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1 **Identification of two new cytochrome P450 genes and RNA interference to evaluate their**
2 **roles in detoxification of commonly used insecticides in *Locusta migratoria manilensis***
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23

24 **ABSTRACT**

25 Cytochrome P450 monooxygenases (cytochrome P450s), found in virtually all living
26 organisms, play an important role in the metabolism of xenobiotics such as drugs, pesticides,
27 and plant toxins. We have previously evaluated the responses of the oriental migratory locust
28 (*Locusta migratoria manilensis*) to the pyrethroid insecticide deltamethrin and revealed that
29 increased cytochrome P450 enzyme activity was due to increased transcription of multiple
30 cytochrome P450 genes. In this study, we identified for the first time two new cytochrome
31 P450 genes, which belong to two novel cytochrome P450 gene families. *CYP409A1* belongs
32 to CYP409 family whereas *CYP408B1* belongs to CYP408 family. Our molecular analysis
33 indicated that *CYP409A1* was mainly expressed in fatbodies, midgut, gastric caecum, foregut
34 and Malpighian tubules of the third- and fourth-instar nymphs, whereas *CYP408B1* was
35 mainly expressed in foregut, hindgut and muscle of the insects at all developmental stages
36 examined. The expression of these two cytochrome P450 genes were differentially affected
37 by three representative insecticides, including carbaryl (carbamate), malathion
38 (organophosphate) and deltamethrin (pyrethroid). The exposure of the locust to carbaryl,
39 malathion and deltamethrin resulted in reduced, moderately increased and significantly
40 increased transcript levels, respectively, of the two cytochrome P450 genes. Our further
41 analysis of their detoxification roles by using RNA interference followed by deltamethrin
42 bioassay showed increased nymph mortalities by 21.1 and 16.7%, respectively, after
43 *CYP409A1* and *CYP408B1* were silenced. These results strongly support our notion that these
44 two new cytochrome P450 genes play an important role in deltamethrin detoxification in the
45 locust.

46

47 Keywords: Cytochrome P450; *Locusta migratoria manilensis*; gene expression; insecticides;
48 RNA interference.

49

50 **1. Introduction**

51 Cytochrome P450 monooxygenases (cytochrome P450s), found in virtually all living
52 organisms from bacteria to human (Feyereisen, 2006), are ubiquitous enzymes. These
53 enzymes constitute an extremely important metabolic system because of their involvement in
54 regulating the titers of endogenous compounds such as hormones, fatty acids, and steroids (Li
55 et al., 2007; Feyereisen, 2011). Additionally, this enzyme system plays a central role in the
56 metabolism of xenobiotics such as drugs, pesticides, and plant toxins (Scott, 2008; Schuler,
57 2011) by catalyzing oxidation reactions (Mizutani and Ohta, 2010; Nielsen and Moller, 2005).
58 In insects, cytochrome P450s play a predominant role in the metabolism of insecticides,
59 which often results in the development of insecticide resistance in insect populations (Zhou et
60 al., 2010).

61 In insects, more than 1000 cytochrome P450 genes have been identified and this
62 number is rapidly increasing due to recently increased insect genome sequences (Ai et al.,
63 2011). Most insect cytochrome P450 genes belong to microsomal CYP4, CYP6, CYP9,
64 CYP28, CYP321 and mitochondrial CYP12 families and many insect CYP genes have
65 frequently been associated with detoxification processes allowing the insect to become
66 tolerant or resistant to insecticides or host plant allelochemicals (Feyereisen, 2005; Li et al.,
67 2007).

68 The oriental migratory locust, *Locusta migratoria manilensis* (Meyen), is a typical
69 hemimetabolous insect and one of the most destructive agricultural pests in the world, due to
70 its ability to form very high populations for highly mobile swarms that lead to severe plagues
71 (Guo and Wang et al., 2011). In recent years, the destructive outbreaks of locusts had been
72 increasing in China, both in frequency and scale, possibly because of environmental changes,
73 such as warmer winters and droughts (Kang et al., 2004; Zhang et al., 2010). Synthetic
74 insecticides are often used to control the locust in management programs. However, extensive

75 applications of insecticides have inevitably resulted in the development of resistance in
76 natural populations of the locust (Ma et al., 2004).

