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Quercetin-metabolizing CYP6AS enzymes of the pollinator *Apis mellifera* (Hymenoptera: Apidae)

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ABSTRACT

Although the honey bee (*Apis mellifera*) genome contains far fewer cytochrome P450 genes associated with xenobiotic metabolism than other insect genomes sequenced to date, the CYP6AS subfamily, apparently unique to hymenopterans, has undergone an expansion relative to the genome of the jewel wasp (*Nasonia vitripennis*). The relative dominance of this family in the honey bee genome is suggestive of a role in processing phytochemicals encountered by honey bees in their relatively unusual diet of honey (comprising concentrated processed nectar of many plant species) and bee bread (a mixture of honey and pollen from many plant species). In this study, quercetin was initially suggested as a shared substrate for CYP6AS1, CYP6AS3, and CYP6AS4, by its presence in honey, extracts of which induce transcription of these three genes, and by *in silico* substrate predictions based on a molecular model of CYP6AS3. Biochemical assays with heterologously expressed CYP6AS1, CYP6AS3, CYP6AS4 and CYP6AS10 enzymes subsequently confirmed their activity toward this substrate. CYP6AS1, CYP6AS3, CYP6AS4 and CYP6AS10 metabolize quercetin at rates of 0.5 ± 0.1, 0.5 ± 0.1, 0.2 ± 0.1, and 0.2 ± 0.1 pmol quercetin/ pmol P450/min, respectively. Substrate dockings and sequence alignments revealed that the positively charged amino acids His107 and Lys217 and the carbonyl group of the backbone between Leu302 and Ala303 are essential for quercetin orientation in the CYP6AS3 catalytic site and its efficient metabolism. Multiple replacements in the catalytic site of CYP6AS4 and CYP6AS10 and repositioning of the quercetin molecule likely account for the lower metabolic activities of CYP6AS4 and CYP6AS10 compared to CYP6AS1 and CYP6AS3.

1. Introduction

Cytochrome P450 monooxygenases (P450s) constitute a large superfamily of heme-thiolate enzymes in a wide range of organisms that catalyze the NADPH-associated reductive cleavage of oxygen to produce a functionalized product and water (http://drnelson.utmem.edu/CytochromeP450.html). P450s catalyze a broad diversity of reactions (Guengerich, 2001) that in insects contribute to the biosynthesis of endogenous pheromones and hormones as well as the detoxification of natural and synthetic xenobiotics (Feyereisen, 2005, 2006; Li et al., 2007). Insect genomes typically contain large numbers of P450 genes, consistent with the hypothesis that this gene superfamily has expanded by multiple gene duplication events associated with "animal–plant warfare" as represented by reciprocal selective responses between herbivores and their host plants (Gonzalez and Nebert, 1990). The available collection of annotated P450s across all available genomes has been subdivided into four major clades. Genes in the CYP3 clade, such as the insect-specific CYP6 family, have been extensively implicated in xenobiotic metabolism (Feyereisen, 2006; Li et al., 2007).

Not all interactions between plants and herbivores, however, constitute "warfare"—since the late Cretaceous many angiosperm plant species have produced sugar-rich nectars for the express purpose of attracting and rewarding mutualistic partner species that contribute to pollination (Brandenburg et al., 2009). For herbivores, the toxicological consequences of consuming nectar differ dramatically from the consequences of consuming chemically well-defended foliar tissues. The first genome sequence of a pollinator species became available in 2006 with the completion of the genome of the western honey bee *Apis mellifera* (Honey Bee Consortium, 2006). Annotation of the complete P450 inventory of this genome (http://drnelson.utmem.edu/CytochromeP450.html) revealed 46 genes, a reduction on the order of 50% or greater in this gene superfamily relative to other insect genomes (Claudianos et al., 2006). Several unique aspects of *A. mellifera* biology are likely to have contributed to this reduction. One aspect is the relatively innocuous nature of this pollinator's diet, which differs considerably from that of typical herbivores. In contrast with foliage-feeding herbivores that consume

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chemically defended plant tissues, the honey bee consumes honey processed from nectar, a plant product manufactured expressly for consumption by mutualistic herbivores. In addition, honey bees consume "bee bread", a mixture of pollen and honey that is aged in the hive, a process that reduces its content of phenolics and other potentially toxic phytochemicals (Liu et al., 2005).

Although the honey bee can be considered a dietary specialist because its diet is restricted to pollen and nectar products, it forages on a tremendous diversity of plant species (Westerkamp, 1991). Many nectars contain trace quantities of biologically active phytochemicals (Adler, 2000) that are ingested in concentrated form in honey. Rather than contributing to a reduction in the number of P450 genes, this exposure to a wide range of phytochemicals over evolutionary time might be expected to result in diversification of xenobiotic-metabolizing P450s (Li et al., 2003). Such diversification has occurred in P450s of the CYP6 family in lepidopteran genomes in the context of detoxification of phytochemicals (Li et al., 2003); the relative dominance of this family in the honey bee genome is suggestive of a role in processing phytochemicals encountered by bees in the diet of both larval and adult stages.

The relatively small inventory of P450 genes in *A. mellifera*, compared with other insects, provides an opportunity to assign functions to particular P450s. Although Claudianos et al. (2006) speculated that an expansion in the CYP6 family generally may be associated with the chemical communication systems associated with eusociality, the expansion in the CYP6AS subfamily, relative to the genome of the parasitic jewel wasp (*Nasonia vitripennis*) (Johnson, 2008), is consistent with an involvement in metabolism of dietary components unique to *A. mellifera*. A considerable amount of previous work documents the involvement of CYP6 enzymes in metabolism of dietary constituents of herbivores (reviewed in Li et al., 2007). Because honey is a food unique to bees and a handful of other social hymenopterans, constituents of honey are candidates for CYP6AS substrates. The fact that three of the 15 CYP6AS genes are upregulated in response to ingested honey (Johnson, 2008) strongly suggests that these particular P450s are involved in the metabolism of phytochemical constituents in honey.

Among the constituents of honey likely to be substrates for *A. mellifera* xenobiotic-metabolizing P450s are flavonoids, which occur as glycosides in nectar and are hydrolyzed to aglycones during the production of many different honeys (Tomás-Barberán et al., 2001). Flavonoids and their glycosylated derivatives are also frequently found in propolis and pollen collected by honey bees (Wiermann, 1968; Gheldof et al., 2002; Azeredo et al., 2003; Viuda-Martos et al., 2008).

Our objective in this study was to characterize CYP6AS subfamily members in *A. mellifera* and determine their enzymatic activity toward dietary constituents found in honey and pollen. With the availability of a molecular model for CYP6AS3, high-throughput screening indicated that flavonoids might be CYP6AS3 substrates; their status as substrates was confirmed by *in vitro* experiments with CYP6AS enzymes heterologously co-expressed with house fly P450 reductase, an essential electron transfer partner for microsomal P450s. Among the four flavonoids tested (quercetin, kaempferol, eriodictyol and taxifolin), quercetin was identified as a shared substrate of CYP6AS1, CYP6AS3, CYP6AS4 and CYP6AS10.

