Identifying Cytochrome P450 Detoxification Enzymes in Navel Orangeworms (NOW) Responsible for Detoxifying Insecticides, Mycotoxins and Hostplant Chemicals

Project No.: **09-ENTO1-Berenbaum**

Project Leader: May R. Berenbaum
Department of Entomology
320 Morrill Hall
University of Illinois
505 S. Goodwin
Urbana, IL 61801-3795
(217) 333-7784
E-mail: maybe@illinois.edu

Project Cooperators and Personnel:
Guodong Niu, Dept. Entomology, University of Illinois
Joel P Siegel, USDA-ARS, San Joaquin Valley Agricultural Sciences Center

Objectives:

A. To characterize at the molecular level the cytochrome P450s encoding the principal detoxification enzymes responsible for metabolizing a set of ecologically important synthetic and natural toxins including insecticides, mycotoxins and plant allelochemicals.
B. To test a potentially safe and sustainable approach for managing navel orangeworm in almond orchards using natural essential oil synergists as cytochrome P450s inhibitors to interfere with this insect's ability to detoxify mycotoxins, phytochemicals and insecticides.
C. Define the inducibility of detoxificative P450s in navel orangeworm's midguts in response to plant chemicals and determine the impact of inducibility on catalytic activity against phytochemicals and pesticides.

Interpretive Summary:

Introduction:

The navel orangeworm (NOW), *Amyelois transitella*, is among the most destructive pests of almonds in California, as well as a serious problem in pistachios, figs and walnuts. Neonates tunnel into the nut and successive instars consume the nutmeat, generating large quantities of frass and webbing. In addition to causing such direct losses, NOW feeding leaves almonds vulnerable to infection by *Aspergillus* spp. that produce toxic aflatoxins (Campbell et al., 2003). NOW adults lay eggs in mummy fruits when new crop nuts are unavailable (Connell, 2001; Molyneux et al., 2007) and the caterpillars have been reported as scavengers on mummified fruits or nuts of at least 25 plant species.

NOW management depends on a combination of control tactics, including cultural practices and insecticide sprays, particularly when infested adjacent crops, such as pistachios, provide immigrants into almond orchards (Higbee and Siegel, 2009). Biological control agents, although identified, cannot yet provide complete control. Chemical sprays for hull splits include organophosphates and pyrethroids; at present, only Entrust (spinosad) sprays are acceptable in organic orchards (Campbell et al., 2003; Molyneux et al., 2007). Virtually nothing is known, however, as to how NOW metabolizes insecticides. Our overall goal in this project is to determine the metabolic basis for insecticide and host plant detoxification and to determine the likelihood of the evolution of resistance in response to insecticide selection pressure as well as the potential for navel orangeworms to colonize new crop host plants. Characterizing the precise enzyme system utilized by NOW in detoxifying pesticides will also facilitate identification of synergists that can be used to enhance pesticide toxicity and to circumvent pesticide resistance should it evolve.

Previous work:
With bioassays, we demonstrated the extremely high tolerance of *A. transitella* to concentrations of aflatoxin B_1 and ochratoxin A and showed that this tolerance exceeds that of other mycotoxin-associated species such as the corn earworm *Helicoverpa zea*. We have confirmed earlier studies that metabolic detoxification by P450s is involved in the ability of *A. transitella* to tolerate very high levels of aflatoxin in its diet. To understand the molecular mechanisms underlying detoxification of phytochemicals, insecticides and mycotoxins by this species, full-length P450 cDNAs from NOW larval midguts were amplified using 3' RACE strategies with a degenerate primer corresponding to the conserved FDPER region in P450s approximately 30 amino acids upstream of the heme-binding region. These 3' RACE clones were subsequently extended to their translation start sites using 5' RACE strategies and gene-specific primers. The isolated P450 cDNAs were identified as CYP6AB11 (516 amino acids), CYP321C1 (496 amino acids), and CYP6B44 (502 amino acids).

