



Article Cytochrome P450 CYP6EV11 in Chironomus kiiensis Larvae Involved in Phenol Stress

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Abstract: Phenol is one of the organic pollutants which can cause water environment pollution. It is not only enriched in aquatic organisms but is also a serious threat to human health. *Chironomus kiiensis* is very sensitive to the contaminants in water and its cytochrome P450s are usually chosen as biomarkers for water pollution. To examine whether *CYP6EV11* plays a role in the oxidative metabolism of phenol, we measured the silencing efficiency of *CYP6EV11* and evaluated larval susceptibility to sublethal phenol levels by RNA interference (RNAi) technology. The results showed that the transcription of *CYP6EV11* was found significantly up-regulated when the 4th instar *C. kiiensis* larvae were exposed to three doses of phenol. However, the transcriptional levels of *CYP6EV11* were significantly suppressed by 92.7% in the 4th instar *C. kiiensis* larvae soaked in *dsGFP* for 6 h. The *CYP6EV11* expression and mortality of the 4th instar *C. kiiensis* larvae with *CYP6EV11* silencing were mostly decreased under phenol stress. Therefore, the *CYP6EV11* gene may be used as a molecular biomarker for earlier warning and monitoring for water pollution.

Keywords: CYP6EV11; phenol stress; Chironomus kiiensis; molecular biomarker

1. Introduction

Phenol (carbolic acid, phenolic acid, phenylic acid or oxybenzene) consists of an aromatic ring linked a hydroxyl group and is widely used as the precursor to produce industrial compounds such as kerosene, phenolic resin and pesticides [1]. The compounds penetrat ecosystems due to the drainage off municipal or industrial sewage to surface water [2]. The Environmental Protection Agency (EPA) specified the standard maximum of phenol contaminant level of 1 mg/L in wastewater [3]. Since the phenolic compounds were stable over a long term and harmful to organisms at low dose, many of them have been classified as hazardous pollutants due to their harmful damage to human health [4]. Although the standards of critical toxic concentration of phenol were much different, and varied from 1.06 μ M to 105.24 μ M (105.24 mM in Malaysia,10.63 mM in the United States, 1.06 mM in Australia) [5–7], there is no doubt that phenol is harmful and should be monitored. Our previous research also found that the enzymatic activities in *Propsilocerus akamusi* were significantly altered in response to phenol [8].

Cytochrome P450s (CYPs) belong to a superfamily, which is found in all organisms that play an important role in many physiological processes such as the metabolism of fatty acids, steroids and vitamin D as well as some other phytochemicals like pesticides [9]. It is well documented that CYPs are associated with the process of detoxification in insects, and the elevated activity of CYP enzymes has the ability to accelerate metabolism of pesticides. The CYP6 families in terrestrial invertebrates have frequently been shown to play a role in the detoxification of xenobiotics and metabolic resistance to insecticides [10–15]. Most CYP6 genes in insects have been shown to enhance metabolic detoxification,

such as CYP6AB37, CYP6AB35, CYP6B53, CYP6AB3, CYP6AB32, CYP6AB33, CYP6AB36, CYP6CT4 and CYP6AN15v1 of Lymantria dispar [15]. It has been reported that CYP6BQ23 in Meligethes aeneus is associated with the resistance to deltamethrin [16]. In several species of Chironomus, different toxic compounds are affected by the expression of the CYP6 gene. For example, the mRNA levels of CYP6B7 in Chironomus riparius are evaluated after exposures to the ultraviolet filters benzophenone-3 (BP3) and 4-methylbenzylidene camphor (4MBC) [17] and the antibacterial agent triclosan [18]; in *C. tentans* the transcriptional activities of CYP6EX3 and CYP6EV1, CYP6EV3 have been studied after atrazine and chlorpyrifos exposures. Moreover, the present study has reported the effects of phenol on the expression of ten CYP6 genes in *C. kiinensis* [19]. On the other hand, other cytochromes in different Chironomus species have been analyzed as possible biomarker genes that could be useful in ecotoxicological studies, risk assessment and bioremediation, such as CYP4G [20], CYP9AT2 [21], CYP4D2, CYP9F2 and CYP12A2 in *C. riparius* [18]; CYP4DG1, CYP4DG2 and CYP9AT1 in *C. tentans* [22,23].

Chironomidae are known as non-biting midges belonging to a family of Diptera: Nematocera. They often distribute in urban and residential areas in close proximity to polluted and eutrophic waters causing a big problem worldwide [24]. *Chironomus kiinensis* is broadly distributed in Malaysia, Japan, USA and South China [25]. *C. kiinensis* could be used extensively for acute or chronic bioassays in fresh water ecosystems as it has a relatively short life cycle, and due to the ease of maintenance of laboratory cultures and relative sensitivity to aquatic contaminants [26]. To provide molecular evidence of CYP gene detoxification that will be of benefit to further monitor water pollution, we: (1) examined the transcriptional responses of *CYP6EV11* in *C. kiiensis* to the exposure of phenol at different concentration; (2) revealed the phenol-induced down-regulation of *CYP6EV11* contributing to decreased toxicity of phenol to *C. kiiensis* using the RNA interference (RNAi) method. These results may potentially develop sensitive molecular markers of Chironomidae for monitoring pesticide exposures in non-target organisms in aquatic systems.

2. Results

2.1. cDNA Cloning and Characterization

In databases, full-length cDNA of *CYP6EV11* was detected in the P450 family genes, with open reading frames (ORFs) of 1476 bp encoding 491 amino acids, with predicted molecular masses of 56.79 kDa and isoelectric points (PI) of 9.12. Besides, there are no signal peptides in *CYP6EV11*.

2.2. Polygenetic Analysis

Based on the identities of *CYP6EV11*, phylogenetic trees were constructed with 22 genes of high homologous amino acids in insects. The *CYP6EV11* and *CYP6EV10* (AHJ10931.1) in *C. kiiensis* shared the highest sequence similarity (71%), and were clustered into a group. All the 22 CYP sequences have been deposited in the NCBI database with their accession numbers as shown in the Figure 1. Five similar motifs were found in typical CYPs, including helix-C, helix-I, helix-K, Meander domain and heme-binding domain from N to C terminal (Figure 2). Helix-C is heme-interacting region with typical sequences WxxxR; AGxET motif is located in helix-I and reportedly to make an oxygen binding pocket; E/SxLR located in helix-K with the hydrogen bonding domain and PxxFxPxxF motif are thought to form a set of salt bridge interactons (E-R-R) for stabilizing the structure of protein [27]; and the P450 heme-binding domain locates at the 3'-end with the FxxGxRxCxG/A sequences [28,29].



Figure 1. Phylogenetic tree of 23 CYP genes are from 7 insects. These genes were downloaded from the National Center for Biotechnology Information (NCBI) databases. The gene accession numbers are parenthesized. The CYP genes are *CtCYP6EV1* (ARO50426.1), *CtCYP6EV2* (ARO50430.1), *CtCYP6EV3* (ARO50428.1), *CtCYP6EV4* (ARO50429.1), *CtCYP6EV5* (ARO50442.1), *CtCYP6EX1* (ARO50434.1), *CtCYP6EX3* (ARO50425.1), *CkCYP6EV9* (AHJ10930.1), *CkCYP6EV10* (AHJ10931.1), *CqCYP4A6* (XP_001867280.1), *CqCYP6A8* (XP_001870174.1), *AaCYP6N3v1* (AAF97936.1), *AaCYP6N3v2* (AAF97937.1), *AaCYP6N3v3* (AAF97938.1), *AdCYP6A8* (ETN65670.1), *AfCYP6M1a* (AFM08393.1), *AfCYP6M1b* (AFM08394.1), *AfCYP6M1c* (AFM08395.1), *AfCYP6M4* (AFM08397.1), *AfCYP6M7* (AIE17403.1), *AfCYP6M8* (AFM08398.1), *LeCYP6JN1* (ALX81394.1), *CkCYPEV11*. *CkCYP6EV11* is a target gene in this study from *Chironomus kiiensis*, which was obtained from transcriptome sequencing in our previous study [26]. These genes are from *Chironomus tentans*, *Chironomus kiiensis*, *Culex quinquefasciatus*, *Aedes albopictus*, *Anopheles darling*, *Anopheles funestus* and *Liposcelis entomophila*.