2. Materials and methods

2.1. Materials

Large fragment DNA polymerase I (9 U/μL), Taq DNA polymerase (5 U/μL), cloned Pfu DNA polymerase (2.5 U/μL), BamH I/Kpn I (10 U/μL), Sf9 insect cells, SF-900 serum-free medium, fetal bovine serum (FBS) and TRIzol Reagent were purchased from Invitrogen (Carlsbad, CA, USA). Penicillin/streptomycin came from Bio-Whittaker (Walkersville, MD, USA). D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase (715 U/mg protein) and candidate flavonoids were obtained from Sigma-Aldrich (St. Louis, MO, USA). Four flavonoids representative of four different structural classes were chosen for evaluation: quercetin (pentahydroxyflavone), kaempferol (tetrahydroxyflavone), eriodictyol (flavanone), and taxifolin (dihydroflavonol). AMV reverse transcriptase (AMV RT) was purchased from Promega (Madison, WI, USA).

2.2. Molecular modeling and in silico substrate prediction

Development of the CYP6AS3 model and quercetin docking were carried out with the methods used for CYP6B33 from *Papilio multicaudatus* (Mao et al., 2007b) using human CYP3A4 (PDB 1TQN; Yano et al., 2004) as the main template and human CYP2C5 (PDB 1N6B; Wester et al., 2003) for the variable FG region as detailed in Baudry et al. (2003, 2006). *In silico* high-throughput docking of prospective ligands was performed using LigandFit implemented in the CERIUS2 program (Version 4.10. Accelrys, San Diego, USA) as previously described in Ke et al. (2005). The energy-minimized model was exported to the CERIUS2 program (Accelrys) as a PDB file and hydrogen atoms were added. Charges were calculated for the heme and protein separately using a charge equilibration algorithm (Rappe and Goddard, 1991) and a Gasteiger algorithm (Gasteiger and Marsili, 1980) under the Dreiding force field (Mayo et al., 1990). Potential binding sites were identified using the SITE SEARCH function of the CERIUS2 program.

The database constructed at the University of Illinois for use in virtual docking calculations contains 14,558 molecules obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) and a list of agrochemical compounds (Compendium of Pesticide Common Names, http://www.alanwood.net/pesticides) augmented with known substrates for plant, insect and vertebrate P450s. Molecules were flexibly docked employing energy minimization and charge calculations using the CFF force field after the calculation of a representative set of conformers for each molecule. Using CERIUS2, which employs a Monte Carlo-based approach for conformational analysis, the number of maximum trials for each run was set to 5000, the number of saved conformations was set to 6 and the number of rigid body minimizations was set to 2 SD (standard deviation) iterations and no BFGS (Broy-den, Fletcher, Goldfarb and Shanno unconstrained optimization) iterations. Other parameters remained at their default values.

Scores for the saved conformation molecules were computed using DOCK, PMF, JAIN, LigScore (variations 1 and 2), and PLP (variations 1 and 2) scoring functions (Gehlhaar et al., 1995, 1999; Jain, 1996; Muegge and Martin, 1999; Krammer et al., 2005). Following the calculation of docking scores, a consensus scoring approach was used to identify compounds that scored among the best 40%, 35% or 30% of the set for each of the seven scoring functions used.

2.3. Sequence alignment and phylogenetic tree construction

For phylogenetic analysis, a protein sequence alignment of the CYP6AS proteins was performed using the CLUSTALW version 1.6 program (Thompson et al., 1994). The SRS (substrate recognition sites) in these proteins were determined by comparison with the aligned eukaryotic P450 templates and inspection of the active site in the CYP6AS3 model. Phylogenetic analyses of the CYP6AS proteins from *A. mellifera* and *N. vitripennis* were conducted using the MEGA version 4.1 program (Tamura et al., 2007) with the alignment generated with the built-in CLUSTALW implementation and a neighbor-joining tree created with 1000 bootstrap replications.

2.4. Cloning of CYP6AS genes

After first strand cDNA synthesis using AMV RT, oligo$(dT)_{18}$ and total mRNA extracted from midguts of honey bees, the cDNAs for

Table 1

Genes	Primers
CYP6AS1	GCGGATCCTATGGATTACTTCCAAATTC BamH I GCGGTACCTTAAATCTTAGTTATTTTTAAGTG Kpn I
CYP6AS3	GCGGATCCTATGGATTATTTTCAACTTCT BamH I GCGGTACCTTAAACTTTCGTTATTCTCAAC Kpn I
CYP6AS4	GCGGATCCTATGCTCCATCATTTTCATAT BamH I GCGGTACCTTAATCAGTTATTTTTTCTATCTTC Kpn I
CYP6AS10	GCGGATCCTATGGCTGCCTTCGAAATTC BamH I GGCTGCAGTTAGTTTTCTATTTTCGTAATTTTTAAAG Pst I
CYP6AS15	GCGGATCCTATGAATATTAGTCTGGAAATTC BamH I GCGGTACCTTAATTCTCCACGTCAGTTATC Kpn I

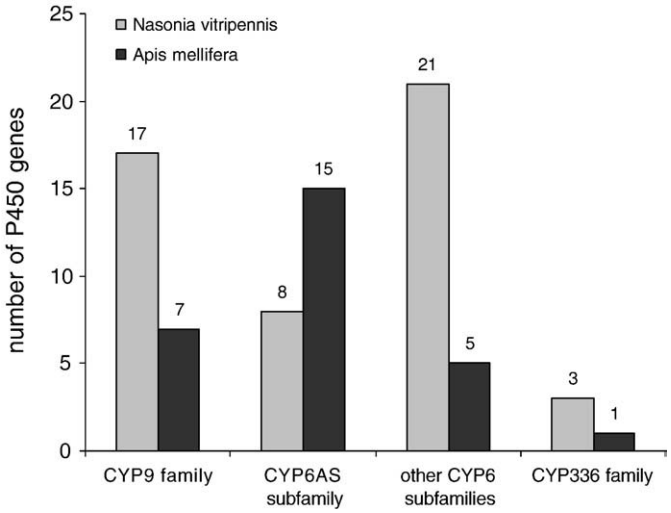


Fig. 1. Composition of clade 3 cytochrome P450s from *Apis mellifera* with those from *Nasonia vitripennis*.

CYP6AS1, *CYP6AS3*, *CYP6AS4*, *CYP6AS10* and *CYP6AS15* were amplified with Pfu and Taq DNA polymerases at a ratio of 10:1 using gene-specific primers for each with varying enzyme sites on their 5′-ends (Table 1) and then inserted into the pFastBac baculovirus expression vector. Positive clones were determined by sequencing the full-length coding regions with vector and internal primers.

