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Full Length Research Paper

Identification and expression analysis of *CYP4G25* gene from the Chinese oak silkworm (*Antheraea pernyi*)

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***CYP450* plays an important role in physiological metabolism. A *CYP4G25* gene of P450 family was cloned from *Antheraea pernyi* using reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA end (RACE-PCR). Sequence analysis revealed that this gene was 2112 bp long and has 97.5% identity with *Antheraea yamamai CYP4G25*. Semi-quantitative polymerase chain reaction (PCR) showed that the expression of *A. pernyi CYP4G25* was found in various tissues with no significant changes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis demonstrated that a 63.6 KD recombinant protein was successfully expressed in *Escherichia coli* cells and its expression was not remarkably changed under induction by different isopropyl-β-D-thiogalactopyranoside (IPTG) concentration.**

Key words: *Antheraea pernyi*, *CYP4G25*, expression, cytochrome P450.

INTRODUCTION

Cytochrome P450s are involved in the metabolism of hormone, fatty acid, steroid, drug insecticide and phytotoxin (Mansuy et al., 1998; Hannemann et al., 2007; Isin et al., 2007; Hassanin et al., 2009). Mammal P450s play a dominant role in clearing ingested compounds and controlling the systemic levels of chemical substrates (Ding and Kaminsky, 2003; Bowles et al., 2006), and plant P450s are essential for the biosynthesis of many compounds including phenylpropanoids, lipids, phyto-hormones and carotenoids (Schuler and Werk, 2003; Inoue, 2004). For insects, P450s have extensive physio-logical functions in growth, development and reproduction through the biosynthesis or catabolism of key hormones like juvenile hormone (JH) and 20-hydroxyecdysone (20E) (Feyereisen, 1999; Chavez et al., 2000; Warren et al., 2002; Gilbert et al., 2004; Helvig et al., 2004; Ono et al., 2006). Some P450s are found to be related with the behavioral phenotypes, insecticide metabolism or inactivation of plant toxins (Dierick and Greenspan, 2006; Wang et al., 2008; Che-Mendoza1 et al., 2009; Ai et al., 2010). For example, Drosophila *CYP6G1* and *CYP4E2* genes are related with dichlorodiphenyltrichloroethane (DDT) resistance (Daborn et al., 2002), while housefly *CYP6D1, CYP6A1* and *CYP6Z1* genes are involved in pyrethroid and organophosphate resistance (Andersen et al., 1994; Kasai and Scott, 2000; Nikou et al., 2003). Up to now, lots of P450 genes have been isolated from more than 40 insect species (Chung et al., 2009), however, the exact roles of P450s in various animals remain to be explored.

Chinese oak silkmoth *Antheraea pernyi* is a kind of silk-producing insects and has excellent economical values (Huang et al., 2002; Zhou and Han, 2006). In this study, a novel cytochrome P450 gene was identified from *A. pernyi* and its expression and biological function were also investigated.

MATERIALS AND METHODS

The experimental insect Chinese oak silkworm variety (Keqing) was introduced from the Sericultural Research Institute of Shandong

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Abbreviations: RT-PCR, Reverse transcriptase-polymerase chain reaction; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **IPTG,** isopropyl-β-D-thiogalactopyranoside; **RACE-PCR,** rapid amplification of cDNA end; **ORF,** open reading frame; **PCR,** polymerase chain reaction.

Table 1. The primers used for PCR.

Primer number	Primer sequence
F1 (-72--51)	5'- TAAAACGTAGGTTGTCGGAGTC -3'
R1 (448-468)	5'- ATTCAAGTGAAATGTGGGTGC -3'
F2 (162-183)	5'- ACCACTACCGATAATAGGAAAC -3'
R2 (983-1003)	5'- CAAGGAGTAAGTCAAGGAAAG -3'
F3 (976-996)	5'- CGCCTAGCTTTCCTTGACTTA -3'
R3 (1361-1381)	5'- GACGACGATGGAGTTTGTATG -3'
F4 (1363-1382)	5'- TACAAACTCCATCGTCGTCC -3'
R4 (1818-1839)	5'- GTACTTTCCCAAAACTATCACC -3'
RC5 (386-406)	5'- TAAGAAGTCCGTTACCGAGCC -3'
RC3 (1364-1384)	5'- ACAAACTCCATCGTCGTCCCG -3'

and reared on the leaves of oak.

