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Biochemistry

A host-inducible cytochrome P-450 from a host-specific caterpillar: Molecular cloning and evolution

(insect P-450 monooxygenase cDNA/xanthotoxin)

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ABSTRACT **Cytochrome P-450 monooxygenases (P-450s) play a critical role in the detoxification of natural and synthetic toxins in a wide range of organisms. We have isolated and sequenced cDNA clones encoding a P-450, *CYP6B1*, from larvae of *Papilio polyxenes* (Lepidoptera: Papilionidae), the black swallowtail butterfly. This P-450, cloned from a herbivorous insect, is highly inducible by xanthotoxin, a secondary metabolite abundant in the host plants of this specialized herbivore. On Northern blots, mRNAs crossreactive with *CYP6B1* were detected in three *Papilio* species that, like the black swallowtail, have high levels of xanthotoxin-metabolic P-450 activity and encounter xanthotoxin or related compounds in their host plants; in contrast, no crossreactive mRNAs were detectable in three papilionid species that never encounter xanthotoxin in their host plants and lack detectable xanthotoxin-metabolic activity. These results provide evidence that new P-450s can arise as herbivores colonize different host plants and support the hypothesis that interactions between herbivores and their toxin-producing host plants have contributed to the diversification of the P-450 superfamily.**

Cytochrome P-450 monooxygenases (P-450s) function in the oxidation of a wide variety of xenobiotic and endogenous substrates (1) and are among the most important enzymes used by herbivores in detoxifying plant secondary metabolites (2). Selective pressure by plant secondary metabolites is thought to be one factor that has led to diversification of the cytochrome P-450 gene superfamily (1, 3, 4), which exhibits great intraspecies and interspecies variability, but there is, as yet, little direct evidence for this hypothesis. Insect herbivores that feed on a narrow range of plants sharing a common secondary chemistry encounter continual selection by a restricted array of secondary compounds and thus represent ideal organisms for investigating the mechanisms by which plant toxins drive P-450 gene evolution.

Larvae of the black swallowtail, *Papilio polyxenes* (Lepidoptera: Papilionidae), feed on only two plant families, the Apiaceae and Rutaceae (5). These plants produce a diversity and an abundance of furanocoumarins, secondary metabolites that occur only rarely in other families (6). One such furanocoumarin, xanthotoxin (8-methoxypsoralen), is highly toxic to generalist insect herbivores (7–9) yet is tolerated at high levels by specialist herbivores that selectively feed on furanocoumarin-containing plants. This tolerance in at least two species, the black swallowtail (10, 11) and the parsnip webworm *Depressaria pastinacella* (Lepidoptera: Oecophoridae) (12), is due to the ability of larvae to detoxify xanthotoxin rapidly by midgut cytochrome P-450 activities. In *P. polyxenes*, this P-450 activity appears to be autoregulated in that xanthotoxin induces the P-450 activity responsible for its own metabolism 7-fold over the endogenous activity present in larvae (13) and is associated with the appearance of a protein of ≈55 kDa in midgut microsomes (14).

The molecular cloning of cDNAs encoding this xanthotoxin-inducible protein has allowed us to confirm its identity as a newly discovered P-450, designated *CYP6B1*. To determine the origin of xanthotoxin-inducible P-450s in the evolution of the subfamily Papilioninae with respect to colonization of furanocoumarin-producing plants, we examined six additional species within this subfamily for their constitutive and xanthotoxin-induced P-450-mediated metabolism of xanthotoxin and screened Northern blots of midgut poly(A)$^+$ RNA for crossreactivity with *CYP6B1*. Of the additional swallowtails examined, *Papilio brevicauda* and *Papilio cresphontes* are specialists on furanocoumarin-producing plants in the Apiaceae and Rutaceae, respectively (5); *Papilio glaucus* is a broadly polyphagous species that only occasionally encounters furanocoumarins in a few rutaceous hosts (15); *Papilio troilus*, *Battus philenor*, and *Eurytides marcellus* are specialists on families that do not produce furanocoumarins, the Lauraceae, Aristolochiaceae, and Annonaceae, respectively (5). Poly(A)$^+$ RNA from *D. pastinacella*, which feeds exclusively on two genera of the Apiaceae high in furanocoumarins (16) but is only distantly related to *P. polyxenes*, was also screened. These experiments were designed to analyze the evolution of P-450 genes with respect to the speciation and host shifts that have occurred in a closely related group of herbivores and to compare P-450 genes in distantly related herbivores specialized to feed on the same plant species. Our results suggest that *CYP6B1* is an adaptation enabling the black swallowtail to feed on furanocoumarin-producing plants and thus that additional P-450s can arise as herbivores colonize new hosts.§

