Identifying Cytochrome P450 Detoxification Enzymes in Navel Orangeworms Responsible for Detoxifying Insecticides and Hostplant Chemicals

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

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Objectives:

A. To characterize the substrate specificities of these P450s using a subtractive method involving extracts of host plants (almond, pistachio, fig) (Mao et al. 2008) and to test their capacity to metabolize insecticides.

B. Define the inducibility of detoxificative P450s in NOW midguts in response to plant chemicals and determine the impact of inducibility on catalytic activity against phytochemicals and pesticides.
C. To investigate allelic variation among hostplant populations of NOW and relate impact of hostplant identity to insecticide metabolism

Interpretive Summary:

Navel orangeworm (NOW) *Amyelois transitella*, is among the most destructive pests of almonds in California. Neonates' tunnel into the nut and successive instars consume most of the nutmeat, generating large quantities of frass and webbing in the process. In addition to direct losses, NOW damage leaves almonds vulnerable to infection by *Aspergillus* fungal species that produce toxic aflatoxins, particularly in soft-shelled late-maturing cultivars with a prolonged hull split period. NOW management depends on a combination of tactics, including chemical control, particularly when infested adjacent crops such as pomegranate or figs provide a source of immigrants into almond orchards. Chemical sprays for hull split include organophosphates, pyrethroids, and newer chemical classes; only Entrust (spinosad) sprays are acceptable for use in organic orchards. Virtually nothing is known, however, as to how NOW metabolize these compounds and thus whether metabolic resistance is likely to evolve rapidly. Our overall goal in this project is to determine the metabolic basis for insecticide and phytochemical detoxification and to determine the likelihood of the evolution of resistance in response to insecticide selection pressure.

We have confirmed earlier studies that metabolic detoxification is involved in the ability of *A. transitella* to tolerate very high levels of aflatoxin in its diet; moreover, we recently demonstrated that certain naturally occurring essential oil constituents (e.g., myristicin) can inhibit metabolism of hostplant phytochemicals and enhance toxicity, and therefore may be of value as insecticide synergists. We tested the synergistic effects of piperonyl butoxide (PBO), a known insecticide synergist, as well as two natural compounds, myristicin (MRSN) and apiole, which share structural similarities with PBO. Performance of NOW consuming diets containing phytochemical or insecticide alone was compared with that of larvae on diets containing the same concentrations of phytochemicals or insecticides supplemented with MRSN, PBO or apiole. Although we obtained consistent evidence that myristicin can act as a synergist of the naturally occurring phytochemical xanthotoxin, results of tests with insecticides were less definitive (**Figure 1**). Concentrations of cypermethrin (CYP) 0.1 µg/g or tau-fluvalinate (FLV) at 2 µg/g for FLV were selected to test for synergistic effects of MRSN (0.5mg/g), PBO (1mg/g) or apiole (1mg/g). Apiole showed a slight synergistic interaction with FLV and effects of PBO on toxicity of CYP were inconsistent in bioassays. This work is continuing with adjustments to concentrations and examination of end points other than percent mortality (e.g., larval duration, pupal weight).

In view of evidence of synergism and metabolic detoxification, identifying the metabolic enzymes responsible for detoxification of pesticides and phytochemicals

and understand their specificity and evolution in NOW are research priorities. We identified three cytochrome P450 monooxygenase enzymes, CYP6AB11, CYP6B44 and CYP321C1, in NOW midguts and we successfully expressed in our heterologous baculovirus Sf9 cell system. We can now assay phytochemicals and insecticides in vivo, with NOW, and in vitro, with our expressed enzymes, to determine toxicity as well as detoxification.

Progress to date:

A. Substrate specificies of detoxification enzymes from *Amyelois transitella*.
We have used three approaches to define substrate specificies of the three P450 enzymes characterized from the midguts of NOW. First, we use a comparative approach, examining substrates known to be metabolized by enzymes in the same P450 subfamilies in other caterpillar species. We also construct computer models of the protein structure of the targeted enzymes and predict substrate structure based on docking results. The third method involves direct tests of extracts of hostplants.