Total RNA extraction and cDNA synthesis

Total RNA was extracted with TRIzol™ Reagent (Transgene) according to the manufacture's instructions. The RevertAid™ H Minus First Strand cDNA Synthesis Kit was used to synthesize cDNAs for reverse transcriptase-polymerase chain reaction (RT-PCR). For rapid amplification of cDNA end (RACE-PCR), the cDNA was synthesized using SMART™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions.

Cloning and sequencing of *Ap-CYP4G25*

Oligonucleotide primers (Table 1) were designed by Primer premier 5.0 software according to P450 sequences from *Antheraea yamamai* and other insects. RT-PCR was performed at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 40 s, 72°C for 1 min and a final step of 72°C for 10 min. The forward primer RC3 and the reverse primer RC5 were designed for RACE-PCR. RACE-PCR was carried out using the program as follows: denaturalization at 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and 30 s. The PCR products were analyzed on 1% agarose gels, then subcloned into the pMD19-T easy cloning vector (Takara) and sequenced at Invitrogen, Shanghai.

Construction of recombinant plasmids and protein expression

Total RNA from fat body was reverse transcribed into cDNA by a First-Strand System Kit (MBI) according to the protocol. The forward primer 5'- GGCGGATCC- ATGAGCTACACCACA-3' and reverse primer 5'- CGCCTCGAGTTATACTTTGGCTTGTT-TCT-3' (restriction enzyme sites *Bam*HI and *Xho*I are underlined) were designed to amplify the open reading frame (ORF) of *CYP4G25* gene by PCR. The PCR product and Pet-28a vector were ligated after being digested with restriction enzymes. The recombinant plasmids (PET-*CYP4G25*) were identified by sequencing and then transformed into competent *Escherichia coli* BL21 (DE3) cells (TransGen) and induced by different concentrations of isopropyl-β-D-thiogalacto-pyranoside (IPTG).

Western blotting

The recombinant proteins from *E. coli* BL21 (DE3) were subjected to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 6% stacking gel and 12% separating gel, and then transferred onto a polyvinylidene difluoride membrane by an electrophoretic transfer system (Bio-Rad). Membranes were blocked with 1% bovine serum albumin (BSA) (diluted with phosphate-buffered saline containing 0.1% Tween 20 (PBST)) for 2 h at room temperature. Membranes were washed with PBST and subsequently incubated with primary antibodies (diluted 1:2000 with PBST) for 2 h at room temperature. After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG antibody for 1 h at room temperature (Zhu and Wu, 2008), and the final detection was performed with a HRP-DAB Detection Kit (Tiangen).

Expression of *Ap-CYP4G25* in different tissues

Mid-intestine, silk gland, hemocytes, fat body, testis integument and ovary were dissected from the larvae at day 3 of the fifth instar and antennae was collected from adult, they were immediately frozen in liquid nitrogen and stored at -72°C. Semi-quantitative PCR was carried out with specific primers F: 5′-GCTCGTGCCGGCT CTCTAATCCT-3′ and R: 5′-ACCGGCAGCTGTTGTATCGTGA-3′ to determine the expression level of *CYP4G25.* The actin gene (GenBank accession number GU073316) was used as an internal reference (with primers F: 5′-TCTGGCACCCCACCTTCTAC-3′ and R: 5′-CCGATTGTGATGACTTGAC-3′). The amplification program used for semi-quantitative PCR was 30 cycles of 94°C for 30 s, 55°C for 35 s and 72°C for 40 s.

RESULTS

Cloning and sequence analysis of *CYP 4G25* cDNA

A cDNA fragment of 2112 bp was obtained by RT-PCR and RACE–PCR. The sequence had been deposited in the GenBank database with accession number GU205081. Nucleotide sequence analysis revealed that *CYP4G25* cDNA contains a 111 bp 5'-untranslated sequence, a putative ORF of 1674 bp, a 326 bp 3′-untranslated region (3′UTR) and a putative poly-adenylation signal. Based on the deduced amino acid sequences, the heme-blinding region (residues 491 to 500), I-helix domain (residues 352 to 361), K-helix domain (residues 416 to 419), C-helix domain (residues 142 to 146) and N-terminal transmembrane anchoring signal (residues 13 to 35) were found using the ExPASy

