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*[^1][^2][^3]

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CYP6AB3v1, a cytochrome P450 monoxygenase in *Depressaria pastinacella* (parsnip webworm), is highly specialized for metabolizing imperatorin, a toxic furanocoumarin in the apiaceous host plants of this insect. Cloning and heterologous expression of CYP6AB3v2, an allelic variant identified in *D. pastinacella*, reveals that it metabolizes imperatorin at a faster rate than does CYP6AB3v1. Comparisons of the NADPH consumption rates for these variants indicate that CYP6AB3v2 utilizes this electron source at a faster rate than does CYP6AB3v1. Molecular modeling of the five amino acid differences between these variants and their potential interactions with P450 reductase suggests that replacement of Val[^8] on the proximal face of CYP6AB3v1 with Ala[^9] in CYP6AB3v2 affects interactions with P450 reductase so as to enhance its catalytic activity. Allelic variation at this locus potentially allows *D. pastinacella* to adapt to both intraspecific and interspecific variation in imperatorin concentrations in its host plants.

Cytochrome P450 monoxygenases (P450s)\(^2\) are heme-containing enzymes that catalyze the NADPH-dependent reductive cleavage of molecular oxygen to produce functionalyzed organic products and a molecule of water, making them crucial for phase I metabolism in a wide range of organisms. Although considerable allelic variation in both the coding and the regulatory regions is known to occur in many vertebrate P450 genes (1–3), the extent of allelic variation in insect P450 loci is not clear.

Much of what is known of allelic variation in insect P450s results from studies of insecticide resistance. Here, most examples of P450-mediated resistance to insecticides involve regulatory changes, often transposon-mediated (4). Only a few examples exist where point mutations in coding regions affect reactivities toward insecticides. Perhaps the best evidence of such allelic variation is the existence of three amino acid variations in CYP6A2 (R335S, L336V, V476L) in the DDT-resistant RDDTR strain of *Drosophila melanogaster*; these substitutions occur near the CYP6A2 active site and increase catalytic efficiency of the CYP6A2 enzyme against DDT, 7-ethoxycoumarin, and 7-benzoxyloxycoumarin (5). Allelic variation in the form of amino acid substitutions has also been documented in CYP6D1, CYP6D3, and CYP6X1 as well as CYP6A2 (6 –8), but the functional contributions of these to resistance have not yet been determined.

In addition to contributing to insecticide resistance, P450s play an important role in mediating resistance to plant allelochemicals (defense compounds) in herbivorous insects (9). However, these allelochemicals present a toxicological challenge to insects that differs in several fundamental ways from that presented by insecticides. Although insecticides are generally single compounds designed to kill and applied in a broadcast fashion, plant defense chemicals tend to occur as mixtures of structurally similar compounds that are idiosyncratically distributed in the environment and expressed in a site-specific manner (10). Thus, although regulatory changes leading to overexpression may be common in P450-mediated insecticide resistance, allelic variations in coding sequences that affect the relative efficiency of substrate metabolism may be of adaptive value in herbivorous insects, particularly those with a narrow range of chemically similar host plants. Such insects are likely to encounter differences in the relative content and composition of toxins among their hosts, and allelic variation in P450-mediated metabolism may allow specialization at the level of insect populations and evolution of host plant responses to herbivory.

*Depressaria pastinacella*, the parsnip webworm, is restricted throughout its range on host plants in the genera *Pastinaca* and *Heracleum* (11, 12). Like many apioid umbellifers, *Pastinaca sativa* (wild parsnip) and *Heracleum lanatum* (cow parsnip) produce phototoxic furanocoumarins capable of intercalating between base pairs in DNA, forming covalent cross-links with the 5,6 double bond of pyrimidines (13). As a consequence of their ability to interfere with DNA replication, furanocoumar-
Pastinaca sativa (wild parsnip)

<table>
<thead>
<tr>
<th>Furanocoumarin</th>
<th>No.</th>
<th>Min.</th>
<th>Max.</th>
<th>Mean</th>
<th>S.D</th>
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</thead>
<tbody>
<tr>
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<td>0.03</td>
<td>16.14</td>
<td>3.59</td>
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<td>1.10</td>
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<tr>
<td>Xanthotoxin</td>
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<td>14.60</td>
<td>3.50</td>
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<td>Sphondin</td>
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<td>0.02</td>
<td>2.30</td>
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<td>0.22</td>
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Heracleum lanatum (cow parsnip)

<table>
<thead>
<tr>
<th>Furanocoumarin</th>
<th>No.</th>
<th>Min.</th>
<th>Max.</th>
<th>Mean</th>
<th>S.D</th>
</tr>
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<td>1.21</td>
<td>0.44</td>
<td>0.38</td>
</tr>
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rins are highly toxic to a wide range of organisms, including insects (14). As shown in Table 1, the amounts of five furano-
coumarins present in fruits of both parsnip webworm hosts differ substantially both within species (12) and between host
plant species (11). As is the case with several other insects, P450s are responsible for detoxifying furanocoumarins pro-
duced in their host plants (15–21).