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CkCYP6EV11	120	GQEWRDLRAR	LSPTFTSGKIKMMPPIVAE	TADRMVEYLRQT	SRSTRDLEMKEIYCEFTT-EV	LASVAF	GLDIKCLGQPDNEFRKVTKYVFDPPFLANLKN	NLLAFAMPNVARFFNVALSPRFVTDFFLNTV	248
CtCYP6EV1	119	GQEWRDLRAR	LSPTFTSGRMRMMFPLVSTI	IADDMIEYLRDI	VERSDTHEMRORFIHLSQQRV:	LASVAF	GLDTKCHGNPNNDFRRMARSIFEPTAMENIKN	LIIISSERISKLENMSENRQETTDFFLRIV	248
CtCYP6EV2	119	GQEWRDLRAR	LSPTFTSGRMRMMFPLVSTI	IADDMIEYLRDI	VERSDTHEMREIYSSFTT-EV:	LASVAF	GLDTKCHGNPNNDFRRMARSIFEPTAMENIKN	LIIISSERISKLENMSENRQETTDFFLRIV	247
CtCYP6EV3	121	LEEWKVLRVK	ISPTFTSGRMRMMFPIVLEC	CVDRMIEHLKG-	-INRGSLEMKEIFSELTT-EV	IGNVAF	GLDVNCIGNPDNEFRKIAKRVLEPSNWDNFKF	PLENLMLPRVARFLNMSINPRFITDYFLEIV	247
CtCYP6EV4	120	GQEWRDLRAK	LSPTFTSGRIKMMFPIVAEA	AADRMADYLRYE	EITHECLEMKEIFASYTT-EV:	IANIAF	GLDTKCLGNHENEFRHITKNIFEPPKLKNFEN	VFLIFSFPKFAKFFNLGINSKHVIEFFMATV	248
CtCYP6EV5	120	GQEWRDLRQR	LSPIFTVGRTKMIFPIIAET	TADRMIEYLKQP	DTNRETIEMKEIFSSFTT-EV	/ANAAF	GLDVKCLGHPDNDFRKATRYIFQPPMWYNLKI	ILLIFSMPEVARPFNMAQNPREVIDFFTRTV	248
CtCYP6EX1	119	GQUWRDRRTK	MSPIFTSGRMRMMFENVDRJ	ISDKLVEVLKKN	IKTSNVHDMKRWSQKFTA-DN	IGNVAF	GLECNCIEDENSAFMKYGRRLLDLKPFEIIKF	PIFAINLPDLARMLKIRSNPKDAGDFFLNTF	247
CtCYP6EX3	122	GQEWRDRRTK	LSPIFTSGRMRMMFEIVDRI	ISDKLAEELGRQ	LKTTSEFEMRNWSQRYTG-DN:	IGNVAF	GIECNCIVNENSDFMKYGRPIFDLSPFDAFMF	FIFTIEFPNLSRKLGLRDNPKESGDFFLNTF	250
CkCYP6EV9	120	EKVWKNLRER	LSPAFTDIHTKLMFPIFNER	KADRMIEYLKQP	DVDHQNLEMKEIFASYTS-EV.	ISSAAF	GMDIKCLGHPDNEFRKCTRFLFEPPMWYNFKI	IFLIFSWPQIAKFLNMAQNPQFVIDFFIKTV	248
CkCYP6EV10	121	GQEWKE LRAK	LTPTFTSGRIKMMFPILSEV	VADRMIEHLKKL	NLSEDGLEMKEIYAEFTT-EV	LANVAF	GLDIKCIGQPDNEFRKIAKSVFDPPKWQHFK	VEMIFALPDVAKFFNMALTPREVTDFFLKTV	249
CqCYP4A6	120	GQEWKNLRAR	LTPTFTSGRMRLMFPIVAD	VAVELKKCLVTE	RDDGGEVELRDVLARYTT-DV	IGRCAF	GLDCNSLENPNAEFREMORKIFTTTPFSIIKI	LFFVQQIKPLARKLGVTVLNQEVTKYFLKAV	248
CqCYP6A8	125	GSRWRNLRHR	LSPTFTSGRMRMMFPTIVA	AGROFNDFLHER	VSHESEFELKDLLARYTT-DV	IGMCAF	GIECNSMKDPDAQFRVMGRKIFETPRGRIKSM	ALTNATPN-FARFIGVKLSIPEVEQFFLRVV	252
AaCYP6N3v1	120	GTRWENLEAR	LTPTFTSGRMKSMYPTIIGV	VAEEFQRMMESE	VGGNTEIEMKDVLARFTT-DV	IGTCAF	GLECNSLYDPDAQFRCMGRKIFAIAKGRFVKI	LIAAQQFRSLARSLHVTLIDKEVSDFFIGAV	248
AaCYP6N3v2	120	GTRWKNLRAK	LTPTFTSGKMKSMYPTIIGV	VAEEFQRMMESE	VGGNTEIEMKDVLARFTT-DV	IGTCAF	GLECNSLHDPDAQFRRMGRKIFAIAKGRFVKI	LIAAQQFRSLARSLHVTLIDKEVSDFFIGAV	248
AaCYP6N3v3	120	GTEWENLEAR	LTPTFTSGKMKSMYPTIIGV	VAEEFQRMMESE	VGGNTEIEMKDVLARFTT-DV	IGTCAF	GLECNSLHDPDAQFRRMGRKIFAIAKGRFVKI	LIAAQQFRSLARSLHVTLIDKEVSDFFIGAV	248
AdCYP6A8	124	GQRWKS LRNK	LSPTFTSGRMRMMFSTIEA	AGROFRD FMEET	VERQNEFELKDLLARFTT-DV:	IGMCAF	GIECNSMRDPEAEFREMGRKIFQQPRGKVRGI	LLTTSMPR-VAKALGITNILPEVSSFFLGVV	251
AfCYP6M1a	126	GQRWKNLRNK	LTPTFTSGRMRMMFPTVVA	AGKQLKEFMDEN	VQRNSELEMEDIMARFTT-DV	IGTCAF	GIECNSMRDPNAEFRAMGKLFVERQPSQFVNJ	IMVQFSHK-LSRMLGIRLIDKEVSTFFLKVV	253
AfCYP6M1b	126	GQHWKHLRNK	LSPTFTSGKMKMMFPTVVA	AGRQLREFMDEN	VQRNSELEMEDIMARFTT-DV	IGTCAF	GIECNSMRDPNAEFRAMGKQFVDRPPSQFVNI	MIQVSPK-LSRMMGIRLIDKEVSTFFLKVV	253
AfCYP6M1c	126	GQHWKNLRNK	LTPTFTSGKMKMMFPTVVA	AGROLKEFMDEN	VQRNSELEMEDIMGRETT-DV	IGTCAF	GIECNSMRDPNAEFRAMGRQFIDRQPSQFVNI	MVQFSTK-LSRMMGIRLIDKEVSTFFLKVV	253
AfCYP6M4	124	GQRWKRLRNK	ISPTFTSGRMRMMFPTIVA/	AGROFENYMEDT	IQQQGEQELKDVLARFTT-DI	EGTCAF	GIECNSMRDPDAKFRVMGRKIFTRTRGTLQQI	LLMNAFPS-VARMVGIRLIVPEVSDFFMRVV	251
AfCYP6M7	124	GQRWKNLRNR	LSPTFTSGRMRMMFPTIIA	AGROFRDFMEET	VHEQVDFELKDVMARFTT-DV	IGMCAF	GIECNSMSNPDAEFRVMGRKIFARPRGKVKSI	LVINSMPR-LAKLIGLRTLDPEVSDFFMKAV	251
AfCYP6M8	125	GQRWRSLRNR	LSPTFTSGRMRMMPPTIVTA	AGROFRDFMEET	VKRENVFELKDLLARFTT-DV	IGMCAF	GIECNSMRNPDAEFRAMGRKIFEISPGTFKTM	ILMNGMPS-LAKMLRMKQTDQDVSDFFMNAV	252
LeCYP6JN1	129	GQRWRNLRIK	LAPTFTSGRMRMMPPTMIT	VGEE LVRVLNES	VGDPDGVEVREMMGRYTT-DV	EASCAF	GIEVSSLKNPDSEFRKMSKALNTRTVIEILG	WLTFIFPQAIRILRLHMIPKQVTKFFMAVV	257
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CkCYP6EV11 CtCYP6EV2 CtCYP6EV3 CtCYP6EV4 CtCYP6EV4 CtCYP6EX1 CtCYP6EX3 CkCYP6EV9 CkCYP6EV10	249 248 248 249 249 249 249 248 251 249 250	RENMEYREKN RENLEYREKN RSNLEYREKN RSNLEYREKN RDNMEYREKN RDNLEFREKN LQTFEYRQRN RENYENREKN RENYENREKN	NIRRNDFFQLLLDIKNSDV- NVKRNDFFQLLINIKNSEI- NVKRNDFFQLLINIKNSE DIRRNDFFQLLINIKNSDV- DIRRNDFFQLLINIKK-DE- NIVRSDFVSLLLGLKH NIQRNDFFQLLINIKK-NQ- NIRRNDFFQLLLDIKNSVV-		CMSFNEIAANSFAFF CMTFNEIAANSFVFF CMTFNEIAANSFVFF CMTINEMAANCFIFF CMSFDEITANSFIFF CMTFNEIAANSFIFF CMTFNEIAAEAFLVF SYTSKELAAEAFLVF SYTSKELAAEAFLVF CMTINEIAANSFAFF	AGFET AGFET AGFET AGFET AGFET AGFET AGFET AGFET	SSSAMTFCTYELALNHGIQDRLRKEIEEVLRU SSSVMTFSTYELALNQDIQDRLRSEIHEVLEI SSSVMTFSTYELALNQDIQDRLRSEIHEVLEI SSSVTFCSYELALNQDIQDRLRTEIDEVIK SSNVMSFCSYELALNQDIQDRLRTEIEVVEI SATAITFCTYELALNQDIQDRLRTEIETVLKI SSTLMTFTLYELALNQEIQDKLREEITTGIEI SSSSIMFCTYELALNQDIQDRLRNEIESVIKI SSSAMTFCTYELALNQDIQDRLRNEIESVIKI	RYNGELTYEAIAEMKYLDMVLNETLRRYPVL KYNGEVSYDAIKEMKYLDMVFFETLRRYPAL KYNGEVSYDAIKEMKYLDMVFFETLRRYPAL RENGEITYDGIKEMKYLDMVFFETLRRYPAL RENGEITYDGIKEMKYLDMVFFETLRYPIF ENDGKLTYEMLFEFKYLDMVINESLRKYPIF KENGEKTYDAIMEMKYLDMVFFETLRRYPIF RYNGELTYDAISEMKYLDMVFFETLRRYPIF	360 359 359 359 359 359 356 359 359 359
