Characterization and Induction of Two Cytochrome P450 Genes, *CYP6AE28* and *CYP6AE30,* in *Cnaphalocrocis medinalis*: Possible Involvement in Metabolism of Rice Allelochemicals

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Two cDNAs specific for P450 genes, *CYP6AE28* and *CYP6AE30*, have been isolated from the rice leaf folder *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae). Both cDNA-predicted proteins have 504 amino acid residues in length, but with molecular masses of 60177 Dalton for CYP6AE28 and 60020 Dalton for CYP6AE30, and theoretical pI values of 8.49 for CYP6AE28 and 8.56 for CYP6AE30, respectively. Both putative proteins contain the conserved structural and functional domains characteristic of all CYP6 members. CYP6AE28 and CYP6AE30 show 52% amino acid identity to each other; both of them have 49–56% identities with CYP6AE1, Cyp6ae12, and CYP6AE14. Phylogenetic analysis showed that the two P450s are grouped in the lineage containing some of the CYP6AE members, CYP6B P450s and CYP321A1. The transcripts of CYP6AE28 and CYP6AE30 were found to be induced in response to TKM-6, a rice variety with high resistance to *C. medinalis*. The results suggest that the two P450s may play important roles in adaptation to the host plant rice. This is the first report of P450 genes cloned in *C. medinalis*.

Key words: *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae), CYP6AE28 and CYP6AE30, Host Rice Resistance

Introduction

It is well established that in insects induction of P450 genes is induced in response to xenobiotics they contact with, such as toxic allelochemicals. For example, the black swallowtail butterfly *Papilio polyxenes* induces the P450 genes *CYP6B1* and *CYP6B3* in response to xanthotoxin produced by plants of the Apiaceae and Rutaceae families, enabling *P. polyxenes* to use these plants as a food source (Petersen *et al*., 2001; Wen *et al*., 2003). Another instance occurs in *Helicoverpa zea*, the cotton bollworm; *CYP6B8* is induced by xanthotoxin and the enzyme metabolizes the toxin (Li *et al*., 2003). In addition, several reports have suggested that some of insect P450s of the CYP4, CYP9, CYP28, and CYP6 families are induced by host plants, or participate in the detoxification of the host-plant allelochemicals (Danielson *et al*., 1997, 1998; David *et al*., 2006; Fogleman *et al*., 1998; Niu *et al*., 2008; Snyder *et al*., 1995; Stevens *et al*., 2000; Yang *et al*., 2007; Zhou *et al*., 2010).

The leaf folder *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae) is a serious pest of rice. The caterpillar affects the rice productivity by attacking the crop at the vegetative and reproductive stage. Traditional rice breeding for leaf-folder resistance was carried out in China as early as in the 1970s; unfortunately, no rice variety with sufficient level of resistance has been developed (Rao *et al*., 2010). Farmers are applying insecticides, which do not control leaf folders very effectively, but usually result in outbreaks (Rao *et al*., 2010). To develop more effective and environment friendly control strategies, the overall molecular response of the leaf folder exposure to host rice resistance should be well understood. In the investigation of this relationship, the pest P450 genes should be studied preferably for the importance of P450s in metabolism.

In this study, we report the cloning and expression analysis of two P450 genes, *CYP6AE28* and *CYP6AE30*, in *C. medinalis* and their potential roles in metabolism. The two P450 genes have been shown to be induced by resistant rice.

Table I. Sequence of primers used for RACE, RT-PCR, and LD-PCR.

Primer	Sequence (5′-3′)	Product length [bp]
CYP6-F	GA(A/G)AC(A/G/C/T)(A/C/T)(C/T)(A/G/C/T)(A/C)G(A/G/C/T)CC(A/G/C/T)(G/T)C	237
CYP6-R	GG(A/G/C/T)CC(A/G/C/T)(G/T)C(A/G/C/T)CC(A/G)AA (A/G/C/T)GG	
GSP1-F	ACGACTGCGTGACCGAGCTGCCGTA	1780
GSP1-R	CTCGCGTGTGATCACGCCAAGAGCTG	1212
GSP2-F	ACGTGGTCACCGAGCTGCCATACACG	1255
GSP2-R	TCGGATCGGAATGTAAATGCGAGTGC	1276
ldprimer1-F	CTGTGCGGACTCAGAGACGC	1885
ldprimer1-R	GAAGTTCACCGGTGACCGGC	
ldprimer2-F	CATCGTGCACTGCCAAGTCC	2058
ldprimer2-R	GGGGGCGCGAGCGAAGCCGC	

F and R indicate forward primer and reverse primer, respectively. Y = C/T, K = T/G.