MATERIALS AND METHODS

Insects. The collection locality of adult swallowtails used to establish, and host plants used to maintain, laboratory colonies were *P. polyxenes*, Port Hope, Ontario, Canada and Champaign County, Illinois, *Petroselinum crispum; P. cresphontes*, Broward County, Florida, *Citrus jambhiri; P. brevicauda*, Shippegan, New Brunswick, Canada, *Ligusticum scotium; P. glaucus*, Champaign County, Illinois, *Prunus serotina; P. troilus*, Vermilion County, Illinois, *Sassafras albidum; B. philenor*, Parke County, Indiana, *Aristolochia durior; E. marcellus*, Crittenden County, Arkansas, *Asimina triloba*. A *D. pastinacella* colony was established from larvae collected in Champaign County, Illinois; larvae were reared on an artificial diet (17).

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§The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M80828 (*CYP6B1v1*, internal *Xho* I site present) and M83117 (*CYP6B1v2*, internal *Xho* I site absent)].

cDNA Cloning. Xanthotoxin-induced midgut microsomes were isolated and subjected to SDS/PAGE as described (14). Gels were stained with Coomassie blue and the ≈55-kDa xanthotoxin-inducible protein was excised with a razor and electroeluted from the gel slices. The eluate was precipitated overnight in 9 vol of acetone/1 mM HCl. A protein sample of ≈100 pmol was submitted to the Biotechnology Center at the University of Illinois at Urbana–Champaign for N-terminal sequencing. A second sample of ≈260 pmol was submitted to the Yale University Protein and Nucleic Acid Chemistry Facility for tryptic digestion and sequencing of an internal fragment.

Fully degenerate primers were designed to portions of the N-terminal and internal amino acid sequences. Poly(A)$^+$ RNA was isolated from the midguts of xanthotoxin-induced fifth instar larvae by use of the FastTrack kit (Invitrogen, San Diego) and reverse transcribed with the internal primer. The resulting cDNAs were amplified by PCR using the N-terminal and internal primers as described (18).

A cDNA library was constructed with the Librarian II kit (Invitrogen) and poly(A)$^+$ RNA isolated from the midguts of xanthotoxin-induced *P. polyxenes* larvae. (All larvae used for library construction were from a laboratory colony established with adults collected in Port Hope, Ontario.) The library was screened at high stringency (50% formamide; 42°C) using a ^{32}P-labeled 1.2-kilobase (kb) product of the reverse transcription/PCR procedure described above. Positive clones with inserts of ≈1.6 kb were characterized by restriction analysis and DNA sequencing using T7 and SP6 vector primers and internal primers of 17–18 bases. Both strands of the *CYP6B1* cDNA clones were completely sequenced.

Assays of Xanthotoxin Metabolism. Final instar larvae were reared for 2–3 days on host plants treated with either acetone (control) or an acetone solution of xanthotoxin at 0.2% (*P. cresphontes, P. polyxenes, P. brevicauda, P. glaucus*) or 0.1% (*P. troilus, B. philenor, E. marcellus*) of foliage (fresh weight); the lower concentration was used for those species that grew poorly on the 0.2% diet. Midgut microsomes were isolated as described (14) but were resuspended in 0.1 M sodium phosphate, pH 7.8/20% (vol/vol) glycerol/1 mM EDTA/0.1 mM dithiothreitol/5 μg of leupeptin per ml/0.5 mM phenylmethylsulfonyl fluoride to improve storage at -80°C.