CkCYP6EV11 CtCYP6EV2 CtCYP6EV3 CtCYP6EV4 CtCYP6EV4 CtCYP6EV5 CtCYP6EX3 CtCYP6EV9 CkCYP6EV10 CqCYP4A6	249 248 248 249 249 249 249 249 251 249 250	RENMEYREKN RENLEYREKN RENLEYREKN RSNLEYREKN RDNNEYREKN RDNLEFREKN LQTFEYRQAY LQTLEYRQAY RENYENREKN RENYENREKN KDTVEYRESN	NIRRNDFFQLLLDIKNSDV- NVKRNDFFQLLINIKNSEI NIQRNDFFQLLINIKNSEQ DIRRNDFFQLLINIKNSDV- NIQRDDFFQLLINIKK-DE- NIVRSDFVSLLIGLKH NIQRNDFVSLLIGLKH NIRRNDFFQMLIDMK-NQ- NIRRNDFFQLLLDIKNSVV-	VDEGSSRSME	GMSENEIAANSFAFF GMTENEIAANSFVFF. GMTENEIAANSFVFF. GMTENEIAANSFVFF. 	AGFET AGFET AGFET AGFET AGFET AGFET AGFET AGFET FAGFET	SSSAMTFCTYELALNHGIQDRLRKEIEEVLRU SSSVMTFSTYELALNQDIQDRLRSEIHEVLEU SSSVMTFSTYELALNQDIQDRLRSEIHEVLEU SSSVLTFCSYELALNQDIQDRLRTEIDEVIK SSTUMTFCTYELALNQDIQDRLRTEIEEVVEU SAIAITFCTYELALNQDIQDRLRTEIETVLKU SSTLMTFTLYELALNQEIQDRLREEITTGIEU SSSIMTFCTYELALNQDIQDRLRBEITTGIEU SSSSAMTFCTYELALNQDIQDRLRBEITTGIEU SSSAMTFCTYELALNQDIQDRLRBEITTGIEU SSSAMTFCTYELALNQDIQDRLRBEITSVIKU	RYNGELTYEAIAEMKYLDMVLNETLRRYPVL KYNGEVSYDAIKEMKYLDMVFRETLRRYPAL KYNGEVSYDAIKEMKYLDMVFRETLRRYPAL KHGEVYTDAIAEMEYLSKVINETMRYPIF RYNGEITYDGIKEMKYLDMVFNETLRRYPIF ENDGKLTYDAIMEMKYLEMVINETLRRYPIF ENDGKLTYDMLFEFKYLDMVVNEALRKYPF RYNGELTYDAIMEMKYLDMVFNETLRYPIF RYNGELTYDAISEMKYLDMVFRETLRRYPIL QH-GSLTYEAIHDMKYLENCIFETLRIYPPA	360 359 359 359 356 359 356 359 361 369
CkCYP6EV11 CtCYP6EV2 CtCYP6EV3 CtCYP6EV4 CtCYP6EV5 CtCYP6EV5 CtCYP6EX3 CkCYP6EV9 CkCYP6EV10 CqCYP6A8 CqCYP6A8	249 249 248 249 249 249 249 250 249 250 249	RENMEYREKN RENLEYREKN RENLEYREKN RSNLEYREKN RDNNEYREKN RDNLEFREKN LQTFEYRQAY LQTLEYRQRN RENNEYREKN RENNEYREKN RENNEYREKN RETLDYRVKN	NIRRNDFFQLLLDIKNSDV- NVKRNDFFQLLINIKNSEI- NIQRNDFFQLLINIKNSEQ- DIRRNDFFQLLINIKNSEQ- NIQRDFFQLLIDVKNSDV- NIQRDDFFQLLINIKK-DE- NIVRSDFVSLLLGLKD NIRRDDFFQMLIDMKN-NQ- NIRRNDFFQLLLDIMKN-NQ- NVRRNDFFQLLLDIMKN-NQ- NVRRNDFFQLLLDIKNSVV-	VDBGSSRSME DTKSDDG-	CMSFNEIAANSFAFF CMTFNEIAANSFVFF, CMTFNEIAANSFVFF, CMTFNEIAANSFVFF, CMSTDEITANSFIFF, CMSTDEITANSFIFF, CMTFNEIAANSFIFL CMTFNEIAANSFIFL CMTINEIAANSFAFF CMTINEIAANSFAFF CMTINEIAANSFAFF CMTINEIAANSFAFF CMTINEIAANSFAFF	AGFET AGFET AGFET AGFET AGFET AGFET AGFET AGFET AGFET	SSSAMTFCTYELALNHGIQDRLRKEIEEVLRU SSSVMTFSTYELALNQDIQDRLRSEIHEVLEU SSSVMTFSTYELALNQDIQDRLRSEIHEVLEU SSSVMTFSTYELALNQDIQDRLRTEIDEVLKU SSNVMSFCSYELALNQDIQDRLRTEIEEVVEN SAIAITFCTYELALNQDIQDRLRTEIETVLKU SSTLMTFTLYELALNQDIQDRLREEITTGIEU SSSSIMTCTYELALNQDIQDRLRBEIESVLKU SSSSMTFCTYELALNQDIQDRLRDEIESVLKU SSSSMTFCTYELALNQDIQDRLRDEIESVLKU SSSIMTCTYELALNQDIQDRARQNVRDVLS(SSTLLTMTLYELALNQDIQDRURQHVKEVLEU	RYNGE LTYEAIAEMKYLDMVLNET LRRYPVL KYNGEVSYDAIKEMKYLDMVFRET LRRYPAL KYNGEVSYDAIKEMKYLDMVFRET LRRYPAL KHGEVTYDAIAEMEYLSKVINET HRRYPIF RENGE TYDGIKEMKYLDMVFRET LRRYPIF ENDGKLTYEMLFEFKYLDMVNEALRKYPFI ENDGKLTYEMLFEFKYLDMVNEALRKYPFIF RYNGE LTYDAIMEMKYLDMVFRET LRRYPIF QH-GSLTYEAIHDMKYLENCIFET LRIYPPA	360 359 359 359 359 359 359 359 361 369 370
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CkCYP6EV11 CtCYP6EV2 CtCYP6EV3 CtCYP6EV4 CtCYP6EV5 CtCYP6EX1 CtCYP6EX3 CkCYP6EX3 CkCYP6EV10 CqCYP4A6 CqCYP6A8 AaCYP6N3v2 AaCYP6N3v3 AdCYP6N3v3 AdCYP6M1a AfCYP6M1a AfCYP6M1a AfCYP6M1 AfCYP6M4	249 248 248 249 249 249 250 249 249 249 249 249 249 249 249 252 254 254 255 254 255 254 255 254 255 254 255 255	RENMEYREKN RENLEYREKN RSNLEYREKN RDNMEYREKN RDNMEYREKN RDNLEFREKN LQTLEYRQAY LQTLEYRQAY LQTLEYRQAY RENMEYREKN RDTYEYRESN RDTIKYRESN RDTIKYRESN RDTIKYREN RDTIDYRVKN RDTIDYRVKN RDTIDYRVKN RDTIDYRVKN RDTIKYRVEN	NIRRND FFQLLLD IKNSDV- NVKRND FFQLLINIKNSEI- NVKRND FFQLLINIKNSE DIRND FFQLLINIKNSE DIRND FFQLLINIKK-DE NIQRND FFQLLINIKK-OB NIQRND FFQLLIDIKNSV- NIQRND FVSLLLGLKD NIRRDD FFQLLD IKNSVV- NVBRND FMDLLIQLD IKNSVV- NVBRND FMDLLD IKNSPD NIBRND FMSLLMKLKKVDNS NIBRND FMSLLMKLKKVDNS NIBRND FMSLLMKLKKVDNS NIBRND FMSLLMKLKKVDNS SIQRND FMDILLIRMRSDK SIQRND FMDLMIRMLQN GIQRND FMDLMIRMLQN GIQRND FMDLMIRMLQN	VDBGSSRSME 	CMSFNEIAANSFAFF CMTFNEIAANSFVFF, CMTFNEIAANSFVFF, CMTFNEIAANSFVFF, CMSTDEITANSFIFF, CMSTDEITANSFIFF, CMTFNEIAANSFIFL CMTFNEIAANSFIFL CMTFNEIAANSFIFL CMTLNEIAANSFAFF CMTLNEIAANSFAFF CMTLNEIAAQAFVFF CLSFNEIAAQAFVFF CALTDEQIAAQAFVFF CLTFNEVAAQAFVFF CSLTFNEVAAQAFVFF	AGFET AGFET AGFET AGFET AGFET IAGET IAGET IAGET IAGFET IAGFET LAGFET AGFET FAGFET FAGFET IAGFET	SSSAMTFCTYELALNHGIQDRLRKEIEEVLRU SSSVMTFSTYELALNQDIQDRLRSEIHEVLEN SSSVMTFSTYELALNQDIQDRLRSEIHEVLEN SSSVTFCSYELALNQDIQDRLRTEIDEVLKN SSNVMSFCSYELALNQDIQDRLRTEIEEVVEN SATAITFCTYELALNQDIQDRLRTEIEEVVEN SATAITFCTYELALNQDIQDRLREEITTGIEN SSSIMFTCTYELALNQDIQDRLRBEITTGIEN SSSSIMFCTYELALNQDIQDRLRBEITTGIEN SSSSIMFCTYELALNQDIQDRLRBEITTGIEN SSSSIMFCTYELALNQDIQDRLRBEITTGIEN SSSSIMFCTYELALNQDIQDRLRBEITTGIEN SSSIMFCTYELALNQDIQDRLRBIESVLRU SSTAMSNCLYELALNQDIQDRLRBIESVLRU SSTAMSNSLYELAQNSELQDKARKSVMDSIKU