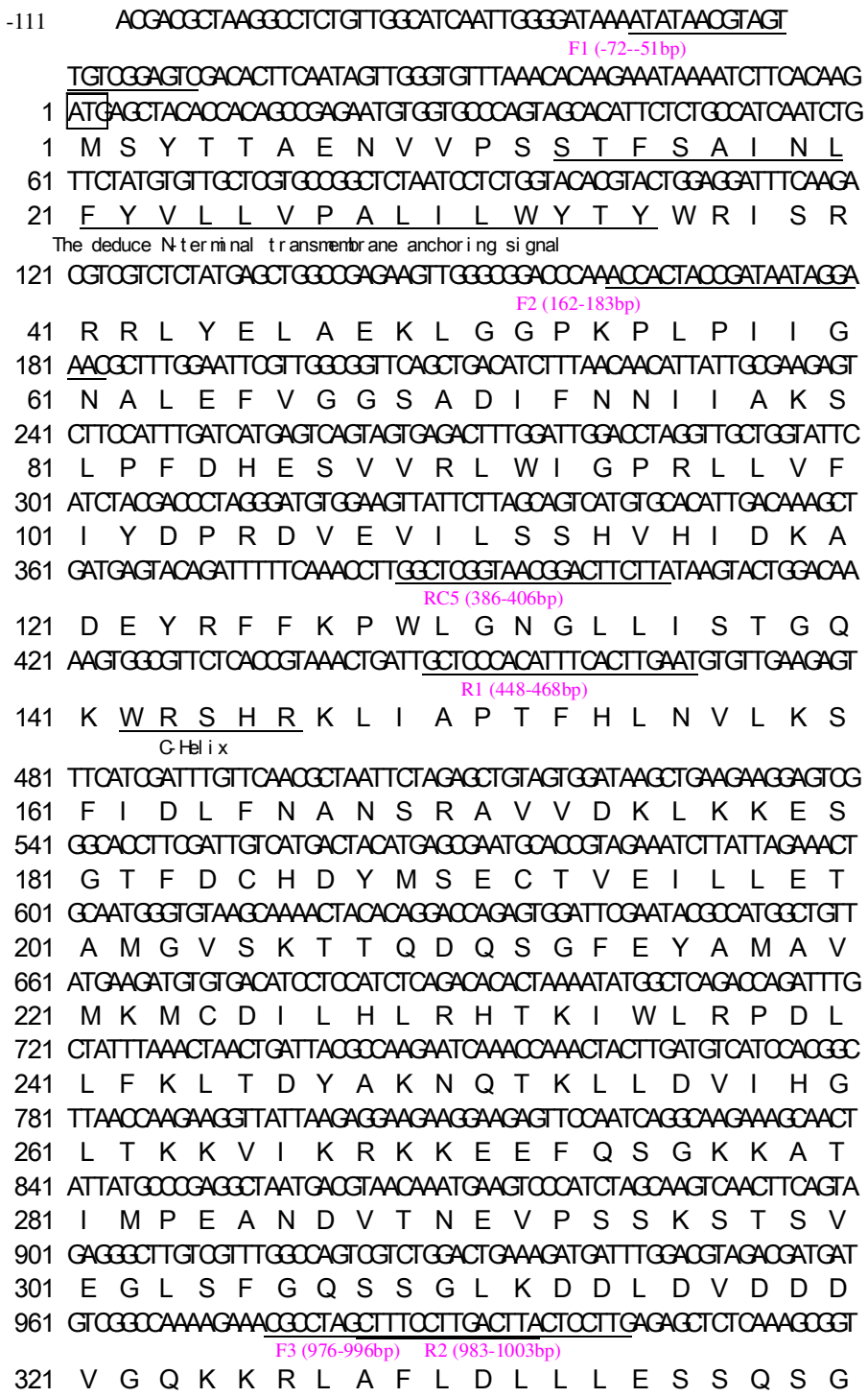

Figure 1. Nucleotide sequence and amino acid sequence of *CYP4G25* from *A. pernyi*. Translation start codon (ATG) and termination codon (TAA) are boxed and the polyadenylation signals AATAAA are double-underlined.

Proteomics tools (Figure 1). Phylogenetic analysis indicated that *A. pernyi CYP4G25* gene has 97.5% identity with *A. yamamai CYP4G25* and 88.3% with *Bombyx mori CYP4G25* (Figures 2 and 3).