Comparisons of SRS regions in P450s with those in their close relatives indicate that amino acid identities in SRS4 (I helix) of these proteins are higher than in other SRS domains (SRS2, SRS3, SRS6), with up to 77% identity among all of selected P450s within each subfamily or family (**Figure 2**). More specifically, in comparisons of CYP6B44 and other CYP6B genes, 39 are absolutely conserved amino acids, 11 are strongly conserved, 11 are weakly conserved and 24 are not conserved (**Figure 1A**). For CYP321C1 and CYP321A1, there are 84 amino acids in the SRS regions, of which 46 are absolutely conserved amino acids, 19 are strongly conserved, 8 are weakly conserved and 11 are not conserved (**Figure 1B**). Of the 88 amino acids in the SRS regions of the CYP6AB proteins, 39 amino acids are absolutely conserved, 15 amino acids are strongly conserved, 3 amino acids are weakly conserved and 31 amino acids are not conserved (**Figure 1C**). We successfully expressed these enzymes in our heterologous baculovirus Sf9 cell system and now are conducting in vivo assays with caterpillars and in vitro assays with enzymes of phytochemicals, mycotoxins and insecticides to determine toxicity and detoxification.

Progress to date

A. Substrate specificies of detoxification P450s from *Amyelois transitella*.

To characterize the substrates for the P450s isolated from NOW, the P450 cDNAs were co-expressed in Sf9 insect cells with house fly P450 reductase cDNA and fruit fly cytochrome *b*5 cDNA. Expression conditions were optimized by adjusting the ratio of P450: P450 reductase:cytochrome *b*5 to achieve maximum activity toward test substrates. Sixteen chemicals including four furanocoumarins (xanthotoxin, angelicin, bergapten, imperatorin), a coumarin (coumarin), a phenylpropanoid (myristicin), four flavonoids (quercetin, kaempferol, flavone, α-naphthoflavone), a phenolic acid (chlorogenic acid), a mycotoxin (AFB1, and two synthetic compounds (the insecticide α-cypermethrin, the synergist PBO) were tested to determine their suitability as substrates based on the predicted structures of these P450s.

Analysis of substrate disappearance in these assays (**Table 1**) indicated that imperatorin is efficiently metabolized by CYP6AB11 (0.88nmol/min/nmol P450) and PBO is turned over by CYP6AB11 at a slower rate (0.11nmol/min/nmol P450). One metabolite was generated and analyzed by HPLC (**Figure 2**). LC-MS analysis was performed to further characterize the structure of the imperatorin metabolite by CYP6AB11. In positive mode electrospray, the metabolite yields four fragments: 203, 269, 287 and 309 (**Figure 3**). This metabolite is identical to that reported for CYP6AB3-mediated metabolism of imperatorin, which also appears to target the double bond in the isoprenyl side chain (Mao et al., 2006). No metabolite of PBO was observed in our GC-MS analysis.

The structure of CYP6AB11, which was predicted using MOE programs (Chemical Computing Group Inc., Montreal, Canada), suggests that its catalytic site contains a doughnut-like constriction over the heme that excludes aromatic rings on substrates and allows only their extended side chains to access the catalytic site. Docking of imperatorin in the active site predicts that imperatorin binds with interaction energy of 38.4 kcal/mol and at a distance from the heme estimated to be 7.2 Å. The Ile310-to-Val308 replacement in CYP6AB11 enlarges the opening to the heme and is predicted to allow imeratorin to access the CYP6AB11 catalytic site (**Figure 4**). That CYP6AB11 can metabolize the principal insecticide synergist piperonyl butoxide (PBO, a methylenedioxyphenyl synergist), in wide use to circumvent pesticide resistance, raises the possibility that, once acquired, insecticide resistance in this species may be difficult to counter.

CYP321C1 did not metabolize any of the 16 possible substrates tested in these assays. Only two compounds, xanthotoxin and aflatoxin B1, were tested with CYP6B44 but neither was metabolized.

B. Effects of naturally occurring and synthetic synergists on the toxicity of insecticides, phytochemicals and mycotoxins to *Amyelois transitella* (Lepidoptera: Pyralidae).

