# Cloning and Characterization of Two Cytochrome P450 CYP6AX1 and CYP6AY1 cDNAs From *Nilaparvata lugens* Stål (Homoptera: Delphacidae)

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Two full-length P450 cDNAs, CYP6AX1 and CYP6AY1, were cloned from the brown planthopper *Nilaparvata lugens* Stål (Homoptera: Delphacidae). Both CYP6AX1 and CYP6AY1 are typical microsomal P450s and their deduced amino acid sequences share common characteristics with other members of the insect P450 CYP6 family. CYP6AX1 and CYP6AY1 show the highest percent identity (36%) of amino acid to each other; both of them have 31–33% amino acid identity with CYP6B1 from *Papilio polyxenes* (Lepidoptera: Papilionidae), CYP6B4 from *Papilio glaucus* (Lepidoptera: Papilionidae), and CYP6B8 from *Helicoverpa zea* (Lepidoptera: Noctuidae). Phylogenetic analysis showed the clustering of CYP6AX1 and CYP6AY1 was in the clade including CYP6AE1 from *Depressaria pastinacella* (Lepidoptera: Oecophoridae) and the CYP6B family members from *Helicoverpa* and *Papilio* species. Northern blot analysis revealed that both of the P450s were induced by the resistant rice variety B5 (*Oryza sativa* L), and CYP6AY1 was expressed at a higher level than CYP6AX1. The results suggest that more than one P450s are likely involved in metabolism of rice allelochemicals and that they are possibly important components in adaptation of *Nilaparvata lugens* to host rice. Arch. Insect Biochem. Physiol. 64:88–99, 2007. © 2007 Wiley-Liss, Inc.

Kerworbs: Brown planthopper *Nilaparvata lugens*; P450; cDNA doning; Northern hybridization analysis; phylogenetic tree

# INTRODUCTION

Cytochrome P450 monooxygenases are microsomal hemoproteins characterized by a highly conserved heme-binding region (F—G—C-G) and a spectral absorbance peak at 450 nm (Omura and Sato, 1964). Insect P450s are involved in metabolism of xenobiotic compounds (i.e., insecticides and plant toxic allelochemicals), as well as endogenous compounds such as juvenile hormones (JHs), ecdysteroids, and pheromones (Hodgson 1985; Berenbaum, 1999; Feyereisen, 1999).

In insects, metabolism of insecticides and toxic natural plant compounds is known to involve members of the cytochrome P450 CYP6 family (Danielson et al., 1997). Previous studies revealed that high levels of P450-mediated xenobiotic metabolism are correlated with the expression of transcripts of CYP6B1 to CYP6B8 in midgut tissue from various Lepidoptera (Cohen et al., 1992; Wang and Hobbs, 1995; Hung et al., 1995a, 1997; Ranasinghe and Hobbs, 1998; Li et al., 2000; Petersen et al., 2001). Insect P450s in other families were also reported to be induced by allelochemicals. Some CYP9 family members from *Manduca sexta* (Lepidoptera: Sphingidae) are induced by xenobiotics and plant allelochemicals (Stevens et al., 2000). Three CYP28 cytochrome P450s are induced by toxic host-plant allelochemicals and phenobarbital, suggesting a potential role of the CYP28 family in

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xenobiotic detoxification (Danielson et al., 1997). In another study of insect P450 induction, CYP4M1 and CYP4M3 in *M. sexta* larvae are activated by chemicals from tomato (Snyder et al., 1995). Clearly, insect cytochrome P450-based metabolism of toxic plant allelochemicals appears to be a key factor in host-plant utilization.

The brown planthopper Nilaparvata lugens Stål (Homoptera: Delphacidae) is a most destructive insect pest of rice (Oryza sativa L.) (Rubia-Sanchez et al., 1999). A previous study revealed that different breeding lines of rice and wild species of Oryza with different N. lugens-resistance levels displayed various effects on the growth, development, and reproduction of the insect (Denno and Roderick, 1990). Comparatively, a broad range of changes in physiology and biochemistry have been observed in rice infested by N. lugens (Rubia-Sanchez et al., 1999; Watanabe and Kitagawa, 2000). In shortterm interactions, some of the N. lugens genes were extensively regulated by host resistance (Yang et al., 2005, 2006). These genes were classified into groups of signaling, stress response, gene expression regulation, detoxification, and metabolism. Some rice genes were significantly induced or repressed in response to N. lugens feeding (Zhang et al., 2004; Wang et al., 2005; Yuan et al., 2005). The well-regulated genes were grouped in the categories of signaling pathways, photosynthesis, macromolecule degradation, oxidative stress/apoptosis, wound-response, and drought-inducible and pathogen-related proteins. Since N. lugens is a specialist feeder in its life span, it may be exposed to a narrow range of allelochemials from host rice, and might be expected to have evolved mechanisms to deal with them. The co-evolutional of N. lugens and its host rice has led to them to become ideal model systems for the study of interactions between plants and sucking herbivorous insects.

We previously obtained five ESTs (GenBank accession numbers: AJ629019, AJ629020, AJ629021, AJ629022, and AJ629023) representing five different P450 alleles from *N. lugens* by degenerate reverse transcription polymerase chain reaction (RT-PCR). The five ESTs all displayed strong regional similarity to insect CYP6 family members.

The expression alterations of one of the five P450 isoforms were confirmed in N. lugens exposed to different rice varieties (Yang et al., 2005). Subsequently, another EST (accession: AJ629002) representing a new P450 in N. lugens was obtained by cDNA-amplified fragment length polymorphism (cDNA-AFLP), and proved to be induced by resistant rice (Yang et al., 2006). The data indicated that there were at least two groups of P450s in N. lugens responding to host resistance. Elucidation of the molecular characteristics of these P450 genes will help in better understanding the ability of N. lugens to adapt to the presence of various allelochemicals in their diet. In this study, we report the cloning and characterization of two full-length P450 cDNAs, CYP6AX1 and CYP6AY1, from N. lugens.

#### MATERIALS AND METHODS

#### Experimental Insects and RNA Isolation

A colony of *N. lugens* (biotype II) were reared on plants of the susceptible rice Taichung Native 1 (TN1) at 25°C with a photoperiod of 16:8 (L:D) h and 80% relative humidity. The seeds of TN1 and the resistant rice variety B5 (Yang et al., 1999) were planted in plastic pots. Fourth-instar nymphs of *N. lugens* were transferred from TN1 plants to B5 and TN1 seedlings in the third leaf stage, and maintained for 24 h. The nymphs were collected separately. Total RNA was isolated by using TRIzol reagent (Invitrogen, La Jolla, CA) according to the manufacturer's instruction.

