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Londono, Diana K.; Siqueira, Herbert A.A.; Wang, Haichuan; Sarath, Gautam; Lydy, Michael J.; and Siegfried, Blair D., "Cloning and expression of an atrazine inducible cytochrome P450, *CYP4G33*, from *Chironomus tentans* (Diptera: Chironomidae)" (2007). *Publications from USDA-ARS / UNL Faculty*. 44. <https://digitalcommons.unl.edu/usdaarsfacpub/44>

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Cloning and expression of an atrazine inducible cytochrome P450, *CYP4G33*, from *Chironomus tentans* (Diptera: Chironomidae)

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Received 8 February 2007; accepted 11 April 2007

Available online 19 April 2007

Abstract

Previous studies performed in our laboratory have measured the effect of atrazine exposure on cytochrome P450-dependent monooxygenase activity and have found increased activity in midge larvae (*Chironomus tentans*) as a result of atrazine exposure (1–10 ppm). Here we report the cloning and expression of a specific *C. tentans* CYP4 gene that is responsive to atrazine induction with an open reading frame of 1678 bp which encodes a putative protein of 559 amino acid residues. Alignments of deduced amino acid sequences with other insect P450 genes and phylogenetic analysis indicated a high degree of similarity to other insect CYP4 genes. Northern blotting analysis employing a fragment of 1200 bp from the CYP4 gene as a probe indicated that the CYP4 gene was expressed in all developmental stages, but was expressed at highest levels in late instar larvae. Additionally, over-expression of CYP4 in *C. tentans* exposed to atrazine (10 mg/l) confirms the ability of atrazine to induce specific P450 genes and provides insight into potential consequences of atrazine exposure in aquatic organisms.

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Keywords: Cytochrome P450; Atrazine; Induction; *Chironomus tentans*; Expression

1. Introduction

Cytochrome P450 (CYP)-dependent microsomal monooxygenases constitute the largest gene superfamily found in nature. The cytochrome P450 enzyme system has been detected in virtually all organisms examined from bacteria to mammals [1]. These enzymes constitute an extremely important metabolic system because of their involvement in regulating the titers of endogenous compounds such as hormones, fatty acids, and steroids. Additionally, this enzyme system plays a central role in the metabolism of xenobiotics such as drugs, pesticides, and plant toxins [1].

In insects, cytochrome P450 involvement in metabolism of insecticides results in either bio-activation or, more fre-

quently, in detoxification, the latter process being enhanced in many insect species that have developed metabolic resistance to insecticides. Additionally, cytochrome P450 is inducible through a mechanism shown to be largely controlled at the transcriptional level [2]. The net result of induction is often observed simply as an increase in enzyme activity. The ecological and physiological significance of induction is uncertain, although with insects, induction is thought to provide versatility in environmental adaptation [3] and may be a protective mechanism whereby the organism can detoxify lipophilic compounds that might otherwise accumulate to potentially toxic levels within cells [4].

The presence of an inducible cytochrome P450 system has been established in a number of different insects [5]. Given the importance of this enzyme system in both activation and detoxification of xenobiotics, induction may play a role in chemical interactions. Several recent studies have shown

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that in larvae of the midge, *Chironomus tentans* (Fabricius) (Diptera: Chironomidae), simultaneous exposure to the triazine herbicide, atrazine and selected organophosphate insecticides caused greater-than-additive toxicity [6–8]. Body residue analysis of midge larvae exposed in vivo to atrazine and chlorpyrifos mixtures indicated that chlorpyrifos was metabolized more rapidly in atrazine-treated midges compared to unexposed controls [7]. Importantly, in vivo metabolism of chlorpyrifos by treated and control midges indicated that the toxic metabolite, chlorpyrifos oxon, was formed more rapidly in atrazine-exposed midges [7].