Protein expression and Western blotting

The ORF of *CYP4G25* was amplified by PCR and ligated to Pet-28a vector for protein expression. A recombinant

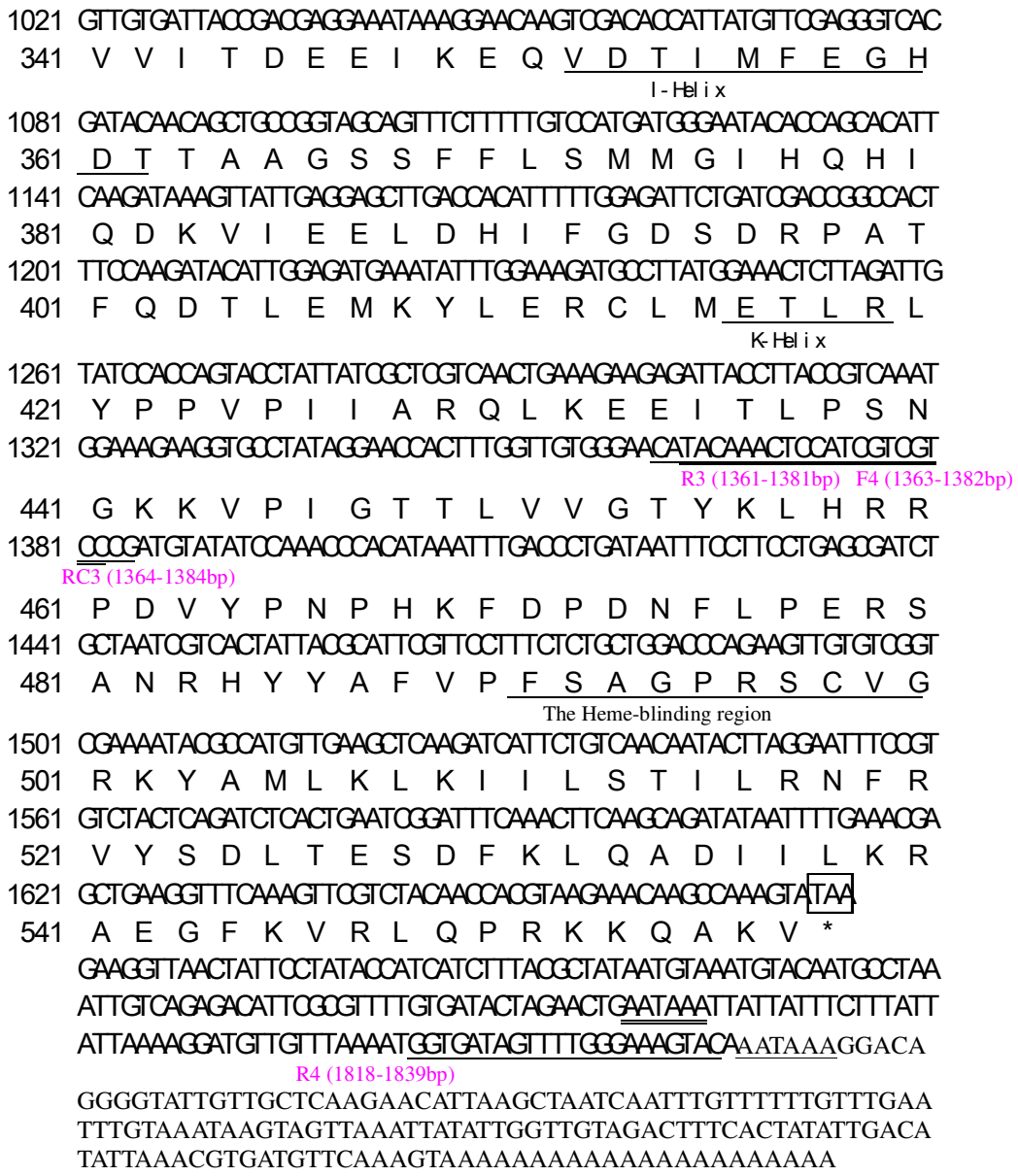

Figure 1. Continued.

protein with a molecular weight of about 63 kDa was detected by SDS-PAGE and the expression was not influenced by different IPTG concentrations (Figure 4).

Western blot analysis of recombinant protein showed that a consensus 63 kDa protein band was detected using anti-His antibody, while there was none in the control group (Figure 5). All this indicate the successful expression of the recombinant *CYP4G25* protein in *E. coli* BL21 (DE3) cells.