2.5. Identification of quercetin as a shared substrate of CYP6AS enzymes

CYP6AS enzymes used in this study were co-expressed in Sf9 cells with house fly P450 reductase at a multiplicity of infection (MOI) ratio of 1:0.5 (P450:P450 reductase), as described for CYP6B33 expression (Mao et al., 2007b). Reaction mixtures for quercetin, kaempferol, eriodictyol and taxifolin were set up with 30 pmol P450 (as defined by carbon monoxide (CO) difference analysis; Omura and Sato, 1964), 5 μL of 5 mM stock solution for each tested flavonoid, 0.5 mg of D-glucose-6-phosphate, 0.5 μL of glucose-6-phosphate dehydrogenase, 50 μL of NADPH (1 mg/mL in 0.1 M phosphate buffer (pH 7.8)) or 50 μL of phosphate buffer (for the no NADPH control) and were adjusted to a final volume of 500 μL with 0.1 M phosphate buffer (pH 7.8). After incubation at 30 °C for 90 min in a shaking water bath, each 500 μL reaction was added to an equal volume of acetone and centrifuged at 10,000*g* for 10 min at room temperature. Reaction products were analyzed with a reverse phase CAPCELL PAK C_{18} column (AG120, 5 μm, 4.6×250 mm; Shiseido Ltd., Tokyo, Japan). A mobile phase of 55% water containing 0.05% formic acid and 45% methanol was used for quercetin, eriodictyol and taxifolin, and a mobile phase of 40% water containing 0.05% formic acid and 60% methanol was used for kaempferol. Absorbances of quercetin and kaempferol were monitored at 373 nm and the absorbances of eriodictyol and taxifolin were monitored at 289 nm using a Waters 996 photodiode array detector. Analyses for quercetin metabolism were repeated three times.

3. Results

3.1. In silico predictions of CYP6AS substrates

Although insect P450s in clade 3 are involved in the metabolism of xenobiotics, their diversity within any one genome makes the functional characterization of individual enzymes challenging. The overall reduction in P450 diversity within clade 3 in the honey bee genome (Fig. 1), as well as the highly specialized diet of this highly eusocial species, increases the likelihood of identifying the natural substrates of xenobiotic-metabolizing P450s. The expansion of the CYP6AS subfamily in the honey bee genome, relative to its size in the *N. vitripennis* genome, and the inducibility of particular loci by extracts of honey, propolis and pollen (Johnson, 2008), led us to hypothesize that these enzymes are involved in metabolism of dietary constituents.

A molecular model of CYP6AS3 constructed using the main template of human CYP3A4 and the FG region of human CYP2C5 allowed us to screen the three-dimensional chemical database of 14,558 compounds using *in silico* high-throughput docking procedures. Ranking of candidate compounds using seven scoring functions within the CERIUS2 program showed that, among the top-ranked 26 compounds having consensus scores in the top 30% to 40% for all seven functions, six were natural compounds sharing the flavonoid core structure: glepidotin A, glepidotin B (2-(2-hydroxy-3-methylbut-3-enyl)-5-(2-phenylethyl)benzene-1,3-diol), tephrowatsin A ((2 S,4R)-5,7-dimethoxy-8-(3-methylbut-2-enyl)-2-phenylchroman-4-ol), chrysosplenetin (quercetagetin 3,6,7,3′-tetramethyl ether), chrysosplenol C (5,6-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3,7-dimethoxychromen-4-one), and fisetin 8-C-glucoside (2-(3,4-dihydroxyphenyl)-3,7-dihydroxychromen-4-one 8-C-glycoside). Of these, fisetin is a structural analogue of quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-chromen-4-one), a widely distributed constituent of honey and pollen.

3.2. Identification of quercetin as a shared substrate of the four CYP6AS enzymes

To confirm the prediction of the CYP6AS3 model, we conducted *in vitro* assays with the individual P450s heterologously co-expressed with house fly P450 reductase. Phylogenetic analysis revealed that the CYP6AS subfamily contains multiple clades: *CYP6AS1*, *CYP6AS3*, *CYP6AS4*, *CYP6AS5*, *CYP6AS10* and *CYP6AS15*, representatives of different lineages within the CYP6AS subfamily (Fig. 2), were cloned from the total RNA extracted from worker midguts by standard RT-PCR methodologies. Sequence analysis of the *CYP6AS5* clone indicated that it is a pseudogene containing a reading-frame shift in the middle of its coding sequence. The remaining CYP6AS proteins, CYP6AS1, CYP6AS3, CYP6AS4, CYP6AS10 and CYP6AS15, share 50.3% to 64.3% identity. All were successfully expressed in the baculovirus system except for *CYP6AS15*, which was expressed in an incorrectly folded P420 form.

In bioassays with the four enzymes co-expressed with house fly P450 reductase at an MOI ratio of 1:0.5, only quercetin was metabolized (Table 2). Activities ranged from 0.20 ± 0.004 to 0.52 ± 0.10 pmol quercetin/pmol P450/min, with CYP6AS1 and CYP6AS3