These results strongly suggest that although atrazine is not acutely toxic, it may act as an inducer of cytochrome P450 activity. We have measured the effect of atrazine exposure on cytochrome P450-dependent monooxygenase activities including aldrin epoxidase [9] and *O*-demethylase [10] and observed increased activity in both assays as a result of atrazine exposure. A 45 kDa protein of increased intensity was also observed after SDS–PAGE of microsomal protein which was similar in size to cytochrome P450 enzymes reported for other insects. Heme staining of SDS–PAGE gels and immunochemical studies using a *Drosophila melanogaster* anti-P450 polyclonal antiserum further supported the cytochrome P450 nature of this inducible 45 kDa protein. A region of a cytochrome P450 family 4 gene was amplified using degenerate primers and sequenced from *C. tentans* larvae, and Northern blot analysis employing the CYP4 gene fragment as a probe indicated over-expression in larvae exposed to atrazine [10].

Atrazine is a herbicide that belongs to a group of pesticides used widely throughout the Midwestern U.S. and has been commonly reported as a contaminant of surface waters [11]. Although a biochemical understanding of atrazine induction of P450 enzymes in *C. tentans* and its potential to interact with other aquatic contaminants is emerging, specific P450 genes and gene products that are induced by atrazine have yet to be identified. In this manuscript, we describe the cloning, expression, and phylogenetic analysis of a novel atrazine-inducible family 4 cytochrome P450 from *C. tentans*.

2. Materials and methods

2.1. Insect population

A colony of *C. tentans* was obtained from Wichita State University, Department of Biological Sciences, and maintained according to U.S. EPA protocols [12] for static cultures with the slight modification that cultures were maintained with a mixture of developmental stages.

2.2. Atrazine exposure

Midge larvae were exposed to atrazine by maintaining groups of 50 third instars in 1 l of moderately hard water in glass beakers at room temperature (20–22 °C) and ambient lighting. Approximately 2 cm of sand was added

to each beaker prior to introducing midges. An experiment consisted of control (without atrazine) and three experimental beakers with atrazine at 10 mg/l. After acclimation of midges for 24 h, 1 ml of technical grade atrazine (99% purity), purchased from Chem Service (West Chester, PA, USA) in ethyl acetate was added to the experimental beakers to achieve a concentration corresponding 10 mg/l. Control treatments consisted of beakers treated with 1 ml of ethyl acetate. After 90 h of exposure, the midges were collected from each beaker for RNA isolation.

2.3. RNA isolation

Total RNA was isolated using TRIzol Reagent from Invitrogen Life Technologies (Carlsbad, CA). *C. tentans* first, second, and third instar larvae, pupae and adult tissue (~100 mg/each) were ground in liquid N₂ and processed with TRIzol to generate total RNA according to manufacturer instructions. The final RNA pellet was dissolved in 50 µl of distilled, autoclaved water. Extracted RNA was diluted in Tris–HCl buffer (5 mM, pH 8.0), quantified spectrophotometrically using the absorbance ratio >1.8 at 260/280 nm [13] and stored at –80 °C until further use.

2.4. RACE PCR, cloning and sequence analysis

Total RNA was used for RACE PCR (Rapid Amplification of cDNA ends) reactions. The SMART RACE cDNA Amplification kit (BD Biosciences, San Diego, CA) was used according to the manufacturer's instructions for both 5' and 3' RACE reactions. The PCRs were performed with oligonucleotide primers designed from the atrazine-inducible CYP4 fragment previously described from *C. tentans* [10]. The following CYP4G33 gene-specific primers were used for the initial 5' RACE and 3' RACE reactions, respectively: 5'-ATAATGATTGTTGTGCCTGCTGG-3' and 5'-TCCAGCAGGCACAACAATCATT-3', both paired separately with the universal primer A mix (UPM) provided in the SMART RACE cDNA amplification kit. Following amplification, RACE products were separated on a 1% agarose gel. Products were excised from the gel and purified using a QIAquick gel extraction kit (QIAGEN, Valencia, CA). Isolated fragments were cloned into the vector pCR2.1 TOPO (Invitrogen, Carlsbad, CA). Positive clones were sequenced at the Iowa State University DNA sequencing facility. By merging the overlapping sequences from the 3' and 5' reactions, a putative full-length cDNA was generated. Sequence analyses of gene fragments were repeated at least three times with different RNA preparations.