Two independent microsomal samples from each species were assayed in triplicate for xanthotoxin metabolism. One-milliliter reaction mixtures contained 0.1 M sodium phosphate (pH 7.8), 0.3 mM NADP, 3.0 mM glucose 6-phosphate, 0.5 unit of glucose-6-phosphate dehydrogenase, 10 μg of xanthotoxin, and 0.05–0.2 mg of microsomes. Reaction mixtures were incubated for 30 min at 30°C, stopped with 200 μl of 2M HCl, and extracted with 2 ml of ethyl acetate. Unmetabolized xanthotoxin was quantified by normal-phase HPLC with a solvent system containing 90% cyclohexane, 6% isopropyl ether, 4% amyl alcohol.

Northern Blotting. Poly(A)$^+$ RNA was isolated, by use of the FastTrack kit, from the midguts of larvae reared as described. One microgram of poly(A)$^+$ RNA was loaded per lane and electrophoresed in a 1% agarose-formaldehyde gel (19). RNA was blotted onto a nylon membrane and sequentially probed with the insert of clone *CYP6B1v1* at both high (40% formamide; 42°C) and low (30% formamide; 25°C) stringency, and with a 0.8-kb fragment of a *Drosophila melanogaster* actin clone (20) at high stringency. Hybridizations were conducted at the specified temperature for 12–16 h in 30% or 40% formamide/0.12 M Na_2HPO_4, pH 7.2/0.25 M NaCl/7% SDS. The membrane was washed once at room temperature for 15 min in 2× standard saline citrate (SSC)/0.1% SDS, and once for 15 min in 0.5× SSC/0.1% SDS. Under these conditions, RNA sequences sharing ≈94% (high stringency) and 69% (low stringency) identity with the probe should be detectable (21).

RESULTS

cDNA Cloning and Characterization. To clone the P-450 responsible for xanthotoxin metabolism in *P. polyxenes*, the 55-kDa midgut microsomal protein induced by xanthotoxin was purified and N-terminal and internal tryptic fragment sequences were determined by microsequencing. The cDNA encoding this xanthotoxin-inducible P-450 was cloned by reverse transcription/PCR amplification of poly(A)$^+$ RNA isolated from xanthotoxin-induced midgut tissue with an internal primer encoding seven amino acids of the internal tryptic fragment and an N-terminal primer encoding six amino acids of the N-terminal fragment (Fig. 1). The major 1.2-kb PCR product generated from this process was subcloned, partially sequenced, and used to probe a xanthotoxin-induced midgut cDNA library. Restriction analysis of the longest clones indicated that two subclasses of cDNA, distinguished by the presence or absence of *Xho* I and *Hin*dIII sites, hybridized at high stringency with the 1.2-kb PCR product.

Sequence analysis of full-length clones from each subclass indicated that the coding regions of representative clones, *CYP6B1v1* (Fig. 1) and *CYP6B1v2*, are 98.2% identical in amino acid sequence. Both cDNAs encode proteins of ≈57 kDa containing the sequence FXXGXXXCXG in the heme-binding region near the C terminus (Fig. 1), which is highly conserved among P-450 proteins (22). Not unexpectedly, the clones contain the N-terminal and internal amino acid sequences obtained from the xanthotoxin-inducible protein, with the exception of tryptophan at position 400, which was misidentified in protein microsequencing as methionine. This extreme level of amino acid identity and genomic DNA analysis (T. Harrison, M.B.C., M.R.B., and M.A.S., unpublished data) indicate that these clones encode alleles of a single locus. On Northern blots (Fig. 2), the *CYP6B1v1* cDNA crossreacts with 1.6-kb mRNAs that are highly induced in black swallowtail larvae fed on xanthotoxin-treated parsley, *Petroselinum crispum*, or on untreated parsnip, *Pastinaca sativa*, a black swallowtail host plant containing high levels of xanthotoxin and other furanocoumarins (23).