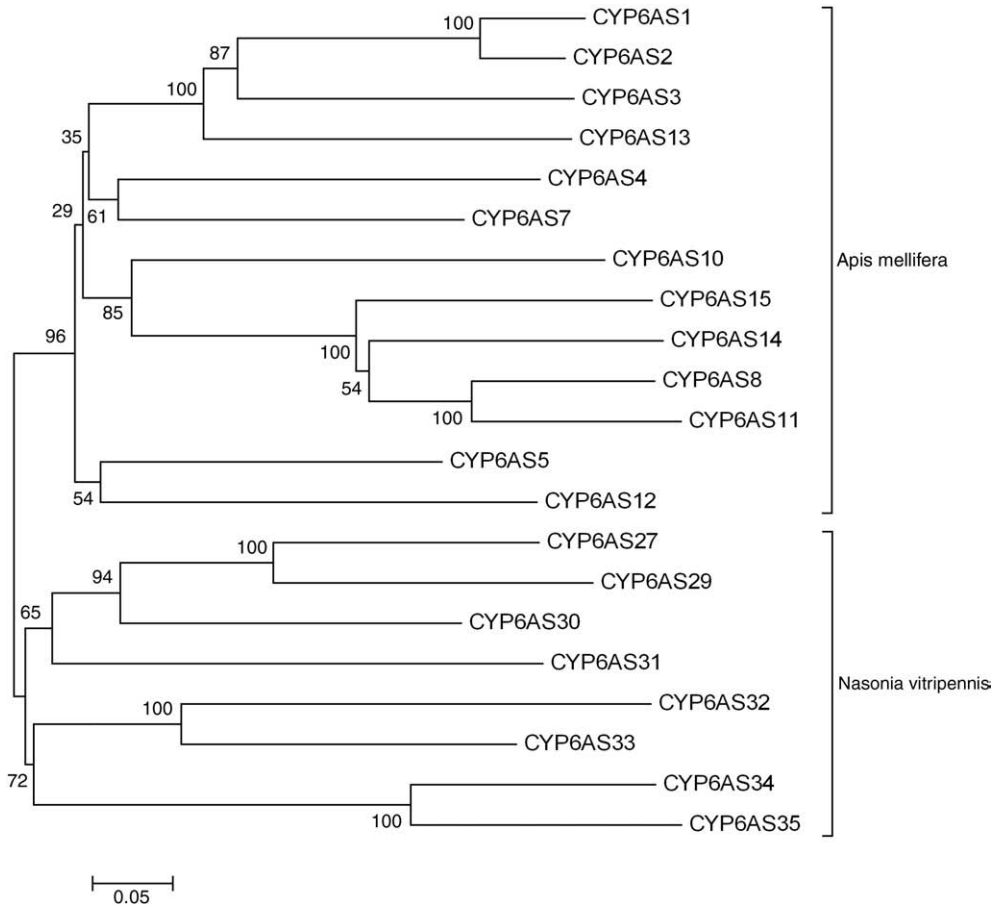


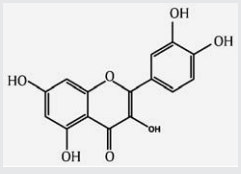
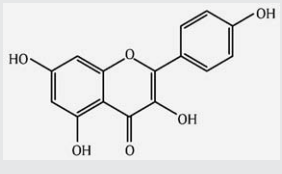
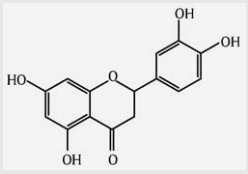
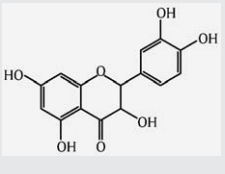
Fig. 2. Phylogenetic comparison of CYP6AS proteins from *Apis mellifera* with those from *Nasonia vitripennis*.

exhibiting higher activity than CYP6AS4 and CYP6AS10. Although all four CYP6AS enzymes were capable of metabolizing quercetin, their catalytic activity was low compared to activities of lepidopteran CYP6 enzymes toward their host plant phytochemicals (Wen et al., 2006a,b; Mao et al., 2007a,b, 2008), including, in the case of the noctuid *Helicoverpa zea*, quercetin (Li et al., 2004).

3.3. Quercetin docking and metabolite prediction

Efforts to characterize quercetin metabolite(s) using our *in vitro* heterologous expression system were unsuccessful, despite the fact that more than 20% of quercetin was transformed in assays containing 30 pmol CYP6AS3. Consequently, we resorted to theoretical substrate docking procedures to predict the possible metabolites of quercetin. Analysis of the 100 possible conformations ranked according to the sum of the ligand's internal energy, van der Waals and electrostatic energy terms of the potential energy function suggested that myricetin might be the hydroxylated product, because the conformation of quercetin with the lowest energy oriented the 5′-carbon of its B ring toward the heme oxygen. Energy minimizations using the MMFF94 force field in MOE with fixed heme coordinates predicted that the 5′-carbon of the B ring on quercetin was 5.64 Å from the

Table 2

P450s	Flavones			
	Quercetin*	Kaempferol	Eriodictyol	Taxifolin
				
CYP6AS1	0.5 ± 0.1	ND**	ND	ND
CYP6AS3	0.5 ± 0.1	ND	ND	ND
CYP6AS4	0.2 ± 0.1	ND	ND	ND
CYP6AS10	0.2 ± 0.1	ND	ND	ND

* Catalytic activity: pmol quercetin/pmol P450/min.
** ND: not detectable.

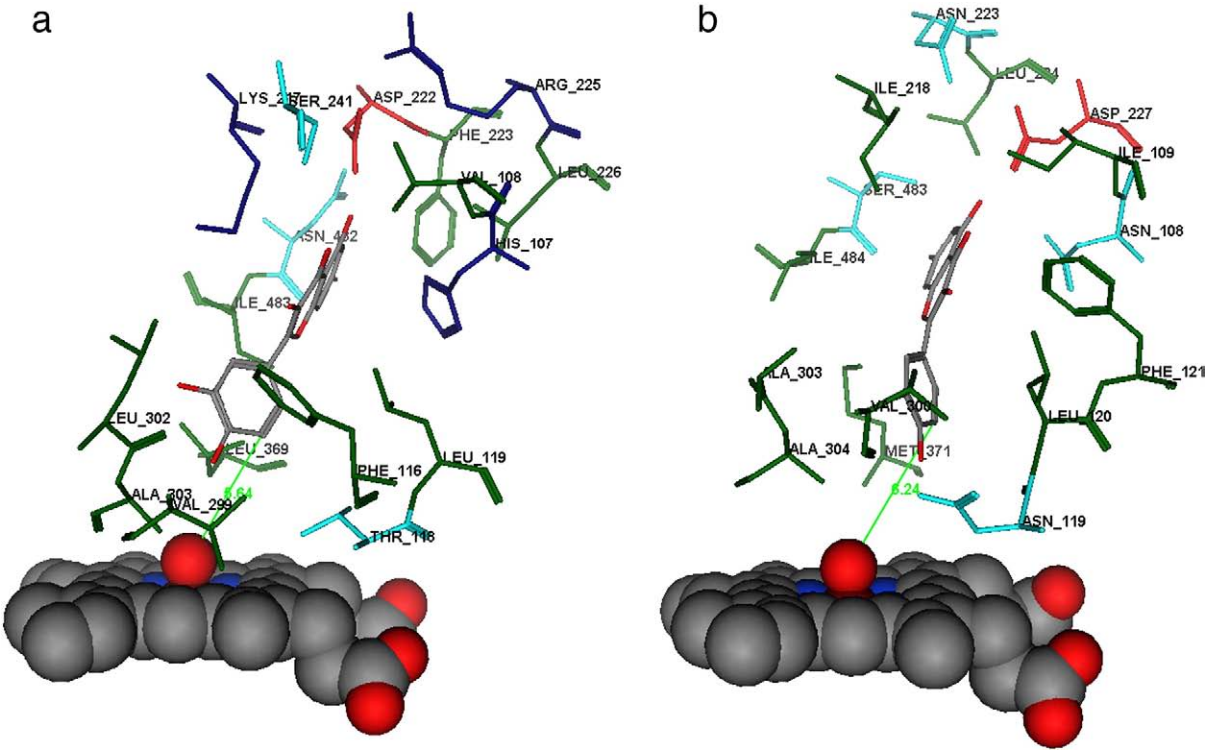


Fig. 3. Docking of quercetin in the CYP6AS catalytic sites. The molecular models of CYP6AS3 (a) and CYP6AS4 (b) are shown with the predicted binding modes for quercetin and the amino acids within 4.5 Å of the substrate coded with red for acidic residues, blue for basic residues, green for hydrophobic residues and cyan for hydrophilic residues.

heme oxygen, with an estimated binding energy of −87.62 kcal/mol (Fig. 3A).