To confirm that the sequences generated by RACE PCR were from the same gene, nearly the full-length cDNA was amplified using gene-specific primers (Fig. 1) complementary to the 5'- and 3' ends of the cDNA sequence using first strand cDNA as template.

gatttcagttttttcattcaattgatagtgataagtagtagttgaaatattttctcatcaaattaaggattaacataccaaca

1 M A V E Q I I Q S S V F S S P L L M P L L A I V F V L A A V
 1 atggctgtg**gagcaaatcattcagagtagtg**gtttctcatctccactactgatgcctctccttgcaattgttttctggtctctgtgtgctgtg

F1

31 H F W Q M S R R E R K I G D L L P G P P T V P I I G N A Y L
 91 cacttttggcaaatgtcaagaaggagagaaaaattggagatttacttccaggaccaccgacagttccaattattggaaacgcttattta

61 F M N G T N H E M F K K A V D L V N C Y G S V V R G W V G H
 181 ttcataaacggcacaacatcatgaaatgttcaaaaagccgctgcacctcgtcaactgctacggaagcgtgctcgtggtgggttagggcac

91 K L L V G L S D P R D V E I I L G S Q V H I D K S D E Y R F
 271 aaacttctcgtcgtctctcagatccacgtgatgttgaatcatcttgggcagtcaagtccacattgataagtcagacgaatacagattc

121 F R P W L G N G L L I S S G D K W R T H R K L I A P A F H M
 361 ttcgaccttggctcgtaattggcttgtaatttcaagtggagataaatggcgaacacatagaaaactcatgcccccagccttccacatg

151 N V L K S F M A T F N D N S R F V I K K L M K E A G K E F D
 451 aatgttttgagagcttcatggcaactttcaatgataaacgctgatttgaatcaagaaatgaaggaagctggaaaggaattcgtat

181 C H D Y M S E A T V D I L L E T A M G S K R T S E S E E G F
 541 tgcatacactatagtgtaagctactgttgatattcttttggaaacagccatgggatcaaagagaacaagtgaagtgaggaagattc

211 K Y A M A V M K M C D I L H R R Q F K I F S R F E P F F T L
 631 aagatgcaatggctgttatgaaaatgtgacacatcctccacagacgtcaattcaaaatcttctcaagattcgaaccattcttccacctg

241 T G M K E Q Q K K L L G I I H G M T Q R V L N E K K A I F D
 721 accggcatgaaggaacaacaaaaaagcttttaggcacattcattcatggtatgacacaaagggtactcaacgaaagaaggcaatctttgat

271 K N L S E G N L P S P S L Q E I I K T D A S V D Q A I K K A
 811 aagaatttatcagaagtaacctcccatcaccatcacttcaagaaattattaaaactgatgcacagttgatcaagcaattaagaaagca

301 K A K A Q N I D A G L H D D L D D I D E N D V G E K R R L A
 901 aaggcaaggcacaacaaatgatgcccgtcttcatgatgatcttgatgatattgatgaaaatgatgttggtgaaagagacgattggca

331 F L D L M I E T S H Y N P Q Q L S Q E E I K Q Q V D T I M F
 991 ttccttgatttgatgattgaaacttcacattataatccacaacaattgtctcaggaggaaattaacaacaagtcgacacaattatgttt

361 E G H D T T A A G S S F T L C M L G C H P D I Q E K V Y Q E
 1081 gaaggacatgacacaactgctgctggatctagttttacattgtgtatgctaggatgctcatccggatccaagaaaaagtttatcaggaa