The majority of furanocoumarin-metabolizing P450 genes identified to date have been members of the CYP6B subfamily, including CYP6B1 from *Papilio polyxenes* (15), the CYP6B4 and CYP6B17 groups from *Papilio glaucus* and *Papilio canadensis* (19, 22), and CYP6B8, CYP6B9, and CYP6B27 from *Helico-
coverpa zea* (23, 24). Furanocoumarin-metabolizing P450s of polyphagous (generalist) species, such as *H. zea* and *P. glaucus*, catalyze metabolism of a wide range of furanocoumarins with lower activities than the P450s of specialist species, such as *P. polyxenes*, which feed exclusively on furanocoumarin-con-
taining host plants (17, 20, 25–29). Molecular modelings of these P450s have indicated that at least some of the P450s in generalist insects are structurally more flexible and capable of binding a more diverse array of compounds when compared with some of the P450s in specialist insects (26, 27).

To date, the P450 associated with furanocoumarin metab-
lolism in *D. pastinacella* CYP6AB3v1 has the narrowest range of substrates among xenobiotic-metabolizing P450s yet identified (21). Molecular analysis has indicated that this P450 transcript is strongly induced by the mixture of furanocoumarins extracted from the reproductive parts of *P. sativa*, allowing it to be cloned along with a few other P450 sequences (30). A molecular model of CYP6AB3v1 contains three-dimensional ele-
ments similar to those predicted in the catalytic sites of the furanocoumarin-metabolizating CYP6B proteins in *P. polyxenes*, *P. glaucus*, and *H. zea* (25–27, 31) including residues in the B-helix and B’-C loop of SRS1, I-helix of SRS4, and the β-turn in β-sheet 4 of SRS6 (30). Amino acids are also con-
served in SRS1, SRS4, and SRS5 that contribute to form the catalytic site of CYP6B1 from the specialist *P. polyxenes*,
CYP6B4 from the generalist *P. glaucus*, and CYP6B8 from the generalist *H. zea*. Specifically, Arg<sup>101</sup>, Val<sup>103</sup>, Glu<sup>111</sup>, Leu<sup>113</sup>, Asn<sup>116</sup>, Phe<sup>118</sup>, and Asp<sup>211</sup> in SRS1 of *D. pastinacella* CYP6AB3 are absolutely conserved in all four of these CYP6B proteins (30); because of small deletions in these other proteins, these CYP6AB3 positions correspond to positions 102–119 in CYP6B1 and CYP6B4 and positions 104–121 in CYP6B8. As well, 9 of 14 positions in SRS4 and three of nine positions in SRS5 are conserved in these four proteins. Among these con-
served positions, Phe<sup>113</sup>, Ala<sup>114</sup>, and Thr<sup>207</sup> in CYP6B1 (corre-
sponding to Phe<sup>218</sup>, Ala<sup>224</sup>, and Thr<sup>318</sup> in CYP6AB3) have been identified as contacts for the furanocoumarin substrates of CYP6B1, and Val<sup>104</sup> is predicted in the catalytic site just out of substrate contact range (25, 31, 32).

The CYP6AB3v1 protein heterologously expressed in SF9 cells is highly specialized for metabolizing imperatorin with V<sub>max</sub> and K<sub>m</sub> of 2.41 pmol/min/pmol of P450 and 94.28 μM, respectively (21). As shown in Table 1, this furanocoumarin is at the same time the most abundant furanocoumarin in both North American hosts of *D. pastinacella* and the most variable in concentration. To determine whether CYP6AB3 allelic vari-
ants exist that metabolize imperatorin more effectively, a cDNA library constructed with RNA from the midguts of par-
spin webworm larvae induced with parsnip seed furanocou-
rins was probed with the original CYP6AB3v1 cDNA. Co-expression of the closely related CYP6AB3v2 cDNA isolated in this process with house fly NADPH-dependent P450 reductase has indicated that this second P450 variant metabolizes impera-
torin significantly more rapidly than CYP6AB3v1. Biochemical analyses coupled with molecular modeling provide insight into the mechanistic reasons for the enhanced activity of CYP6AB3v2, which differs in only five positions from CYP6AB3v1. The fact that mutagenesis of Val<sup>29</sup> to Ala<sup>29</sup> in CYP6AB3v1 caused it to metabol-
ize imperatorin nearly as effectively as CYP6AB3v2 indicates that this amino acid switch is the one primarily respon-
sible for the enhanced activity of CYP6AB3v2 toward imperatorin.