We tested the effects of a naturally occurring plant-derived chemical, myristicin (MYR), and a synthetic inhibitor of cytochrome P450 monoxygenases (P450s), piperonyl butoxide (PBO), on the toxicity of three insecticides (α-cypermethrin, τ-fluvalinate and

methoxyfenozide), a phytochemical (xanthotoxin) and a mycotoxin (AFB1) to *A. transitella*. The results of the insecticide assays show that α-cypermethrin and methoxyfenozide are more toxic than τ-fluvalinate to the newly hatched larvae (56-fold and 9.6-fold at 48 hr, respectively). The PBO and myristicin synergism bioassays with insecticides show that PBO significantly synergizes cypermethrin and fluvalinate and increases their toxicity to NOW, while myristicin synergizes only cypermethrin. Neither compound has any significant synergistic effects on methoxyfenozide. Synergism bioassays with xanthotoxin demonstrate that PBO significantly synergizes the toxicity of this phytochemical over time and that myristicin only slightly increases mortality after 7 days. None of the potential synergists tested enhanced AFB1 toxicity to NOW larvae. In view of these findings and the limited availability of environmentally safe synthetic insecticides for sustainable management of NOW, myristicin should be explored as a field treatment to reduce survival of this pest species and aflatoxin contamination in orchard situations.

C. Induction of detoxification P450s in NOW midguts in response to host plant chemicals.

The first instar larvae were fed with artificial diets containing an insecticide, α-cypermethrin, and supplemented with host plant chemicals such as quercetin, rutin, or chlorogenic acid. The mortality of the tested larvae was recorded daily. Quercetin significantly improved detoxification of the insecticide, as evidenced by reduced mortality compared with the larvae fed diets containing α-cypermethrin alone. Semi-quantitative RT-PCR gel blot analyses will be performed with RNA isolated from fifth instars fed for 48 hr on diets containing quercetin, rutin, or chlorogenic acid. Induced CYP6B44, CYP6AB11 and CYP321C1 transcripts will be compared with their low basal level in DMSO-treated control larvae, to determine the capacity of hostplant constituents to alter pesticide detoxification capacity.

References:

Campbell BC, Molyneux RJ. Schatzki TF, 2003 Current research on reducing pre- and post-harvest aflatoxin contamination of US almond, pistachio, and walnut. Journal of Toxicology Toxin Reviews, 22: 225-66.

Connell JH. 2001. Leading edge of plant protection for almond. Hort Technology, . 12:619-622.

Higbee BS, Siegel JP, 2009. New navel orangeworm sanitation standards could reduce almond damage. Calif Agric., 63: 24-28.

Mao W, Rupasinghe S, Zangerl AR, Schuler MA, Berenbaum MR, 2006. Remarkable substrate-specificity of CYP6AB3 in *Depressaria pastinacella*, a highly specialized caterpillar. Insect Mol Biol., 15:169-79.

Molyneux RJ, Mahoney N, Kim JH, Campbell BC, 2007. Mycotoxins in edible tree nuts. Int J Food Microbiol., 119:72-8.

Schatzki TF, Ong MS, 2000. Distribution of aflatoxin in almonds. 2. Distribution in almonds with heavy insect damage. J. Agric. Food Chem. 48:489-492.

Publications resulting from Almond Board support:

Niu, G., 2010. Toxicity of mycotoxins to insects and underlying molecular and biochemical mechanisms. Doctoral dissertation, University of Illinois at Urbana-Champaign.

Niu, G., H. S. Pollock, A. Lawrance, J. Siegel, and M. R. Berenbaum. Effects of naturally occurring and synthetic synergists on the toxicity of insecticides, phytochemicals and mycotoxins to *Amyelois transitella* (Lepidoptera: Pyralidae). J. Econ. Entomol., submitted 07/10.

Niu, G., S. G. Rupasinghe, A. R. Zangerl, J. P. Siegel, M. A. Schuler, and M. R. Berenbaum, A substrate-specific cytochrome P450 monooxygenase, CYP6AB11, from the polyphagous navel orangeworm (*Amyelois transitella*). Insect Biochem. Mol. Biol, submitted 08/10).