Full-length amino acid sequence comparisons indicate that *CYP6B1* is 32% identical to *CYP6A1*, a phenobarbital-inducible P-450 from the housefly (24), and 29–32% identical to members of the *CYP3* family.

Xanthotoxin Metabolism and Occurrence of *CYP6B1* in Other Lepidoptera. To gain insight into the evolutionary origin of *CYP6B1*, we surveyed species closely related to *P. polyxenes* for xanthotoxin-metabolic activity and mRNA crossreactivity with *CYP6B1*. In addition to *P. polyxenes*, xanthotoxin-metabolic P-450 activities occur in three species that consistently or occasionally encounter furanocoumarins in their host plants: *P. brevicauda, P. cresphontes*, and *P. glaucus* (Table 1). No xanthotoxin metabolism was detectable in the remaining three swallowtails—*P. troilus, B. philenor*, and *E. marcellus*—which never feed on furanocoumarin-producing plants in nature.

High-stringency Northern blot analysis (Fig. 2*A*) revealed crossreactivity of *CYP6B1v1* only with xanthotoxin-inducible mRNAs in *P. brevicauda*, the closest relative of *P. polyxenes* examined (Fig. 3) and a species that also feeds on furanocoumarin-containing Apiaceae. Low-stringency Northern blots demonstrated crossreactivity with *CYP6B1*-related mRNAs in two other species—*P. cresphontes*, a specialist on furanocoumarin-containing Rutaceae, and *P. glaucus*, a generalist that encounters furanocoumarin-containing plants occasionally. No closely related mRNAs were detected in the three other papilionid species examined.

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1     GTAGTGACCGCGTTCAACATGTTGTATCTTTTAGCTCTTGTTACGGTTTTGGCAGGCCTTTTACATTACTACTTTACCAGGACTTTTAATTACTGGAAGAAAAGAAATGTCGCTGGA
1                                M  L  Y  L  L  A  L  V  T  V  L  A  G  L  L  H  Y  Y  F  T  R  T  F  N  Y  W  K  K  R  N  V  A  G

118   CCCAAACCAGTACCATTCTTTGGCAACTTAAAAGATTCCGTCCTCAGACGGAAACCTCAGGTAATGGTCTACAAAAGTATTTACGACGAATTTCCCAATGAAAAAGTTGTCGGAATTTAC
34    P  K  P  V  P  F  F  G  N  L  K  D  S  V  L  R  R  K  P  Q  V  M  V  Y  K  S  I  Y  D  E  F  P  N  E  K  V  V  G  I  Y

238   AGAATGACAACACCATCTGTGTTGTTACGTGATTTGGACATAATCAAGCATGTGCTCATCAAAGACTTCGAATCATTTGCCGACAGAGGAGTCGAATTCAGTCTCGATGGTCTCGGAGCC
74    R  M  T  T  P  S  V  L  L  R  D  L  D  I  I  K  H  V  L  I  K  D  F  E  S  F  A  D  R  G  V  E  F  S  L  D  G  L  G  A

358   AACATTTTCCACGCAGATGGCGACCGGTGGAGATCCTTAAGGAATCGTTTCACTCCTCTCTTCACCTCCGGAAAGCTCAAGTCCATGCTGCCCTTGATGTCGCAAGTCGGCGACAGGTTT
114   N  I  F  H  A  D  G  D  R  W  R  S  L  R  N  R  F  T  P  L  F  T  S  G  K  L  K  S  M  L  P  L  M  S  Q  V  G  D  R  F

478   ATAAACAGCATTGATGAAGTAAGTCAAACACAACCGGAACAGTCAATTCATAATCTAGTTCAGAAATTCACAATGACTAATATCGCAGCCTGTGTATTCGGTCTCAACTTGGATGAAGGA
154   I  N  S  I  D  E  V  S  Q  T  Q  P  E  Q  S  I  H  N  L  V  Q  K  F  T  M  T  N  I  A  A  C  V  F  G  L  N  L  D  E  G