**EXPERIMENTAL PROCEDURES**

**Reagents**—XbaI and BamHI restriction enzymes were bought from Invitrogen. Angelicin, bergapten, isopimpinellin, sphondin, and xanthotoxin were purchased from Indofine Chemical Co. (Belle Mead, NJ). Imperatorin was bought from Feinbiochemica (Heidelberg, Germany). NADPH was obtained from Sigma. SF9 insect cells, SF-900 serum-free medium, pFast-
Bac1 vector, and DH10BAC competent cells were purchased from Invitrogen. Penicillin/streptomycin was obtained from Bio-
Whittaker (Walkersville, MD). HPLC solvents were bought from Fisher.

**cDNA Library Selection**—The full-length cDNA library used by Li et al. (30) was rescreened with the CYP6AB3v1 cDNA at medium stringency (65 °C, 5× SSC, 1% SDS, 5× Denhardt’s solution). From 2000 clones, five positive clones over 1.6 kb in length were sequenced using T3 and T7 vector primers and CYP6AB3-specific internal primers. All sequences were defined by sequencing both cDNA strands.

**Expression of CYP6AB3v2 and CYP6AB3v1 V92A Mutant in SF9 Cells**—The CYP6AB3v2 cDNA was inserted into the pFast-
Bac vector using a PCR strategy with two primers synthesized against the N- and C-terminal ends of the coding sequence. The CYP6AB3v2 forward primer, C3-R (5′-GGCGGATCCGATG-
TATTTTTTAAATGCAATAG-3′) contains the translation start codon and a BamHI site on its 5′ end, and the reverse primer, C3-L (5′-CGCTCAGATTTACCTTTCTTGCA-
CACAATG-3′), contains the stop codon and an XbaI site on its 5′
end. After PCR amplification of the CYP6AB3v2 clone, the PCR products were digested with BamHI and XbaI and ligated into BamHI-XbaI-cut pFastBac vector.

The CYP6AB3v1 V92A mutant was constructed using the CYP6AB3v1-containing pFastBac clone described in Mao et al. (21) as a template for single-stranded DNA site-directed mutagenesis (33). For this, the 5'-GAACATATAAAGAG-GGTCATGGCTACGGATTTCATACTTCCCAC-3' primer (mutant nucleotides are underlined) was used instead of the pair of primers in the QuikChange™ site-directed mutagenesis kit developed by Stratagene (La Jolla, CA). The PCR amplification was performed using the method of Humma et al. (33) except that annealing was done at 60 °C. The final mutants were confirmed by sequencing of the full length of the coding region with vector and internal primers.

All procedures for expression of the recombinant viruses in Sf9 cells were performed as described by Invitrogen protocols. As described previously for CYP6AB3v1 (21), one plate (100 × 20 mm) was seeded with 8 × 10⁶ cells in 10 ml of SF-900 serum-free medium supplemented with 8–10% fetal bovine serum, 50 μg/ml streptomycin sulfate, and 50 units/ml penicillin and coinfect 1 h later with the recombinant CYP6AB3 viruses and house fly P450 reductase virus at varying multiplicities of infection (m.o.i.). Hemin was added to a final concentration of 2 μg/ml 24 h after the initial infection. Baculovirus-infected insect cells were harvested 72 h after infection by centrifugation at 1000 × g for 5 min and washed two times with 0.1 x ice-cold phosphate buffer (pH 7.8) (PB). The cell pellet was resuspended in one-fifth volume of ice-cold cell lysate buffer (0.1 M phosphate buffer (pH 7.8), 1.1 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml (w/v) leupeptin, 20% glycerol) and lysed by sonication on ice (3 × 15 s). After the sonicated extract was centrifuged at 3200 × g for 5 min at 4 °C, the supernatant was re centrifuged at 112,500 × g for 1 h at 4 °C to collect microsomes, and these were resuspended in one-tenth volume of cold lysate buffer. The concentration of expressed P450s was measured by carbon monoxide (CO) difference analysis (34), and aliquots of the expressed P450s were stored at −80 °C.