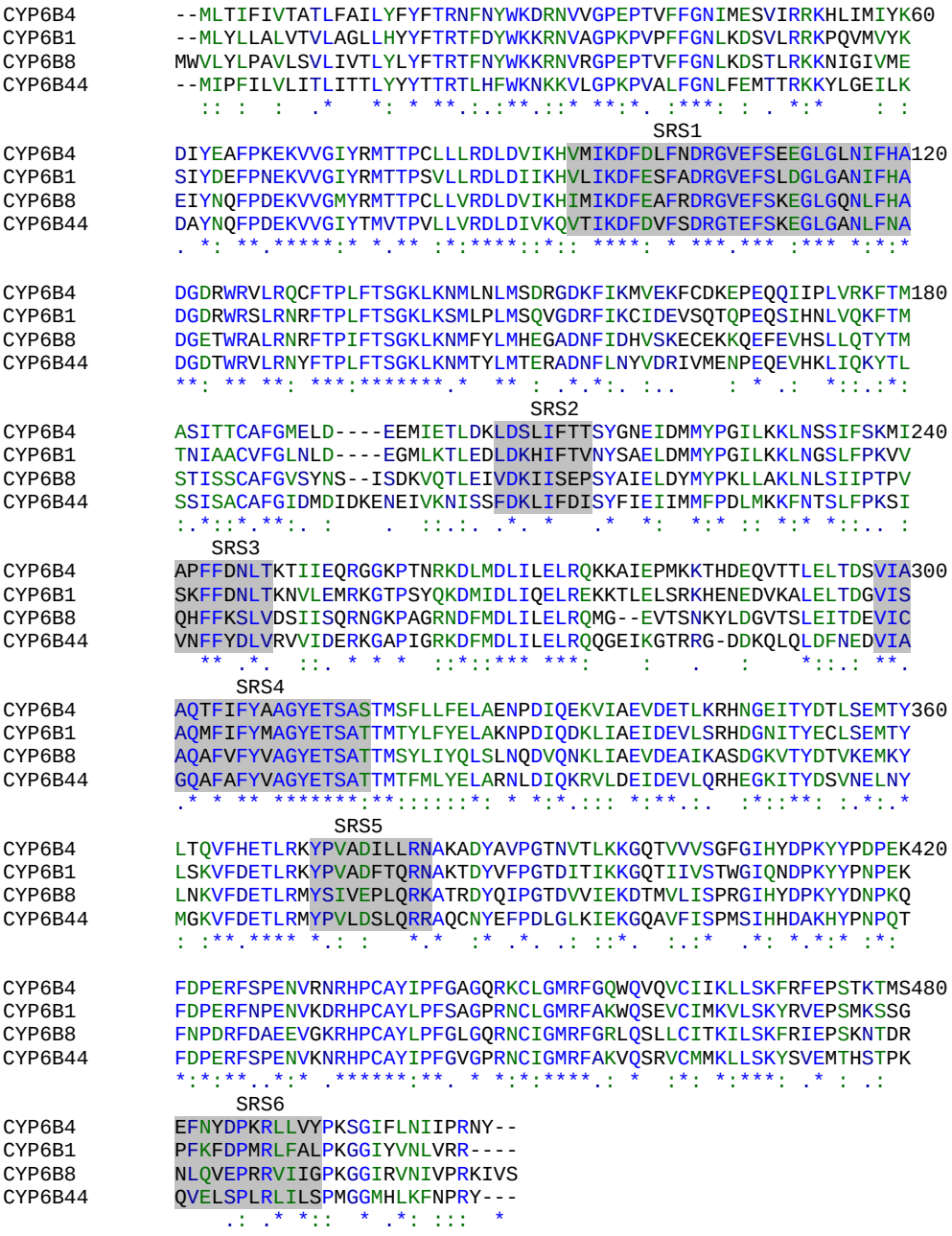


Figure 1. The protein alignment with the labeled SRS domains of the P450s from insects including CYP6AB (A), CYP6B(B) and CYP321(C) subfamilies. The sequences of CYP6B1 (*Papilio polyxenes*)**,** CYP6B8 and CYP321C1 (*Helicoverpa zea*); CYP6B4 (*Papilio glaucus*), CYP6B44, CYP321C1 and CYP6B11v1 (*Amyelois tansitella*); CYP6AB3v1 and CYP6AB3v2 (*Depressaria pastinacella*) were found from the NCBI protein data base. SRS indicates the substrate recognition sites and the amino acids in gray are within SRS regions. Consensus key symbols as follows: "*"single, fully conserved residue; ":"conservation of strong groups; "."conservation of weak groups; "no label" represents no consensus.


CYP321A1 MLQLPLILLGLVLLLTWYLIGRHNENYWKARGVKFYSKNKVIGPSWDYLFTKGAMFEKFG
CYP321C1 MFVQIILLIFIVFLFLIYTTGKHNENYWKKRGVKLHKKNKVTGLSWDFFTTDKPVFQIIG
SRS1
CYP321A1 ELYKAYRNEPVVAIGQILTPSLFVIDAKNVQHVLSSDFQSFNHRGLDSVEGDQLTESITL