#### The 5' and 3' Rapid Amplification of cDNA Ends (RACE)

Gene-specific primers (GSPs) for 5' and 3' RACE were designed based on the sequences of two cloned P450-like gene cDNA fragments (accession nos: AJ629022 and AJ629019). They were as follows: GSP1-1 (5'-GSP from AJ629022 for CYP6AX1), GSP1-2 (3'-GSP from AJ629012 for CYP6AX1), GSP2-1 (5'-GSP from AJ629019 for CYP6AY1), and GSP2-2 (3'-GSP from AJ629019 for CYP6AY1). The primer sequences are listed in Table 1 (see also Fig. 2). The cDNA synthesis and RACE were per-

TABLE 1.	Sequence	of Primers	Used for	RACE and	LD-PCR

Primer	Sequence (5'-3')
GSP1-1	TGGGTCGAACTTTTGAGGGTCGGGGTA
GSP1-2	GATTGCCGGCTCACTGATAAGGCGTTG
GSP2-1	CCGAAGGGCAGGTACGTGTAATTGATGG
GSP2-2	CCAGGGACATCAGTGGCCATTCCAG
LD1-1	ACCAGTCAAGAGCTGGTTTTGATGAC
LD1-2	GTGAGTTTGAAAATCGCTTCCCGTTG
LD2-1	GCTTGCTGATAATAGTGTGCTTGCTC
LD2-2	GGATGAGTTGGCTCCCTCTTTATTG

formed exactly following the instruction manual of the SMART RACE cDNA Amplification Kit (BD Bioscience Clontech, USA). Briefly, the 5'-RACE-Ready cDNA was synthesized at 42°C for 1.5 h in a 10-µL reaction containing 1 µL 5'-CDS, 1 µL BD SMART A oligo, 1  $\mu$ L total RNA (1  $\mu$ g/ $\mu$ L), 2  $\mu$ L  $ddH_2O_1$  2 µL of 5 × First-Strand Buffer, 1 µL of 20 mmol/L DTT, 1 µL of 10 mmol/L dNTP mix, and 1 µL BD PowerScript Reverse Transcriptase. The 3'-RACE-Ready cDNA was synthesized at 42°C for 1.5 h in a 10-µL reaction containing 1 µL 3'-CDS primer A, 1  $\mu$ L total RNA (1  $\mu$ g/ $\mu$ L), 3  $\mu$ L ddH<sub>2</sub>O,  $2 \mu L \text{ of } 5 \times \text{First-Strand Buffer, } 1 \mu L \text{ of } 20 \text{ mmol/L}$ DTT, 1 µL of 10 mmol/L dNTP mix, and 1 µL BD PowerScript Reverse Transcriptase. The 5'-ends of cDNA were amplified in a 50-µL reaction containing 34.5  $\mu$ L ddH<sub>2</sub>O, 5  $\mu$ L of 10 × BD Advantage 2 PCR Buffer, 1 µL of 10 mmol/L dNTP mix, 1 µL of  $50 \times BD$  Advantage 2 Polymerase Mix, 2.5  $\mu$ L 5'-RACE-Ready cDNA (diluted 10-fold with Tricine-EDTA Buffer), 5  $\mu$ L of 10 × UPM, 1  $\mu$ L of 10  $\mu$ mol/L GSP1-1 or GSP2-1. The 3'-ends of cDNA were amplified in a 50-µL reaction containing 34.5 µL  $ddH_2O_1$  5 µL of 10 × BD Advantage 2 PCR Buffer, 1  $\mu$ L of 10 mmol/L dNTP mix, 1  $\mu$ L of 50  $\times$  BD Advantage 2 Polymerase Mix, 2.5 µL 3'-RACE-Ready cDNA (diluted 10-fold with Tricine-EDTA Buffer), 5  $\mu$ L of 10  $\times$  UPM, 1  $\mu$ L of 10  $\mu$ mol/L GSP1-2 or GSP2-2. Touchdown amplification profiles for 5' and 3' RACE were used as follows: 94°C for 30 s, 72°C for 3 min for 5 cycles, then 94°C for 30 s, 70°C for 30 s and 72°C for 3 min for 5 cycles, and then 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min for the remaining 28 cycles. Finally, the reaction was kept at 72°C for 7 min to complete synthesis of all DNA strands. The PCR

reactions were performed in a GeneAmp PCR System 2400 machine (Perkin Elmer). PCR products were checked by electrophoresis on 0.8% W/V agarose gel in TAE buffer (40 mmol/L Tris–acetate, 2 mmol/L Na<sub>2</sub>EDTA · 2H<sub>2</sub>O) and the resulting bands were visualized by ethidium bromide staining.

Amplified fragments were routinely cloned into pGEM-T vector (Promega, Madison, WI), and sequenced from both ends by using M13 forward and M13 reverse universal primers and BigDye™ terminator cycle sequencing version 2.0 ready kit (PE Applied Biosystems, Foster City, CA), on an ABI 377 automatic sequencer (PE Applied Biosystems). More than 4 independent clones of 5' and 3' end of cDNAs were sequenced to eliminate possible PCR mutations, respectively. The nucleotide sequences of the 5' and 3' RACE products were aligned to form two full-length cDNA sequences and they were designated as CYP6AX1 and CYP6AY1, respectively. To confirm that the 5' and 3' cDNA ends have been amplified from the same genes, two pairs of primers for long-distance polymerase chain reaction (LD-PCR) were designed from the two full-length cDNA sequences, respectively. The primers were forward primer LD1-1 and reverse primer LD1-2 from CYP6AX1, forward primer LD2-1 and reverse primer LD2-2 from CYP6AY1 (Table 1). The primers were optimized and picked from five sets of candidate primers in view of the annealing temperature and G/C content.