598   ATGTTAAAAACTTTGGAAGACCTAGATAAGCATATATTCACTGTAAATTACAGCGCTGAGCTCGATATGATGTACCCGGGTATATTGAAAAAACTTAACGGTTCTCTCTTCCCGAAGGTT
194   M  L  K  T  L  E  D  L  D  K  H  I  F  T  V  N  Y  S  A  E  L  D  M  M  Y  P  G  I  L  K  K  L  N  G  S  L  F  P  K  V

718   GTTAGTAAATTTTTTGACAATCTAACAAAAAACGTACTCGAAATGAGAAAAGGAACGCCATCATATCAAAAGGATATGATCGACTTAATCCAAGAATTAAGGGAAAAGAAAACACTTGAG
234   V  S  K  F  F  D  N  L  T  K  N  V  L  E  M  R  K  G  T  P  S  Y  Q  K  D  M  I  D  L  I  Q  E  L  R  E  K  K  T  L  E

838   TTGTCGAGAAAACACGAGAACGAGGATGTGAAAGCCCTCGAGCTCACCGACGGGGTGATCTCTGCACAGATGTTCATATTCTACATGGCTGGCTACGAGACTAGCGCCACTACAATGACT
274   L  S  R  K  H  E  N  E  D  V  K  A  L  E  L  T  D  G  V  I  S  A  Q  M  F  I  F  Y  M  A  G  Y  E  T  S  A  T  T  M  T

958   TACCTGTTCTACGAACTAGCGAAGAATCCTGATATACAAGATAAACTTATTGCTGAAATAGACGAAGTTCTTTCCCGTCATGATGGCAATATAACCTACGAATGTTTGAGCGAAATGACA
314   Y  L  F  Y  E  L  A  K  N  P  D  I  Q  D  K  L  I  A  E  I  D  E  V  L  S  R  H  D  G  N  I  T  Y  E  C  L  S  E  M  T

1078  TATTTGAGTAAGGTGTTTGATGAGACATTAAGGAAATATCCCGTCGCAGACTTCACGCAGCGCAACGCTAAAACTGACTACGTGTTCCCCGGTACCGACATCACTATCAAGAAAGGGCAA
354   Y  L  S  K  V  F  D  E  T  L  R  K  Y  P  V  A  D  F  T  Q  R  N  A  K  T  D  Y  V  F  P  G  T  D  I  T  I  K  K  G  Q

1198  ACGATCATTGTATCCACGTGGGGCATTCAGAACGACCCAAAATACTATCCTAATCCAGAAAAATTTGACCCCGAACGTTTTAATCCGGAAAATGTAAAAGACAGACACCCCTGCGCTTAC
394   T  I  I  V  S  T  W  G  I  Q  N  D  P  K  Y  Y  P  N  P  E  K  F  D  P  E  R  F  N  P  E  N  V  K  D  R  H  P  C  A  Y

1318  CTACCATTTAGTGCAGGTCCTAGAAACTGCCTAGGTATGCGGTTTGCTAAGTGGCAGTCTGAAGTTTGCATCATGAAGGTACTATCGAAATACCGTGTGGAGCCCTCAATGAAGTCCTCT
434   L  P  F  S  A  G  P  R  N  C  L  G  M  R  F  A  K  W  Q  S  E  V  C  I  M  K  V  L  S  K  Y  R  V  E  P  S  M  K  S  S

1438  GGTCCATTTAAATTTGATCCTATGCGTCTCTTTGCTCTCCCTAAAGGAGGCATTTATGTAAACCTTGTCCGCAGATAAGCTTTTTTGTATTATGTATGATAAATGTTTGACTATGCTCTT
474   G  P  F  K  F  D  P  M  R  L  F  A  L  P  K  G  G  I  Y  V  N  L  V  R  R  *

1558  TAAATAAATTATTATATTCGTTTCAAATAAATAACATTTAACATATCAGCACAAAAAAAAAAAAAA
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FIG. 1. Sequence of *CYP6B1v1*. Amino acid sequences obtained from the xanthotoxin-inducible protein are underlined; fully degenerate primers were designed to the corresponding double-underlined nucleotide sequences. Amino acid positions highly conserved among P-450s are boxed. The internal *Xho* I site, absent in *CYP6B1v2*, is double underlined.