CYP321C1 DLYKEYREEPAVGFGNFLTPSLLVNDATNIQHIFQTDFQSFNHRGFEINEGDHLADNILF
CYP321A1 LNGPKWKLMRKTMTPLFTSSKLKNMYYIMDKCAQDFVTYLKRDPKLWKGNFFESAMLYCN
CYP321C1 MNGNRWKLVRQSMTPIFTSTKLKQMFHIMDKSGRDFITYLKDNPERLNGNAIDSISTFCC
SRS2
CYP321A1 AAVCAAIFGIGEQSTFDSPFLKFAKDVSISSFKNNMKFTFFSLAPKLFTMLGLKVFKEHE
CYP321C1 AAIGAAVFGVESESVFESPFLDVAKRAMKFSVTMNIKALIMNVFPWLSKKLNFKLFSEHE
SRS3
SRS4
CYP321A1 DFFVGAIGDVIKQREEENVKRHDFADLCVQLQKNGTLKDQATGYELEPTTGLLSAQAFFF
CYP321C1 DFFIGAVKRIIRQREQDKVQKHDFADMCVSLQRNGTLKDRDTGLELEPTDELLSGQAFFF
CYP321A1 FTAGVEPAADGIFATFALLSQHPEILQKVHQEIDEYFEKYDGKITYDVVCDMEYVDKVLS
CYP321C1 FTAGVDPSAAAIFGCLLELGRNPEHLKKVHNEIDTAFEKLNGNLSYESVMEMEYLEKVLC
SRS5
CYP321A1 ESLRMFPPIGFLTRQCIHDTVLPVGNVKVAKGTKLFTPIYEIHHDPKYYPDPEVFDPERF
CYP321C1 ESLRMWPPIGVLNRQCVADTVLPVGNIKIDKDTKVYAAVYELHHDPKYYPNPEVFDPERF
SRS6
CYP321A1 FKERRPNDDLYMPFGMGNRTCIGARYFKLQLLAAIVHVLRSFTLKPIPESQQKKVTFLRH
CYP321C1 SSTSAQDSNVYMPFGKGNRLCVGMRFARLQVKTGLIHFLRNFTVKTKVDGD--RIRFKKD
CYP321A1 AIGVRIGNVKVELIPRDTK
CYP321C1 PVQVRPVNVDIEFIWRNI-

