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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.

KEYWORDS: Brown planthopper *Nilaparvata lugens*; P450; cDNA cloning; 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 short-term 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 AJ629022 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	GGATGAGTTGGCTCCCTCTTTTATTG

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 μL total RNA (1 μg/μL), 2 μL ddH_2O, 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 μL total RNA (1 μg/μL), 3 μL ddH_2O, 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 5′-ends of cDNA were amplified in a 50-μL reaction containing 34.5 μL ddH_2O, 5 μL of 10 × BD Advantage 2 PCR Buffer, 1 μL of 10 mmol/L dNTP mix, 1 μL of 50 × BD Advantage 2 Polymerase Mix, 2.5 μL 5′-RACE-Ready cDNA (diluted 10-fold with Tricine-EDTA Buffer), 5 μL of 10 × UPM, 1 μL of 10 μmol/L GSP1-1 or GSP2-1. The 3′-ends of cDNA were amplified in a 50-μL reaction containing 34.5 μL ddH_2O, 5 μL of 10 × BD Advantage 2 PCR Buffer, 1 μL of 10 mmol/L dNTP mix, 1 μL of 50 × BD Advantage 2 Polymerase Mix, 2.5 μL 3′-RACE-Ready cDNA (diluted 10-fold with Tricine-EDTA Buffer), 5 μL of 10 × UPM, 1 μL of 10 μmol/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 $Na_2EDTA \cdot 2H_2O$) 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 inde-

pendent 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 pI/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^+ (Amersham, Arlington Heights, IL) nylon membrane and hybridized with the two LD-PCR products representing CYP6AX1 and CYP6AY1 labeled with [α-^{32}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

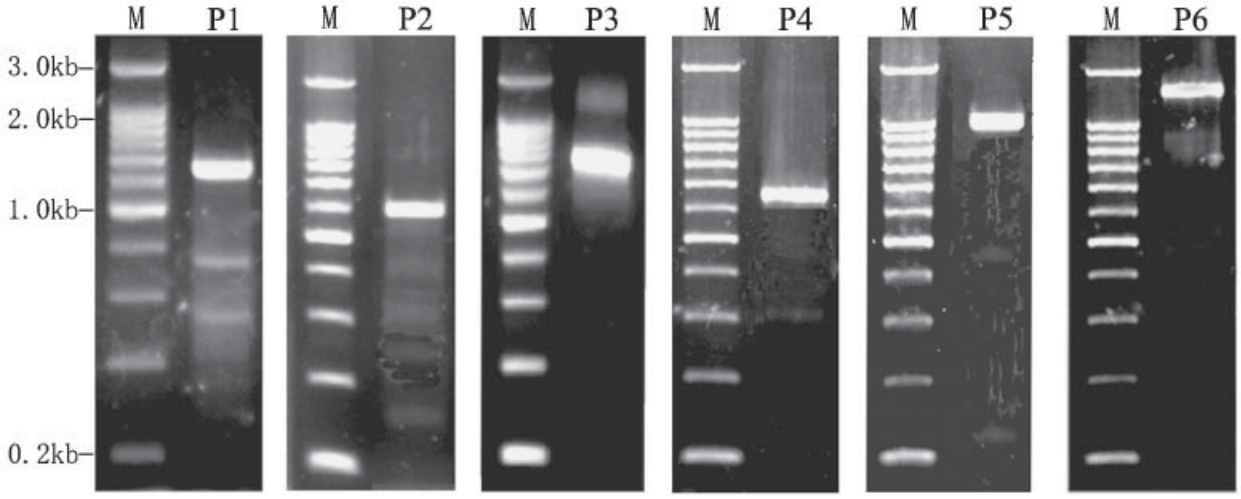

Fig. 1. Amplified products of CYP6AX1 and CYP6AY1 by 5′ and 3′ rapid amplification of cDNA ends (5′, 3′ RACE) and long distance-polymerase chain reaction (LD-PCR). The products were separated on a 0.8% agarose gel and stained with ethidium bromide. M: 200-bp DNA ladder; P1: 5′ RACE product of CYP6AX1; 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 85-bp 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),