# Cloning the Full-Length cDNAs (CYP6AX1 and CYP6AY1) by LD-PCR

The two pairs of LD-PCR primers were used to amplify the two P450 cDNAs containing the putative open reading frames and part segments of 5'and 3'- untranslated regions from *N. lugens*, respectively. An LD-PCR reaction mixture was similar to the amplification of the 5'-ends of cDNA, except that the primers were replaced by LD-PCR primers. The mixture was heat-denatured at 95°C for 1 min and then amplified for 35 cycles (95°C for 30 sec, 65°C for 30 sec, and 72°C for 3 min). Amplified fragments were cloned into pGEM-T vector (Promega) and sequenced from both ends. More than 4 independent clones of each amplified cDNA were sequenced to eliminate possible PCR mutations.

### Computer-Assisted Analysis of P450 cDNAs

Clustal X v1.8 (Thompson et al., 1997) and DNAtools 6 were used for analyzing the alignment and edition. Molecular mass and isoelectric point were predicted by Compute pl/Mw tool (http:// us.expasy.org/tools/pi\_tool.html). N-terminal signal anchor of the deduced proteins was 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 = 1,000) (Page, 1996).

## Northern Blot Analysis

Twenty microgram of total RNAs from *N. lugens* exposed to the susceptible variety TN1 and the resistant variety B5 of rice were electrophoresed on formaldehyde denatured agarose gels (1.5%). Total RNA was blotted onto a Hybond-N<sup>+</sup> (Amer-

sham, Arlington Heights, IL) nylon membrane and hybridized with the two LD-PCR products representing CYP6AX1 and CYP6AY1 labeled with [ $\alpha$ -<sup>32</sup>P]-dCTP (Perkin Elmer Life Sciences, Oak Brook IL), 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 exposed to X-ray films (FUJI medical X-ray films, Japan) at -20°C with an intensifying screen. The experiment was repeated three times.

# RESULTS

#### **cDNA** Cloning

To clone two full-length P450 cDNA sequences from *N. lugens*, gene-specific primers from the EST AJ629022 and AJ629019 were designed for amplification of the 5' and 3' cDNA ends. The amplified products were electrophoresed on agarose gel, and each showed a clear main band (Fig. 1). A DNA band with the length of approximately 1,300 bp in 5' RACE and a band with the length of approximately 1,200 bp in 3' RACE were obtained

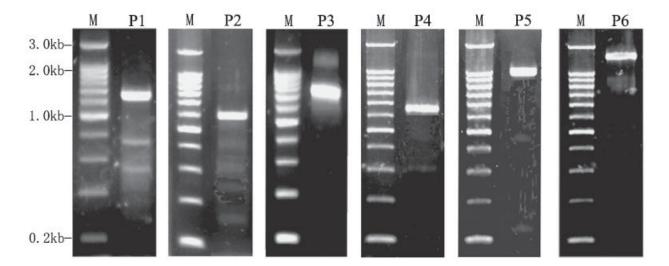


Fig. 1. Amplified products of CYP6AX1 and CYP6AY1 by 5' and 3' rapid amplification of cDNA ends (5', 3' RACE) and long distance-polymerase chain reaction (LD-PCR). The products were separated on a 0.8% agarose gel and stained with ethidium bromide. M: 200-bp DNA lad-

der; P1: 5' RACE product of CYP6AX1; P2: 3' RACE product of CYP6AX1; P3: 5' RACE product of CYP6AY1; P4: 3' RACE product of CYP6AY1; P5: LD-PCR product of CYP6AX1; P6: LD-PCR product of CYP6AY1.

by using GSP1-1 along with UPM, and GSP1-2 with UPM, respectively. Another 5' RACE product approximately 1,500 bp in size and a 3' RACE product approximately 1,300 bp in size were amplified by using GSP2-1 with UPM, and GSP2-2 with UPM, respectively. The PCR products were subcloned. Recombinant plasmids were purified from colonies and the nucleotide sequences of inserted PCR products sequenced. A cDNA contig of 2,395 bp was obtained by aligning the 5' and 3' cDNA ends amplified by using GSP1-1, GSP1-2, and UPM. Similarly, the second cDNA contig with 2,720 bp in length was formed by aligning the 5' and 3' cDNA ends amplified by GSP2-1, GSP2-2, and UPM. Both of the cDNA contigs exhibited similarity to known P450 genes when compared with the published sequences in GenBank.

To exclude the 5' and 3' RACE products that may have been obtained from different genes, LD-PCR primers were designed from the 2,395-bp and 2,720-bp cDNA sequences, and used to directly clone the cDNA fragments containing the full coding regions and part of the 5'- and 3'-untranslated regions. Each of the amplified products gave a clear single band (Fig. 1). The amplified fragments were cloned and sequenced. One of the LD-PCR products 2,108 bp in length, amplified by using primers LD1-1 and LD1-2, proved to be the slightly internal sequence of the 2,395-bp cDNA contig. Another LD-PCR product 2,601 bp in length was amplified by using primers LD2-1 and LD2-2, and was consistent with the internal sequence of the 2,720-bp cDNA contig in the nucleotide sequence arrangement (Fig. 2). The 2,395-bp gene was designated CYP6AX1, and the 2,720-bp gene was designated CYP6AY1 by the nomenclature committee (D. R. Nelson and D. W. Nebert), and deposited in the GenBank with accession numbers AJ852422 for CYP6AX1 and AJ852423 for CYP6AY1, respectively.

# **cDNA** Characterization

The consensus nucleotide sequences and their conceptual amino acid translations are shown in Figure 2A and B. The CYP6AX1 cDNA contains a 209-bp 5' untranslated region (5' UTR), a 1,542-

bp open reading frame encoding 514 amino acid residues, and a 641-bp 3' UTR. This extended 3' UTR contains four consensus polyadenylation signal sequences (AATAAA) upstream from a 12-bp poly(A) tract. The CYP6AY1 cDNA contains an 85bp 5' UTR, a 1,503-bp open reading frame encoding 501 amino acid residues, and a 1,129-bp 3' UTR. This extended 3' UTR contains two consensus polyadenylation signal sequences (AATAAA) upstream from a 19-bp poly(A) tract. Such multiple polyadenylation signals have been reported in a number of P450 genes including CYP6D1 (Tomita and Scott, 1995), CYP28A1 and CYP28A2 (Danielson et al., 1997), and CYP6B8 (Li et al., 2000).