SSTAMSNSLYELAQNSELQDKARKSVMDSIKU SSTAMSNSLYELAQNSELQDKARKSVMDSIKU SSTLLTTLYELALNPEIQBKURQCVKEILEN SSTLLTWTLYELALNPEIQBKURQCVKEILEN SSTLLTWTLYELALNPEIQBKURQCVKEILEN SSSLLTWTLYELALNPEIQBKURQCVKEVLDU	RYNGELTYEAIAEMKYLDMVLN ET LRRYPVL KYNGEVSYDAIKEMKYLDMVFRET LRRYPAL KYNGEVSYDAIKEMKYLDMVFRET LRRYPAL KYNGEVSYDAIKEMKYLDMVFRET LRRYPAL RENGEITYDGIKEMKYLDMVFRET LRRYPIF RENGEITYDGIKEMKYLDMVFRET LRRYPIF ENDGKLTYEMLFEFKYLDMVTN ET LRRYPIF KNGELTYDAISEMKYLDMVFRET LRRYPIF RYNGELTYDAISEMKYLDMVFRET LRRYPIF RYNGELTYBAIGDMCYLDMVFRET LRRYPIF RYNGELTYBAIGDMCYLDQILSSLRRYPPA KH-GSLTYEAIGDMQVIDQCIN ESLRYPPA KH-GSLTYEAIGDMQVIDQCIN ESLRYPPA KH-GSLTYEAIGDMQVIDQCIN ESLRYPPA KH-GSLTYEAIGDMQVIDQCIN ESLRYPPA KH-GSLTYEAIGDMQVIDQCIN ESLRYPPA KH-GSLTYEAIGDMQVIDQILSLRYPPA KH-GSLTYEAIGDMQVIDQILSLRYPPA KHNGEMTYEAILDMKYLDQILNESLRYPPV KHNGEMTYEAILDMKYLDQILNESLRYPPV KHNGEMTYEAILDMKYLDQILNESLRYPPV KHNGEMTYEAILDMKYLDQILNESLRYPPV KHNGEMTYEAILDMKYLDQILNESLRYPPV RHNGELTYBAALEMOYLDRVIRECLRYPPV RHGELTYBAALEMVYLDQILNESLRYPPV RHGELTYBAALEMVYLDQILNESLRYPPV RHGELTYBAALEMVYLDQILNESLRYPPV RHGELTYBAALEMVYLDQILNESLRYPPV	360 359 359 359 359 356 359 361 369 368 368 368 368 368 368 369 369 369 369 369 369 369
CkCYP6EV11 CtCYP6EV2 CtCYP6EV3 CtCYP6EV4 CtCYP6EV5 CtCYP6EX1 CtCYP6EX3 CkCYP6EX3 CkCYP6EV9 CkCYP6EV9 CkCYP6EV10 CqCYP6A8 AaCYP6N3v2 AaCYP6N3v2 AaCYP6N3v2 AaCYP6N3v4 AfCYP6M1a AfCYP6M1a AfCYP6M1a AfCYP6M1 AfCYP6M7 AfCYP6M7	249 249 248 249 249 250 249 253 249 259 249 249 249 249 249 252 254 252 254 255 255 255 255 255 255	RENMEYREKN RENLEYREKN RSNLEYREKN RSNLEYREKN RDNLEFREKN LQTFEYRQXN RENYENREKN RENYENREKN RENYENREKN RSTIDYRVKN KDTIKYREEN KDTIKYREEN RDTIKYRVEN KDTIDYRVKN KDTIDYRVKN RDTIDYRVKN RDTIDYRVKN RDTIKYRVEN RDTIKYRVEN RDTIKYRVEN	NIRRND FFQLLLD IKNSDV- NVKRND FFQLLINIKNSEI- NVKRND FFQLLINIKNSEI- DIRND FFQLLINIKK-DE- DIRND FFQLLINIKK-DE- NIVRSD FFQLLINIKK-DE- NIVRSD FFQLLIQLKH NIRRND FFQLLLGLKH NIRRND FFQLLLGLKH NIRRND FFQLLLD IKNSVV- NVFRND FMDLLIQMRSPD NIBRND FMSLLMKLKKVDMS NIBRND FMSLLMKLKKVDMS NIBRND FMSLLMKLKKVDMS NIBRND FMSLLMKLKKVDMS NIBRND FMSLLMKLKKVDMS SIQRND FMSLLMKLKKVDMS SIQRND FMSLLMKLKKVDMS SIQRND FMSLLMKLKKVDMS SIQRND FMSLLMKLKKVDMS NVQRND FMSLLMKLKKVDMS SIQRND FMSLLMKLKKVDMS NVQRND FMSLLMKLKKVDMS NVQRND FMSLLMKLKKVDMS NVQRND FMSLLMKLKKVDMS NVQRND FMSLLMKLKKVDMS NVQRND FMSLMRMLQN SIQPND FMSLMRMLQN NVQRND FMSLLMKASDK KVKRND FVSLLITMMSKD	VDEGSSRSME DTKSDDG SDN-TGEDSE SDN-TGEDSE SDN-TGEDSE TENPEE - - - - - - - -	CMSFNEIAANSFAFF CMTFNEIAANSFVFF CMTFNEIAANSFVFF CMTINEMAANCFIFF CMSFDEITANSFIFF CMTFNEIAANSFIFF CMTFNEIAANSFIFF CMTFNEIAAASFIFF CMTFNEIAAQAFVFF CMTLNEIAAQAFVFF CMTLNEIAAQAFVFF CMTLNEIAAQAFVFF CMTLNEQIAAQAFVFF CMTLNEQIAAQAFVFF CMTLNEQIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF CMTLTNEIAAQAFVFF	AGFET AGFET AGFET AGFET AGFET IAGLET IAGLET IAGFET IAGFET LAGFET FAGFET FAGFET IAGFET IAGFET IAGFET IAGFET	SSSAMTFCTYELALNHGIQDRLRKEIEEVLRU SSSVMTFSTYELALNQDIQDRLRSEIHEVLEI SSSVMTFSTYELALNQDIQDRLRSEIHEVLEI SSSVMTFCSYELALNQDIQDRLRTEIDEVIKU SSSVLTFCSYELALNQDIQDRLRTEIEEVVEI SATAITFCTYELALNQDIQDRLRTEIETVLKU SSTLMTFTLYELALNQDIQDRLREEITTGIEI SSSSIMFCTYELALNQDIQDRLRBEITTGIEI SSSSIMFCTYELALNQDIQDRLRBEITTGIEI SSSSIMFCTYELALNQDIQDRLRBEITTGIEI SSSSIMFCTYELALNQDIQDRLRBEITTGIEI SSSTAMSSLYELALNQDIQDRLRBEITTGIEI SSTAMSNSLYELALNQDIQDRARQNVRDVLSU SSTAMSNSLYELAQNSELQDKARKSVMDSIKU SSTAMSNSLYELAQNSELQDKARKSVMDSIKU SSTAMSNSLYELAQDSELQDKARKSVMDSIKU SSTLTWTLYELALNPEIQBKORQCVKEILEI SSTLLTWTLYELALNPEIQBKORQCVKEILEI SSTLLTWTLYELALNPEIQBKORQCVKEILEI SSTLLTWTLYELALNPEIQBKORQCVKEILEI SSSLLTWTLYELALNPEIQBKORQCVKEILEI SSSLLTWTLYELALNPEIQBKORQCVKEILEI SSSLLTWTLYELALNPEIQBKORQCVKEILEI SSSLLTWTLYELALNPEIQBKORQCVKEILEI SSSLLTWTLYELALNPEIQBKORQCVKEILEI SSSLLTWTLYELALNPEIQBKORQCVKEILEI SSSLLTWTLYELALNPEIQBKORQCVKEILEI	RYNGELTYEAIAEMKYLDMVLN ET LRRYPVL KNNGEVSYDAIKEMKYLDMVFRET LRRYPAL KNNGEVSYDAIKEMKYLDMVFRET LRRYPAL KNNGEVSYDAIKEMKYLDMVFRET LRRYPAL ERNGEITYDGIKEMKYLDMVFRET LRRYPI RFNGEITYDGIKEMKYLDMVFRET LRRYPI ENDGKLTYEMLFEFKYLDMVIN ET LRKYPIF ENDGKLTYDAIKEMKYLDMVFRET LRRYPI KHNGEVTYDAIMEMKYLDMVFRET LRRYPIF RYNGELTYDAISEMKYLDMVFRET LRRYPIF RYNGESTYDAISEMKYLDMVFRET LRRYPIF RYNGESTYDAISEMKYLDMVFRET LRRYPIF RYNGESTYDAISEMKYLDMVFRET LRRYPIF RYNGESTYDAISEMKYLDMVFRET LRRYPIF RYNGESTYDAISEMKYLDMVFRET LRRYPIF RYNGESTYDAISEMKYLDMVFRET LRRYPIF RYNGESTYDAISEMKYLDQIN ESLRKYPPV RH-GSLTYEAIQDMQVIDQCIN ESLRKYPPV RH-GSLTYEAIQDMQVIDQCIN ESLRKYPPV RHNGEMTYEAILDMKYLDQILREALRKYPPV RHNGEMTYEAILDMKYLDQILREALRKYPPV RHNGEMTYEAILDMKYLDQILREALRKYPPV RHNGEMTYEAILDMKYLDQILRESLRKYPPV RHNGELTYDAAVEMMYLDQILRESLRKYPPV RHNGELTYDAAVEMMYLDQILRESLRKYPPV RHNGELTYDAAVEMMYLDQILRESLRKYPPV RHNGELTYDAAVEMMYLDQILRESLRKYPPV RHNGELTYDAAVEMMYLDQILRESLRKYPPV RHNGELTYDAAVEMMYLDQILRESLRKYPPV RHNGELTYDAAVEMMYLDQILRESLRKYPPV	360 359 359 359 359 359 359 359 359 369 368 368 368 368 368 369 369 369 369 369 369 369 369 369 369