77 Our previous studies have evaluated the effect of different insecticides, including
78 carbaryl (carbamate), malathion (organophosphate) and deltamethrin (pyrethroid), on the
79 expression of 15 cytochrome P450-like genes in *L. migratoria migratoria* (Guo and Zhang et
80 al., 2011). We have found that the increased cytochrome P450 enzyme activity is likely due
81 to increased transcription of multiple cytochrome P450 genes in response to deltamethrin
82 exposures, whereas malathion and carbaryl did not have significant effect on cytochrome
83 P450 deethylation activity. However, the effect of carbaryl and malathion on the expression
84 of each of the cytochrome P450 genes at the transcriptional level has not been determined
85 and the specific detoxification functions of these genes are still elusive.

86 In this paper, we report two novel cytochrome P450 genes (*CYP409A1* and *CYP408B1*)
87 identified from *L. migratoria manilensis* and their detoxification roles by using RNA
88 interference (RNAi) to silence each of the two genes followed by insecticide bioassays. Our
89 findings shed new light on functional importance of each of these two genes in the
90 detoxification of insecticides. Such an approach may be applicable to other detoxification
91 genes in other organisms exposed to various environmentally toxic chemicals.

92

93 2. Materials and Methods

94

95 *2.1. Insects*

96 Eggs of *L. migratoria manilensis* were provided by the Insect Protein Co., Ltd.
97 Cangzhou, China and were incubated in a growth chamber (MGC-350NR2, Shanghai
98 Permanent Science and Technology Co., Ltd., China) at 28±1°C and 50% relative humidity
99 (RH) with a 14:10-h light: dark photoperiod. After hatching, locust nymphs were reared on
100 fresh wheat sprouts under the same temperature and light conditions.

101

102 *2.2. cDNA cloning and sequence analysis of CYP409A1 and CYP408B1*

103 Based on the conserved motifs of cytochrome P450 genes in insects, the database of
104 expressed sequence tags (ESTs) of *L. migratoria manilensis* was analyzed using
105 bioinformatics methods. Two cDNA fragments, LMC_001998 and LMC_003797, were
106 selected for subsequent work. Specifically, the midguts of fifth-instar nymphs were used to
107 extract total RNA by using RNAiso^{TM} Plus (Takara, Dalian, China). mRNA was isolated
108 using PolyATtract® mRNA isolation systems (Promega, Madison, WI, USA). cDNA was
109 synthesized from 1 μg mRNA using the SMART^{TM} RACE cDNA amplification kit (Clontech,
110 Mountain View, CA , USA) according to the manufacturer's instructions. For amplification
111 of 3'-end and 5'-end cDNA sequences, SMART^{TM} RACE cDNA amplification kit (Clontech)
112 was applied according to the manufacturer's protocol. Amplified products from each reaction
113 were purified using Gel Mini purification kit (Tiangen, Beijing, China), and the isolated
114 amplification products were quantified and subcloned into pGEM-T easy vector (Promega)
115 and then sequenced.

116 To confirm that the sequences generated by RACE-PCR were from the same gene, the
117 full-length cDNA was amplified using gene-specific primers complementary to the 5'- and
118 3'- ends of the cDNA sequence using first-strand cDNA as template. The PCR primer
119 sequences are shown in the Supplementary Information. The following cycling parameters
120 were used: 94 °C for 1 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C
121 for 2 min, followed by a final extension step of 72 °C for 7 min. PCR products were purified
122 by 1% agarose gel, subcloned into pGEM-T easy vector (Promega) and then sequenced in
123 both directions by Beijing AuGCT Biotechnology Co., Ltd (Beijing, China).