The predicted isoelectric points of the two cDNA-deduced P450 proteins are 8.56 and 6.17, respectively. The molecular weights (MW) of the two P450s are 59,602 and 57,616, respectively, and are in the range (46-60 kDa) of other reported cytochrome P450s (Nelson et al., 1993). Both CYP6AX1 and CYP6AY1 are typical microsomal P450s, for there are hydrophobic transmembrane regions in the N-terminals of the two deduced amino acid sequences (Fig. 2). The deduced amino acid sequences of CYP6AX1 and CYP6AY1 contain important conserved domains common to CYP6 members (Nelson et al., 1993), such as hydrophobic N-terminal transmembrane anchors (positions 13 to 35 in CYP6AX1, 7 to 25 in CYP6AY1), the oxygen binding motif (helix I) of P450 (AG-T, positions 317 to 321 in CYP6AX1, 307 to 311 in CYP6AY1), the helix K (E—R—P, positions 374 to 380 in CYP6AX1, 364 to 370 in CYP6AY1), the heme-binding motif (PF—G—C-G—F, positions 498 to 461 in CYP6AX1, 438 to 451 in CYP6AY1), and the conserved amino acids sequence (P-F-P, positions 425 to 430 in CYP6AX1, 415 to 420 in CYP6AY1) near the carboxyl ends (Fig. 2). Therefore, the obtained 2 full-length cDNAs should be the members of CYP6 family, although they do not belong to the same subfamily.

Homologous analysis of amino acid sequences revealed that CYP6AX1 has the highest percent amino acid identity with CYP6AY1 (36%), CYP6B1 (33%) from *P. polyxenes* (Cohen et al., 1992), CYP6B4 (32%) from *P. glaucus* (Hung et al., 1997),

в

TAT AMA ACC ABT CAA GAB CTG GTT TTG ATG ACA ACT CAG TCA GTG GAT CTA TGT TAT CAA 62 CAA ACT TAG GIT ATG AAA TTG TAG TGT GTG TGT CTG TGT GCC TTC ACA TGG TGA ATG TTT 122 CTC TAA GTT TAG GTC ATT GAC AGT GAA TTG AAC CTT GTT ATA ATT ATA TAG TGA TTG AAG 182 GAA TAT TGA AGA GAA AAA AAA COG AAA ATG AAC GOC TTC GGT TAC GAA GTA TCA TGG TTC 242 M N A F G Y E V S W 11 GAC ATC TTC TTC CTA GTT GTC AGC CTA GTG TCC ATA ATC TAC TAC TTC GGC ATC AAC ACA 302 DIFFLVVSLVSIIYYFGINT 31 TTC AGC CAC TEG GAG AAA AAG GEC ATC AAA TTC ATG AAA CCG GTE CCG TTC TTC GEG AAC 362 SHW EKKGIKFWKPVPFF 51 ACC CTG GAT CTG GCC ATA GOC CGA CAA CAC ATC ACC ACC TGT CAT CAG GTC ATC TAT GAC 422 **DLAIGROHITTCHOVIY** 71 TAC TTC GCT GAT GAC GCC TAT GGT GGC TGG CTC GCC AAC CGA TCT CCC CTA CTT ATG GTA 482 A D D A Y G G W L A N R S P L L AAA GAT CCC GAA CTC ATA CAA CAG ATC CTT GTC AAA GAT TTT GTA CAT TTC TAT GAT CGC PEL IQQILVKDFVHFYD 111 GGA ACA ACA GTT GAT ATG AAG CTA GAC CCA CTG AAC GCG AAT CTG TTA AAC ATG ACG GGA 602 T V D W K L D P L N A N L L N M т 131 6 CAA COC TOG AAA GCT CTG CGA CAA AAA CTG ACA CCA GCG TTC AGT TCT GGA AAA CTG AAA 662 KALRQKLTPAFSSGK CTG ATG TCC CCC CAG TTC AAC GAG TGT GTC GAT GAT TTA TGC GGA TTG ATC GAC GAG AAA 722 M S P Q F N E C V D D L C G L I D E 171 TCA AAC AAA AAA GAG TTG ATC GAC GTG CAA GAA TCT ATG TCA AAA CTG GCC ACC GAC GTG 782 KKEL I D V Q E S M S K L ATA GOC TCC TGC GCG TTT GGT CTG CAA TTC AAT TCC CTG AAA AAC CCA GAA TCC GAG TTT 842 G S C A F G L O F N S L K N P F S F F 211 COT COC ATG GGA COC GAC ATA CTG AGA CCT TCG TGG COC TTC AAG GTG AGG ACT TTC ATG 902 R M G R D I L R P S W R F K V R TF 231 AGE GTC ATC AGT GAC AGT CTG COG TCC TTE CTC GGG GTC AAG GCC TTC GAC AAA TCC AAG R V I S D S L P S L L G V K A F D K S K 251 GAG GAC TTT TTC ATC AAC CTG GTC AAC GAC ACC ATG AAG TAT AGA GAG GAC AAC AAG GTG 1022 FFINLVNDTMKYREDNK 271 GAA COC AAT GAC TTC ATT CAG ATT TTG ATG AAT CTC AAG AAA ATC GAC GAG AAT ATG GAG 1082 NDFI QILMNLKKIDENME 291 ATA GAT CCC AAC AAC GAA AGT CAT GTT ATT CTT GAC GAC AAA CTA CTA GCA GCC AAC ACA 1142 D P N N E S H V I L D D K L L A A N T 311 TTT ATC TTC TTC ATT GCB GGB TTT GAG ACC ACC GCC ACA CTA ACA TTC AGC ATG TAC 1202 FIFFI<u>AOFET</u>TATTLTFSMY Helix I 331 GAA CTG GCA GTC AAC CAA GAG ATA CAA GAC AAA CTG CGT CAA GAG GTG CAG ACA ACC TTT 1262 E L A V N Q E I Q D K L R Q E V Q T T F 351 GAG AAA TAT GGA GCA ATA AAC TAT GAC TCA ACT AAA GAT ATG GAC TAC TTG GAC CGC GTC 1322 YGAINYDSTKDMDYLDR 371 ATT TET GAA ACA ETC ADA AAA TAC COS ATT GEC GBE TEA ETG ATA ADE CET TOC ACT AAG 1382 \* <u>E T L R K Y P</u> Helix K IAGSLIRRCT 391 GCA TEG CAG GTE CCC GES GCA AAG GET AAA CTE GAA GTE GET GAC AGE GTE GTE ATT CCE 1442 W Q V P G A K G K L E V G D R V V 411 BTC TAC CCC ATC CAC CAT BAC CCC AAA TAC TAC CCC BAC CCT CAA AAB TTC BAC CCA BAA **ҮРІНН РКҮҮ** CYP6 specific region P 431 COS TTC ACT CCA GAA AAC AAG ADG TCT AGA CCC CCT TGC ACC TAT ATG CCG TTT GGA GAT 1562 R F T P E N K R S R P P C T Y M P F G D 451 GGG CCT CGC ATT TGT ATT GGA GCA AGA TTC GCC CTC CAG GAG CTG AAA ACG ACT CTA AGC 1622 IGARFALQELKTTLS R ¢. Heme binding region TOT ATA CTG CTG CAT TAT ANG TTG ACA CTG AAT GAA AAA AGC AAA GCA ACT CTA CCA CTG 1682 ILLHYKLTLNEKSKAT AAA ATG AAA CCT CGA CGA ATA CTA ATG CAG TCT GAG CAA GAA ATT TAC ATT AAT TTC ACC 1742 **MKPRRILMQSEQEIY** I N F 511 AMA ATA AMG TAG ATT AAT TCA CAA AMA AAT TAG TAA GTA TTC TAT GTT TTG AGT TTT ACC 1802 AAG AAT GAG AAA TGT TGG ACT TTC AAG GAT CTG CAT CTT TTC TAT GGT TTC ACT ATA AAT 1862 AAG ATA TTT AAA AAA AAT GAA TGA CCA ACA TTA TGT ATT GTA CTG AGT GAC CAA AAC ATT 1922 ATG TAT AGT ACT TGA TAC ATA TTT TTA GTA GAT TCT ATA ACC TTC TAA ATT GAA CAG TGT 1982