Figure 1B


CYP6AB3v2 MYFLIALGILLIILYLYGIKNHKYWEKKGVPYVKPIPFFGTNFKVFMQRICISDQLCKYY
CYP6AB3v1 MHFLIALGILLIILYLYGIKNHKYWEKKGVPYVKPIPFFGTNFKVFMQRICISDQLCKYY
CYP6AB11 MIIPIAVIVICLLLYYYGTRNFKYWQKRGIIHDKPIPFFGNNIDGYLFRKSVTQIATDMY
SRS1
CYP6AB3v2 EQFPNEKFVGAFIGDRIGLVLREPELIKRVMATDFQYFHPRGVNPHKTVYEPLLKNLFTG
CYP6AB3v1 EQFPNEKFVGAFIGDRIGLVLREPELIKRVMVTDFQYFHPRGVNPHKTVYEPLLKNLFTG
CYP6AB11 RKYPNEKVVGFYRANIPELVVRDPETIKRVLVTDFEHFYPRGIN-HKEVVEPLMRNLFFA
CYP6AB3v2 DGDMWKLLRQRITPAFTSGKLKAMFPLIVERAERLQIIAATAAQSHGEVDVRELMARFTT
CYP6AB3v1 DGDMWKLLRQRITPAFTSGKLKAMFPLIVERAERLQIIAATAAESHGEVDVRELMARFTT
CYP6AB11 DGDLWRLLRQRMTPAFTSGKLKAMFPLIIDCADRLQKRALTVSAAGKKLDARDLMARYTT
SRS2
CYP6AB3v2 DFIGACGFGIDADTLNDEESTFRRLGKRIFTLTRRDGFVFMLKTIAPEIFKNLHMFAPEI
CYP6AB3v1 DFIGACGFGIDADTLNDEESTFRRLGKRIFTLTKRDGFVFMLKTIAPEIFKNLHMFAPEI
CYP6AB1 DFIGACGFGVNADSLGDEDSAFRKLGAQIFQPRVQDLIVAILKEVFPDTFKHLKYLS-RL
SRS3
CYP6AB3v2 EKTTVDLVTSIMQQRKYKHSGRNDFIDFLLELKGKGKIVGESVEKRNPDGTPKIVEMELD
CYP6AB3v1 EKTTVDLVTSIMQQRKYKHSGRNDFIDFLLELKGKGKIVGESVEKRNPDGTPKIVEMELD
CYP6AB11 EDDFKKFVSDVLHLRNYKPSGRNDFIDLMLECKQKGTIVGESIERRDKEGRPEKATVELD
SRS4
CYP6AB3v2 DMLMAAQVFIFFAAGFETSSSTTSYTLHQLAFHPEEQKKCQDQIDEVLSRHGGKLSYEAI
CYP6AB3v1 DMIMAAQVFIFFAAGFETSSSTTSYTLHQLAFHPEEQKKCQDQIDEVLSRHGGKLSYEAI
CYP6AB11 DTLIAAQVFVFFAAGFETSSSATSYTLHELAFNPEIQERVQKEIDTVLAKHDNKLSYDAV
SRS5
CYP6AB3v2 KEMTYLDMIFKESMRMYPSLGILTRRCVQKYTFPGTNLTIDEDVLVCIPVHALHNDEKYF
CYP6AB3v1 KEMTYLDMIFKESMRMYPSLGILTRRCVQKYTFPGTNLTIDEDVLVCIPVHALHNDEKYF
CYP6AB11 KEMTYLEWTFKEAMRMLPSLGFLIRQSVRPYTFPELGLSIDADVGIMIPLQALHTDPEYF
CYP6AB3v2 DEPEKFKPERFSPENIKNIPKYVYLPFGDGPRACIGERLGHMQSLAGLAALLSKFSVAPS
CYP6AB3v1 DEPEKFKPERFSPENIKNIPKYVYLPFGDGPRACIGERLGHMQSLAGLAALLSKFSVAPS
CYP6AB11 DNPMEFRPERFDPENFTRKQRDIYLPFGTGPRACIGERLGLMQSLAGLAAVLSKFSVSPA
SRS6
CYP6AB3v2 KNTLRQPITDPTCTVVKSIKGGLPLSLVARKV------
CYP6AB3v1 KNTLRQPITDPTCTVVKSIKGGLPLSLVARKV------
CYP6AB11 ADTKRWPEIDPRSDIVQTIVGGLPLQFSERKRNVVNFN