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CkCYP6EV11	361 DYHIRKSYKPFKIPNTE	LIIPSOVPIFIFTGAIHNDERYF	PERFDPERI	NDENITRHVPFSYLP	SEGPRICIO	LRFOVMQARIOMIKLLKNFKLSPCSKTLIPMKFAPNANFQSPLOGMWIKVET	488
CtCYP6EV1	361 DNONRKSTHDFKIPNSI	CLIIPAGTNIIIPVHOLHNDERFY	NPERFDPERI	SEENIKERPSFVYIP	SECORMCIO	YRFOTMESKVOLIKLFSVFRILPSEKTSIPFOFFPTVFFQTPLOGMOLKLOR	488
CtCYP6EV2	360 DNQNRKSTHDFKIPNSI	CLIIPAGTNIIIPVHGLHNDERFY	INPERFOPERI	SEENIKKRPSFVYIP	PSBOORMCIO	YRFOTMESKVGLIKLLSVFRILPSEKTSIPIQFSPTASLQTPLGGMWLKLQR	487
CtCYP6EV3	360 DTAMRQSSRDFRTPNSH	UVIPGRSMIMIPTIGIHNDDRFY	OPDREDPERI	NEENVORRHPLAFIP	PSEOPRICLO	LRFALMBIRISMVYLLRNFRILPSNRTLIPMQYSLTSSFQSPRHGMGLRLBB	487
CtCYP6EV4	361 DTHVRRCTRDFKIPKT	LVIPAGVTVMIPAIGLHHDERYP	NPDVFDPNR	SDENVKKLVSHTFIP	FSEGERMCIO	QRFGTMQIKIGLVKLLRNFRIFPCNKTLVFMKYTPNSGFQSALGGMWLKLEH	488
CtCYP6EV5	360 EVQVRRCTREFTMRMPDSE	LAIPIGTPVLIPVAGIHNDERYFI	OPERFORER	SEGNIKHLVPHTFIF	FSEGPRNCIC	ISFGLMQIKLGLVKFLKNFRIMPCSKTIIPMKFTPNYSFQSPLGGMWIKIES	489
CtCYP6EX1	357 PILFRRCVRDYRIPOTI	LIIPTOIKALINVYSLHHDPEYFT	PERSKPDPRR	SDENTRNIKSCTFLP	FOBOORNCIO	MRFGQMQSRMAILKLVKNFKFSPCDKTLIPMKFETFAPFISPKOCMWLKVER	184
CtCYP6EX3	360 AENLRECIEDYEIPOTI	LVIPROTSIDINIESLHRDPEYE	DPERFOPERI	SEENIKNIRPFTFLP	FOEOPRNCIO	MRFGQMQSKIGITKLIKNFKFSPCDKTPIPMVFDPKSLFTAPKGGMWLKVEK	1 487
CKCYP6EV9	360 EVQIRECTERYKIPGT	LIIHSOMPILIPVIGIHMDERVF1	INPERFOPERI	NEENVERLVPHTYVP	FSBOPRNCIO	TSPOTMQARVOLVKFLRNFRVHPSNKTLIPMKYAPNYSFQSPLOOMWLRMQS	487
CKCYP6EV10	362 DSHMRRCVRDYKIPNT	ILVIPSOVTILVPVYAIQNDEEYF"	NPERFOPER	TEENVERQIPETYME	FSBOPRTCIO	LRFOTMQTKIGLIKILRNFRILPSKKTLIPMKFAPNAGFQLPFOCMWIKLEN	489
CqCYP4A6	370 SILFRTATQDYRVPNTI	FTIERGTATNIPVLAIHRDPEIY	PMRFDPER	NADQVARRHPFAYLP	FGEGPRVCIO	MREALMOTRVGLATLLONFRETVSPRTRIPAKINPASGIIMAEGGLYLOVDR	497
CqCYP6A8	371 PIHEREVARDYQVPNT	CSVLEAOTQVFIPVYGIHHDPEVF	PERFOPOR	SPEQEANRNPYAWTP	POBOPRICIO	LRFOMMQARIOLAYLLTNFRFSIGERCKVPLELNKKSFILAPEOGLWLKVER	498
AaCYP6N3v1	369 SNLTRIVSKDYKLPNCI	VVLQQGSTIIVPVYALHHDAEYY	NPERYDPDR	TPEEVAKRNPYCFLP	FGEGPRNCIC	MRFGIMQARVOLAYLLEDFSFTLSSETPVPLEISPHNPILTSEGGLWLNVER	: 496
AaCYP6N3v2	369 SNLTRIVSKDYKLPNCI	VVLQQGSTIIVPVYALHHDAEYYI	THERYDPDR	TPEEVARRNPYCFLP	FOBOPRNCIO	MRFGINQARVOLAYLLKOFSFTLSSKTPVPLKISPHNPILTSEGGLWLNVKK	: 496
AaCYP6N3v3	369 SNLTRIVSKDYKLPNCI	VVLQQGSTIIVPVYALHHDAEYYI	PERYDPDR	TSEEVARRNPYCFLP	FOROPRNCIO	MRFCHMQARVOLAYLLKOFSFTLSSKTPVPLKISPHNPILTSEGGLWLNVEK	1 496
AdCYP6A8	370 PVHFRVTSKEYQVPGEI	CTVLEAGTSVMI PVYAIHRDPENFI	PERYDPDR	SPEEEARRHPYAWTP	FGEGPRICVO	LRFGMMQARIGLAYLLNSFQFSRGVKTVVPLKLDVKSFILSPEGGMWLKVEK	497
AfCYP6M1a	370 PIHIRVARODY RVPNTI	SVIEAGTLVLVPIYAIQRDPDIF	PERFOPERS	SPEEEARRHPFAWIP	POBOPRVCIO	LRFOMMQARIOLAYLLQGFSFAPYERTSVPMRFVTNNILLSPRDGLWLRVNR	497
AfCYP6M1b	370 PMHERMITQDYHVPNTI	SIIEAGTRVLIPTFAIQRDPDIF	PERFOPERI	SPEEZARRHPFAWIP	CEGPRVCIC	LRFGMMQARIGLAYLLQGFSFTPYERTSVPRRFIRMHIFLSPRDGLWLRVNR	: 497
AfCYP6Mic	369 PMHERTAAQDYHVPNTT	SIIEAOTMILTPTFAIQRDPDIF	PERFOPER	SPEEEAKRHPFAWIP	POBOPRVCIO	LRFOMMQARIGLAYLLQGFSFAPYEKTSVPMKFVTNSFMLAPRDGLWLKVNK	496
AfCYP6M4	370 SVHFRITARDYLVPGTH	SILERGTSVMIPVLGIHRDAEHF	PREREDEDRI	TPEQEARRHPYAWTP	PGEGPRICVO	LRFOMMQARIGLIHLLTSPRFACCSRTPIPMRFDITHTILSPROGMWLRMER	(497
AfCYP6M7	370 PVHERTTSKEYQVPGT	CTVLEAGTSVMVPVYAIHRDPEHF1	POLEDPDRI	TPEEEARRHPYAWTP	FOEGPRICVO	LRFGMMQARIGLAYLLTGFRFVPGAKTMVPMKLDVKSLS	484
AfCYP6M8	371 FVHFRVASRDYQVPOT	KSVLEAGTAVMVPVHAIHHDPAVF	PERYDPER	SPEQEAKRHPYAWTP	POBUPRICVU	LRFOMMQARIOLAYLLDGFQFAPSSKTVIPMELSTESFIMAPKGGLWLKVDK	498
LeCYP6JN1	388 FVLLRECTEDYTIPETH	IVIERGTRVIIPTLGIORDPDIY	PERFDPDR	SPEEKEKRDRYAYLP	FGEOPRICIO	NRFOLTOTKVOLALIFKNENLVVCKRTPIPFSLDPKIDTLTPKETIYLKVEK	515