Fig. 2. Nucleotide and deduced amino acid sequences of CYP6AX1 and CYP6AY1 cDNAs from *N. lugens*. The transmembrane region in the N-terminal anchor of each predicted protein is boxed. Regions of conservation associated with the helix I, helix K, CYP6 family-specific region, and heme-binding decapeptide are underlined and marked with corresponding italic words. Putative polyadenylation

GTA GTC AGT CGG TTG TGG GAC TCT GGT TGG TGT GGA GCG GTC GGT ACC TAT TAT TCT CAG 61 CAG CTG TTC AAC AAT GAA CAC GCA ATG ATC GAG ATT GGC TTG CTG ATA ATA GTG TGC TTG 121 M I E I G L L I V C L 12

CTC ATT TAC CAC TAC TGC ACA GCA ACA TTC AGG TAT TGG AAG CAA CGC AGT GTG CCA CTT 181 I Y H Y C T A T F R Y W K Q R GTA GAA COG ACT GCT CTA TTC GGC AAC TAT TAC GAC GTA GTC TCA TTG AAA ACA TCT CCG 241 G N Y D 1 s L GCC GCT TGC CAT GAA TCA ATT TAC AGA AAC TTT CCC AAT GAG AAA TAC GTT GGA ATG ATC HES YRNFPNEK c Y ٧ G м 72 CAA TTE AGA ACA CCA GCA CTE CTE ATC CGA TET CCA GAA ATE ETC AAA CAE ATT CTE ETE RTPALLIRCPENVKO . AAA GAC TTC AAC CAT TTC ATG GAT CGC GGA TTT CAT GCT GAT GAA GAA CGC GAG CCA ATC 421 D F N H F M D R G F H A D E E R E P ACC GCA CAC CTG GTC AAC CTT CAG GGC GAA AAA TGG CGA ATG CTG AGG CAG AAG ATT TCA 481 H L V N L Q G E K W R H L R Q K CCG GTT TTC ACC TCT GGA AAA CTA AAA GOC ATG TTT CCA CTA CTT GAA ACA TGC TCA TCA 541 к LKAMF s Ε L L CAG TTA TCC AAT TAC ATT GAG AGT GCT TTG GOC GAG CAA GAC AGC AGC TTA CTG GAA ATG 601 IESALGEQDSSLL E M 172 N Y COT GAC GTC ATG GCA CGT TTC ACG ACA GAC GTG ATC GGA TCG GTG GCC TTC GGA CTG CAT MARF т TDVI GSVA G L 192 TTC AAC TCT TTC ACC GAB AAA GAA TCC GAC TTC CAA CTG ATG GGG CGA CGT GTC CTC GAC N S F T E K E S D F O L N O R R V L 212 TOT TOT CAG AGG TOT GTT ATC ACC AAA GOG ATA AGA GTC TTT TTC COT CAG CTG TTT CAC 781 Q T S V I T K A I R V F TTC TTG AGA CTC CGC ACG TTT CCT GAG GAG ATA GCT ACG TTC TTC CAG ACA GTA ATC CAT 841 P GAC ACC ATT GAA AAT COG GAG AGA AAC GAC GTA CAA COC AAT GAC TTC ATA COG CTA CTG 901 ENR ERND VQRNDF LL ATG CAS CTG AGA AAG AAG AGT CCC GAC TAT GAT GGA ACG GAG GCT AAT GAT TTA GAA ATA 961 R K K S P D Y D G T E A N D L E 292 0 L GAG AGT GTT ATA GCA GCT CAA GCT TTT GTA TTC TTC ATG GCA GGA TAT GAA ACA TCC 1021 ACA GAG AGT GTT ATA GGA GGT CAA GUT IT GTA THE STATUS THE STATUS A A O A F V F F M <u>A G Y E</u>Helix IE T S 312

TCG ACA ACG TTG AGC TTT TGC TTG TAT GAG TTG GCC AAA AAT CTG GAC GTT CAG GAA AAA 1081 S T T L S F C L Y E L A K N L D V 0 E K 332 GCT TGC AAT GAG ATT AAA AAA ATT CTG AAC AAG CAC GGG AAG CTG AGC CAT GAG GCA TTG 1141 A C N E I K K V L N K H G K L S H E A L 352 ATG GAT TTG GAC TAT TTG GAG ATG ATC CTA TTG GAA AGC ATG AGG AAA TAC CCA CCT GTA 1201 M D L D Y L E M I L L E T M R K Y P P V 372  $\frac{1}{Hclix K}$ 