Figure 1C

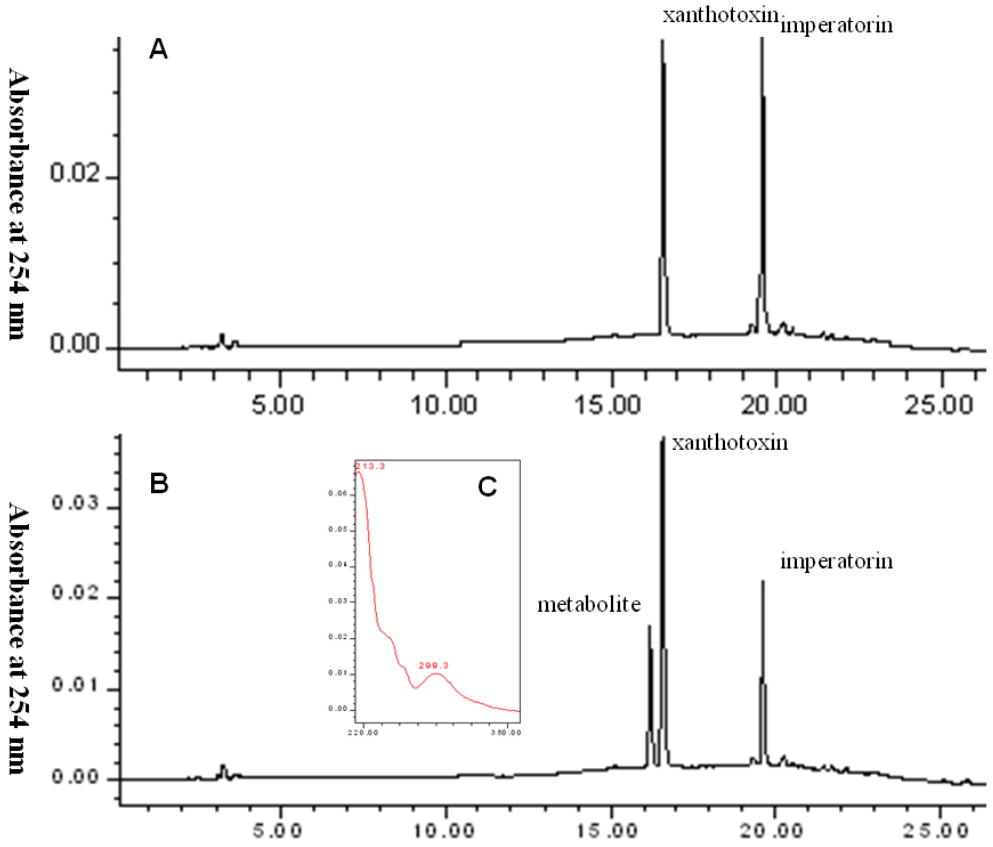


Figure 2. Reverse-Phase HPLC chromatogram showing imperatorin metabolite produced by heterologously expressed CYP6AB11 in insect cells. Metabolic reactions were carried out as described in Materials and Methods. The metabolite of imperatorin was eluted at 16.1 min in the reaction with addition of NADPH (B) compared with the reaction without addition of NAPDH (A). Xanthotoxin as internal standard was eluted at 16.6 min. The imperatorin metabolite was scanned with the HPLC photodiode array detector and the spectrum of the imperatorin was shown in **Figure 3**.