Figure 2. The alignment of deduced amino acid sequences from 23 CYP genes from seven insects. The five important conserved motifs of the CYPs were framed by only partial sequence alignment. The purple box shows helix-C with conserved sequences WxxxR; blue box indicates helix-I with typical sequences AGxET; and dark box highlights helix-K with sequences E/SxLR. The heme-binding domain is boxed in pink with typical residues FxxGxxxCxG/A and the conserved Meander domain was boxed in red with conserved sequences PxxFxPxxF. The similarity of *CYP6EV11* with *CkCYP6EV10*, *CtCYP6EV4*, *CtCYP6EV5*, *CkCYP6EV9*, *CtCYP6EV2*, *CtCYP6EV3*, *CtCYP6EV1*, *CtCYP6EX3*, *AfCYP6M8*, *CqCYP6A8*, *AfCYP6M4*, *AdCYP6A8*, *AaCYP6N3v3*, *AaCYP6N3v2*, *AaCYP6N3v1*, *AfCYP6M7*, *CqCYP4A6*, *AfCYP6M1b*, *AfCYP6M1a*, *CtCYP6EX1*, *LeCYP6JN1*, *AfCYP6M1c* is 71%, 64%, 63%, 61%, 58%, 56%, 56%, 47%, 43%, 44%, 43%, 42%, 41%, 42%, 43%, 42%, 45%, 45%, 44%, 41%, 44%, respectively.