124

125 *2.3. Sequence characterization and phylogenetic analysis*

126 The prediction of the open reading frames (ORFs) and the translation of the cDNA

127 sequences into amino acid sequences were performed using the translation tool in ExPaSy
128 (http://www.expasy.org/tools/dna.html). The molecular mass and isoelectric point (pI) were
129 predicted based on their amino acid sequences. The deduced amino acid sequences of the two
130 new locust cytochrome P450 genes (*CYP409A1* and *CYP408B1*) were aligned with *CYP6H1*
131 (AF115777), the only known cytochrome P450 gene of the locust, and *CYP408A1*
132 (GQ911996.1) of *Nilaparvata lugens* by using GENEDOC software (Nicholas et al., 1997)
133
134 *2.4. Analysis of tissue- and stage-dependent expression patterns*
135 Total RNA from various tissues and developmental stages of the locust were prepared
136 by using RNAisoTM Plus (Takara). Tissues used for RNA extraction were foregut, midgut,
137 gastric caecum, hindgut, Malpighian tubules, fatbodies, muscles, ovary and testis from
138 fifth-instar nymphs. Total RNA were collected from seven developmental stages, including
139 eggs, first-, second-, third-, fourth-, and fifth-instar nymphs and adults; all were 3-day old of
140 their developmental stages. To remove potential genomic DNA contaminations, the extracts
141 were treated with RNase-free DNase I (Promega). Subsequently, the first-strand cDNA was
142 synthesized from 4 μg RNA with an oligo(dT) primer using MLV reverse transcriptase
143 (Takara).
144 The sequences of the primers used for semi-quantitative PCR analysis are shown in the
145 Supplementary Information. The PCR amplifications were carried out in a final volume of a
146 25-μL reaction mixture containing 2 μL of 10× diluted template cDNA, 12.5 μL Taq Master
147 Mix (Tiangen), 0.3 μL (10 μM) of each primer, and sterilized water to reach the final volume.
148 *β-actin* was used as a reference gene for its implication with the same templates. The
149 experiment was repeated with three biological replications. The thermal cycling profile
150 consisted of initial denaturation at 94 °C for 1 min, 28 cycles (22 cycles for *β-actin*) of 94 °C
151 for 30s, 60 °C for 30s, and 72 °C for 45s, followed by a final extension step of 72 °C for 5
152 min. Aliquots of 7.5-μL PCR products were analyzed on 2% agarose gel.

153
154 *2.5. Insecticide exposures*

155 Three insecticides, carbaryl, deltamethrin and malathion, each with three doses (LD_{10},
156 LD_{30} and LD_{50}), were used in this study as described by Guo and Zhang et al. (2011). In each
157 treatment, 15-20 third-instar nymphs were topically applied with 3 μL of each dose or
158 acetone (control) in the abdomen between the second and third sterna. Each treatment was
159 repeated three times. After 24 h, surviving locusts were quickly frozen in liquid nitrogen for
160 subsequent experiments.

161 For time-dependent study, four time points (6, 12, 24 and 48 h) were used to examine
162 the effect of each insecticide on the expression of *CYP409A1* and *CYP408B1* in the locust
163 after exposed to each of three insecticides at the dose of LD_{15}. The locusts treated with
164 acetone were used as controls. After the treatments, the surviving locusts were collected to
165 determine the expression of the two genes at each time point. Three biological replicates were
166 used for each treatment.

167
168 *2.6. Real-time quantitative PCR (qPCR) analysis*

169 The transcript levels of *CYP409A1* and *CYP408B1* were quantified by qPCR using a
170 Biosystems 7300 real-time PCR system (Applied Biosystems Inc, Foster, CA, USA) and
171 SYBR® Premix Ex Taq TM II kit (Takara). The thermal cycling profile consisted of initial
172 step at 95 °C for 10s followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. The primers
173 were the same as those used for semi-quantitative PCR analyses. The PCR mixture (20 μL)
174 contained of 10 μL SYBR GREEN PCR mix (Takara), 0.4 μL ROX reference Dye II, 0.8 μL
175 of each primer, and 2 μL of 1:20 diluted cDNA templates, according to the manufacturer's
176 instructions. Each qPCR experiment consisted of three independent biological replicates,
177 each with two technical replicates. *β-actin* was used as a reference gene to normalize the
178 target gene expression levels among the samples.