The instability of myricetin in the basic solutions used for our enzyme bioassay system is likely the reason that no hydroxylated metabolite of quercetin was observed in our *in vitro* reactions containing CYP6AS3. Time course analyses of myricetin degradation at pH 7.4 and pH 7.8 indicated that over 90% of the starting material was degraded after 10 min at pH 7.4 and 100% was degraded at pH 7.8. Use of shorter incubation times for our reactions was not feasible since just 10% of the substrate was transformed by CYP6AS3/1 after 90 min of incubation and decreasing the incubation time would not have generated sufficient metabolite to characterize despite possible gains in product stability.

3.4. Contacting residues

Although the active sites of most insect P450s are packed primarily with hydrophobic amino acids, the active pocket of CYP6AS3 contains both polar and hydrophobic amino acids. This structure is well suited to the specific properties of quercetin, a relatively small hydrophobic compound with five hydroxyl groups, one carbonyl group, and one ether oxygen. Two positively charged amino acids, His107 in SRS1 and Lys217 in SRS2, and the carbonyl group of the backbone between Leu302 and Ala303 in SRS4 appear important for holding quercetin in a proper conformation in the active site (Fig. 3A). His107 and Lys217 are located on either side of the A and C rings, with the imidazole ring of His107 having the potential to form pi-stacking interactions. The carbonyl group is predicted to associate with the two hydroxyl groups on 3′- and 4′-carbons of the B ring, so that the 5′-carbon is oriented toward the heme oxygen.

For comparative purposes, a quercetin-docked model of CYP6AS4 was built using the same hybrid template and docking procedures as for CYP6AS3. This model (Fig. 3B) predicts that quercetin is positioned in the active site with the carboxyls of Asn108 and Asp227 and the carbonyl group between Ser483 and Ile484 associated with the two hydroxyls on the A ring. The carbonyl group between Ser299 and Val300 as well as the carboxyl group of Asn119 associate with the hydroxyls on the 3′- and 4′-carbons of the B ring. Among residues in this catalytic site predicted to contact this substrate, Leu120 in SRS1 and Ala304 in SRS4 are the only residues consistently conserved among all four CYP6AS proteins (Fig. 4). In comparison with CYP6AS3, which also positions the 5′-carbon of the B ring toward the heme oxygen, the distance to quercetin and its interaction energy are greater for CYP6AS4 (6.24 Å, −63.43 kcal/mol) than CYP6AS3 (5.64 Å, −87.63 kcal/mol). The reduced catalytic activities of CYP6AS4 and CYP6AS10 toward quercetin likely result from multiple replacements throughout the catalytic site that cause this substrate to bind less effectively.

To obtain a mechanistic understanding of the differential metabolic capabilities of CYP6AS3 and CYP6B8 with respect to quercetin, we compared our CYP6AS3 model with our existing CYP6B8 model, updated to use the same criteria (Baudry et al., 2003, 2006; Rupasinghe et al., 2007). Substrate docking experiments with the CYP6B8 model (Fig. 5) indicate that quercetin binds much closer to the heme (3.84) than in CYP6AS3 but with similar interaction energy (−67.9 kcal/mol). Both models suggest that CYP6B8 and CYP6AS3 use substrate access channel pw2a, as designated in Wade et al. (2004), for quercetin. Comparison of the whole cavity, the active site and the channel connecting it to the outside surface of the CYP6AS3 and CYP6B8 models indicate that the CYP6B8 cavity lies almost parallel to the heme surface (Rupasinghe et al., 2007), while the cavity in CYP6AS3 lies at a distinct angle to the heme surface (Fig. 5). This topological difference in the active sites seems to be the main reason for the differences in rates of quercetin metabolism between the two proteins. This configuration causes the quercetin molecule to bind closer to the heme in CYP6B8 and slightly further away in the CYP6AS3 model. Further investigation of the binding modes indicate that the residues stabilizing the unreactive distal A ring of the quercetin molecule lie further away from the heme molecule in CYP6AS3 (His107 and Lys217) when compared to CYP6B8 (Lys110 and Glu211) (Fig. 5).

4. Discussion

This study, the first to assign a function to any honey bee P450, demonstrates that the CYP6AS subfamily is involved in the metabolism of quercetin, a flavonoid component of honey and pollen. The catalytic activities of four CYP6AS enzymes characterized here toward quercetin are comparable to the functions of other CYP6 family members, such as the CYP6B and CYP6AB proteins that metabolize host plant furanocoumarins (Cohen et al., 1992; Li et al., 2000, 2003; Mao et al., 2007a,b, 2008) and flavonoids (Li et al., 2004).

The CYP6AS subfamily, which is apparently unique to species in the order Hymenoptera, is represented in two wasp (*Philotrypesis pilosa*, *N. vitripennis*) and one ant (*Solenopsis invicta*) genomes (http://drnelson.utmem.edu/CytochromeP450.html). CYP6AS constitutes the largest subfamily within the CYP6 family in the *A. mellifera* genome, comprising 15 genes and accounting for approximately 54% of 28 genes in all of clade 3 and 75% of 20 genes in the CYP6 family. In contrast, the 8 CYP6AS genes in the *N. vitripennis* genome comprise ca. 16% of 49 genes in clade 3, and 28% of 29 genes in the CYP6 family (Fig. 2). Although the CYP6AS genes in both genomes may have arisen from a common ancestral gene, they separated very early, after which serial duplications occurred in each lineage. As a social insect consuming relatively non-toxic honey and pollen as food (Claudianos et al., 2006), *A. mellifera* is thought to encounter a narrower array of xenobiotics than *N. vitripennis*, which in its larval stages lives inside the pupae of filth flies infesting carrion. Ostensibly, expansion of the CYP6AS subfamily may have resulted through gene duplication events during the long period of association with flowering plants, contacting, processing, and consuming honey, pollen and propolis.

Quercetin is a main component of the flavonoids contained in honey and pollen. As potent enzyme inhibitors (Kitamura et al., 2005; Schewe et al., 2002), quercetin and related flavonoids present a toxic

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CYP6AS3       -MDYFQLLCVIGALLFAIYYYLTLTFDTWKNRGIPGPKPTIFFGNFQEVILKKISLAEKT
59
CYP6AS1       -MDYFQILCAISIVILTIYYYYSSKYTFWKKRGISGPKPIIFFGNFVDSIIQKRSTSEAV
59
CYP6AS4       MLHHFHILTAFVAIFLALYYYLTSKFDFWKNRGVSGPRPVPFFGNAKDVLLRKIGIGSFI
60
CYP6AS10      -MAAFEILCGFIIFIFAFYYYLIKPQEYWKNRGVPGPKPIPIFGNFFRLTFARISIGDLM
59
                :  *.:*  :  .::::***       **:**:.**:*  :***     : : . ..