Analysis of Wild-type and Mutant Metabolic Activities—Reaction mixtures of 500 μl were set up with 4 μl of a 5 mM furanocoumarin stock in methanol, 50 μl of NADPH (5 mg/ml in 0.1 M PB, pH 7.8) for different treatments or 50 μl of PB for the control without NADPH, 30 pmol of CYP6AB3v2 expressed at an m.o.i. ratio of 1:0.1 (CYP6AB3v2/house fly P450 reductase), and a suitable volume of 0.1 M PB (pH 7.8). After incubating for 30 min in a shaking water bath at 30 °C, the reactions were terminated by incubating for 5 min at 70 °C to avoid breakdown of imperatorin derivatives. After this inactivation, 5 μl of 1 mM psoralen was added as an internal control, and unmetabolized furanocoumarins were extracted with one 500-μl aliquot of ethyl acetate and analyzed by normal phase HPLC with a solvent system containing 55% cyclohexane, 42% isopropyl ether, 3% amyl alcohol.

CYP6AB3v2 and CYP6AB3v1 V92A mutant activities at varying substrate concentrations were determined using final concentrations of 10, 20, 30, 40, 60, and 80 μM imperatorin in reactions that were terminated at 15 min. Assays were replicated four times, and the data were analyzed using Prism 3.03 software.

Variations in m.o.i. Ratios—Five m.o.i. ratios of the recombinant CYP6AB3 virus to recombinant house fly P450 reductase virus (1:0.01, 1:0.05, 1:0.1, 1:0.5, 1:1) were used to infect Sf9 cells in six plates of 8 × 10⁶ cells/plate to determine the effect of varying ratios on CYP6AB3v2 protein levels. Four ratios (1:0, 1:0.1, 1:0.25, 1:0.5) were used for experiments on CYP6AB3v2 and CYP6AB3v1 V92A mutant activities with each treatment replicated three times. After preparing microsomal samples, their P450 concentrations and activities were determined as above using imperatorin as the substrate with the rates of metabolism expressed as pmol of imperatorin metabolized/ min/pmoll of P450, and means were compared by analysis of variance.

Comparison of NADPH Consumption Rates—To compare the NADPH consumption rates of CYP6AB3v2, CYP6AB3v1, and its V92A variant co-expressed with house fly P450 reductase at m.o.i. ratios of 1:0.25, 5:0.25, and 1:0.25, respectively, 500-μl reaction mixtures containing 1 μl of 2.5 mM imperatorin in methanol, 20 pmol of a P450, and a suitable volume of 0.1 M PB (pH 7.8) were mixed and loaded into a 500-μl microcuvette. For all, the sample baseline was monitored at 340 nm on a Cary 100 spectrophotometer at room temperature until it stabilized and zeroed. After monitoring for 1 min at 340 nm, the reaction was initiated by the addition of 1 μl of 2.5 mg/ml NADPH (67 μM final concentration), and NADPH consumption was monitored for another 9 min.

Purification and Characterization of Imperatorin Metabolites—Imperatorin metabolism reactions by wild-type CYP6AB3v1 and CYP6AB3v2 proteins were carried out in 500-μl reactions as for substrate determinations except that CYP6AB3v1 was co-expressed at an m.o.i. ratio of 1:0.5 and CYP6AB3v2 was expressed at an m.o.i. ratio of 1:0.1. After 50 μl of the ethyl acetate-extracted products and remaining substrate was dried in a SpeedVac centrifuge, the residue was dissolved with 500 μl of methanol, and 50 μl of this solution was loaded on reverse phase 4.7 × 150-mm C-18 Novapak column (Waters Corp., Franklin, MA). The sample was gradient-eluted at a flow rate of 1 ml/min with solvent A composed of 5% MeOH, 1.9% glacial acetic acid, 93.1% water by volume and solvent B being 100% acetonitrile. The CYP6AB3v2 imperatorin metabolite peak was collected and MS-MS analyzed at the Mass Spectrometry Service Facility at the University of Illinois using solvent A alone for 5 min followed by a linear gradient changing from 0 to 100% solvent B over 50 min and then 100% solvent B for 5 min. The absorbance at 254 nm was monitored using a Waters 996 photodiode array detector.

Homology Modeling—The molecular model of CYP6AB3v3 was constructed using MOE programs (Chemical Computing Group Inc., Montreal, Canada) by introducing four amino acid replacements (V92A, E164Q, K214R, I303L listed with the residue in CYP6AB3v1 first) into the CYP6AB3v1 model described in Mao et al. (21) and reminizming the model using the CHARMM22 force field.