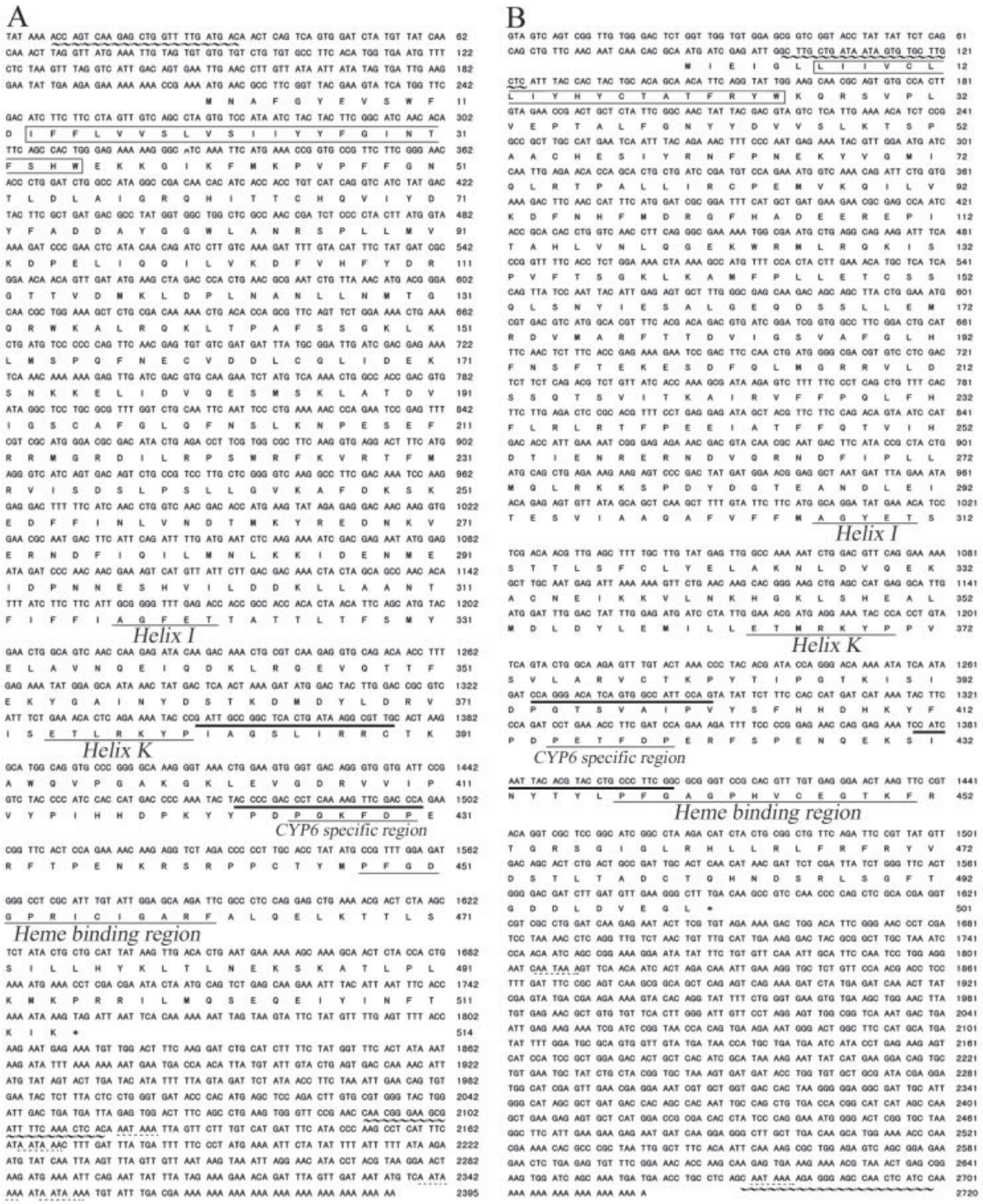

Fig. 2. Nucleotide and deduced amino acid sequences of CYP6AX1 and CYP6AY1 cDNAs from *N. lugens*. The transmembrane region in the N-terminal anchor of each predicted protein is boxed. Regions of conservation associated with the helix I, helix K, CYP6 family-specific region, and heme-binding decapeptide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