                                                                  SRS 1
CYP6AS3       KQLYQEYKNELVFGIFQGRTPILVINDLEMIKDVLIRDFSVFPDRGIHVNPKVEPIFQTL
119
CYP6AS1       KKWYDDYKHESVFGIFGGTTPLLVINDLDMIKDVLIRDFSLFVDRGFHIFPKIEPLSEHL
119
CYP6AS4       AELYKRYDNEAMFGIFIGRSPNLVLRDLDLIKDVLIKDFSIFDNRGLNIPERAEPFSVNL
120
CYP6AS10      TKFYKEYKHEPVFGLYMRNVRVLAINNPDLIKTVLIKDFSKFAHRGLALNEVTEPLSQHL
119
               : *. *.:* :**::      *.:.: ::** ***:*** * .**: :    **:   *

CYP6AS3       FSLKSKTWRPLRMKLSPVFTSGKLKDMFPLILDCAKNLEEFVEKVRNSGEPVDCRDMAAK
179
CYP6AS1       FLLEAERWRPMRMKLSPIFTSGKLKEMFFLIMESAGNLEKYLDEVIKKDEMVECRELAAK
179
CYP6AS4       FSVDATRWRPLRMRLSPVFTSGKLKEMFPLILECAEHLEQCLEDAVKRGGPVDCFEIPAR
180
CYP6AS10      FVLEPKRWRPLRTKLSPIFTSGKLKDMFSLIIECSNTLENYVEHLISKNDRVEVRDLAAK
179
              * :..  ***:* :***:*******:** **::.:  **: ::.  . .  *:  ::.*:

                                                        SRS 2
CYP6AS3       FTTDVIGSCAFGVCMNSLSPEGSEFRRMGEQLGKFSFKKLARDFTRLYMPFLFDIIGGYL
239
CYP6AS1       FMTDVIGSCAFGINTNSLLEEDSEFRRMGKKISTPNLKVMLGNICKEFFPPLYEIVGSIF
239
CYP6AS4       YTTDVIGSCAFGINMNALSDERSEFRKMGRNMFDQNMIKFTRNLLRDFFPRFYNLLGFVL
240
CYP6AS10      FTTDVIGSCGFGVDMNAMSDVQCKFRDIGREFFGPSFKQILKIRLRENLPRLYTFLGYIL
239
              : *******.**:  *::    .:** :*.::   .:  :     :  :* :: ::*  :

              SRS3
CYP6AS3       QSHEVNNFFINLIRDSIKYRQENNVYRPDFVNTLKELKEHPEKLENIELTDALLTSQALV
299
CYP6AS1       TLKDVNEFFINLVSDTMKYRKDNNIIRSDFINMLMQLKEHPEKMENIELTNTLLTAQAVV
299
CYP6AS4       PYTESTVFMTKLIKGTIKYREENDVVRPDFVNLLMELKKHPEKLKNIEITDTLLAAQASV
300
CYP6AS10      PRDETTTFFTNVVLDMIKYRKTNDIYRPDFINALINIQNHPEKLD-IELTEPLLVAQAFL
298
                 : . *: ::: . :***: *:: *.**:* * ::::****:. **:*:.**.:** :
```

Fig. 4. Sequence alignments of CYP6AS1, CYP6AS3, CYP6AS4 and CYP6AS10. Putative SRS domains are underlined. The amino acids within contact distance of quercetin in each of these catalytic sites are highlighted in yellow. Asterisks, colons and dots under the alignment represent identical, conserved and semi-conserved residues, respectively.

```
                SRS4
CYP6AS3         FFLAGFETSSTTISNALYELAQNPEMQDKLRKEIKEVYENNGGALSYTDVKEMKYLDKVF
359
CYP6AS1         FFVAGFETSSSTMAFSLYELAQNQEIQDKLREEIRNMHEKNKGVLTYTDVKEMKYLDKVF
359
CYP6AS4         FFAAGFETSSTTMAHALYEMALNPDIQDKLRNEMKEFHAKNNGNLKYEDIKEMKYLDKVF
360
CYP6AS10        FFVAGFETSSLTIATALYELAQNQDIQDKLRDEITEHHKLNNGEWQYENIKNMPYLDAVF
358
                ** ******* *:: :***:* * ::*****.*: : :  * *   * ::*:* *** **

                      SRS5
CYP6AS3         KETLRKYPVLAALSRQATENYTFKDTKIKISKGTRIWIPVYGIQHDPNIYPEPEVFDPER
419
CYP6AS1         KETLRKYPILPMLFRQAMENYTFKDTKITIPKGMKLWVPVHGIHHDPNIYPEPEVFDPER
419
CYP6AS4         RETLRKYPPGMLLRRKCNSNYTFHGTKVSIPAGTSVIIPLYAIQIDPKFYENPDVFDPER
420
CYP6AS10        KETLRKYVPLTVLMRQSLEDYTFESINLTIPKDTRIFIPIYAIHRDPDIYPNPEVFDINR
418
                :******     * *:. .:***.. ::.*. .  : :*::.*: **.:* :*:*** :*

                                heme binding
CYP6AS3         FEDDAFASRHPMTYLPFGDGPRNCIGARFAHYQSKVGLITILRNNKVEVCAKTLIPYKSE
479
CYP6AS1         FEDDAFASRHPMSYLPFGDGPRNCIGARFAHYQSKVGLITILRHHKVNVCEKTTIPFKAD
479
CYP6AS4         FNEDAVAARHPMTYLPFGDGPRNCVGARFAVYQTKVGLIKILQNFRVDVCEKTMIPYVKK
480
CYP6AS10        FSKEAEATRHPMHYLPFGDGPRNCIGARFAIFQTKIGLIKILRTYKVDVCNETQIPFINE
478
                *..:* *:**** ***********:***** :*:*:***.**:  :*:** :* **:  .