2.3. Expression Profiling under Phenol Stress

QRT-PCR analysis was performed to compare the transcription levels of *CYP6EV11* under the three doses of phenol stresses. The *CYP6EV11* was significantly up-regulated by 1 and 100 μ M of phenol, respectively. However, *CYP6EV11* was suppressed by phenol at the dose of 10 μ M at 6 h. After the larvae were stressed for 12~96 h, the expressions of *CYP6EV11* were significantly up-regulated under three doses of phenol stresses. As the results of induced expression of *CYP6EV11* at 1 μ M phenol stress, 1 μ M was chosen as the dose of treatment to explore the effects of gene silencing on development and response to phenol stress (Figure 3).



Figure 3. Transcriptional profiles of *CYP6EV11* in the 4th instar *C. kiiensis* larvae following exposure to three doses of phenol (1, 10 and 100 μ M) during a 96-h period. The larvae without phenol treatment were regarded as controls. The standard error (SE) bars were calculated based on three experimental replicates. The bars with different letters (a–c) are significantly different at *p* < 0.05 based on one-way ANOVA followed by Duncan multiple comparisons. All of the relative expression levels were log2 transformed.

2.4. Gene Silencing Analysis

To determine whether dsCYP6EV11 could inhibit the expression of CYP6EV11, the 4th instar larvae of *C. kiiensis* were soaked in dsCYP6EV11 and larvae soaked in dsGFP were chosen as the controls; qRT-PCR analyses showed that the *CYPEV11* transcript level was reduced at three of four times, especially, the *CYPEV11* transcript level was reduced by 92.7% at 6 h compared with those soaked in dsGFP (p < 0.05). However, the *CYPEV11* transcript level was increased by 182.3% at the time point of 24 h (Figure 4).

Meanwhile, the mortality rate was recorded to explore the effects of gene silencing on the growth of larvae. The results showed that the mortality rate of each treatment gradually increased with the increasing of silencing time. However, there were no statistical differences in the mortality rates between the larvae soaked in *dsCYPEV11* and *dsGFP* at the period of 3~24 h. The mortality rate of larvae soaked in *dsCYP6EV11* reached the highest at 72 h, which was 24.9% more than observed for the *dsGFP* treatment (Figure 5).



Figure 4. The *CYPEV11* transcriptional level in *C. kiiensis* soaked in *dsCYPEV11* compared with those soaked in *dsGFP*. The asterisk (*) on the SE bars indicate significant differences between treatments and controls (p < 0.05).



Figure 5. The mortality rate of *C. kiiensis* larvae with *CYP6EV11* gene silencing and the groups soaked in *dsGFP*.

2.5. Effects of Gene Silencing on Development and in Response to Phenol Stress

Since the transcript levels of *CYP6EV11* were successfully suppressed in larvae soaked in *dsRNA*, we further examined whether the suppression of *CYP6EV11* transcript had effects on *CYP6EV11* in response to phenol stress. *CYP6EV11* was mostly suppressed after 3-h treatment, the transcript level was reduced by 99.9% compared with what was observed in those soaked in *dsGFP* (p < 0.05). However, the *CYPEV11* transcript level was increased by 180.5% compared with the larvae soaked in *dsRNA* for 6 h. After 72 h of stress, the susceptibility of larvae to phenol gradually decreased and the transcript level was reduced by 77.9% (Figure 6).

The mortality rates of dsCYP6EV11 and dsGFP treatments gradually increased with the increase of silencing time. After the larvae were treated with a mixture (phenol at 1 μ M and dsRNA at 2 μ g/ μ L) for 24 h, all the mortalities of dsCYP6EV11 groups were significantly higher than those of dsGFP groups

(p < 0.05, Figure 7). Especially, the mortality of *dsCYPEV11* treatment increased by 70.5% compared with the larvae soaked in *dsGFP* at the 72-h time point.



Figure 6. Transcriptional levels of *CYP6EV11* in silenced *C. kiiensis* larvae in response to phenol stress. The larvae treated with *dsGFP* were regarded as control. The SE bars were calculated based on three experimental replicates. The asterisk (*) on the SE bars indicate significant differences between treatments and controls (p < 0.05).



Figure 7. The mortality rates of *C. kiiensis* larvae with *CYP6EV11* gene silencing under phenol stress. The larvae were soaked in the mixture phenol at 1 μ M and *dsRNA* at 2 μ g/ μ L, and the groups soaked in *dsGFP* were regarded as controls to compare the susceptibility to phenol after *CYP6EV11* gene silencing.

3. Discussion

Insect cytochrome P450s are known to play an important role in detoxifying insecticides and plant toxins [15,30]. The up-regulation of CYPs, especially the members of the CYP6 family, has been confirmed to be associated with enhanced metabolic detoxification of insecticides in insects [16,31–34].

In this study, the transcripts of *CYP6EV11* treated with phenol were found to be significantly up-regulated compared to those in the untreated groups. The increased expression of *CYP6EV11* may imply an enhanced ability to metabolize exogenous compounds. Our pervious study also found that the *CYP6FV2* and *CYP6FV1* in 4th instar *C. kiinensis* larvae were mainly up-regulated during a 96-h phenol exposure [19]. As a result of stress, similar differential transcriptional expression levels have been reported in *C. tentans*. Tang et al. (2018) reported stress-related genes in *C. tentans*, including two cytochrome P450 genes (*CYP6EV1* and *CYP4DG2*), have considerable potential as sensitive biomarkers for the diagnosis of chlorpyrifos contamination [22]. The expressions of *CYP6EX3* and *CYP6EV3* in *C. tentans* can also be significantly up-regulated by atrazine at 1000 and 5000 mg/L, respectively [23]. Thus, upregulation of the *CYP4G* gene in *Chironomus riparius* was found after exposures to TBTO (1 ng/L 24 h–0.1 ng/L 96 h) [20].