179

180 *2.7. Functional analysis of CYP409A1 and CYP408B1 by RNAi*

181 To explore biological functions of *CYP409A1 and CYP408B1*, RNAi was performed by
182 injecting sequence-specific dsRNA to second-instar locust nymphs followed by insecticide
183 bioassay. In order to obtain specific RNAi effects for each target gene, we designed PCR
184 primers for dsRNA syntheses based on the sequences of two different domains of *CYP409A1*
185 and *CYP408B1*, where showed low similarities between the two genes. Templates for *in vitro*
186 transcription reactions were prepared by PCR amplification using dsRNA synthesis primers
187 of *CYP409A1 and CYP408B1* as shown in the Supplementary Information. The PCR
188 products of *CYP409A1* and *CYP408B1* were subcloned and sequenced to confirm their
189 identities. Then the expected fragments were examined by 1% agarose gel and excised and
190 purified with Wizard® SV gel and PCR clean-up system (Promega). After the concentration
191 of the purified fragments was determined by a SpectraMax 190 microplate reader and
192 SOFTmax software (Molecular Devices, Sunnyvale, CA, USA), they were used for *in vitro*
193 transcription with T7 RNA polymerase. Double-stranded RNA *(*dsRNA) was synthesized
194 using T7 RiboMAX™ Express RNAi System (Promega) following the manufacturer's
195 instructions. The synthesized dsRNAs were dissolved in nuclease-free water, and examined
196 by 1.5% agarose gel. The final concentration of dsRNA was adjusted to 1.5 μg/μL.

197 Second-instar nymphs (2-day old) were used for dsRNA injection experiments. In each
198 treatment or control, each of 15 nymphs was injected with 2 μL dsRNA (3 μg/insect) of each
199 gene or nuclease-free water (control) into the abdomen between the second and third
200 abdominal segments using a microinjector (Ningbo, China). Because our preliminary studies
201 did not show any visible difference between the locusts injected with 2 μL of the green
202 fluorescent protein (GFP) dsRNA and nuclease-free water (Zhang et al., 2010), the locusts
203 injected with deionized water were used as negative controls for our RNAi experiment. Each
204 treatment or control was repeated three times. To assess the transcript level of each gene in

205 the dsRNA-injected or control locusts, the whole body of the nymphs was used for

206 subsequent RNA extraction. For each group, five nymphs were used for RNAi efficiency test

207 at each of three different time points (12, 24 and 48 h) after injection by using

208 semi-quantitative PCR and qPCR as described in Section 2.4 and 2.6.

209 For insecticide bioassays after RNAi, 60 nymphs from each dsRNA-injected group or

210 the control group at the time point showing highest RNAi efficency were separated into three

211 subgroups as replicates and were topically applied with 3 μL of each insecticide solution to

212 the abdomen between the second and third sterna. The mortalities of the treated nymphs were

213 assessed at 24h after the insecticide treatment.

214

215 *2.8. Statistical analysis*

216 The gene expression data were analyzed by using unpaired Student's *t*-test and one-way

217 analysis of variance (ANOVA) in combination with a Fisher's least significant difference

218 (LSD) multiple comparison test by using the SPSS statistics program (Chicago, IL, USA). All

219 data were expressed as mean ±SE and statistical differences were considered significant at

220 $P<0.05$.

221

222 **3. Results**

223

224 *3.1. Identification of two new cytochrome P450 genes in* L. migratoria manilensis

225 Two full-length cDNAs putatively encoding cytochrome P450 proteins were sequenced

226 by using 5' and 3' RACE with the primers designed based on the two putative cytochrome

227 P450 cDNA fragments from LocustDB. These cytochrome P450 genes were named

228 *CYP409A1* and *CYP408B1* (GenBank accession numbers: HM153425 and HM153426,

229 respectively) by the P450 Nomenclature Committee (Dr. D. Nelson, personal

230 communication). The cDNA sequences of *CYP409A1* and *CYP408B1* have open reading

231 frames of 1548 and 1536 nucleotides encoding proteins of 516 and 512 amino acid residues,
232 respectively.

233 Based on the translated amino acid sequence, *CYP409A1* has a theoretical pI value of
234 7.14 and molecular mass of 58485.5 Da. It is the first member of CYP409 family in locust.
235 The longest ORF began with the first ATG codon at position 159 and ended with a TGA
236 termination codon at nucleotide (nt) 1707 (Fig. 1A). A polyadenylation signal, AATAAA,
237 was present in the 3' untranslated region. The 5'-UTR and 3'-UTR of *CYP409A1* were 158
238 and 188 bp, respectively. The heme-binding sequence motif was FGLGARTCLG by amino
239 acid residues 450-460. Other conserved motifs of the cytochrome P450 superfamily were also
240 identified.