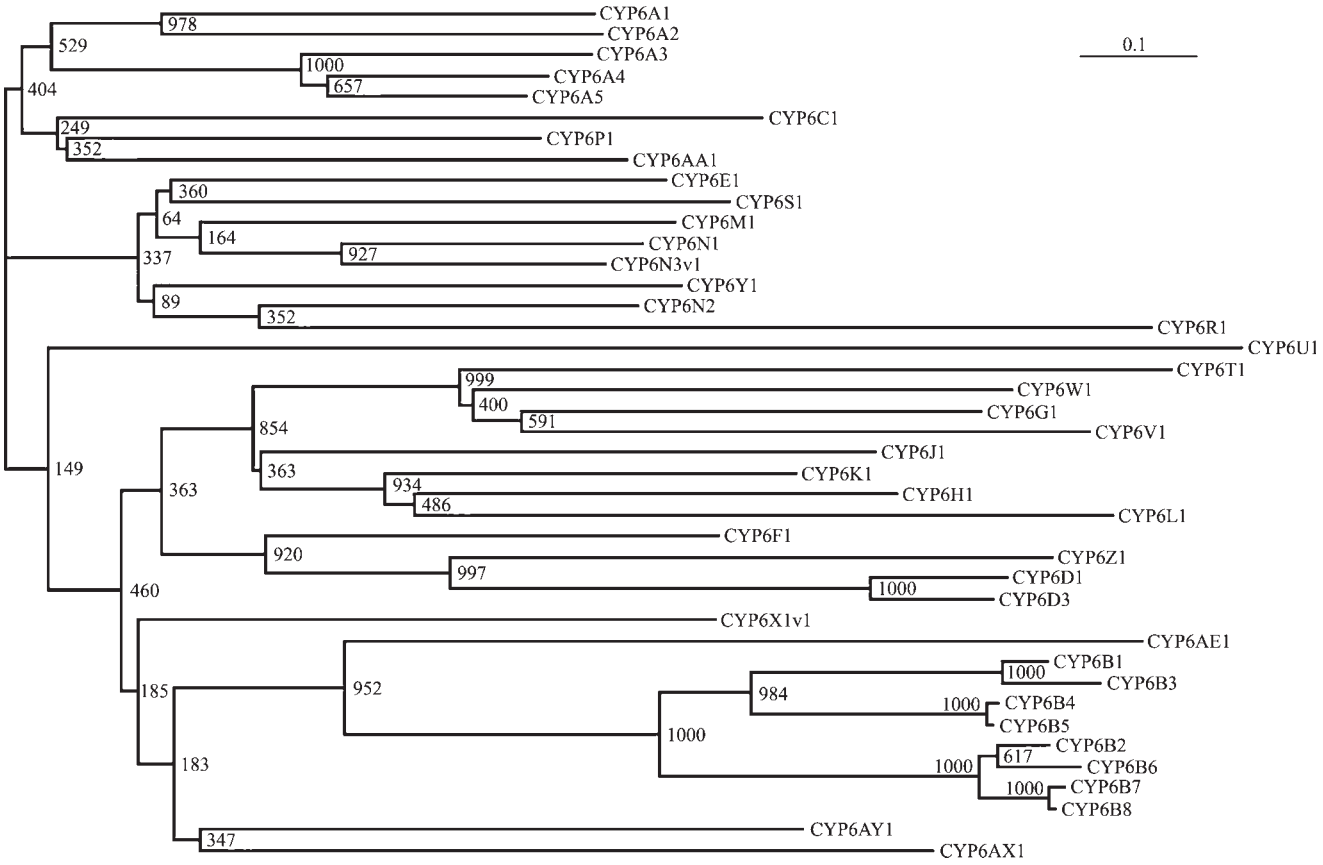

Fig. 3. Phylogenetic trees of full-length amino acid sequences of deduced CYP6AX1 and CYP6AY1 and the other insect CYP6 members are constructed by the neighbor-joining method. The bootstrap values with 1,000 trials are indicated on branches. The scale bar represents 0.05 nucleotide substitutions per 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 ingestion 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.

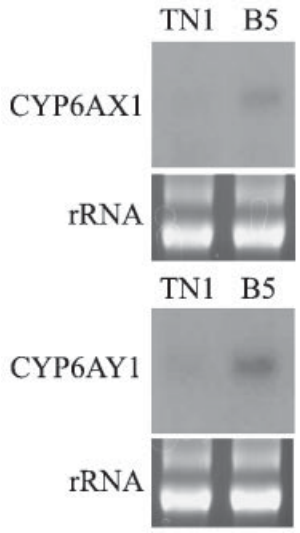

Fig. 4. Expression levels of CYP6AX1 and CYP6AY1 in *N. lugens* nymphs exposed to susceptible (TN1) or resistant plants (B5) as analyzed by northern blot 24 h after initiation of exposure. Total RNA (15 μg per lane) was separated on 1.5% formaldehyde denatured agarose gel. The blot was hybridized with the probes labelled by random primer using α-[^{32}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.