Therefore they are possible key factors in the adaptation of the leaf folder to the host plant rice.

Material and Methods

Experimental insects and RNA isolation

The 2nd instar *C. medinalis* larvae were collected from a rice field in Wuchang District, Wuhan City, China. The larvae were reared on TN1, a leaf folder-susceptible rice variety. When developed to the 5th instar stage, the larvae were exposed to TKM-6, a highly resistant *indica* rice culture (Khan and Joshi, 1990), and collected after 0, 12, 24, 36, 48, 60, 72, and 84 h, respectively. Larvae midguts were dissected on ice under an ordinary stereomicroscope. The midgut luminal content was discarded, and the midgut was rinsed several times in 50 mM K_3PO_4, 150 mM NaCl, pH 7.8, and immediately used for total RNA isolation using TRIzol reagent (Invitrogen, Carsbad, CA, USA) according to the manufacturer's instruction.

Cloning of the full cDNAs

Two degenerate oligonucleotide primers (CYP6-F and CYP6-R, Table I) were designed for reverse transcription-polymerase chain reaction (RT-PCR) to amplify cytochrome P450 family 6 genes (Kasai *et al.*, 2000). Two P450 clones representing two novel family 6 genes were obtained after cloning and sequencing (data not shown). Gene-specific primers (GSPs) for 5' and 3' rapid amplification of cDNA ends (RACE) were designed based on the sequences of the two cDNA clones. They were as follows: GSP1-R, GSP1-F, GSP2-R, and GSP2-F (Table I). The cDNA synthesis and RACE were performed exactly following the instruction manual of the SMART RACE cDNA amplification kit (BD Bioscience Clontech, Palo Alto, CA, USA).

Amplified fragments were routinely cloned into pGEM-T vector (Promega, Madison, WI, USA) and sequenced. The nucleotide sequences of the 5' and 3' RACE products were aligned to form two full-length cDNA sequences, and the cDNA-predicted proteins were designated as CYP6AE28 and CYP6AE30, respectively. Two pairs of primers (Table I) for long-distance-polymerase chain reaction (LD-PCR) were designed to amplify the internal sequences of the full cDNAs, respectively. The PCR system was heated at 95 °C for 1 min and then amplified for 35 cycles (95 °C for 30 s, 65 °C for 30 s, and 72 °C for 3 min). Amplified fragments were cloned and sequenced

Computer-assisted analysis of P450 cDNAs

ClustalX v1.8 (Thompson *et al.*, 1997) was used for analysing the alignment. Molecular masses and isoelectric points were predicted by Compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html). The N-terminal transmembrane anchors of the deduced proteins were predicted by the TMHMM Server v. 1.0 (http://www.cbs.dtu.dk/services/TMHMM/). A molecular phylogenetic tree was constructed by the ClustalW Server (http://crick.genes.nig.ac.jp/homology/clustalw-e.shtml) using the bootstrap N-J tree option (number of bootstrap trials = 1000) (Page, 1996).