CYP6AB11 is the first P450 for which we have successfully identified substrates. This enzyme can metabolize myristicin, a natural methylenedioxphenyl (MDP) compound (MRSN), as well as its synthetic analogue piperonyl butoxide (PBO). CYP6AB11 shares 56% similarity with CYP6AB3v2 in *Depressaria pastinacella* (parsnip webworm), an enzyme that is highly specialized for metabolizing MRSN as well as the furanocoumarin imperatorin (Mao et al., 2007) (**Figure 2**). The metabolism rate for MRSN is 0.06pmol/min/pmol, substantially lower than that of CYP6AB3v2; CYP6AB11 can metabolize PBO at a slightly higher rate of 0.11 nmol/min/nmol protein. Aligning the protein sequences of these two P450s shows that the CYP6AB11 has a Val^{92} where CYP6AB3v2 has Ala^{92}; the identity of the amino acid at this position is crucial in enhancing catalytic activity and the substitution may explain the difference in metabolism rate between these two P450s. CYP6AB11 in our expression system cannot metabolize several coumarins (xanthotoxin, bergapten, coumarin, and angelicin), flavanoids (naringinin, quercetin), chlorogenic acid, flavone or α-nathoflavone. Functional studies of the other two P450s (CYP6AB11 and CYP321C1) are ongoing.

B. Catalytic activity against phytochemicals and pesticides and inducibility of detoxificative P450s in response to plant chemicals
The toxicity of three insecticides, including the pyrethroids cypermethrin (CPM) and tau-fluvalinate (FLV) and the ecdysone agonist methoxyfenzide (MXF) has been evaluated in laboratory bioassays assessing mortality of newly hatched larvae fed with artificial diets containing different concentrations of the insecticides. In these assays, CPM and MXF are more toxic than FLV. The LC_{50} (defined as the concentration which can cause 50% mortality in treated larvae in 4 days) of FLV was determined as 3.7 [2.58-4.91] µg/g (**Figure 3**). CPM and MXF at 500 ng/g caused >90% mortality in first instar larvae in 4 days while this concentration of FLV caused no mortality (**Figure 4**). Tolerance of insecticides increased with larval development. More than 90% of third instars survived when treated with 100 ng/g CPM or MXF and FLV at 500ng/g had no detectable effects on third instar larvae(data is not shown).

Toxicity of insecticides and other toxins to caterpillars is well known to vary with hostplant species; this effect of diet is of particular interest in the management of a highly polyphagous pest such as the navel orangeworm. Dietary effects on toxicity are generally the result of induction of detoxification enzymes by phytochemical constituents of hostplants (Li et al., 2002). Among the many chemicals identified from NOW's host plants, compounds such as coumarins, flavonoids, chlorogenic acid, and simple phenols have been shown to influence pesticide toxicity by inducing detoxification enzymes in other species; we are now testing potential effects of hostplant identity on insecticide toxicity in NOW. These tests are now underway. To date, the flavonoids quercetin and rutin have been examined; these two compounds do not affect insecticide toxicity (data not shown). Tests on coumarin, xanthotoxin, caffeic acid and chlorogenic acid are underway.

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Mao W, Rupasinghe SG, Zangerl AR, Berenbaum MR, Schuler MA, 2007. Allelic variation in\the Depressaria pastinacella CYP6AB3 protein enhances metabolism of plant allelochemicals by altering a proximal surface residue and potential interactions with cytochrome P450 reductase. J Biol Chem., 282(14):10544-52.

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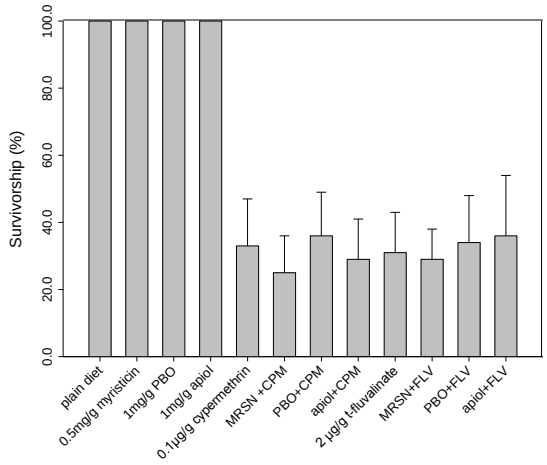

Figure 1. Effects of synergists on toxicity of α-cypermethrin and tau-fluvalinate to 3rd instar *Amyelois transitella*. Third instars within 12 hr of molt were fed 0.1 µg/g for CPM or 2 µg/g for FLV, or insecticide supplemented with 0.5 mg/g MRSN, 1mg/g PBO or 1mg/g apiole. Survivorship of the treated larvae in 7 days was recorded.