241 The putative protein of *CYP408B1* showed a predicted molecular mass of 57152.6 Da
242 with a theoretical pI of 6.09. Further analysis of the *CYP408B1* nucleotide sequence indicated
243 that the start codon, ATG, was located at positions 62-64 and the termination codon, TGA, at
244 positions 1,598-1,600 (Fig. 1B). The 5'-UTR and 3'-UTR of *CYP408B1* were 61 and 317 bp,
245 respectively. Although the putative polyadenylation signal, AATAAA, was not shown in its
246 cDNA sequence, it does not necessarily indicate the lack of such a signal sequence. Because
247 the mRNA that we used to synthesize the cDNA was isolated by using PolyATtract® mRNA
248 isolation systems, its cDNA sequence is expected to contain a poly(A) sequence. Thus, the
249 lack of a polyadenylation signal in the Cyp408B1 cDNA sequence could be due to a long
250 3'-UTR, which make it difficult to be obtained by 3'-RACE. In addition, this family is special
251 in that it does not have a Cys at the heme signature region F××G×R×C×G, but has a sequence
252 of FGLAGSNTAN. The sequence motif of P××F×P and a K-helix motif E××R××P were also
253 identified.

254 *CYP409A1* and *CYP408B1* share only 19% identity in their deduced amino acid
255 sequences with each other but 34 and 21% with that of *CYP6H1*, the only known cytochrome

256 P450 gene of the locust (Fig. 2). On the other hand, *CYP408B1* is relatively similar to a *N.*
257 *lugens* cytochrome P450 gene, *CYP408A1* (GQ911996.1) with an amino acid identity of 38%.
258 Although its overall identity to *CYP408A1* is less than 40%, they are consigned to the same
259 family because of its conserved sequence in heme signature region (Dr. D. Nelson, personal
260 communication).
261
262 *3.2. Tissue dependent expression patterns of CYP409A1 and CYP408B1*
263 The relative expression profiles of the *L. migratoria manilensis CYP409A1* and
264 *CYP408B1* genes were determined in different tissues of the fifth-instar nymphs by using
265 semi-quantitative PCR (Fig. 3A). *β -actin* was used as an internal reference gene. The
266 relatively high expression of *CYP409A1* was observed in the fatbodies, followed by the
267 midgut, gastric caecum, foregut and Malpighian tubules of the nymphs, but its expression
268 was relatively low in the hindgut, muscle, ovary and testis. In contrast, the expression of
269 *CYP408B1* was relatively high in the foregut, hindgut and muscle, low in the ovary, testis,
270 Malpighian tubules and fatbodies, and lowest in the midgut and gastric caecum of the
271 nymphs.
272
273 *3.3. Stage-dependent expression patterns of CYP409A1 and CYP408B1*
274 The expression patterns of *CYP409A1* and *CYP408B1* at different developmental stages
275 of the locust were examined in whole body by using semi-quantitative PCR (Fig. 3B). The
276 expression was relatively high in the third- and fourth-instar nymphs and low in the
277 first-instar nymphs, but no expression was detected in eggs. Furthermore, the expression
278 pattern of *CYP408B1* was different from that of *CYP409A1*. Specifically, *CYP408B1*
279 transcript can be detected and remained to be highly expressed in all developmental stages.
280
281 *3.4. Expression response of CYP409A1 and CYP408B1 to insecticide exposures*
282 All the three insecticides influenced the expression of *CYP409A1* and *CYP408B1*,

283 ranging from induction to repression (Fig. 4). Deltamethrin at all tested concentrations
284 increased gene expression of *CYP409A1* and *CYP408B1* by 1.22- to 2.86-fold and 1.12- to
285 1.69-fold ($P < 0.05$), respectively. The maximum effect of deltamethrin on the expression of
286 the two cytochrome P450 genes occurred at the dose of LD_{30} (2.4 ng per gram of body
287 weight). However, similar treatments with malathion and carbaryl show different results.
288 Exposures of the insects to malathion at the concentrations of LD_{10}, LD_{30} and LD_{50} for 24 h
289 did not show significant effect on the expression of the two genes. However, exposures of the
290 insects to carbaryl at the concentration of LD_{50} for 24 h suppressed the expressions of
291 *CYP409A1* by about 65% and *CYP408B1* by 37%.