391 Q K A I F G D S D R D C T F A D T L E M K Y L E R V I F E T
 1171 caaaaagcaatttttgggtgattcagatcgagactgtacattcgcagatacattggagatgaaatatttggaaagctcatttttgaaca

421 L R M Y P P V P L I A R K I N K D I R L A S C D Q V V P A G
 1261 ttgaggatgtaccaccagttccacttattggcagaaaaatcaacaagacatccgtcttgcacatgtgatcaagttgttccagcaggc

451 T T I I I A T V K I H R R P D I Y P N P D K F D P D N F L P
 1351 acaacaatcattattgtctacagttaaaattcatcgacgacctgatctctccaaccagacaaaatttgatcctgacaacttttacc

481 E R T S N R H Y Y G F I P F S A G P R S C V G R K Y A M L K
 1441 gaagaacatcaaacagacattattatggcttcattccattcagtgctggaccacgatcatgtgttgagcgaataacgcaatggtgaa

511 L K V L L S T I V R N F Y V K S T V P E K D F K L Q A D I I
 1531 ttgaaagtctctcagtagcaattgtgaggaacttctacgtaagagtacagttccagagaagatttcaagcttcaagctgatattatc

541 L K R T D G F R I K L E P R K T K A N *end
 1621 ttgaagagaactgatggattcagaattaaattggaa**ccaagaagaccaagctaact**aaattgacaaatattaatattaatagcaaatg

R2

1711 acttatgatgaccttgaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Fig. 1. Full length cDNA sequence of *CYP4G33* (Accession No. AY880065) and the conceptual translation of this gene. Both amino acids and nucleotides are numbered on the left. Gene specific primers used to confirm sequences from RACE PCRs are in bold and amino acids in conserved regions within P450 underlined. Both start codon (atg) and stop codon (taa) are double underlined.

2.5. Sequence and phylogenetic analyses

Assembly of sequence fragments, sequence confirmation and amino acid translations were conducted using Vector NTI ContigExpress (Invitrogen, Carlsbad, CA). Sequence alignment of the deduced *C. tentans* CYP4G33 protein with other CYP4 proteins was performed using ClustalW [13] within the MEGA3.1 program [14] with default parameters. Computer-assisted phylogenetic analysis was conducted with the MEGA3.1 program [14], using the bootstrapping N–J tree (1000 trials) with the Jones–Tay-

lor–Thornton (JTT) matrix, pairwise deletion of gaps and missing data, and a uniform rate of evolution. A consensus tree was obtained which shows all the branches that are supported at the default cutoff bootstrap confidence limits (BCL) of ≥50%.

2.6. Northern blotting

A 1.2 kb fragment corresponding to the cytochrome P450 family 4 gene isolated from *C. tentans* was labeled by direct dioxigenin (DIG)-labeling of DNA fragments

generated by PCR amplification using the PCR DIG Probe Synthesis Kit (Roche Biochemical; Mannheim, Germany) according to the manufacturer's instructions. Northern blot-

ting was performed with RNA fixed to nylon membranes (Zeta-Probe; Bio-Rad, Hercules, CA) by capillary transfer following denaturing formaldehyde agarose electrophoresis

Table 1

Percentage of amino acid identity among 24 insect CYP4 full genes with atrazine inducible *CYP4G33* from *C. tentans*