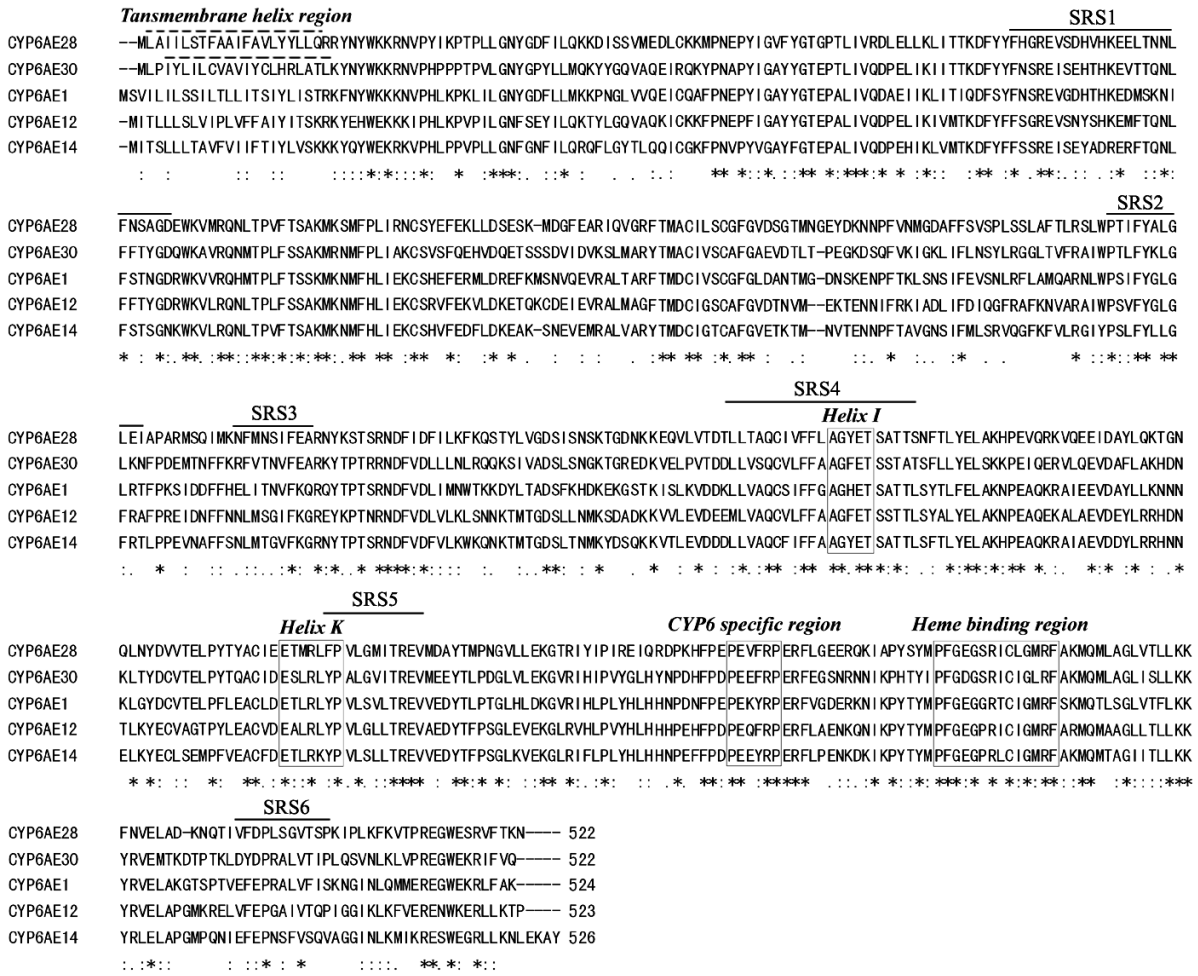

Fig. 1. Amino acid alignment of CYP6AE28 (CAX94849) and CYP6AE30 (CBB07053) with three CYP6 members: *D. pastinacella* CYP6AE1 (AAP83689), *H. armigera* CYP6AE12 (ABB69054), and CYP6AE14 (ABI84381). The proposed substrate recognition sites, denoted as SRS1 – SRS6, are covered with lines (Gotoh, 1992). The predicted transmembrane helix regions in the N-terminals are covered with discrete lines. The helix I, helix K, the heme-binding motifs, and the 6-residue sequences specific to CYP6 members are boxed and denoted with the corresponding names in italics. Identical amino acids are indicated by asterisks and conservative substitutions by dots.

Northern blot analysis

10 μg of total RNA pooled from midguts of *C. medinalis* larvae exposed to TKM-6 plants for the indicated time were subjected to electrophoresis on formaldehyde-denatured agarose gel (1.5%). Total RNA was blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and hybridized with the two LD-PCR products representing *CYP6AE28* and *CYP6AE30* labeled with [α-^{32}P]-dCTP (Perkin Elmer Life Sciences, Boston, MA, USA), respectively. The membranes were hybridized overnight at 65 °C, washed in 1 × SSC, 0.2% (w/v) SDS at 65 °C for 15 min, and in 0.5 × SSC, 0.1% (w/v) SDS at 65 °C for another 15 min. Then the membranes were scanned in a Typhoon 9400 Scanner (Amersham Biosciences, Piscataway, NJ, USA) to detect hybridization signals. Hybridizing bands were quantified by the software provided with the scanner. The constitutive 18S ribosomal RNA (FN820292) present in each lane was used as an

