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Cytochrome P450 genes from the aquatic midge *Chironomus tentans*: Atrazine-induced up-regulation of *CtCYP6EX3* enhanced the toxicity of chlorpyrifos

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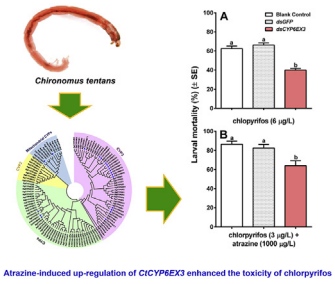
HIGHLIGHTS

- Open reading frames of 19 cytochrome P450 genes were sequenced from *C. tentans*.
- Atrazine up-regulated *CtCYP6EX3*, *CtCYP6EV3*, *CtCYP9AT1* and *CtCYPEX1*expressions.
- Chitosan/dsRNA nanoparticle-based RNAi was used to study the function of *CtCYP6EX3*.
- Atrazine-induced up-regulation of *CtCYP6EX3* enhanced the toxicity of chlorpyrifos.

GRAPHICAL ABSTRACT



Atrazine-induced up-regulation of *CtCYP6EX3* enhanced the toxicity of chlorpyrifos

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ABSTRACT

The open reading frames of 19 cytochrome P450 monooxygenase (*CYP*) genes were sequenced from *Chironomus tentans*, a commonly used freshwater invertebrate model. Phylogenetic analysis of the 19 CYPs along with a previously reported CYP (*CtCYP4G33*) revealed that they belong to three different clans, including 3 in CYP4, 15 in CYP3, and 2 in mitochondria clan. When third-instar larvae were exposed to atrazine at 5000 μg/L, the transcription of *CtCYP6EX3*, *CtCYP6EV3*, *CtCYP9AT1* and *CtCYPEX1* was significantly up-regulated. To examine whether *CtCYP6EX3* played a role in oxidative activation of chlorpyrifos to chlorpyrifos-oxon, we evaluated larval susceptibility to chlorpyrifos after *CtCYP6EX3* transcript was suppressed by RNAi. The larvae fed chitosan/ds*CtCYP6EX3* nanoparticles showed a significantly decreased *CtCYP6EX3* transcript (53.1%) as compared with the control larvae fed chitosan/ds*GFP* nanoparticles. When the *CtCYP6EX3*-silenced larvae were exposed to chlorpyrifos at 6 μg/L or its binary mixture with atrazine (chlorpyrifos at 3 μg/L and atrazine at 1000 μg/L), the larvae became less susceptible to the pesticides as their mortalities decreased by 24.1% and 20.5%, respectively. These results along with our previous findings suggested that the increased toxicity of chlorpyrifos was likely due to an enhanced oxidative process from chlorpyrifos to chlorpyrifos-oxon by CtCYP6EX3 as RNAi of *CtCYP6EX3* led to decreased susceptibility of *C. tentans* larvae to chlorpyrifos alone and the binary mixture of atrazine

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and chlorpyrifos. However, further study would be necessary to validate our results by functional assays using heterologously expressed CtCYP6EX3 enzyme.

1. Introduction

Cytochrome P450 monooxygenases (CYPs) are found in all kingdoms of life and constitute a large superfamily of heme proteins sharing a common, ancient evolutionary origin (Feyereisen, 2012). CYPs mainly catalyze oxidation reactions that metabolize a wide variety of substrates including both endogenous substances (e.g., steroids, fatty acids, prostaglandins) and exogenous substances such as pesticides and drugs (Feyereisen, 1999). Some CYPs have highly conserved substrate-specificity (e.g., human CYP1A2), whereas others have diverse substrate preferences. For example, human liver CYP3A4 can metabolize over 50% marketed pharmaceuticals (Nerurkar et al., 1993; Otyepka et al., 2012). Furthermore, some CYPs produce only a single metabolite from a given substrate, whereas others can produce multiple metabolites (Otyepka et al., 2012).

Insects, the most diverse group of organisms in terms of number of species and number of individuals, dominate all habitats on the earth. The species diversity reflects the diversity of CYPs in insects. Over 1800 CYP sequences have been identified so far and they were packed into four clans which can be further subdivided into 67 families based on 11 sequenced insect species genomes (Baldwin et al., 2009; Ai et al., 2011; Nelson, 2011; Feyereisen, 2012). Insect CYPs are detected in a wide range of tissues. Highest CYP enzyme activities are usually associated with the midgut, fat bodies and Malpighian tubules (Hodgson, 1983). Dramatic variations of the CYP enzyme activities and their gene expression also occur during the development of the insects. In general, total CYP activity levels are undetectable in eggs, rise as larvae develop, become undetectable in pupae, and show lower expression levels in adults (Agosin, 1985).