TCA GTA CTG GCA ADA GTT TGT ACT AAA COC TAC ACG ATA CCA GGG ADA AAA ATA TCA ATA 1201 S V L A R V C T K P Y T I P G T K I S I 392 GAT CGA GGA ACA TCA TCA ATA TCA TCA 1201 D P G T S V A I P V Y S F H H D H K Y F 412 CCA GAT CCT GAA CCT GAT CCT GAT

AAT TAC AGE TAC CTG CCC TTC BOC BCB BET COB CAC BTT TBT BAB BBA ACT AAB TTC CBT 1441 N Y T Y L P F G A G P H V C E G T K F R 452

Heme binding region

ACA GET COC TCC GOC ATC GOC CTA AGA CAT CTA CTE COS CTE TTC AGA TTC CET TAT GTT 1501 8 8 6 1 6 1 RHLLRLFR R 472 GAD AGD ACT CTG ACT GOD GAT TGC ACT CAA CAT AAC GAT TCT CGA TTA TCT GGG TTC ACT L T A D C T Q H N D S R L S G F π 492 GOG GAC GAT CTT GAT GTT GAA GGG CTT TGA CAA GCC GTC CAA CCC CAG CTC GCA CGA GGT GDDL DVEGL OBT OBC CTG GAT CAA GAG AAT ACT TOB TGT AGA AAA GAC TGG ACA TTC GGG AAC CCT CGA 1681 TCC TAA AAC CTC AGG TTG TCT AAC TGT TTG CAT TGA AAG GAC TAC GCG GCT TGC TAA ATC 1741 CCA ACA ATC AGC COG AAA GGA ATA TAT TTC TGT GTT CAA ATT GCA TTC CAA TCC TGG AGG 1801 AAT CAA TAA AGT TCA ACA ATC ACT AGA CAA ATT GAA AGG TGC TCT GTT CCA ACG ACC TCC TTT GAT TTC CGC AGT CAA GCS GCA GCT CAG AGT CAG AAA GAT CTA TGA GAT CAA ACT TAT 1921 CGA GTA TGA CGA AGA AAA GTA CAC AGG TAT TTT CTG GGT GAA GTG TGA AGC TGG AAC TTA 1981 THE GAR AAC HET ATH THE THE ACT HAR ATT HET OCT AND ANT THE CHA AAT HAC THA 2041 ATT GAS AMA AMA TOS ATC COS TAA CCA CAS TOA ASA AMT GOS ACT GOC TTC CAT GCA TGA 2101 TAT TIT GGA TGC GCA GTG GTT GTA TGA TAA CCA TGC TGA TGA ATC ATA CCT GAG AAG AGT 2161 CAT CCA TOC GCT GGA GAC ACT GCT CAC ATC GCA TAA AAG AAT TAT CAT GAA GGA CAG TGC 2221 TOT GAA TOC TAT CTG CTA COO TOC TAA AGT GAT GAT ACC TOS TOT GCT GOS ATA COA GOA TOD CAT COA GTT GAA COA GGA AAT COT GCT GGT GAC CAC TAA GGG GGA GOC GAT TGC ATT 2341 GGG CAT AGC GCT GAT GAC CAC AGC CAC AAT TGC CAG CTG TGA CCA CGG CAT CAT AGC CAA 2401 GCT GAA GAG AGT GCT CAT GGA CCG CGA CAC CTA TCC CAG GAA ATG GGB ACT CGG TGC TAA 2481 GGC TTC ATT GAA GAA GAG AAT GAT CAA GGA GGG CTT GCT TGA CAA GCA TGG AAA ACC CAA 2521 CGA AMA CAC GCC CGC TAA TTG GCT TTC ACA ATT CAA AMG CGC TGG AGA GTC AGC GGA GAA 2581 GAA CTC TGA GAG TGT TTC GGA AAC ACC AAG CAA GAG TGA AAG AAA ACG TAA ACT GAG CGG 2641 AAG TGG ATC AGC AAA TGA TGA AGC TGC CTC AGC AAT AAA AGA GGG AGC CAA CTC ATC CAA ------2720

signals (AATAAA) in the 3'-untranslated sequence are underlined with dots. The GSP sequences are marked with bold underline. The LD-PCR primer sequences are marked with wavy underline. A: Nucleotide and deduced amino acid sequences of CYP6AX1. B: Nucleotide and deduced amino acid sequences of CYP6AY1.