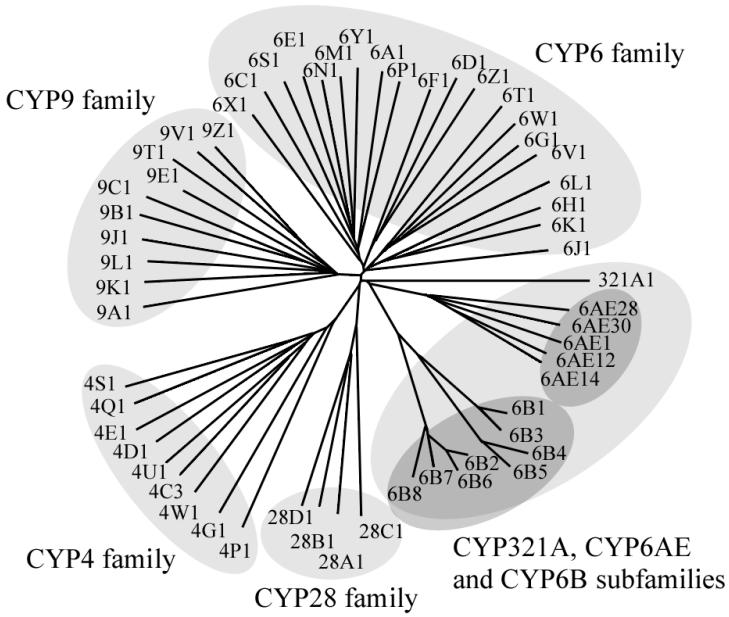

Fig. 2. Phylogenetic trees of full-length amino acid sequences of deduced CYP6AE28 and CYP6AE30 and some members from CYP4, 6, 9, 28, and 321 families constructed by the neighbour-joining method. The tree was constructed with the full-length sequences of the P450s. To give a good view of the figure, the bootstrap values were omitted. The GenBank accession numbers of the P450 genes in a reduced version of the neighbour-joining tree are: *Musca domestica* CYP6A1 M25367, *Cnaphalocrocis medinalis* CYP6AE28 FN356971, *C. medinalis* CYP6AE30 FN544262, *Depressaria pastinacella* CYP6AE1 AY295774, *Helicoverpa armigera* CYP6AE12 DQ256407, *H. armigera* CYP6AE14 DQ986461, *Papilio polyxenes* CYP6B1 M80828, *H. armigera* CYP6B2 U18085, *P. polyxenes* CYP6B3 U25819, *Papilio glaucus* CYP6B4 U47059, *P. glaucus* CYP6B5 U65490, *H. armigera* CYP6B6 AY950636, *H. armigera* CYP6B7 AF031468, *Helicoverpa zea* CYP6B8 AF102263, *M. domestica* CYP6C1 U09233, *M. domestica* CYP6D1 AF081288, *Culex quinquefasciatus* CYP6E1 AB001323, *Culex pipiens pallens* CYP6F1 AY662654, *Drosophila melanogaster* CYP6G1 NM_136899, *Locusta migratoria* CYP6H1 AF115777, *Blattella germanica* CYP6J1 AF281325, *Blattella germanica* CYP6K1 AF281328, *B. germanica* CYP6 L1 AF227531, *Anopheles gambiae* CYP6M1 AAL58569, *A. gambiae* CYP6N1 AY028786, *A. gambiae* CYP6P1 AY028785, *A. gambiae* CYP6S1 AY028784, *D. melanogaster* CYP6T1 NM_134613, *D. melanogaster* CYP6 V1 NM_134559, *D. melanogaster* CYP6W1 NM_136362, *Lygus lineolaris* CYP6X1 AAL15173, *A. gambiae* CYP6Y1 AF487536, *A. gambiae* CYP6Z1 AF487535, *Heliothis virescens* CYP9A1 U23506, *D. melanogaster* CYP9B1 NM_078921, *D. melanogaster* CYP9C1 NM_079126, *Dasyprocta punctata* CYP9E1 AY509245, *A. gambiae* CYP9L1 XM_320243, *Aedes aegypti* CYP9J1 AF390099, *A. gambiae* CYP9K1 AF487533, *A. gambiae* CYP9 L1 AF487781, *Ips confusus* CYP9T1 EU915209, *Leptinotarsa decemlineata* CYP9V1 DQ117460, *Tribolium castaneum* CYP9Z1 XM_970288, *D. melanogaster* CYP4C3 NM_079859, *D. melanogaster* CYP4D1 NM_166932, *D. melanogaster* CYP4E1 NM_080032, *D. melanogaster* CYP4G1 NM_080292, *D. melanogaster* CYP4P1 NM_080089, *Tribolium castaneum* CYP4Q1 XM_966145, *H. armigera* CYP4S1 EF591060, *Carpobrotus acinaciformis* CYP4U1 AF046010, *Boophilus microplus* CYP4W1 AF081807, *Drosophila mettleri* CYP28A1 U89746, *M. domestica* CYP28B1 AF355144, *Drosophila yakuba* CYP28C1 XM_002101334, *D. melanogaster* CYP28D1 NM_135068, *H. zea* CYP321A1 AY113689.

