among different organisms. Being a member of the housekeeping genes, *tpi* is required for cell growth and maintenance (Brown *et al.*, 1985). That is consistent with the expression of *Hatpi* in all examined in the present study.

After the 21 intron positions reported randomly in the *tpi* coding region (Logsdon *et al.*, 1995), none of the identified sequence variations affected the amino acid composition of the TPI protein, hence they are unlikely to impact the catalytic activity of the enzyme (Sun *et al.*, 2008). Yet in the four introns of *Hatpi* gene, only one intron shared a position (between Lys111 and Val112) that was summarized in the 21 known intron positions (Logsdon *et al.*, 1995). Relationships between this variation and the function of *Hatpi* remain unclear until now. A recent study in *Drosophila* reveals that the pathogenesis of TPI deficiency could result from proteasomal degradation of the apparently functional enzyme (Seigle *et al.*, 2008). Moreover, *tpi* had been located on Z chromosome in Lepidoptera insect *Heliconius erato* (Kapan *et al.*, 2006), which may facilitate the future pest control by constructing specific transposal strains T (W, Z) or transgenic lines as that in *Bombyx mori* (Marec *et al.*, 2005). More interest could be focused on in the future.

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棉铃虫磷酸甘油醛异构酶基因 *Hatpi* 的克隆及分析

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摘要：棉铃虫 *Helicoverpa armigera* 是一种严重危害棉花等经济作物的鳞翅目害虫，开展分子水平研究对防控害虫将具有重要参考意义。本研究利用 RACE（rapid amplification of cDNA ends）技术克隆了棉铃虫磷酸甘油醛异构酶（triosephosphate isomerase）基因 *Hatpi*（GenBank 登录号为 AY736358）。该基因 cDNA 全长为 1 149 bp，编码 248 个氨基酸，预测等电点为 5.82，分子量为 16.4 kD。HaTPI 含有磷酸甘油醛异构酶类蛋白的典型$(\beta\alpha)_8$结构、保守的活性位点（Lys12，His94 和 Glu165）和小肽序列（AYEPVWAIGTG 和 GGASLKPEF）等。RT-PCR 检测分析发现 *Hatpi* 在棉铃虫卵巢、幼虫、蛹、成虫均有表达，提示该基因可能在棉铃虫的不同发育阶段均起作用。

关键词：棉铃虫；磷酸甘油醛异构酶；基因；RACE；序列分析；表达

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