Insect species	Family	% Identity	Source
<i>Aedes aegypti</i>	Similar to CYP4G17 ^a	64	EAT39885
<i>Anopheles gambiae</i>	CYP4G17	62	^a
<i>Apis mellifera</i>	CYP4G11	58	ABB36785
<i>Bombyx mori</i>	CYP4G25	58	ABF51415
<i>Leptinotarsa decemlineata</i>	CYP4G29	58	AAZ94273
<i>Tribolium castaneum</i>	Similar to CYP4G15	57	XP973423
<i>Aedes aegypti</i>	Similar to CYP4G16 ^a	56	EAT44585
<i>Anopheles gambiae</i>	CYP4G16	56	^a
<i>Antheraea yamamai</i>	CYP4G25	56	BAD81026
<i>Ips paraconfusus</i>	CYP4G27	55	ABF06553
<i>Tribolium castaneum</i>	Similar to CYP4G15 isof-1	55	XP966683
<i>Blatella germanica</i>	CYP4G19	54	AAO20251
<i>Drosophila melanogaster</i>	CYP4G1	54	AAF45503
<i>Drosophila melanogaster</i>	CYP4G15	52	AAF76522
<i>Musca domestica</i>	CYP4G13	50	AAK40120
<i>Blaberus discoidalis</i>	CYP4C1	38	AAA27819
<i>Drosophila melanogaster</i>	CYP4C3	36	AAF57098
<i>Coptotermes acinaciformis</i>	CYP4U1	35	AAC03111
<i>Mamestra brassicae</i>	CYP4L4	34	AAL48300
<i>Manduca sexta</i>	CYP4M2	34	AAC21661
<i>Diabrotica virgifera virgifera</i>	CYP4AJ1	31	AAF67724

^a <http://drnelson.utmem.edu/anopheles.fasta.html>.

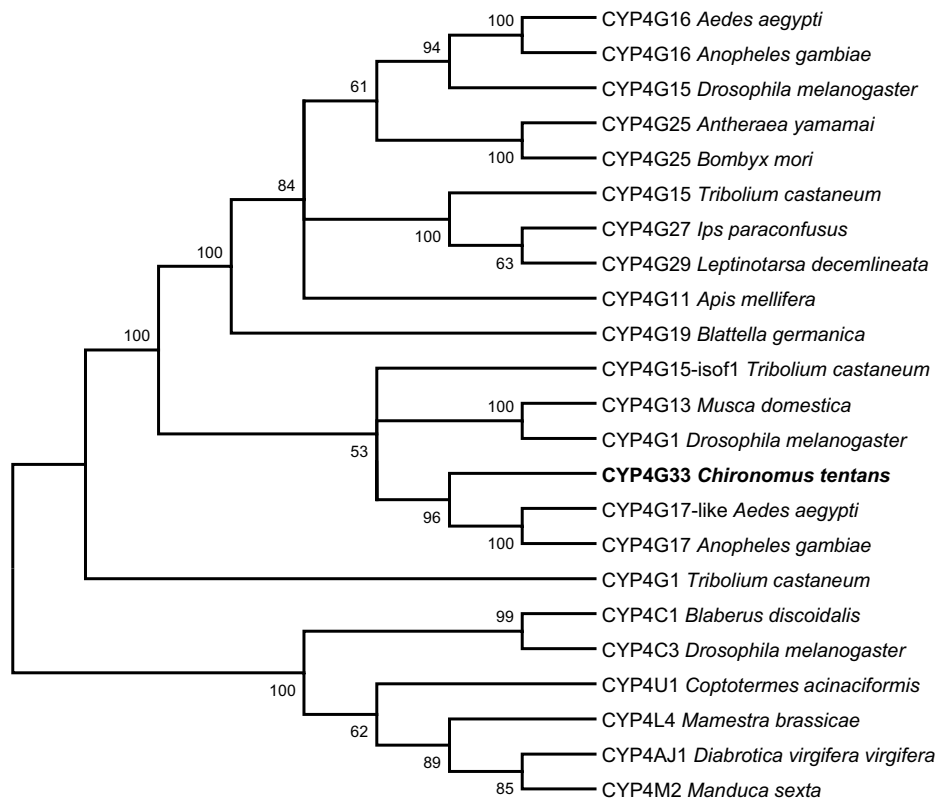


Fig. 2. Phylogenetic tree of aligned *Chironomus tentans* CYP4G33 with other CYP4 sequences from different insect orders. Alignment was performed with ClustalW within the MEGA 3.1 package. See Section 2 for details of analysis. The numbers close to the nodes correspond to the bootstrap values.