The homology model of the FMN-binding domain of house fly P450 reductase was generated in MOE by aligning the house
fly P450 reductase sequence with the FMN-binding domain of Bacillus megaterium P450 reductase (Protein Data Bank code 1BVY) (35) and rat P450 reductase (Protein Data Bank code 1AMO) (36). Ten models were generated using 1AMO as the primary template, the best model (as ranked by MOE) was selected, and the FMN coordinates from the rat P450 reductase were copied into it. The model was energy-minimized using the CHARMM22 force field, and the complex between CYP6AB3v2 and the FMN domain of house fly P450 reductase was constructed by overlaying the CYP6AB3v2 and the P450 reductase models with the crystal structure of the CYP102-P450 reductase FMN domain (1BVY) (35).

RESULTS

cDNA Cloning and Characterization—Of the 2000 clones screened from the furanocoumarin-induced parsnip webworm midgut cDNA library (31) with the existing CYP6AB3v1 cDNA, 14 positive clones were obtained including four CYP6AB3v1 (C4, 8, 11, 26), five CYP6AB3v2 (C3, 6, 7, 10, 23), and four CYP6AB7 clones (C1, 2, 12, 18). Alignments of these nucleotide sequences (Supplemental Fig. 1) indicate that individual cDNAs have the same lengths in their coding region and significantly different lengths to their polyadenylation sites. Alignments of the CYP6AB3v1, CYP6AB3v2, and CYP6AB7 protein sequences (Fig. 1) indicate that CYP6AB3v2 shares 99 and 87.9% amino acid identity with CYP6AB3v1 and CYP6AB7, respectively. Differences between the CYP6AB3v1 and CYP6AB3v2 variants are limited to five amino acids (H2Y, V92A, E164Q, K214R, I303L listed with the residue in CYP6AB3v1 first) with only one of these (K214R) occurring in SRS2.

Effect of Co-expressed House Fly P450 Reductase on Expression and Bioactivity of CYP6AB3v2—For comparison of wild-type CYP6AB3v2 activities with and without co-expressed insect P450 reductase, CYP6AB3v2 recombinant virus was expressed in Sf9 cells with varying m.o.i. between 1/0.01 and 1/1 (CYP6AB3v2/P450 reductase) (Fig. 2). As in the expression of the first CYP6AB3v1 variant (21) and other P450s in Sf9 cells, the expression level of CYP6AB3v2 as monitored by carbon monoxide (CO) difference analysis decreased as the m.o.i. of recombinant house fly P450 reductase increased; at an m.o.i. ratio of 1:1, the P450 concentration of CYP6AB3v2 was just 0.1/49262 M (Fig. 2a). However, in contrast to the many other P450s that we have analyzed, this variant metabolizes imperatorin at a rate of 8.0 pmol/min/pmol of P450 when supplemented with even the lowest levels of P450 reductase (m.o.i. ratio 1:0.1), and this activity remains constant as m.o.i. ratios increase to 1:0.5 (Fig. 2b). In the absence of co-expressed P450 reductase, CYP6AB3v2 metabolizes imperatorin at a rate of 2.39 pmol/min/pmol of P450.

Substrate Reactivities, Kinetics, and NADPH Consumption Rates of the Wild-type CYP6AB3v2 and CYP6AB3v1 Proteins—Analysis of other furanocoumarins (xanthotoxin, bergapten, isopimpinellin, angelicin, sphondin) indicated that none were metabolized by CYP6AB3v2 even when P450 reductase was co-expressed at an m.o.i. ratio of 1:1. Additional experiments performed to define the kinetics of CYP6AB3v2 enzyme indicated that it metabolizes imperatorin with Vmax and Km values of 14.18 pmol/min/pmol of P450 and 63.22/49262 M, respectively, at an m.o.i. ratio of 1:0.25 (Fig. 3). In comparison, wild-type CYP6AB3v1 metabolizes imperatorin with Vmax and Km values of 2.41 pmol/min/pmol of P450 and 94.28/49262 M, respectively, at non-limiting P450 reductase levels (21).
As the above experiments indicate, CYP6AB3v2 reaches its highest activity when co-expressed with very low levels of P450 reductase (m.o.i. ratio of 1:0.1), suggesting that CYP6AB3v2 couples more efficiently with P450 reductase than CYP6AB3v1 with P450 reductase. Imperatorin-dependent NADPH consumption rates were monitored in microsomes derived from Sf9 cells co-expressing CYP6AB3v1, CYP6AB3v2, or CYP6AB3v1 V92A mutant and P450 reductase at m.o.i. ratios of 5:0.25, 1:0.25, and 1:0.25, respectively. In these, the rate of NADPH consumption was significantly faster for CYP6AB3v2 and CYP6AB3v1 V92A reactions containing equal P450 concentrations (as monitored by CO difference analyses) than for CYP6AB3v1 (Fig. 4).