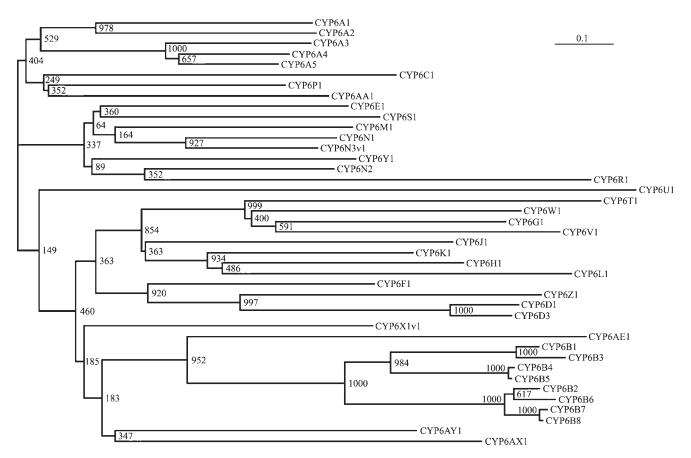


Fig. 3. Phylogenetic trees of full-length amino acid sequences of deduced CYP6AX1 and CYP6AY1 and the other insect CYP6 members are constructed by the neighborjoining method. The bootstrap values with 1,000 trials are indicated on branches. The scale bar represents 0.05 nucleotide substitutions per site. Sequences used: CYP6A1 (from Musca domestica, GenBank accession number: L27241); CYP6A2 (Drosophila melanogaster, M88009); CYP6A3 (Musca domestica, U09231); CYP6A4 (Musca domestica, U09232); CYP6A5 (Musca domestica, U09343); CYP6B1 (Papilio polyxenes, M80828-1); CYP6B2 (Helicoverpa armigera, U18085); CYP6B3 (Papilio polyxenes, U25819); CYP6B4 (Papilio glaucus, U47059); CYP6B5 (Helicoverpa armigera, Q95036); CYP6B6 (Helicoverpa armigera, Q95031); CYP6B7 (Helicoverpa armigera, AAC09227); CYP6B8 (Helicoverpa zea, AF285828-1); CYP6C1 (Musca domestica, U09233); CYP6D1 (Musca domestica, U15168); CYP6D3 (Musca domestica, AF283257); CYP6E1 (Culex pipiens quinquefasciatus, AB001323); CYP6F1 (Culex pipiens quinquefasciatus, AB001324); CYP6G1 (Drosophila melanogaster, AAL89788); CYP6H1 (Locusta migratoria, AF115777); CYP6J1 (Blattella germanica, AF281325-1); CYP6K1 (Blattella germanica, AF281328); CYP6L1 (Blattella germanica, AF227531); CYP6M1 (Anopheles gambiae, AY062208); CYP6N1 (Anopheles gambiae, AY028786); CYP6N2 (Anopheles gambiae, AY028782); CYP6N3v1 (Aedes albopictus, AF283836); CYP6P1 (Anopheles gambiae, AY028785); CYP6R1 (Anopheles gambiae, AY028783); CYP6S1 (Anopheles gambiae, AY028784); CYP6T1 (Drosophila melanogaster, AY071245); CYP6U1 (Drosophila melanogaster, AY069834); CYP6V1 (Drosophila melanogaster, AAF50889); CYP6W1 (Drosophila melanogaster, AY069121); CYP6X1v1 (Lygus lineolaris, AY054411-1); CYP6Y1 (Anopheles gambiae, AF487536); CYP6Z1 (Anopheles gambiae, AAL93296); CYP6AA1 (Anopheles gambiae, AY095933); CYP6AE1 (Depressaria pastinacella, AY295774). The tree was constructed with the full-length sequences of the P450s.

and CYP6B8 (31%) from *H. zea* (Li et al., 2000); while CYP6AY1 shares 32% amino acid identity with CYP6B1, 33% with CYP6B4, and 32% with CYP6B8, respectively. The relatedness of the CYP6AX1 and CYP6AY1 sequences to each other is also revealed by the fact that they form an independent cluster contained in the clade including CYP6AE1 from *D. pastinacella* (Li et al., 2004) and the CYP6B family members from *Helicoverpa* and *Papilio* species (Fig. 3) (Li et al., 2000).

#### Expression Analysis of CYP6AX1 and CYP6AY1

Expression level of CYP6AX1 and CYP6AY1 was compared in *N. lugens* exposed to two rice varieties (TN1 and B5) by northern analysis (Fig. 4). There was no detectable hybridization signal in *N. lugens* feeding on TN1, suggesting both of the P450 genes were expressed at a very low level with in-

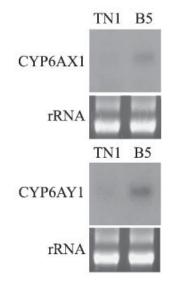


Fig. 4. Expression levels of CYP6AX1 and CYP6AY1 in *N. lugens* nymphs exposed to susceptible (TN1) or resistant plants (B5) as analyzed by northern blot 24 h after initiation of exposure. Total RNA (15  $\mu$ g per lane) was separated on 1.5% formaldehyde denatured agarose gel. The blot was hybridized with the probes labelled by random primer using  $\alpha$ -[<sup>32</sup>P] dCTP. Equivalent loading of total RNA in each lane was verified by rRNAs stained with ethidium bromide. TN1 indicates the insect nymphs feeding on TN1 plants and B5 indicates the nymphs feeding on B5 plants for 24 h.

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gestion of susceptible rice. But they were activated and expressed at a higher level in *N. lugens* on B5. Notably, the mRNA transcript of CYP6AY1 is accumulated approximately two times higher than that of CYP6AX1.

#### DISCUSSION

The SMART RACE along with the LD-PCR technique enabled us to successfully clone full-length cDNA sequences for the P450 genes, CYP6AX1 and CYP6AY1, in N. lugens (Fig. 1). The predicted amino acid sequences of the two P450s possess the characteristics present in all other CYP6 family members, including the helix I (AG—T), the helix K (E-R-P), the CYP6 specific region (P-F-P), the heme-binding region (PF-G-C-G-F), and the N-terminal hydrophobic transmembrane region (Fig. 2). Conservation around the helix I, helix K, and the heme-binding regions shows the evolutionary relatedness of CYP6AX1 and CYP6AY1 to CYP6 family members. Historically, according to the cytochrome P450 nomenclature committee, all members of the same P450 family should share >40% amino acid identity (Nelson et al., 1993). However, with more and more P450 members being obtained from various organisms, people have shifted to focus more on conserved regional sequence identity and results of phylogenetic analyses (Danielson et al., 1997; Nelson, 2006). The cockroach CYP4C1 was included in the CYP4 family despite having only 32-36% amino acid identity with its vertebrate homologs (Bradfield et al., 1991). Similarly, conservation around the hemebinding decapeptide shows the evolutionary relatedness of CYP6A1 and CYP6B2 despite their <40% global sequence identity (Nelson et al., 1993). Thus, the two obtained P450s in this study should be included in the CYP6 family, and represent a distinct branch of host-inducible cytochrome P450s, despite their global sequence identity below 36%.

It is well known that the insect CYP6B subfamily is involved in the metabolism of host plant toxin (reviewed in Scott et al., 1998). Some CYP6B members (CYP6B1-8) are speculated to metabo-