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 heme-binding 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-

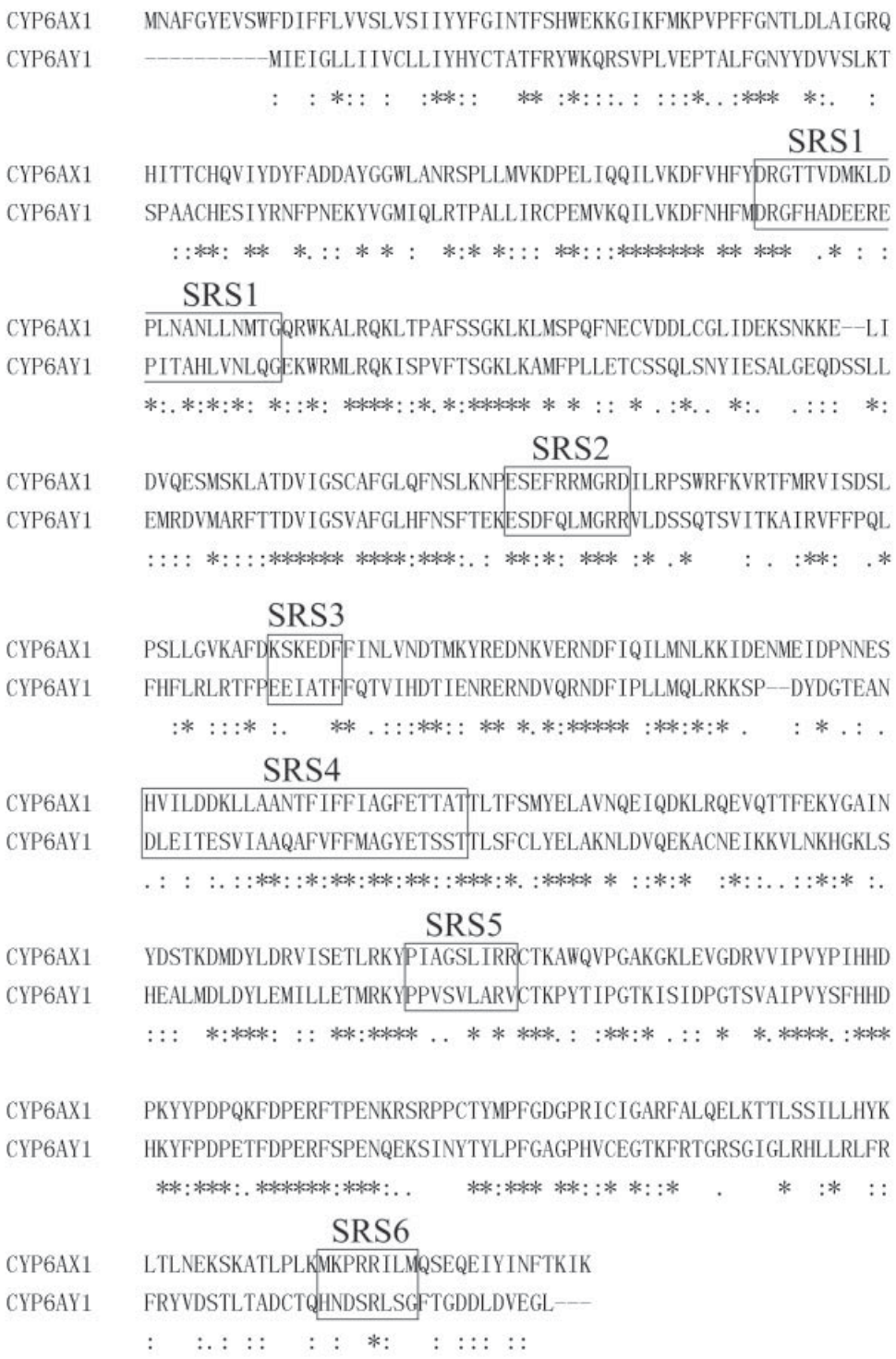

Fig. 5. Amino acid alignment of CYP6AX1 and CYP6AY1. The proposed substrate recognition sites, denoted as SRS1-6, are boxed (Gotoh, 1992). Identical amino acids are indicated by asterisks and conservative substitutions by dots.

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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