To reveal the role of *CYP6EV11* in pollutant metabolism, RNAi technology was used in this study. RNAi has been successfully used in most insects like Lepidoptera and Coleoptera, but rarely used in Chironomidae [35–37]. Lu et al. (2012) had successfully revealed two acetylcholinesterase genes (TcAce1 and TcAce2) in Tribolium castaneum by the method of gene silencing [38]. Knockdown of CYP6EV11 was successfully conducted by this method, and a similar method was also applied for CYP6EX3 silencing to investigate the susceptibility of C. tentans larvae to chlorpyrifos [23]. Compared with dsGFP groups, CYP6EV11 expression was significantly decreased in C. kiiensis larvae soaked in dsCYP6EV11 under phenol and non-phenol stresses. The results showed that CYP6EV11 played a role in oxidative metabolism to phenol. Interestingly, the C. kiiensis larvae decreased susceptibility to phenol when the CYP6EV11 was silenced resulted in low mortality indicating other CYP family genes may be triggered to increase resistance to phenol stress. This result is consistent with the report of Cao et al. (2016) [19]. RNAi technology with *dsRNA* soaking was used to further study gene function of Chironomidae. Our results have provided, for the first time, crucial evidence with regard to which CYP6EV11 in C. kiiensis may be a new molecular biomarker for monitoring phenol pollution and, therefore, the extension in other species is available. Further studies should validate the metabolism ability by heterologously expressed CYP6EV11 in C. kiiensis.

4. Materials and Methods

4.1. Experimental Midge Rearing

The *Chironomus kiiensis* were obtained from Shenzhen Municipal Water Affairs Bureau, China, and were cultured according to the method of Cao et al. (2013) [26]. Briefly, the *C. kiiensis* were reared in mixed-age cultures by generation to generation under the condition of 20 ± 2 °C and L16:D8. The midges were fed with goldfish granules (Beijing San You Beautification Free TECH. Co., Ltd., Beijing, China) and maintained in a glass tank (50 cm \times 20 cm \times 30 cm) that was covered with nylon net.

4.2. Cloning and Identification of CYP6EV11

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's guidelines and treated with RNase-free DNase I (Qiagen, Madison, WI, USA). RNA concentrations were measured using a spectrophotometer, and RNA integrity was checked by analysis on a 1.0% w/v agarose gel. The *C. kiinensis* transcriptome was profiled by conducting Solexa sequencing at the Beijing Genomics Institute (BGI) (Shenzhen, China) [26].

The cDNA of *CYP6EV11* was cloned by the method of RACE using 3'-Full RACE Kit and 5'-Full RACE Kit (TaKaRa, Kyoto, Japan), and was purified using E.Z.N.A. Gel Extraction Kit (Omega, Norcross, GA, USA). After purity and quality checks, the open reading frames (ORFs) were confirmed using the ORF finder (Available online: http://www.ncbi.nlm.nih.gov/gorf.html). The molecular masses, isoelectric points (PI) and the conserved domains were derived using ProtParam (Available online: http://au.expasy.org/tools/protparam.html) and Conserved Domains (Available online: http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) of NCBI, respectively. SignalP3.0 Server

(Available online: http://www.cbs.dtu.dk/services/SignalP) was used to compute signal peptide of the CYP6EV11 h.

4.3. Multiple Sequence Alignment and Polygenetic Analysis

Amino acid sequences corresponding to CYP6 in other insects were retrieved from the NCBI database (Available online: http://www.ncbi.nlm.nih.gov/BLAST/) for multiple sequence alignment using CLUSTALX 1.83(Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkrich, France). The phylogenetic tree was constructed by the neighbor-joining method and bootstrapped with 1000 replicates to evaluate the branch strength using MEGA 5.1 software(CEMI, Temp, AZ, USA) [39].

4.4. C. kiiensis Larvae Stress and RNAi Analysis

The larvae were exposure to phenol (0, 1, 10, 100 μ M) with ten replicates of each treatment. Thirty 4th instar larvae with similar size and body color were randomly assigned in each replicate. The controls were maintained without any exposure to chemicals for the different durations and concentrations along with the phenol-exposed samples. After exposure, two living larvae were randomly collected from each replicate at 6, 12, 24, 48, 72 and 96 h, and immediately frozen in liquid nitrogen before being stored at -80 °C Twenty frozen midges were randomly selected from each treatment at each time interval for RNA preparations.

The *dsRNAs* were synthesized with cDNA of *CYP6EV11* and *GFP* using MEGAscript T7 Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions and were purified with ammonium acetate, water saturated phenol and chloroform. The *dsRNA* was resuspended in RNase-free water, and quantitated at 260 nm using Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). The quality and integrity of *dsRNA* were verified by 1.0% agarose gel electrophoresis. The larvae were soaked in *dsRNA* of *CYP6EV11* ($2 \mu g/\mu L$), ten replicates containing thirty 4th instar larvae in each replicate. The controls were soaked in *dsRNA* of *GFP* ($2 \mu g/\mu L$) along with the *dsRNA* of *CYP6EV11* samples. Two living larvae were collected for each replicate at 3, 6, 24 and 72 h, respectively, and then stored at -80 °C for RNA extraction to measure the silence efficacy. To explore *CYP6EV11* silencing in response to phenol stress, the survival larvae after soaking with the 1 μ M phenol for 1 h were soaked in the mixture of 2 $\mu g/\mu L$ *CYP6EV11 dsRNA* and 1 μ M phenol. Twenty healthy larvae were collected at 3, 6, 24, 48, and 72 h, respectively, and stored at -80 °C for RNA extraction to obtain *CYP6EV11* gene expression profiles. The groups soaked in the mixture of 2 $\mu g/\mu L$ *GFP dsRNA* and 1 μ M phenol were regarded as controls. All mortalities were recorded among treatments.

4.5. Real-time RT-PCR Analysis

Approximately 1 µg of total RNA was reverse transcribed to cDNA using 1 µM oligodeoxythymidine primer. Synthesized cDNAs were diluted to 100 µL with sterile water and used as template for real-time PCR. The following primers were designed for amplification of the *CYP6EV11* gene, F: 5'-GGCGGACAAGAATGGAAAGA-3' and R: 5' -GGCTGTCCAAGACACTTGAT-3'. The Actin (F: 5'-AATGGGATCGCTTGGGTGCTTT-3' and R: 5'-TCAGCTTCACCCAATGTTGCCT-3') was selected as internal controls to calculate the relative expression level by the method of delta–delta CT method [40] and $2^{-\Delta\Delta Ct}$ [41]. Amplifications were performed with the following parameters: 94 °C for 30 s followed by 45 cycles at 94 °C for 12 s, 60 °C for 30 s, 72 °C for 40 s, and 82 °C for 1 s for plate reading.

5. Conclusions

The *CYP6EV11* in *C. kiiensis* was firstly identified and was found to be mostly upregulated under phenol stress. Compared with *dsGFP*, the *CYPEV11* was effectively 92.7% silenced by RNAi in 4th instar *C. kiiensis* larvae soaked in *dsCYP6EV11* for 6 h. The *CYP6EV11* transcript level and susceptibility of the *C. kiiensis* larvae were markedly decreased under phenol stress after *CYP6EV11*

silencing. Therefore, the *CYP6EV11* gene may be used as a sensitive molecular marker for phenol pollution monitoring.

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