As in *P. polyxenes* and *P. brevicauda*, the *CYP6B1*-related mRNAs in *P. glaucus* were also xanthotoxin induced, while the inducibility of those in *P. cresphontes* is unclear because the control RNA was overloaded (Fig. 2*C*).

Crossreactive mRNAs of ≈1.6 kb or larger are faintly visible in the remaining three swallowtails and in *D. pastinacella* (Fig. 2*B*). These weakly crossreactive mRNAs do not appear to be induced in the xanthotoxin-treated samples and, as yet, their identity is unclear. mRNAs of ≈1.3 kb are also visible in most lanes of swallowtail RNA (Fig. 2*B*) but are unlikely to encode P-450s. The smallest known eukaryotic P-450 is ≈480 amino acids long (3) and the minimal size of an mRNA encoding such a protein, excluding 3′ and 5′ nontranslated regions, would be 1.4 kb.

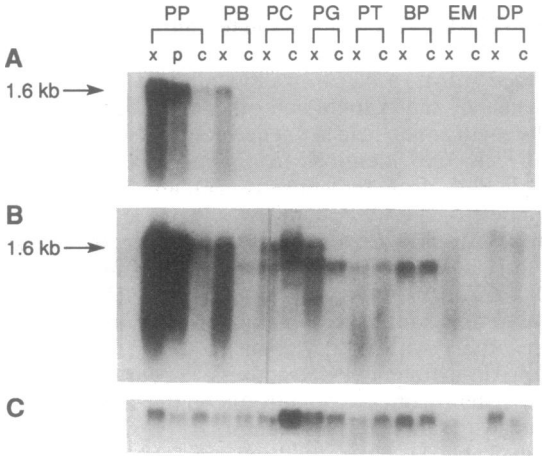

FIG. 2. Northern blot analysis of poly(A)$^+$ RNA isolated from midguts of control and xanthotoxin-induced caterpillars. PP, *P. polyxenes*; PB, *P. brevicauda*; PC, *P. cresphontes*; PG, *P. glaucus*; PT, *P. troilus*; BP, *B. philenor*; EM, *E. marcellus*; DP, *D. pastinacella*. Final instar larvae were reared for 2–3 days on parsnip (p), control (c), or xanthotoxin-supplemented (x) (0.2% of fresh weight, *P. polyxenes, P. brevicauda, P. cresphontes, P. glaucus*, and *D. pastinacella*; 0.1% of fresh weight, *P. troilus, B. philenor*, and *E. marcellus*) diets. One microgram of poly(A)$^+$ RNA was loaded per lane, electrophoresed in a 1% agarose-formaldehyde gel, and transferred to a nylon membrane. (*A*) Membrane probed with ^{32}P-labeled insert of *CYP6B1v1* at high stringency (40% formamide; 42°C). (*B*) Membrane probed at low stringency (30% formamide; 25°C). (*C*) Membrane probed with 0.8-kb *Sal* I fragment of a *D. melanogaster* actin clone (20) to indicate relative amounts of RNA loaded per lane.