inner reference. *C. medinalis* larvae reared on TN1 plants were used as control (0 h). The experiments were repeated three times.

Results

cDNA cloning

Two P450 cDNAs with 2903 bp and 2373 bp in length from *C. medinalis* were amplified by degenerate RT-PCR, RACE, and LD-PCR strategies. Both of the cDNA contigs exhibited similarity to known CYP6 family genes when compared with the sequences published in GenBank.

One of the LD-PCR products with 1885 bp in length, amplified with primers ldprimer1-F and ldprimer1-R, proved to be the internal sequence of the 2903 bp cDNA contig. Another LD-PCR product with 2058 bp in length was amplified

with primers ldprimer2-F and ldprimer2-R, and was well consistent in nucleotide sequence arrangement with the internal sequence of the 2373-bp cDNA contig. The 2903-bp cDNA-predicted P450 was designated CYP6AE28 and the 2373-bp cDNA-encoded protein was designated CYP6AE30 by the P450 nomenclature committee (D. R. Nelson and D. W. Nebert, personal communication). The two cDNAs were deposited in the GenBank with accession numbers: FN356971 for *CYP6AE28* and FN544262 for *CYP6AE30*, respectively.

cDNA characterization

The *CYP6AE28* cDNA contains a 42-bp 5' untranslated region (5' UTR), an 1569-bp open reading frame encoding 522 amino acid residues, and an 1292-bp 3' UTR. This extended 3' UTR contains a consensus polyadenylation signal sequence (AATAAA) upstream from a 9-bp poly(A) tract. The *CYP6AE30* cDNA contains a 33-bp 5' UTR, an 1569-bp open reading frame encoding 522 amino acid residues, and a 770-bp 3' UTR. This 3' UTR contains a polyadenylation signal sequence (ATTAAA) upstream from an 18-bp poly(A) tract (not shown).

The predicted isoelectric points of the two cDNA-deduced P450 proteins are 8.49 and 8.56, respectively. The molecular masses of the two P450s are 60177 Da and 60020 Da, respectively, and are in the range 46–60 kDa of other reported cytochrome P450s (Nelson *et al.*, 1993). Both CYP6AE28 and CYP6AE30 have a hydrophobic transmembrane anchor in the N-terminal and are typical microsomal P450s (Fig. 1). Both P450s contain important conserved domains common to CYP6 family members, such as the oxygen-binding motif (helix I), the helix K, the heme-binding motif, and a 6-residue sequence (PEXXRP) specific to family 6 members (Nelson *et al.*, 1993).

Homology analysis revealed that CYP6AE28 shares the highest amino acid identities, *i.e.* 56%, with CYP6AE1 (AY295774) of *Depressaria pastinacella*, 55% with CYP6AE12 (DQ256407) of *Helicoverpa armigera*, and 51% with CYP6AE14 (DQ986461), a gossypol-induced cytochrome P450 of *H. armigera*. CYP6AE30 is most similar to CYP6AE28 with amino acid identity of 52%, with the second highest identity level (51%) to CYP6AE1 and with the next highest identity level (49%) to CYP6AE12 and CYP6AE14, respectively. Therefore, the two cloned genes should be members of the *CYP6AE* subfamily in the *CYP6* family.

The relatedness of CYP6AE28, CYP6AE30, CYP6AE1, CYP6AE12, and CYP6AE14 to each other is also revealed by the fact that they form an independent cluster contained in the clade including CYP6B members. A higher lineage containing the members of the CYP6AE and CYP6B subfamilies and CYP321A1 is formed in the phylogenetic tree. Each of the CYP9, CYP6, CYP4, and CYP28 family proteins forms an independent lineage (Fig. 2).