**Imperatorin Metabolites Generated by CYP6AB3v1 and CYP6AB3v2**—Considering that the K214R replacement in CYP6AB3v2 occurs in one of the six SRS regions that potentially affect substrate orientation and reactivities, the metabolites of imperatorin generated by CYP6AB3v2 were determined and compared with those generated by CYP6AB3v1. HPLC analyses of these metabolites (Fig. 5) provided the initial evidence that those produced by CYP6AB3v1 and CYP6AB3v2 were identical. Comparisons of the spectra for these metabolites with that of imperatorin indicated that the absorption peak at 222.6 nm was decreased and that the absorption peaks at 249.7 and 301.6 nm remained constant as typical for furanocoumarin spectra (Fig. 5). Subsequent tandem MS-MS analysis of the imperatorin metabolite at m/z 286.9 indicated that it fragmented to three peaks at m/z 269.2, 245.1, and 203.2 (Fig. 6a). Among these, the peak at m/z 203 contains the furanocoumarin core structure, the peak at m/z 269.2 corresponds to the loss of one oxygen atom from the core structure, and the peak at m/z 245.1 corresponds to the loss of a CH2(CH3)2 group (Fig. 6b). These data indicate that the metabolite arises as a result of cleavage of the double bond on the isoprenyl side chain of imperatorin and the formation of an epoxide intermediate as shown previously for CYP6AB3v1 (21). We have concluded that the metabolites generated by CYP6AB3v1 and CYP6AB3v2 are the same.

**Homology Modeling**—To compare these two P450 variants in the most effective manner, the three-dimensional model of CYP6AB3v2 was generated by computationally mutating the CYP6AB3v1 model (21) and minimizing the CYP6AB3v2 replacement model. Overlays of these models indicate that the V92A residue varying between CYP6AB3v1 and CYP6AB3v2 occurs in part of the B-helix on the proximal surface of these proteins. Relevant to the differences observed in NADPH consumption rates, this V92A variation occurs in a region predicted to interact with the FMN domain of the P450 reductase and lies close to the heme (Fig. 7). The model of the
CYP6AB3v2-FMN domain complex that we have built indicates that several hydrogen bonds are possible between Ala92 in CYP6AB3v2 and P450 reductase in this region (not shown). In this position, substitution of Ala92 in CYP6AB3v2 for the larger Val92 in CYP6AB3v1 is likely to disrupt this hydrogen-bonding network and other interactions important for electron transfer, thereby reducing substrate turnover and NADPH consumption rates in CYP6B1v1. Of the three other variations, the E164Q variation is located at the C terminus of the D-helix on the outer surface of the protein with no possible contacts with P450 reductase, the K214R variation is located at the C-terminal of the F-helix on the outer surface with no possible contacts with P450 reductase, and I303L is located at the C terminus of the I-helix near predicted exit pathways 1 and 2C (designated as in Wade et al. (37)) with no possible contacts with P450 reductase. Of these last two residues, there is some possibility that residue 214 contacts the substrate and residue 303 affects product exit.

**Substrate Reactivity of the CYP6AB3v1 V92A Mutant**—To conclusively determine whether the V92A replacement in the CYP6AB3v1 variant is responsible for the higher activity of the CYP6AB3v2 variant, this single site change was introduced into the wild-type CYP6AB3v1 protein, and the rate of imperatorin metabolism by the CYP6AB3v1 V92A mutant was monitored at varying house fly P450 reductase virus levels. Like the CYP6AB3v2 protein expressed without P450 reductase supplementation, the CYP6AB3v1 V92A mutant protein also metabolizes imperatorin but at a lower velocity (0.5 pmol/min/pmol of P450) than for the CYP6AB3v2 protein (Fig. 2b). Supplementation with increasing amounts of P450 reductase virus enhances activity of the CYP6AB3v1 V92A mutant protein but not as significantly at the lowest P450 reductase viral titer (1:0.1) as seen for the CYP6AB3v2 protein. Its activity remains constant (6.0–6.4 pmol/min/pmol of P450) at m.o.i. ratios higher than 1:0.25 and slightly lower than the activity of CYP6AB3v2 (7.8–8.2 pmol/min/pmol of P450). Further analyses shown in Fig. 3 have determined that the CYP6AB3v1 V92A mutant protein metabolizes imperatorin with $V_{max}$ and $K_m$ values of 11.38 pmol/min/pmol of P450 and 32.10 μM, respectively. NADPH consumption assays have indicated that the CYP6AB3v1 V92A mutant protein consumes NADPH at a rate substantially more similar to the CYP6AB3v2 protein than to wild-type CYP6AB3v1 protein (Fig. 4).