[15]. Hybridization occurred overnight at 42 °C followed by three washes at room temperature with a final wash at 68 °C under mild agitation. Luminescent detection was accomplished using the DIG High Prime Detection Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. A control probe for an actin gene was amplified from a *C. tentans* cDNA mix using the forward primer, 5'-TCAGGGTGTGATGGTAGG-3' and reverse primer, 5'-CTCTTTCTGCTGTGGTGGTG-3' to generate a 560 bp fragment. RNA used in blotting experiments was extracted as described previously, quantified spectrophotometrically and standardized loadings were confirmed by hybridization with an actin probe specific for *C. tentans* (Accession No. DQ176317).

3. Results and discussion

3.1. Cloning and sequencing

CYP4 gene fragments corresponding to the 3' and 5' cDNA ends were successfully cloned, and the overlapping fragments generated an open reading frame of 1678 bp with a deduced protein of 559 amino acids (AA) and a predicted molecular mass of 63.8 kDa (Fig. 1). Gene-specific primers that flanked most of the ORF resulted in a fragment of the expected size and sequence. The full-length cDNA of this CYP4 gene has been named *CYP4G33* by the P450 Nomenclature Committee (Accession No. AY880065). CYP4 genes from several different insect orders share a high degree of sequence similarity with *CYP4G33* (Table 1) and support its assignment to the CYP4 P450 family. The highest percent AA identities (Table 1) were with *CYP4G17* (64%) from *Aedes aegypti* and *CYP4G17* (62%) from *Anopheles gambiae*, and all three genes appear to be closely related (Fig. 2).

The deduced AA sequence of *CYP4G33* contains important domains that are conserved among microsomal P450s (underlined AAs, Figs. 1 and 3). The P450 protein signa-

ture motif in the heme binding region, FxxGxRxCxG [16] is present at AA residues 494–503 (Fig.1). The deduced AA sequence also shares a number of common characteristics with other members of the P450 superfamily, such as the charge pair consensus (ExxR) [17] within the K-helix, the consensus (WxxxR) in the C-helix and the consensus sequence (A/G/E)GxxT (Figs. 1 and 3) [18,19].

A partial AA sequence alignment in regions conserved among P450 enzymes indicates a highly conserved AA sequence that appears as 12 residues (QVDTIMFEGHDTT) (354–361). This conserved region is in contrast to the 13-residue motif (EVDTFMFEGHDTT) previously reported as invariant among CYP4 family members [20,21]. The differences at positions 354 (E/Q), 356 (D/G/N), and position 358 (F/I) indicate that this region is less conserved than previously thought.

3.2. Determination of CYP4 atrazine induction by Northern blotting

Following atrazine exposure for 90 h at 10 mg/l, mRNA was isolated from exposed and un-exposed third instar larvae from *C. tentans*. A single 695 bp mRNA band hybridized with the actin probe (control) and a 1.9 kb mRNA

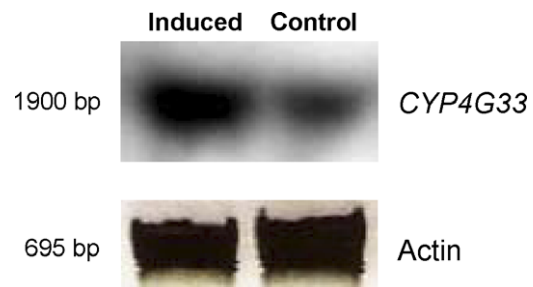


Fig. 4. Northern analysis of *C. tentans* CYP4 expression in induced (exposed to 10 mg/l atrazine in solution for 90 h) and unexposed 3rd instars in comparison with *C. tentans* actin expression in control and atrazine-exposed 3rd instar larvae.