Expression analysis of CYP6AE28 and CYP6AE30

Northern blot analyses revealed that *CYP6AE28* and *CYP6AE30* were constitutively expressed at relatively low levels in the midgut of the 5th instar *C. medinalis* larvae fed on TN1 plants (0 h), but remarkably induced after exposure of the larvae

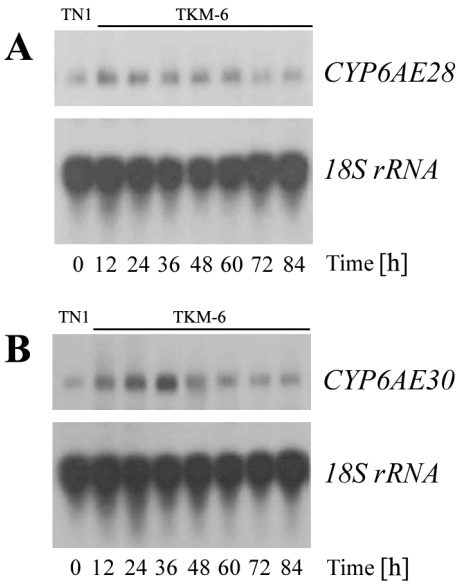

Fig. 3. Temporal expression levels of (A) *CYP6AE28* and (B) *CYP6AE30* in *C. medinalis* exposed to susceptible (TN1) or resistant (TKM-6) plants as analysed by Northern blot hybridization. Total RNA (10 μg/lane) was separated on 1.5% formaldehyde-denatured agarose gel. The blot was hybridized with the probes labelled by random priming using α-[^{32}P]-dCTP. Equivalent loading of total RNA in each lane was verified by 18S rRNAs (FN820292). TN1 indicates the insect feeding on TN1 plants and TKM-6 indicates the insect feeding on TKM-6 plants.

to TKM-6 plants (Fig. 3). An obvious induction of *CYP6AE28* was found from 0 to 12 h; the expression remained at a relatively constant level from 12 to 60 h, and then declined from 72 to 84 h (Fig. 3A). A gradual increase of the expression of *CYP6AE30* was observed from 0 to 36 h, which peaked at 24 h, and then the expression was declined from 36 to 48 h and remained at a stable level from 48 to 84 h (Fig. 3B).

Discussion

In this work, we successfully cloned two full-length cDNAs for the P450 genes *CYP6AE28* and *CYP6AE30* encoding P450 proteins in *C. medinalis*. The P450 protein sequences deduced from the two cDNAs have the characteristics present in all family 6 P450s. Protein sequence alignment analysis revealed that both of the two P450s are highly homologous to the CYP6AE subfamily P450s. The two P450 genes should be grouped into the *CYP6AE* subfamily according to the rules of the cytochrome P450 nomenclature committee.

In the phylogenic tree, the lineage (containing the rice folder P450s, CYP6AE1, CYP6AE12, CYP6AE14, CYP321A1, and the CYP6B members) is independent of the rest of the CYP6 family members, mostly derived from dipterans, which form another distinct lineage. In the lepidopteran lineage, CYP321A1 contributes to the resistance of *H. zea* larvae to toxic furanocoumarins and insecticides (Sasabe *et al.*, 2004). In *Papilio* and *Helicoverpa*, CYP6B transcripts are expressed at a basic level and dramatically induced by a subset of host furanocoumarins (Li *et al.*, 2000, 2001, 2002). Many members of the CYP6B subfamily have been confirmed to metabolize furanocoumarins by heterologeous expression analyses (Chen *et al.*, 2002; Wen *et al.*, 2003; Li *et al.*, 2004a). CYP6AE1 is a P450 in *D. pastinacella* proven to detoxify furanocoumarin from the wild parsnip (*Pastinaca sativa*) (Li *et al.*, 2004b). CYP6AE14 was confirmed to detoxify gossypol from cotton (Mao *et al.*, 2007). The phylogeny suggests a close relationship and similar physiological functions of these lepidopteran P450s. Northern blot hybridization analyses indicated that CYP6AE28 and CYP6AE30 transcripts are expressed at a significant basal level and are highly induced by resistant rice. Taken together, it appears most likely that CYP6AE28 and CYP6AE30 should contribute to adaptation of *C. medinalis* to the host plant rice by metabolism of the allelochemicals.

Further studies on the two heterologously expressed CYP6 enzymes will help in understanding their catalytic activity, and, furthermore, will facilitate the search for more reasonable control strategies. For example, silencing the P450 genes by RNA strategy combining planting resistant rice cultivars should be a preferable choice.

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