292 In contrast, the transcript levels of *CYP409A1* and *CYP408B1* varied significantly
293 among different insecticide exposure times (Fig. 5). For example, the two genes were induced
294 at various levels in deltamethrin and malathion treated locusts as compared with acetone
295 treated control locusts. Significant increases (1.4- to 3.0-fold for *CYP409A1* and 2.0- to
296 2.7-fold for *CYP408B1*) were observed when the locusts were treated with deltamethrin at all
297 the exposure times. Malathion at the concentration of LD_{15} induced the expression of
298 *CYP409A1* at the exposure times of 6 h (2.0-fold), 12 h (2.2-fold) and 24 h (1.5-fold) but did
299 not significantly induce its expression at 48 h. However, malathion induced the expression of
300 *CYP408B1* at the exposure times of 6 h (1.6-fold), 24 h (1.6-fold) and 48 h (1.7-fold) but did
301 not significantly induce its expression at 12 h ($P < 0.05$). However, carbaryl at the
302 concentration of LD_{15} did not show significant effect on the expression of the two
303 cytochrome P450 genes in *L. migratoria manilensis* (Fig. 5).

304

305 *3.5. Functional analysis of CYP409A1 and CYP408B1 by RNAi*

306 To evaluate the RNAi efficiency of *CYP409A1* and *CYP408B1*, the corresponding
307 sequence-specific dsRNA for *CYP409A1* and *CYP408B1* were synthesized *in vitro* and
308 injected into the second-instar nymphs of the locust. Semi-quantitative PCR and qPCR

309 analyses at different time points (12, 24 and 48h) after the injection of dsRNA for each target
310 gene showed significantly decreased transcript levels of *CYP409A1* and *CYP408B1* (Fig. 6A
311 and B). The different expression levels of *CYP409A1* at different time points after injection of
312 ddH_2O were observed, suggesting its possible development-related expression changes. The
313 transcript levels of *CYP409A1* and *CYP408B1* were reduced by about 99% in the nymphs
314 injected with respective dsRNA as compared with those in the controls at 24 h. These results
315 indicate an extremely high efficiency of silencing these two cytochrome P450 genes by
316 RNAi.
317 As both the *CYP409A1* and *CYP408B1* transcripts were significantly repressed in the
318 locusts by RNAi, we assessed the susceptibility of the dsRNA-injected locusts to different
319 insecticides. The mortalities of the locusts injected with nuclease-free water (control), dsRNA
320 of *CYP409A1* and dsRNA of *CYP408B1* after exposed to deltamethrin at the dose of 1.0 ng
321 per gram of body weight were 22.9, 44.0 and 39.6%, respectively (Fig. 6C). These results
322 represent an increased mortality of the locusts to deltamethrin by approximately 2-fold after
323 the repression of each of the two cytochrome P450 genes by RNAi. In contrast, similar
324 treatments with malathion and carbaryl in the locusts after RNAi for these genes did not show
325 significant effects on the susceptibility of the locusts to these insecticides (data not shown).
326
327 **4. Discussion**
328 In insects, cytochrome P450s enzymes are known to be involved in the metabolism of
329 plant allelochemicals and insecticides, resulting in bioactivation or detoxification of these
330 compounds (Feyereisen, 1999). Therefore, identification and characterization of new
331 cytochrome P450 genes have become a very attractive research area. To date, however, only a
332 single cytochrome P450 gene (*CYP6H1*) from *L. migratoria manilensis* has been reported.
333 This gene has been functionally characterized as a microsomal ecdysone 20-hydroxylase of