It is well known that the elevated activity of CYP enzymes accelerates metabolism of pesticides and is considered as a mechanism for insecticide-tolerance and/or resistance in insects (Scott, 1999; Li et al., 2007; Schuler, 2011). The induced CYP enzyme activity was also reported in *Chironomous tentans*, a model organism to assess freshwater sediment quality, under pesticide exposures (Keddy et al., 1995; Anderson and Zhu, 2004). The toxicities of organophosphate insecticides (e.g., chlorpyrifos) and triazine herbicides (e.g., atrazine), and the synergistic effect of pesticides mixture (e.g., binary mixture of chlorpyrifos and atrazine) on *C. tentans* have been extensively documented (Anderson and Lydy, 2001; Lydy and Austin, 2004; Anderson and Zhu, 2004; Jin-Clark et al., 2002, 2008; Rakotondravelo et al., 2006). Atrazine, an extensively used herbicide in agricultural and residential areas, has been routinely detected in many surface and ground waters (USGS, 1999). Although atrazine alone doesn't pose much acute toxicity to *C. tentans* even at the concentration $\geq$1000 μg/L, atrazine is known to induce CYP enzyme activities (Londono et al., 2004; Anderson and Zhu, 2004; Jin-Clark et al., 2008). Increased CYP activity can either synergistically or antagonistically affect the toxicity of organophosphate insecticides to *C. tentans* (Miota et al., 2000; Anderson and Lydy, 2001; Jin-Clark et al., 2002; Anderson and Zhu, 2004; Rakotondravelo et al., 2006), depending on the chemical structures of the insecticides (Anderson and Zhu, 2004). Further research confirmed the up-regulation of a particular CYP gene (*CtCYP4G33*) in *C. tentans* exposed to atrazine at very high concentration (10 mg/L, Londono et al., 2007).

To provide molecular evidences on the effect of atrazine on the toxicity of organophosphate insecticides, we: 1) identified and sequenced the open reading frames of 19 CYP genes from *C. tentans*; 2) phylogenetically analyzed the deduced amino acid sequences of these genes along with a previously reported CYP gene (*CtCYP4G33*) in *C. tentans*; 3) examined the transcriptional responses of these genes to the exposure of atrazine at different concentrations; and 4) revealed the atrazine-induced up-regulation of a new CYP gene (*CtCYP6EX3*) contributing to increased toxicity of chlorpyrifos to *C. tentans* by using a nanoparticle-based RNA interference (RNAi) method.

2. Materials and methods

2.1. Insects

An aquatic midge, *C. tentans*, colony (mixed-age brood) was maintained in a culture tank contained reconstituted water and silica sand substrate at 25 ± 1 °C with a 16L:8D photoperiod according to the standard culturing procedures (USEPA, 1993; Anderson and Zhu, 2004).

2.2. Pesticides

Chlopyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate, 99.9% purity) and atrazine [6-chloro-*N*-ethyl-*N*'-(1-methylethyl)-1,3,5-trazine-2,4-diamine, 99.9% purity] were purchased from Chem Service Inc. (West Chester, PA, USA).

2.3. Sequence analysis of CYPs

Nineteen CYP genes were identified and sequenced from our *C. tentans* EST library whereas one (*CtCYP4G33*, Londono et al., 2007) was obtained from the NCBI databases. The full-length open reading frames (ORFs) of some CYPs were obtained either by re-sequencing their cDNA clones from the cDNA library or by performing 3′- and 5′-RACE (rapid amplification of cDNA ends) using Clontech SMART™ RACE cDNA amplification kit (Mountain View, CA). In 5′-RACE of *CtCYP6EX3*, 1.0 μg of total RNA extracted from a group of five fourth-instar larvae was used for 5′-RACE first strand cDNA synthesis. A touchdown PCR was performed using *CtCYP6EX3* gene-specific primers (Table 1) and a universal primer provided by Clontech RACE cDNA kit. The PCR products were subcloned using Invitrogen TA-vector system (Invitrogen Inc. Carlsbad, CA) and sequenced using ABI 3700 DNA sequencer at the Kansas State University DNA Sequencing Facility (Manhattan, KS).

Multiple amino acid sequence alignments of the conserved domains and active sites of the 20 CtCYPs were performed in Clustal Omega (http://www.ebi.ac.uk). In phylogenetic analysis, 96 *Anopheles gambiae* CYPs from the NCBI databases and 20 *C. tentans* CYPs were used because *C. tentans* and *A. gambiae* are closely related dipteran species and *A. gambiae* CYP genes have been well annotated (Wiegmann et al., 2011). The phylogenetic tree was constructed by the neighbor-joining algorithm with bootstrapping (1000 replications) using Mega 5.0 software (Tamura et al., 2011).