Species	CYP4 Gene	362	419	470	494
<i>Chironomus tentans</i>	CYP4G33	LSQEEIKQ	QVDTIMFEGHDTTAAAGSSF...	FETLRM...PNE	DKFDPD...IPFSAGPRSCVGR
<i>Spodoptera litura</i>	CYP4G32	-----	QVDTIMFEGHDTTAAAGSSF...	LETLRM...KDE	DTFNPD...IPFNAGPRNCIGQ
<i>Spodoptera litura</i>	CYP4G31	-----	QVDTIMFEGHDTTAAAGSSF...	LESLRM...KDE	NTFNPD...IPFTAGPRNCIGQ
<i>Spodoptera litura</i>	CYP4G30	-----	QVDTIMFEGHDTTAAAGSSF...	LETLRM...KDE	DTFNPD...IPFTAGPRNCIGQ
<i>Leptinotarsa decemlineata</i>	CYP4G29	INDEEIKE	QVDTIMFEGHDTTAAAGSSF...	METLRM...PNE	DKFDPD...IPFSAGPRSCVGR
<i>Antheraea yamamai</i>	CYP4G25	ITDEEIKE	QVDTIMFEGHDTTAAAGSSF...	METLRL...PNE	HKFDPD...VPFSAGPRSCVGR
<i>Bombyx mori</i>	CYP4G25	ISDEEIKE	QVDTIMFEGHDTTAAAGSSF...	METLRL...PNE	KNKFPD...VPFSAGPRSCVGR
<i>Anopheles funestus</i>	CYP4G21	-----	QVDTIMFEGHDTTAAAGSSF...	FETLRM...PHE	PETFNPD...IPFTAGPRNCIGQ
<i>Blatella germanica</i>	CYP4G19	LTDEEIKE	QVDTIMFEGHDTTAAAGSSF...	LEALRM...PNE	DVYDPD...VPFSAGPRSCVGR
<i>Anopheles gambiae</i>	CYP4G17	-----	QVDTIMFEGHDTTAAAGSSF...	FETLRM...PHE	PETFNPD...IPFTAGPRNCIGQ
<i>Aedes aegypti</i>	CYP4G17	LTDEEIKE	QVDTIMFEGHDTTAAAGSSF...	FETLRM...PNE	DVFNPD...IPFSAGPRSCVGR
<i>Drosophila melanogaster</i>	CYP4G15	ITDTEIKE	QVDTIMFEGHDTTAAAGSSF...	METLRM...ANP	NVDFPD...VPFSAGPRSCVGR
<i>Tribolium castaneum</i>	CYP4G15	LNDEEIRE	QVDTIMFEGHDTTAAAGSSF...	LETLRM...PNE	DKFDPD...IPFSAGPRSCVGR
<i>Musca domestica</i>	CYP4G13	WTDKDVMD	QVDTIMFEGHDTTAAAGSSF...	CETQRL...PDE	PEKFPD...IPFSAGPRSCVGR
<i>Apis mellifera</i>	CYP4G11	LTDKEVKE	QVDTIMFEGHDTTAAAGSSF...	LETLRM...PNE	DVDFPD...VPFSAGPRSCVGR
<i>Solenopsis invicta</i>	CYP4AB1	LTDVDIRE	QVDTIMFEGHDTTAAAGSSF...	KESLRL...PNE	DVDFPD...IPFSAGPRNCIGQ
<i>Blaberus discoidalis</i>	CYP4C1	MSDTDIRE	QVDTIMFEGHDTTAAAGSSF...	KESLRL...PNE	PEAFNPD...VPFSAGPRNCIGQ
<i>Helicoverpa zea</i>	CYP4M6	ISDQVQVE	QVDTIMFEGHDTTAAAGSSF...	KESLRL...KNE	TSFDPD...IPFSAGPRNCIGQ
Conserved AA		GxxT.....ExxR.....PxxxxP.....
				FxxGxRxxxx

Fig. 3. Comparison of *CYP4G33* to other CYP4 genes in regions conserved among P450 enzymes. Shaded amino acid residues are absolutely conserved in P450 enzymes and the motif (EVDTFMFEGHDTT) conserved in CYP4 family. Numbers are in reference to *CYP4G33* sequence. Amino acids shaded in black are those that vary among CYP4 P450s.