Expression of *Ap-CYP4G25* in different tissues

Semi-quantitative PCR was carried out to detect the expression of *CYP4G25*. The *CYP4G25* gene ubiquitously expressed in fat body, integument, midintestine, hemocytes, silk glands, antennae, testis and ovary with no obvious difference (Figure 6). These results suggest that the *CYP4G25* plays an important role in the growth and development of *A. pernyi*.

DISCUSSION

In this study, a full-length cDNA encoding *CYP 4G25* gene was identified from *A. pernyi*. The cDNA is 2112 bp long and contains an open reading frame of 1674 bp. The predicted protein consists of 557 amino acids with a

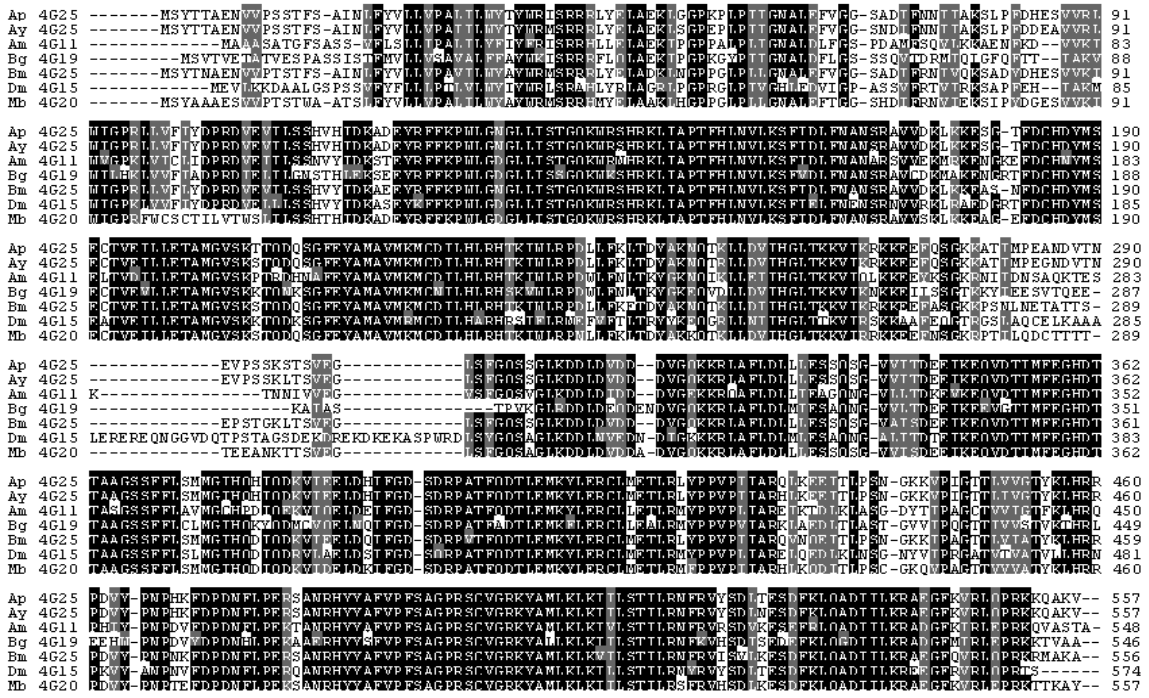

Figure 2. Sequence alignment of the *A. pernyi CYP4G25* proteins with its homologues. The *CYP* proteins from *A. yamamai CYP4G25* (BAD81026), *Apis mellifera CYP4G11* (ABB36785), *Blattella germanica CYP4G19* (AAO20251), *B. mori CYP4G25* (ABF51415), *Drosophila melanogaster CYP4G15* (AAF76522) and *Manduca sexta CYP4G20* (ADE05582) were included.