                SRS6
CYP6AS3         PRNILMIPKGGKVELRITKV-- 499
CYP6AS1         ERSFLLTLKGG-VHLKITKI-- 498
CYP6AS4         INSITLAPRDG-IFLKIEKITD 501
CYP6AS10        PRTFTLAPKHD-LTLKITKIEN 499
                 ..: :  : . : *:* *:
```

Fig. 4 (*continued*).

challenge to *A. mellifera*, which may explain why many subgroups of CYP6AS genes metabolize this compound. In comparison with the flavonoid-metabolizing CYP6B8 protein in *H. zea*, CYP6AS enzymes have lower activities toward quercetin, ranging from 0.20 ± 0.004 to 0.52 ± 0.10 pmol quercetin/pmol P450/min, which ecologically might relate to the low concentrations of quercetin in honey and pollen (e.g., 1.1 to 49.8 μg/100 g in heather honey: Ferreres et al., 1996) relative to concentrations found in foliage (e.g., up to 200,000 ppm, Duke, 1992).

Docking analysis of the amino acids comprising the CYP6AS3 active pocket showed that the two positively charged amino acids (His107,

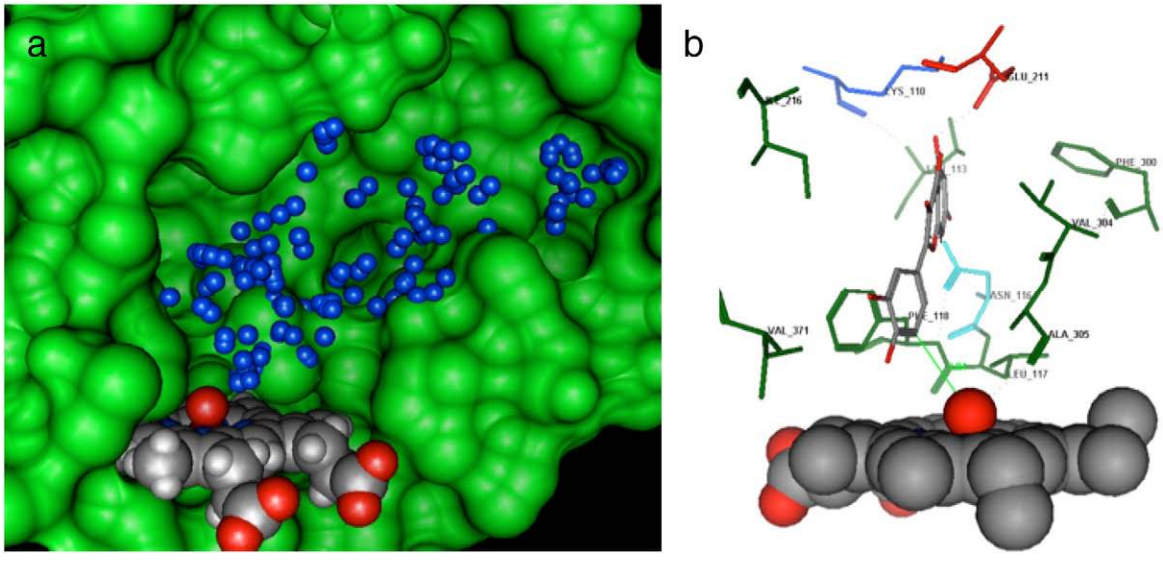

Fig. 5. Predicted active site cavity in the CYP6AS3 model (a) and predicted binding mode for quercetin in the CYP6B8 model with amino acids within 4.5 of the substrate (b).

Lys217) and the carbonyl group of the backbone between Leu302 and Ala303 are likely to moderate the positioning of quercetin in the CYP6AS3 catalytic site. Replacements of His107, Lys217 and other amino acids that serve as substrate contacts are predicted to affect the conformation of quercetin in the catalytic site and likely explain why the activities of CYP6AS4 and CYP6AS10 toward quercetin are lower in comparison with the activities of CYP6AS1 and CYP6AS3. These amino acid substitutions, however, may increase the catalytic activity of these enzymes toward other flavonoids and phenolics that can also be found in pollen and nectar. The redundancy of CYP6AS genes in the honey bee genome may provide sufficient breadth of catalytic activity to allow honey bees to process the range of phenolics and flavonoids encountered in the nectar of the many different floral species that this species can utilize.

Thus, honey bees appear to be well-equipped with P450s to process the phytochemicals that are predictably found in their principal foods (i.e., honey and pollen). *A. mellifera*, however, is relatively unusual among pollinators by virtue of its ability to forage for nectar from a wide range of species and its ability to process and alter the chemical properties of nectar by converting it into honey. This attribute characterizes both *A. mellifera* and *Apis cerana*, the two species within the genus *Apis* with the largest colonies; the expansion of the CYP6AS inventory coincides with the evolutionary separation of these two species from the remaining species in the genus (Claudianos et al., 2006). The P450 inventories of solitary bees, which consume nectar and not honey, generally from a narrower range of floral sources, may be even more reduced in number than in the honey bee genome; resolution of this question awaits sequencing of additional bee genomes displaying a range of social and feeding behaviors.

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References

Adler, L.S., 2000. The ecological significance of toxic nectar. Oikos 91, 409–420.
Azeredo, L.da C., Azerodo, M.A.A., de Souza, S.R., Dutra, V.M.L., 2003. Protein contents and physicochemical properties in honey samples of *Apis mellifera* of different floral origins. Food Chem. 80, 249–254.
Baudry, J., Li, W., Pan, L., Berenbaum, M.R., Schuler, M.A., 2003. Molecular docking of substrate and inhibitors in the catalytic site of CYP6B1, an insect cytochrome P450 monooxygenase. Protein Eng. 16, 577–587.
Baudry, J., Rupasinghe, S., Schuler, M., 2006. Class-dependent sequence alignment strategy improves the structural and functional modeling of P450s. Protein. Ent. Des. Sel 19, 345–353.
Brandenburg, A., Dell'Olivo, A., Bshary, R., Kuhlemeier, C., 2009. The sweetest thing: advances in nectar research. Curr. Opin. Plant Biol. 12, 486–490.
Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M.A., Berenbaum, M.R., Feyereisen, R., Oakeshott, J.G., 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Mol. Biol. 15, 615–636.
Cohen, M.B., Schuler, M.A., Berenbaum, M.R., 1992. A host-inducible cytochrome P450 from a host-specific caterpillar: molecular cloning and evolution. Proc. Natl. Acad. Sci. U.S.A. 89, 10920–10924.
Duke, James A., 1992. Handbook of Phytochemical Constituents of GRAS Herbs and Other Economic Plants. CRC Press, Boca Raton, FL.
Ferreres, F., Andrade, P., Gil, M.I., Tomas-Barberan, F.A., 1996. Floral nectar phenolics as biochemical markers for the botanical origin of heather honey. Z. Lebensm. Unters. Forsch. 202, 40–44.
Feyereisen, R., 2005. Insect cytochrome P450. In: Gilbert, L.I., Latrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Insect Science, Vol. 4, Chapter 1. Elsevier, Oxford, pp. 1–77.
Feyereisen, R., 2006. Evolution of insect P450. Biochem. Soc. Trans. 34, 1252–1255.
Gasteiger, J., Marsili, M., 1980. Iterative partial equalization of orbital electronegativity—a rapid access to atomic charges. Tetrahedron 36, 3219–3228.
Gehlhaar, D.K., Verkhivker, G.M., Rejto, P.A., Sherman, C.J., Fogel, D.R., Fogel, L.J., Freer, S.T., 1995. Molecular recognition of the inhibitor AG1343 by HIV-1 protease: conformationally flexible docking by evolutionary programming. Chem. Biol. 2, 317–324.
Gehlhaar, D.K., Bouzida, D., Rejto, P.A., 1999. Fully automated and rapid flexible docking of inhibitors covalently bound to serine proteases. Evol. Program. 1998, 449–461.