DISCUSSION

Although cDNA sequences of >150 P-450s have been reported (27), *CYP6B1* is the first from a specialized herbivore and the first from a herbivorous insect. The data that we have collected to date suggest that *CYP6B1* evolved as an adaptation for detoxification of xanthotoxin and possibly other furanocoumarins as an ancestor of the black swallowtail colonized furanocoumarin-producing plants and, thus, provide support for the hypothesis that plant secondary com-

Table 1. Xanthotoxin metabolism by midgut microsomes isolated from seven species of swallowtail caterpillars

Species	Xanthotoxin metabolized, nmol per min per mg of protein	
	Control diet	Xanthotoxin diet
P. cresphontes	3.44 ± 3.34	6.55 ± 0.49
P. polyxenes	1.26 ± 0.04	5.74 ± 1.74*
P. brevicauda	0.99 ± 0.37	4.29 ± 0.11*
P. glaucus	0.48 ± 0.43	2.83 ± 0.47*
P. troilus	†	†
B. philenor	†	†
E. marcellus	†	†

Results are expressed as means ± SD (n = 2).
*Xanthotoxin treatment significantly greater than control at $P \leq 0.05$; one-tailed t test.
†Metabolism <0.20 nmol per min per mg of protein.

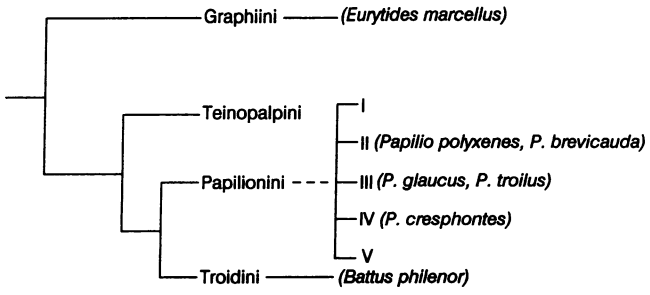

FIG. 3. Phylogenetic relationships of tribes of the subfamily Papilioninae (25) and the sections of the genus *Papilio* (26).

pounds have influenced the diversification of the cytochrome P-450 gene superfamily.

Levels of CYP6B1 mRNAs (Fig. 2) and P-450-mediated metabolism of xanthotoxin (27) are higher in black swallowtail larvae reared on parsnip, a black swallowtail host high in xanthotoxin and other furanocoumarins (23), than in larvae reared on parsley, a host with lower levels of these compounds (6). CYP6B1 mRNAs (Fig. 2) and protein (14) and P-450-mediated metabolism of xanthotoxin (13) are also induced when larvae ingest parsley treated topically with additional xanthotoxin, suggesting that this compound is at least partly responsible for CYP6B1 induction when larvae feed on parsnip. Expression of *CYP6B1* in baculovirus-transformed Tn5 cells to evaluate its substrate specificity indicates that the *CYP6B1* cDNA encodes the ability to metabolize xanthotoxin (R. Ma, M.B.C., M.R.B., and M.A.S., unpublished data).

Additional information on the evolution of *CYP6B1* with regard to the colonization of furanocoumarin-producing plants was gained by examining species related to *P. polyxenes* for the occurrence of this gene. On Northern blots, mRNAs closely related to *CYP6B1* were detected in the related species, *P. brevicauda*, that has a high level of xanthotoxin-metabolic P-450 activity and consistently feeds on furanocoumarin-producing plants. P-450 mRNAs related to *CYP6B1* also exist in two other swallowtail species that feed on furanocoumarin-producing plants, *P. cresphontes* and *P. glaucus*. In *P. brevicauda* and *P. glaucus*, both the crossreactive mRNAs and P-450-mediated metabolism of xanthotoxin were inducible, while levels of mRNA and metabolic activity in *P. cresphontes* were constitutively high. *P. brevicauda* and *P. cresphontes* are restricted to plants in the furanocoumarin-producing families Apiaceae and Rutaceae, respectively, while the larvae of *P. glaucus* are highly polyphagous and are able to survive on at least two rutaceous species (15). In *P. troilus* and *B. philenor*, species that do not feed on furanocoumarin-producing plants, xanthotoxin metabolism was low and not inducible (Table 1), and crossreaction of midgut mRNAs with *CYP6B1* was weak. *E. marcellus*, which also does not feed on furanocoumarin-containing plants, appears to have little *CYP6B1* crossreactive mRNA, although this sample may be underrepresented by this Northern blot analysis.