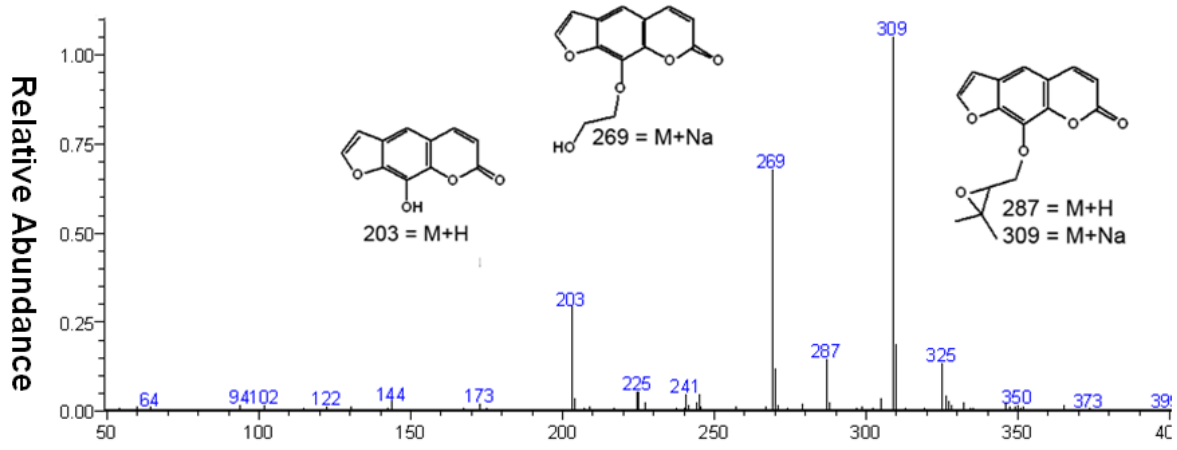


Figure 3. LC-MS spectrum of the imperatorin metabolite by CYP6AB11.

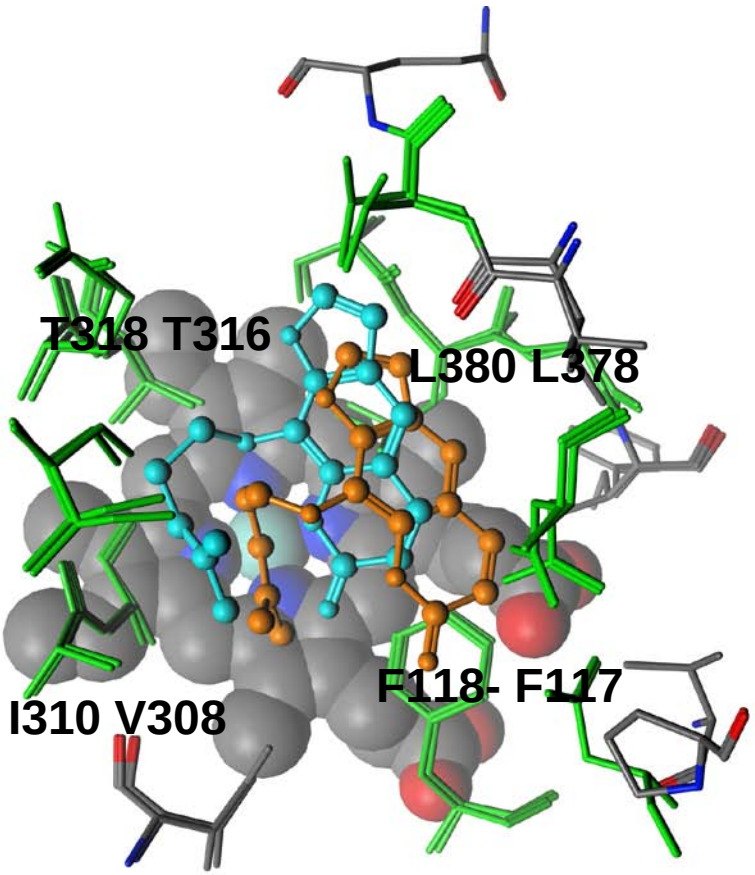
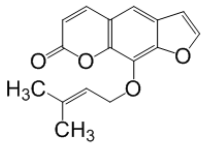
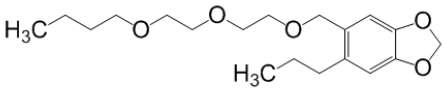


Figure 4. Predicted imperatorin binding in CYP6AB11 and CYP6AB3 models. Residues around a 4.5 Å radius are shown. Conserved residues are shown in green while non-conserved residues are shown in elemental colors. Imperatorin binding conformation in CYP6AB3 is shown in aqua and the conformation in CYP6AB11 is shown in orange.

Table 1. Metabolism rates by CYP6AB11.

substrate	structure	metabolism rate (nmol/min/nmol P450)	error
imperatorin		0.88	0.11
piperonyl butoxide		0.11	0.01