**DISCUSSION**

Advances in x-ray crystallography, homology modeling, and site-directed mutagenesis have yielded crucial insights into the structural basis of P450 catalytic efficiencies. With all previous studies identifying residues in insect P450 catalytic sites affect-

![FIGURE 5. Reaction products derived from wild-type CYP6AB3v1 and CYP6AB3v2 proteins. The products derived from imperatorin in reactions containing wild-type CYP6AB3v1 and CYP6AB3v2 co-expressed with house fly P450 reductase at m.o.i. ratios of 1:0.5 and 1:0.1, respectively, were fractionated on a reverse phase C18 HPLC column and compared with control reactions lacking NADPH. The spectra of imperatorin and its metabolites are shown in the insets above and below one another.](http://www.jbc.org/)

CYP6AB3v2-FMN domain complex that we have built indicates that several hydrogen bonds are possible between Ala92 in CYP6AB3v2 and P450 reductase in this region (not shown). In this position, substitution of Ala92 in CYP6AB3v2 for the larger Val92 in CYP6AB3v1 is likely to disrupt this hydrogen-bonding network and other interactions important for electron transfer, thereby reducing substrate turnover and NADPH consumption rates in CYP6B1v1. Of the three other variations, the E164Q variation is located at the C terminus of the D-helix on the outer surface of the protein with no possible contacts with P450 reductase, the K214R variation is located at the C-terminal of the F-helix on the outer surface with no possible contacts with P450 reductase, and I303L is located at the C terminus of the I-helix near predicted exit pathways 1 and 2C (designated as in Wade et al. (37)) with no possible contacts with P450 reductase. Of these last two residues, there is some possibility that residue 214 contacts the substrate and residue 303 affects product exit.

**Substrate Reactivity of the CYP6AB3v1 V92A Mutant**—To conclusively determine whether the V92A replacement in the CYP6AB3v1 variant is responsible for the higher activity of the CYP6AB3v2 variant, this single site change was introduced into the wild-type CYP6AB3v1 protein, and the rate of imperatorin metabolism by the CYP6AB3v1 V92A mutant was monitored at varying house fly P450 reductase virus levels. Like the CYP6AB3v2 protein expressed without P450 reductase supplementation, the CYP6AB3v1 V92A mutant protein also metabolizes imperatorin but at a lower velocity (0.5 pmol/min/pmol of P450) than for the CYP6AB3v2 protein (Fig. 2b). Supplementation with increasing amounts of P450 reductase virus enhances activity of the CYP6AB3v1 V92A mutant protein but not as significantly at the lowest P450 reductase viral titer (1:0.1) as seen for the CYP6AB3v2 protein. Its activity remains constant (6.0–6.4 pmol/min/pmol of P450) at m.o.i. ratios higher than 1:0.25 and slightly lower than the activity of CYP6AB3v2 (7.8–8.2 pmol/min/pmol of P450). Further analyses shown in Fig. 3 have determined that the CYP6AB3v1 V92A mutant protein metabolizes imperatorin with $V_{max}$ and $K_m$ values of 11.38 pmol/min/pmol of P450 and 32.10 μM, respectively. NADPH consumption assays have indicated that the CYP6AB3v1 V92A mutant protein consumes NADPH at a rate substantially more similar to the CYP6AB3v2 protein than to wild-type CYP6AB3v1 protein (Fig. 4).

**DISCUSSION**

Advances in x-ray crystallography, homology modeling, and site-directed mutagenesis have yielded crucial insights into the structural basis of P450 catalytic efficiencies. With all previous studies identifying residues in insect P450 catalytic sites affect-
ing substrate recognition, turnover, and product release (25, 31, 38, 39), this study is the first to identify a residue affecting interactions with P450 reductase, the one electron transfer partner essential to endoplasmic reticulum-localized P450s. The natural evolution of residue 92 in CYP6AB3v1 from valine to the smaller alanine side chain enhances the NADPH consumption rate of the CYP6AB3v2 protein 2.4-fold when the initial slopes on these curves are compared.