P. polyxenes and *P. brevicauda* are part of the *Papilio machaon* species complex, a clearly monophyletic group of about eight species (28). All members of this species complex feed primarily on Apiaceae, and *CYP6B1* may have arisen as the ancestral member of the *P. machaon* complex colonized plants in this family. Their high degree of identity indicates that xanthotoxin-inducible P-450s from *P. polyxenes* and *P. brevicauda* are almost certainly orthologous—i.e., "correspond[ing] to the ancestral gene which existed before the evolutionary divergence of the two species" (29)—and *CYP6B1* is likely to occur in all members of the *P. machaon* complex.

By similar reasoning, the crossreactive 1.6-kb mRNAs in *P. cresphontes* and *P. glaucus* probably also encode more divergent orthologues of *CYP6B1*. Rutaceae-feeding is widely distributed within the genus *Papilio* (5) and it is possible that *P. polyxenes*, *P. brevicauda*, *P. cresphontes*, and *P. glaucus* shared a common Rutaceae-feeding ancestor in which *CYP6B1* arose in response to the presence of xanthotoxin or other furanocoumarins in host foliage. It has also been argued (30) that Rutaceae-feeding arose independently in the three sections of *Papilio* (II, III, and IV) in which these species are placed. If so, the crossreactive P-450s in these species may be descended from a common ancestral P-450 predisposed toward xanthotoxin metabolism because of its original specificity against structurally related compounds. This shared ancestral P-450 probably arose after *P. polyxenes*, *P. brevicauda*, *P. cresphontes*, and *P. glaucus* diverged from *B. philenor* and *E. marcellus*; it may have been secondarily lost from *P. troilus*.

The results in Fig. 2 must be interpreted cautiously, however, for at least two reasons. The first is the confounding effect of phylogenetic distance. The mRNAs from *P. cresphontes* may crossreact more strongly with *CYP6B1* than do mRNAs from *B. philenor*, for example, simply because of the more recent divergence of *P. polyxenes* and *P. cresphontes*, rather than because these two species feed on furanocoumarin-producing plants, while *B. philenor* does not. The second reason for caution is the large and complex nature of the cytochrome P-450 gene superfamily. P-450s similar in function may differ greatly in overall sequence, while by contrast a single amino acid difference between two P-450s may result in great differences in substrate specificity (31). Molecular cloning of the crossreactive cDNAs from *P. cresphontes* and *P. glaucus*, followed by sequencing and analysis of their substrate specificity, will clarify the relationship of these P-450s to *CYP6B1* and, we hope, ultimately add to our understanding of the interaction between P-450 evolution and colonization of new host plants. The search for and characterization of cytosolic receptors for furanocoumarins or other plant secondary compounds are also of great interest because of the role played by these proteins in the induction of some P-450 genes (22).

The evolution of P-450s specialized to metabolize host plant compounds also has implications for improved control of herbivorous insect pests. If selective inhibitors (32) for P-450s such as CYP6B1 can be developed, then oligophagous insects like *P. polyxenes* could be rendered susceptible to toxins already present in their hosts, possibly without effects on nontarget organisms. Such inhibitors would be of enhanced value if they were effective against several pests of a given plant species. We are consequently interested in characterizing xanthotoxin-metabolizing P-450s from *D. pastinacella*, an oligophagous species that shares with *P. polyxenes* wild parsnip as a host plant but that is only a distantly related member of the same order, Lepidoptera. There is weak crossreactivity between *CYP6B1* and mRNAs of ≈1.6 kb from *D. pastinacella*, although these do not appear to be xanthotoxin induced (Fig. 2*B*). The overall sequence similarity between CYP6B1 and xanthotoxin-metabolizing P-450s from *D. pastinacella* will probably be low, but analysis of these P-450s and possible xanthotoxin receptors may reveal shared features that may serve as targets for common inhibitors.

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