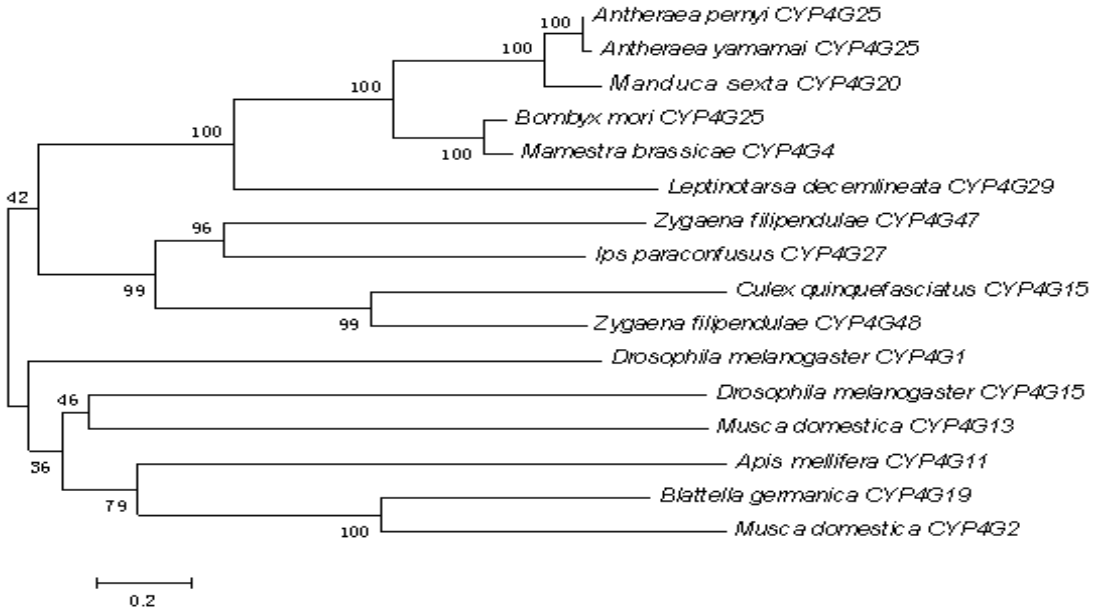

Figure 3. Phylogenetic analysis was performed by MEGA (version 4.0) program based on the *CYP4G25* amino acid sequences from various insects. The phylogenetic tree was constructed using the neighbor-joining algorithm method and bootstrap values (1000 repetitions) of the branches are indicated. The *CYP* proteins from other organisms are: *A. yamamai CYP4G25* (BAD81026), *A. mellifera CYP4G11* (ABB36785), *B. germanica CYP4G19* (AAO20251), *B. mori CYP4G25* (ABF51415), *D. melanogaster CYP4G1* (ABY20430) and *CYP4G15* (AAF76522), *M. sexta CYP4G20* (ADE05582) and *Mamestra brassicae CYP4G* (AAR26517), *Culex quinquefasciatus CYP4G15* (EDS33030), *Leptinotarsa decemlineata CYP4G29* (AAZ94273), *Zygaena filipendulae CYP4G47* (ACZ97413) and *CYP4G48* (ACZ97414), *Ips paraconfusus CYP4G27* (ABF06553), *Musca domestica CYP4G2* (ABV48808) and *CYP4G13* (AAK40120).