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CYP6AB11        MIIPIAVIVICLLLYYYGTRNFKYWQKRGIIHDKPIPFFGNNIDGYLFRKSATQIATDMY
CYP6AB3v2       MYFLIALGILLIILYLYGIKNHKYWEKKGVPYVKPIPFFGTNFKVFMQRICISDQLCKYY
                * : **: :: ::** ** :*.***:*:*: : *******.*:. :: * . ::   . *

CYP6AB11        RKYPNEKVVGFYRANIPELVVRDPETIKRVLVTDFEHFYPRGIN-HKEVVEPLMKNLFFA
CYP6AB3v2       EQFPNEKFVGAFIGDRIGLVLREPELIKRVMATDFQYFHPRGVNPHKTVYEPLLKNLFTG
                .::****.** : .:   **:*:** ****:.***::*:***:* ** * ***:**** .

CYP6AB11        DGDLWRLLRQRMTPAFTSGKLKAMFPLIIDCADRLQKRALTVSAAGKKLDARDLMARYTT
CYP6AB3v2       DGDMWKLLRQRITPAFTSGKLKAMFPLIVERAERLQIIAATAAQSHGEVDVRELMARFTT
                ***:*:*****:****************:: *:***  * *.: :  ::*.*:****:**

CYP6AB11        DFIGACGFGVNADSLGDEDSAFRKLGAQIFQPRVQDLIVAILKEVFPDTFKHLKYLS-RL
CYP6AB3v2       DFIGACGFGIDADTLNDEESTFRRLGKRIFTLTRRDGFVFMLKTIAPEIFKNLHMFAPEI
                *********::**:*.**:*:**:** :**    :* :* :** : *: **:*: :: .:

CYP6AB11        EDDFKKFVSDVLHLRNYKPSGRNDFIDLMLECKQKGTIVGESIERRDKEGRPEKATVELD
CYP6AB3v2       EKTTVDLVTSIMQQRKYKHSGRNDFIDFLLELKGKGKIVGESVEKRNPDGTPKIVEMELD
                *.   .:*:.::: *:** ********::** * **.*****:*:*: :* *: . :***

CYP6AB11        DTLIAAQVFVFFAAGFETSSSATSYTLHELAFNPEIQERVQKEIDTVLAKHDNKLSYDAV
CYP6AB3v2       DMLMAAQVFIFFAAGFETSSSTTSYTLHQLAFHPEEQKKCQDQIDEVLSRHGGKLSYEAI
                * *:*****:***********:******:***:** *:: *.:** **::*..****:*:

CYP6AB11        KEMTYLEWTFKEAMRMLPSLGFLIRQSVRPYTFPELGLSIDADVGIMIPLQALHTDPEYF
CYP6AB3v2       KEMTYLDMIFKESMRMYPSLGILTRRCVQKYTFPGTNLTIDEDVLVCIPVHALHNDEKYF
                ******:  ***:*** ****:* *:.*: ****  .*:** ** : **::***.* :**

CYP6AB11        DNPMEFRPERFDPENFTRKQRDIYLPFGTGPRACIGERLGLMQSLAGLAAVLSKFSVSPA
CYP6AB3v2       DEPEKFKPERFSPENIKNIPKYVYLPFGDGPRACIGERLGHMQSLAGLAALLSKFSVAPS
                *:* :*:****.***:..  : :***** ********** *********:******:*:

CYP6AB11        ADTKRWPEIDPRSDIVQTIVGGLPLQFSERKRNVVNFN
CYP6AB3v2       KNTLRQPITDPTCTVVKSIKGGLPLSLVARKV------
                 :* * *  ** . :*::* *****.:  **
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Figure 2. Protein sequences of CYP6AB11 from *Amyelois transitella* and CYP6AB3v2 from *Depressaria pastinacella*. Consensus key symbols: "*"single, fully conserved residue; ":"conservation of strong groups; "."conservation of weak groups; "no label" represents no. The amino acid on position of 92 was marked in grey.