Gheldof, N., Wang, X., Engeseth, N.J., 2002. Identification and quantification of antioxidant components of honeys from various floral sources. J. Agric. Food Chem. 50, 5870–5877.
Gonzalez, F.J., Nebert, D.W., 1990. Evolution of the P450 gene superfamily: animal–plant 'warfare', molecular drive and human genetic differences in drug oxidation. Trends Genet. 6, 182–186.
Guengerich, F.P., 2001. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chem. Res. Toxicol. 14, 611–650.
Honeybee Genome Sequence Consortium, 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443, 931–949.
Jain, A.N., 1996. Scoring noncovalent protein–ligand interactions: a continuous differentiable function tuned to compute binding affinities. J. Comp. Aided Mol. Des. 10, 427–440.
Johnson, R.M. 2008. Toxicogenomics of *Apis mellifera*. Doctoral dissertation, University of Illinois at Urbana-Champaign.
Ke, N., Baudry, J., Makris, T.M., Schuler, M.A., Sligar, S.G., 2005. A retinoic acid binding cytochrome P450: CYP120A1 from *Synechocystis* sp. PCC 6803. Arch. Biochem. Biophys. 436, 110–120.
Kitamura, A., Mizuno, Y., Natsui, K., Yabuki, M., Komuro, S., Kanamura, H., 2005. Characterization of human cytochrome P450 enzymes involved in the in vitro metabolism of perospirone. Biopharm. Drug Dispos. 26, 59–65.
Krammer, A., Kirchhoff, P.D., Jiang, X., Venkatachalam, C.M., Waldman, M., 2005. LigScore: a novel scoring function for predicting binding affinities. J. Mol. Graph. Model. 23, 395–407.
Li, X., Berenbaum, M.R., Schuler, M.A., 2000. Molecular cloning and expression of *CYP6B8*: a xanthotoxin-inducible cytochrome P450 cDNA from *Helicoverpa zea*. Insect Biochem. Mol. Biol. 30, 75–84.
Li, W., Schuler, M.A., Berenbaum, M.R., 2003. Diversification of furanocoumarin-metabolizing cytochrome P450 monooxygenases in two papilionids: specificity and substrate encounter rate. Proc. Natl. Acad. Sci. 100, 14593–14598.
Li, X., Baudry, J., Berenbaum, M.R., Schuler, M.A., 2004. Structural and functional divergence of insect CYP6B proteins: from specialist to generalist cytochrome P450. Proc. Natl. Acad. Sci. U.S.A. 101, 2939–2944.
Li, X., Schuler, M.A., Berenbaum, M.R., 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu. Rev. Entomol. 52, 231–253.
Liu, F., He, J., Fu, W., 2005. Highly controlled nest homeostasis of honey bees helps deactivate phenolics in nectar. Naturwiss. 92, 297–299.
Mao, W., Rupasinghe, S.G., Zangerl, A.R., Berenbaum, M.R., Schuler, M.A., 2007a. 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, 10544–10552.
Mao, W., Schuler, M.A., Berenbaum, M.R., 2007b. Cytochrome P450s in *Papilio multicaudatus* and the transition from oligophagy to polyphagy in the Papilionidae. Insect Mol. Biol. 16, 481–490.
Mao, W., Zangerl, A.R., Berenbaum, M.R., Schuler, M.A., 2008. Metabolism of myristicin by *Depressaria pastinacella* CYP6AB3v2 and inhibition by its metabolite. Insect Biochem. Mol. Biol. 38, 645–651.
Mayo, S.L., Olafson, B.D., Goddard, W.A., 1990. DREIDING: a generic force field for molecular simulations. J. Phys. Chem. 94, 8897–8909.
Muegge, I., Martin, Y.C., 1999. A general and fast scoring function for protein–ligand interactions: a simplified potential approach. J. Med. Chem. 42, 791–804.
Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. J. Biol. Chem. 239, 2379–2385.
Rappe, A.K., Goddard III, W.A., 1991. Charge equilibration for molecular dynamics simulations. J. Phys. Chem. 95, 3358–3363.
Rupasinghe, S.G., Wen, Z., Chiu, T.L., Schuler, M.A., 2007. *Helicoverpa zea* CYP6B8 and CYP321A1: different molecular solutions to the problem of metabolizing plant toxins and insecticides. Protein Eng. Des. Sel. 20, 615–624.
Schewe, T., Kühn, H., Sies, H., 2002. Flavonoids of cocoa inhibit recombinant human 5-lipoxygenase. J. Nutr. 132, 1825–1829.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Tomás-Barberán, F.A., Martos, I., Ferreres, F.I., Radovic, B.S., Anklam, E., 2001. HPLC flavonoid profiles as markers for the botanical origin of European unifloral honeys. J. Sci. Food Agric. 81, 485–496.
Viuda-Martos, M., Ruiz-Navajas, Y., Fernandez-Lopez, J., Perez-Alvarez, J.A., 2008. Functional properties of honey, propolis, and royal jelly. J. Food Sci. 73, R117–R124.
Wade, R.C., Winn, P.J., Schlichting, I., Sudarko, J.A., 2004. A survey of active site access channels in cytochromes P450. Inorg. Biochem. 98, 1175–1182.
Wen, Z., Schuler, M.A., Berenbaum, M.R., 2006a. Inhibition of CYP6B1-mediated detoxification of xanthotoxin by plant allelochemicals in the black swallowtail (*Papilio polyxenes*). J. Chem. Ecol. 32, 507–522.
Wen, Z., Rupasinghe, S., Niu, G., Berenbaum, M.R., Schuler, M.A., 2006b. CYP6B1 and CYP6B3 of the black swallowtail (*Papilio polyxenes*): adaptive evolution through subfunctionalization. Mol. Biol. Evol. 23, 2434–2443.
Wester, M.R., Johnson, E.F., Marques-Soares, C., Dansette, P.M., Mansuy, D., Stout, C.D., 2003. Structure of a substrate complex of mammalian cytochrome P450 2C5 at 2.3 Å resolution: evidence for multiple substrate binding modes. Biochemistry 42, 6370–6379.
Westerkamp, C., 1991. Honeybees are poor pollinators—why? Plant Syst. Evol. 177, 71–75.
Wiermann, R., 1968. Phenylpropanoid metabolism by pollen I. Survey of flavonoid components isolated from gymnosperms and angiosperms. Ber. Dtsch. Bot. Ges. 81, 3–16.
Yano, J.K., Wester, M.R., Schoch, G.A., Griffin, K.J., Stout, C.D., Johnson, E.F., 2004. The structure of human microsomal cytochrome P450 3A4 determined by x-ray crystallography to 2.05-Å resolution. J. Biol. Chem. 279, 38091–38094.