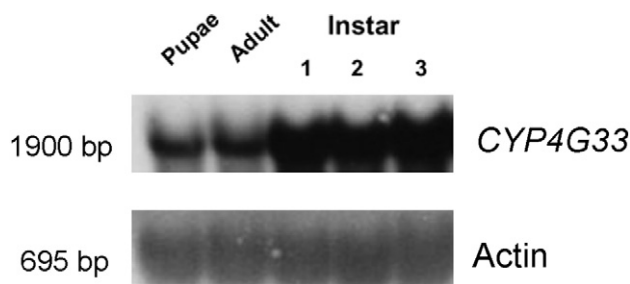


Fig. 5. Northern analysis of *C. tentans* CYP4G33 expression in different developmental stages in comparison with the *C. tentans* actin gene.

band hybridized with the CYP4G33 probe (Fig. 4). The signal for the CYP4 gene was more intense for atrazine exposed relative to unexposed midges confirming that cytochrome P450 family 4 gene expression was induced by atrazine (Fig. 4). Previous studies employing a partial family 4 sequence as a probe indicated that two P450 family 4 genes were induced by atrazine [10]. This could be a result of multiple P450 family 4 genes induced by atrazine or a single gene with an alternative splicing [19]. The longer probe used in the present study is likely to have provided a higher degree of specificity and could explain the detection of a single band.

3.3. Determination of CYP4 expression levels by Northern blotting

CYP4G33 expression was compared among the different developmental stages of *C. tentans*. A single 695 bp mRNA band hybridized with the actin probe (control) and a 1.9 kb mRNA band hybridized with the CYP4 probe in the Northern blot (Fig 5). A signal of the CYP4 gene was highest in all larval stages with much lower expression in adults and pupae (Fig. 5).

Induction of cytochrome P450 by herbicides and the consequences to insecticide toxicity are not well documented. Kao et al. [22] have shown that incorporation of the herbicides atrazine and 2,4-D into the diet of southern armyworm (*Spodoptera eridania*) resulted in induction of both cytochrome P450 activity and total P450 content [22]. Additionally, atrazine has been shown to synergize the toxicity of a number of different insecticides in *D. melanogaster* [23], although the exact mechanism of this synergism has not been determined.

Insect CYP4 genes are suggested to be involved in toxin metabolism, and some CYP4 enzymes have been implicated in the metabolism of steroids and xenobiotics [24]. Family 4 cytochrome P450's exhibit a high degree of structural diversity and have been identified from numerous invertebrates, although for most of these enzymes a specific function has yet to be described [22].

Results from the present research are important because knowledge of the cytochrome P450 diversity in insects especially for those species that have potential to serve as bio-indicators, may provide important tools for future

development of biochemical and molecular markers associated with adaptation and resistance to chemicals. Our data suggest that *C. tentans* CYP4G33 is a reliable marker for atrazine-exposure at 10 mg/l in this species and provide some evidence for a role of this protein in pesticide metabolism. Future research should test the reliability of this marker at lower more environmentally relevant concentrations. Given the large number of genes in the CYP superfamily, assigning even putative functions assists in clarifying the roles of specific proteins (e.g. CYP4G33) in cellular metabolism. Identification of a specific inducible cytochrome P450 will improve our understanding of the molecular and chemical basis of cytochrome P450 family 4 mediated detoxification of atrazine in aquatic organisms.

Acknowledgments

Terence Spence provided invaluable assistance with colony maintenance. Support for the project was obtained from a USDA National Research Initiative Grant # 2003-35102-13545 to M.J. Lydy, K.Y. Zhu and B.D. Siegfried.

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