As early as 1988, Nadler and Strobel (40) found that chemical modification of P450 reductase affected electrostatic interactions between vertebrate P450s and P450 reductase that were necessary for docking and electron transfer step(s) between these proteins. Structural comparisons of the vertebrate P450s with crystal structures of bacterial CYP101 and CYP102 available in the earliest of these investigations suggested that residues in the B-, C-, G-, and L-helices might interact with residues in P450 reductase. Subsequent analyses of site-directed replacements on several vertebrate endoplasmic reticulum-localized P450s effectively demonstrated that residues in the B-, J- and L-helices of human CYP17A1 (41, 42), in the C-helix, F-G loop and the heme-binding region of rabbit CYP2B4 (43, 44) and in the B- and G-helices, G-H loop, and β2 (2) and β1 (3) strands of CYP1A1 and CYP1A2 (45, 46) were involved in the formation of complexes with P450 reductase and/or cytochrome b5 (47). The V92A replacement in our newly defined CYP6AB3v2 variant exists in a region of the B-helix that is predicted to be positioned on the external surface of the CYP6AB3 protein in close proximity to residues in the β2-α3 loop and the β1-α2 loop and N terminus of α4.

The electrostatic potential of the loop between strand 5 and helix F on the P450 reductase surface is largely negative (36) with clusters of negatively charged residues proposed to be involved in the recognition of specific positively charged residues on their associated P450 (48). Mutagenesis of specific residues within two negatively charged clusters in the rat P450 reductase (cluster I: Asp207-Asp208-Asp209; cluster II: Glu213-Glu214-Asp215) has now demonstrated that negatively charged residues within the FMN domain are important for the interaction of rat P450 reductase with P450s (49). Positively charged residues on the proximal P450 surface play an important role in this interaction cluster near the center of the proximal face and heme-binding domain and consist of a four-helix bundle (the D-, E-, I-, and L-helices), the J- and K-helices and the “meander” region (50–52).

Site-directed mutagenesis and chemical modifications have identified basic residues important for the P450 reductase interactions on the proximal surface of the P450. These residues are located in three distinct secondary structural elements and include the B-helix, the C-helix, and the loop between the K”-helix and heme-binding region. Specifically, Arg122, Arg126, Arg133, and Lys139 in the C-helix and Lys433, Arg422, and Arg443 in the loop between the K”-helix and heme-binding region located on the proximal surface of CYP2B4 participate in the interaction with the P450 reductase (43). Lys99 and Lys105 in the
B-helix, Lys^{440}, Lys^{457}, Lys^{463}, and Arg^{455} in the loop between the K'-helix and heme-binding region, as well as an Arg cluster (Arg^{135}, Arg^{136}, Arg^{137}) in the C-helix on the proximal surface of CYP1A2 might also be involved in the interaction with P450 reductase (45). Chemical modification of Lys^{884} (N-terminal of the β1–3 strand), Arg^{422}, Lys^{433}, and Arg^{473} (loop between the K'-helix and heme-binding region) in CYP2B1 (corresponding to the same residues in CYP2B4) caused 95% loss of benzphetamine demethylation activity (46, 53). Basic arginine and lysine residues on the surface of CYP6AB3 including Lys^{126}, Arg^{129}, Arg^{131}, Lys^{140}, Lys^{142}, Lys^{441}, Arg^{442}, and Arg^{458} correspond to some of these clusters in CYP2B4 and CYP1A2. Of these, Lys^{126}, Arg^{129}, Arg^{131}, Lys^{140}, and Lys^{142} locate to the C-helix, and Lys^{441} and Arg^{458} locate to the loop between the K'-helix and the heme-binding region. In the three-dimensional P450 structure, the B-helix and the K'-helix to the heme-binding region loop come close to each other on the proximal surface, bringing Val^{32} (B-helix) close to the basic residue cluster on loop between the K'-helix and heme-binding region and likely account for the dramatic effects that this particular residue has in defining the activity of CYP6AB3. The 2.4-fold increase in NADPH consumption rate by the CYP6AB3v2 protein is in the same range as the 4.3-fold increase in $V_{max}$ for this enzyme. Other factors contributing to the higher activity of this variant may be slight differences in substrate binding affinity but, because inhibitor does not induce strong spectral shifts upon binding to these proteins, substrate binding affinities cannot be reliably measured. Allelic variation at the CYP6A3B locus may be instrumental in allowing D. pastinacella to utilize host plants that differ quantitatively and qualitatively in furanocoumarin composition; although all of its host plants in North America contain high concentrations of furanocoumarins, the precise content and composition vary between species and among populations within species. The parsnip webworm displays a remarkable capacity for chemical phenotype matching, according to which the distribution and abundance of furanocoumarins in host plants is mirrored by the detoxification profile of the local webworm population (12, 54, 55). Given the extreme substrate specificity of CYP6A3B relative to furanocoumarins, there are likely other P450s in this species that contribute to furanocoumarin metabolism; allelic variation at these other loci may allow for the rapid local adaptation that takes place in this herbivore.

REFERENCES

**Insect P450 Alleles**