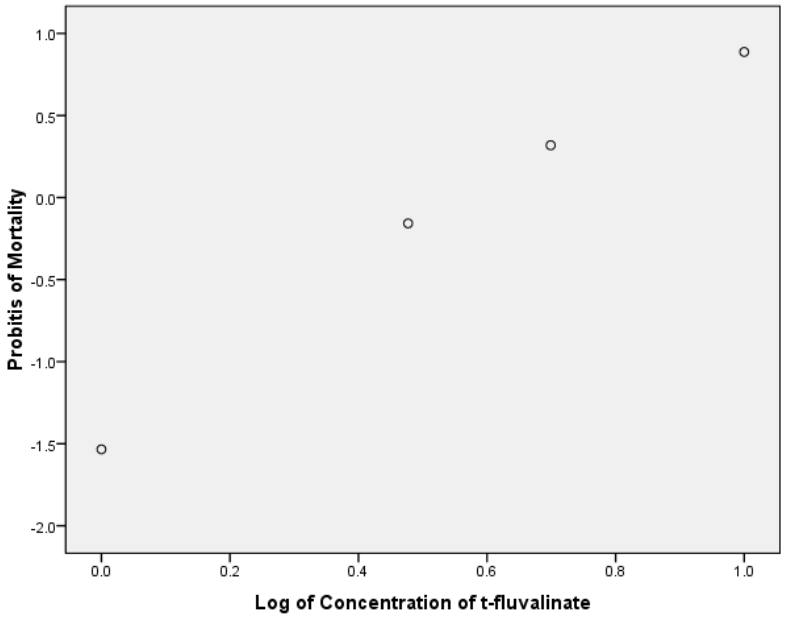

Figure 3. Determination of LC_{50} of tau-fluvalinate to 1st instar larvae of *Amyelois transitella*. LC_{50} is defined as the concentration of FLV that causes 50% mortality of the treated larvae in 4 days and is calculated based on probit analysis. Artificial diets containing 1, 3, 5, 7, 10 and 15 µg/g of FLV and control diet containing methanol were fed to newly hatched larvae and mortality in 4 days was recorded. LC_{50} with 95% confidence intervals is 3.7 [2.58-4.91] µg/g.

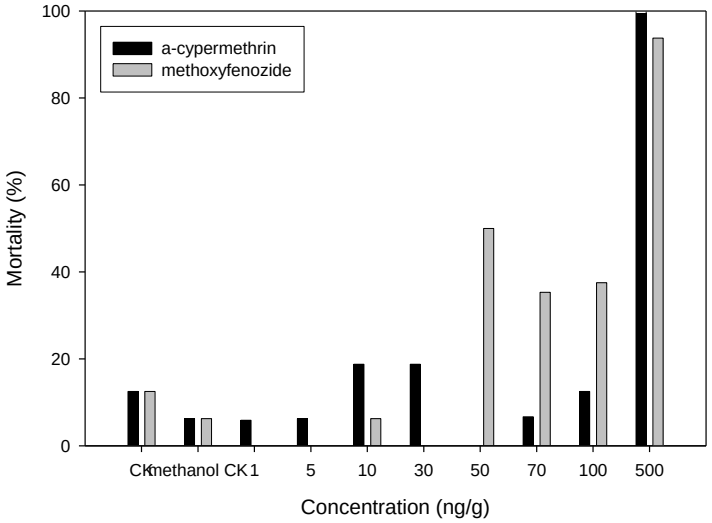

Figure 4. Toxicity of α-cypermethrin and methoxyfenozide to 1st instar larvae of *Amyelois transitella*. Artificial diets containing 1, 5, 10, 30, 50 70, 100, 500 ng/g of FLV and the control diet containing methanol or without methanol were fed to newly hatched larvae and mortality in 4 days was recorded.