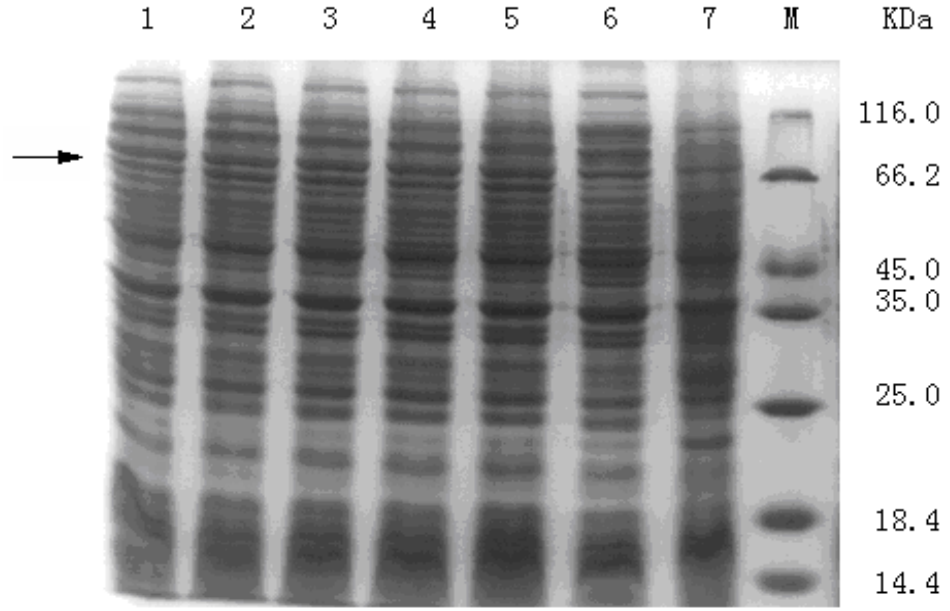

Figure 4. Analysis of recombinant *Ap-CYP4G25* protein on 12% SDS-PAGE gels. The protein amount used for SDS-PAGE was 30 µg in each lane and the gels were revealed by Coomassie blue R-250 staining. Bacterial proteins were collected after 4 h induction with different IPTG concentration. Lanes 1 to 5, after induction with 0.2, 0.4, 0.6, 0.8 and 1.0 mM IPTG, respectively; Lane 6, before induction; Lane 7, *E. coli* BL21(DE3); M, molecular weight marker.

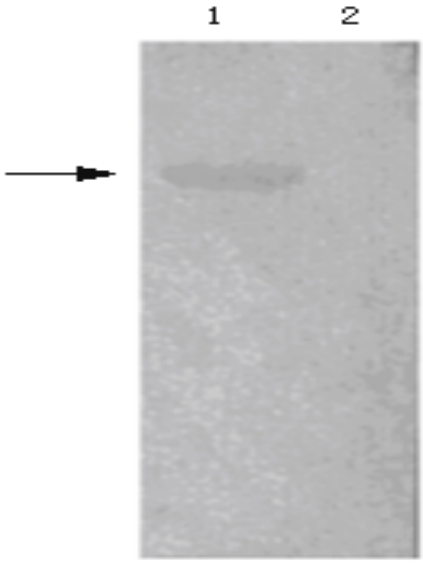

Figure 5. Western blot analysis of recombinant proteins with anti His-tag antibody. A total of 30 µg recombinant protein was used for Western blotting and a protein band with a molecular mass of about 63.6 kDa was detected by Western blotting using anti His-tag antibody. No immunoreactive band was found in the control group. Lane 1, After IPTG induction; lane 2 , no IPTG induction.

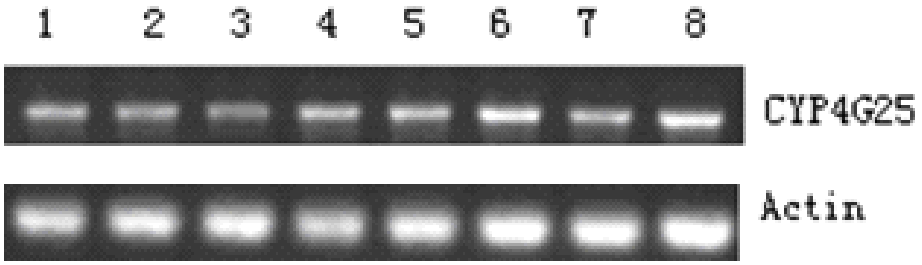

Figure 6. Expression analysis of *Ap-CYP4G25* by semi-quantitative PCR. Lanes 1 to 8, expression of *Ap-CYP4G25* in mid-intestine, silk gland, hemocytes, fat body, testis integument, ovary and antennae, respectively. The expression of Ap-actin was used as a control.

calculated molecular mass of 63.6 kDa, which is somewhat larger than the size of other known vertebrate and invertebrate P450s (55 to 60 kDa). Phylogenetic analysis indicated that *A. pernyi CYP4G25* was highly homologous to that of *A. yamamai* and a heme-binding domain (FXXGXRXCXG) which serves as fifth ligand to the heme iron and a K helix was found in the protein sequence (Werk and Feyereisen, 2000).

The *CYP4* is a member of the most ancient P450s, and many *CYP4* subfamilies have been identified in arthropods and their enzymatic activities had been determined (Snyder et al., 1995; Pittendrigh et al., 1997). According to the reports, the new gene *CYP4G20* may be associated with the diversity of odorants (Maibeche et al., 2005), while *CYP4G15* is probably important for the metabolism of endogenous compounds (Maibeche et al., 2000), and *CYP4G25* in *A. yamamai* is associated with diapause (Yang et al., 2008). Furthermore, P450s are involved in the detoxification of many xenobiotics (Feyereisen, 1999; Isin et al., 2007). However, the exact biological function of *CYP4G25* in *A. pernyi* remains unknown.

All together, *A. pernyi CYP4G25* was characterized in this experiment and it was ubiquitously expressed in all examined tissues, and the prokaryotic expression of this protein was also successfully performed, we hope these results will provide some information for further studies.

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