CYP6AX1	MNAFGYEVSWFDIFFLVVSLVSIIYYFGINTFSHWEKKGIKFMKPVPFFGNTLDLAIGRQ				
CYP6AY1	MIEIGLLIIVCLLIYHYCTATFRYWKQRSVPLVEPTALFGNYYDVVSLKT				
	: : *:: : :**:: ** :*:::: :::*:*** *:. :				
	SRS1				
CYP6AX1	HITTCHQVIYDYFADDAYGGWLANRSPLLMVKDPELIQQILVKDFVHFYDRGTTVDMKLD				
CYP6AY1	SPAACHESIYRNFPNEKYVGMIQLRTPALLIRCPEMVKQILVKDFNHFMDRGFHADEERE				
	::**: ** *.:: * * : *:* *::: **:::********				
	SRS1				
CYP6AX1	PLNANLLNMTGQRWKALRQKLTPAFSSGKLKLMSPQFNECVDDLCGLIDEKSNKKELI				
CYP6AY1	PITAHLVNLQGEKWRMLRQKISPVFTSGKLKAMFPLLETCSSQLSNYIESALGEQDSSLL				
	*:.*:*:*: *::*: *akaka::*.*:*akakakak * * :: * .:* *:::: *:				
	SRS2				
CYP6AX1	DVQESMSKLATDVIGSCAFGLQFNSLKNPESEFRRMGRDILRPSWRFKVRTFMRVISDSL				
CYP6AY1	EMRDVMARFTTDVIGSVAFGLHFNSFTEKESDFQLMGRRVLDSSQTSVITKAIRVFFPQL				
	:::: *::::******* ****::***::: **:*: *** :* .* .* : . :**: .*				
	SRS3				
CYP6AX1	PSLLGVKAFDKSKEDFFINLVNDTMKYREDNKVERNDFIQILMNLKKIDENMEIDPNNES				
CYP6AY1	FHFLRLRTFPEEIATFFQTVIHDTIENRERNDVQRNDFIPLLMQLRKKSPDYDGTEAN				
	:* :::* :. ** .:::**:: ** *.*:***** :**:*:* . : * .: .				
	SRS4				
CYP6AX1	HVILDDKLLAANTFIFFIAGFETTATTLTFSMYELAVNQEIQDKLRQEVQTTFEKYGAIN				
CYP6AY1	DLEITESVIAAQAFVFFMAGYETSSTTLSFCLYELAKNLDVQEKACNEIKKVLNKHGKLS				
	.: : :.::**::*:**:**:**:***:***:********				
	SRS5				
CYP6AX1	YDSTKDMDYLDRVISETLRKYPIAGSLIRRCTKAWQVPGAKGKLEVGDRVVIPVYPIHHD				
CYP6AY1	HEALMDLDYLEMILLETMRKYPPVSVLARYCTKPYTIPGTKISIDPGTSVAIPVYSFHHD				
	::: *:****: :: **:***** * * ****.: :**:* .:: * *.*****.:****				
CYP6AX1	PKYYPDPQKFDPERFTPENKRSRPPCTYMPFGDGPRICIGARFALQELKTTLSSILLHYK				
CYP6AY1	HKYFPDPETFDPERFSPENQEKSINYTYLPFGAGPHVCEGTKFRTGRSGIGLRHLLRLFR				
onomi	**:***:.*****:***: **:*** **::* *::*				
	SRS6				
CYP6AX1	SKSO LTLNEKSKATLPLKMKPRRILMQSEQEIYINFTKIK				
CYP6AY1	FRYVDSTLTADCTQHNDSRLSGFTGDDLDVEGL				
	1: 11:1 dd 2:00 1:5 2 555 83				

Fig. 5. Amino acid alignment of CYP6AX1 and CYP6AY1. 6, are boxed (Gotoh, 1992). Identical amino acids are in-

The proposed substrate recognition sites, denoted as SRS1- dicated by asterisks and conservative substitutions by dots.

lize xanthotoxin in Helicoverpa and Papilio species (Li et al., 2000). CYP6AE1 is a P450 cloned from D. pastinacella, the parsnip webworm, and is supposed to be capable of cytochrome P450-mediated detoxification of furanocoumarin (Li et al., 2004). Figure 3 shows the tree of the aligned full-length sequences for CYP6AX1 and CYP6AY1, and most of the insect CYP6 family members; these P450s at present have been linked to xenobiotic metabolism. Bootstrap analyses revealed that CYP6AX1 and CYP6AY1 formed a separate monophyletic cluster with bootstrap values of 645 (Fig. 3). The clustering of the two P450 sequences in the clade including CYP6B members and CYP6AE1 suggests both evolutionary relatedness and the possibility of similar physiological functions.

The substrate recognition sites (SRSs) have been shown to be critical for various mammalian P450s in defining metabolism of substrates (Gotoh, 1992). Similar studies indicated that amino acids within the SRS2, SRS4, SRS5, and SRS6 regions of the CYP2B1 (Kedzie et al., 1991; He et al., 1994; Szklarz et al., 1995) and CYP3A4 (Harlow and Halpert, 1997; He et al., 1997; Domanski et al., 1998) proteins are critical for metabolism of particular substrates. The 8 full-length sequences (CYP6B1-8) of CYP6B subfamily are 51 to 99% identical to each other. The SRS1 and SRS4 among these P450s are 75 to 100% identical with each other, coinciding with their catalytic activity towards the same or similar substrates (Li et al., 2000). In the same regions of CYP6AX1 and CYP6AY1 (Fig. 5), the SRSs identities range from 12.5% (SRS6) to 60% (SRS2). Except for SRS2 (60%) and SRS4 (38.4%), the identities of the other four SRSs are lower than the identity level (36%) for the two full-length proteins. Based on the weak sequence identity between the two P450 proteins and within their SRS domains, it appears highly likely that the two P450s probably possess different catalytic characteristics. Clearly, determination of the substrates for CYP6AX1 and CYP6AY1 is needed to resolve this speculation.

The expression level of CYP6AX1 and CYP6AY1 were analyzed simultaneously. Both of them were expressed at an undetectable level in *N. lugens* on

the TN1 variety. After the insects were exposed to the B5 variety, an obvious difference in the mRNA levels of the two P450s was observed. The expression of CYP6AY1 was approximately two times higher than CYP6AX1. A typical expression pattern in three separate experiments was shown in Figure 4. The result implied that the transcription regulatory elements of the two genes are very likely different and regulated in a distinct manner. Based on the data and the previous work (Yang et al., 2006), it is speculated that multiple P450 genes from various families are possibly promoted in N. lugens in response to resistant rice. Except CYP6AX1 and CYP6AY1, full-length cDNAs of the other four P450s have not been isolated successfully to date and we can not determine the relationship between these P450s. Further cloning of the full-length cDNAs will help in classifying the P450 members and unveiling their biological roles in N. lugens.

In this study, we report the successful cloning of two P450 genes, CYP6AX1 and CYP6AY1, from N. lugens for the first time. Both CYP6AX1 and CYP6AY1 are typical microsomal P450s with characteristics common to other members in CYP6 family. They were expressed differentially in N. lugens responding to resistant rice B5, suggesting the two P450s are important components of defense mechanisms against resistant rice. Although the data can provide valuable clues about the molecular characteristics of the two P450 genes, our results provide very limited information about the substrates for the two enzymes. It is of interest and necessity to determine the exact substrates upon which they act by employing either a suitable expression system or using specific antisera in the future.

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