334 the locust (Winter et al., 1999). By searching the EST database of *L. migratoria manilensis*,
335 sequencing the cDNAs after RACE-PCR and analyzing the cDNA and deduced amino acid
336 sequences, we obtained the full-length cDNA sequences of two new cytochrome P450 genes
337 (*CYP409A1* and *CYP408B1*). Phylogenetic analysis showed that these two genes were
338 clustered in the CYP3 clade that mainly includes CYP6 and CYP9 gene families that are
339 known to play important roles in xenobiotic metabolism and insecticide resistance, and are
340 inducible by phenobarbital, pesticides and natural products (Feyereisen, 2006). The CYP6
341 genes in this clade are related to the vertebrate CYP3 and CYP5 families. By using BLASTp,
342 we found that CYP408B1 is very closely related to CYP3 family from mammals (Feyereisen,
343 2006). *CYP408B1* belongs to the family with a slightly different but conserved sequence in
344 heme signature region and missing domain WxxxR. Although the lack of the CYS residue
345 and domain WxxxR may affect the function of these enzymes, our RNAi experiment clearly
346 indicated that *CYP408A1* should be functional.
347 It has been known that the expression profiles of cytochrome P450 genes are highly
348 diverse in insects (Scott and Wen, 2001). Because different developmental stage or
349 tissue-specific expression patterns of cytochrome P450 genes in animals may imply their
350 specific functions (Chung et al., 2009), we used semi-quantitative PCR to analyze expression
351 patterns of the two genes in different tissues of the fifth-instar nymphs and in different
352 developmental stages. *CYP409A1* was mainly distributed in midgut and Malpighian tubules,
353 whereas *CYP408B1* mRNA was detected in foregut, hindgut and muscle of nymph and
354 mainly in the fifth-instar nymphs. Several studies have demonstrated that the midgut of
355 insects to be one of the major organs involved in detoxification of xenobiotics (Cohen et al.,
356 1992; Snyder et al., 1995). Our results of tissue-specific expression patterns are consistent
357 with this notion for *CYP409A1* but not so much for *CYP408B1*.
358 Furthermore, we found that the expression of *CYP409A1* was higher in fourth-instar

359 nymphs than the locusts of other developmental stages. In particular, we did not detect the
360 expression of *CYP409A1* in eggs. The gradually increased expression of this gene from the
361 egg to adult may imply an adaptive ability of the insect to metabolize xenobiotics upon
362 exposure (Gong et al., 2005). On the other hand, however, the expression of *CYP408B1* was
363 detected at high levels in all life stages examined, suggesting that this gene probably plays
364 other roles in insect physiology. Indeed, although cytochrome P450 enzyme can generally
365 metabolize many different substrates, individual cytochrome P450 enzyme has multiple roles
366 during insect development (Feyereisen, 1999).
367 The induction of cytochrome P450 enzymes by various chemicals has been reported in
368 a number of insect species (Fuchs et al., 1994). Indeed, the normal regulatory network would
369 be changed through mutations for an inducible cytochrome P450 gene, which could cause
370 higher constitutive expression of the gene, and therefore lead to resistance (Le Goff et al.,
371 2006). Thus, the inducibility of a detoxification enzyme by xenobiotics may represent a risk
372 factor for developing resistance to insecticides in insect populations. Nevertheless, due to
373 their toxicity, insecticides generally are unlikely to cause the induction (Ranasinghe et al.,
374 1997). For example, a study in *D. melanogaster* showed that six chemically distinct
375 insecticides did not induce the expression of cytochrome P450 genes with an exception of
376 DDT. Even with DDT, only a marginal induction of a single cytochrome P450 gene was
377 observed (Willoughby et al., 2006).
378 In our study, we found significantly different responses of *CYP409A1* and *CYP408B1* at
379 the transcriptional level to the exposures of *L. migratoria manilensis* to three different
380 insecticides including malathion (organophosphate), carbaryl (cabamate) and deltamethrin
381 (pyrethroid). Deltamethrin significantly induced the expression of the two cytochrome P450
382 genes at 12 h and LD_{30}, which is in agreement with the maximum induction of the
383 cytochrome P450 enzyme activity by the same insecticide as observed in other insect species

384 (Fisher et al., 2003; Scott et al., 1996; Stevens et al., 2000). However, the induction is less
385 pronounced at LD_{50}. Under such a high concentration, deltamethrin could play an important
386 role in intoxication rather than induction. Furthermore, the induction of cytochrome P450
387 enzyme activity by deltamethrin appears to be due to the increased expression of its gene
388 through a mechanism that is largely controlled at the transcriptional level (Batard et al., 1997;
389 Gong et al., 2005). If the two deltamethrin-inducible genes *CYP409A1* and *CYP408B1* are
390 involved in the detoxification of the same or other insecticides, such an induction could lead
391 to an elevated tolerance to these insecticides, which consequently contributes to a difficulty in
392 controlling *L. migratoria manilensis* in the field.
393 Malathion appeared to induce the expressions of *CYP409A1* and *CYP408B1* when its
394 concentration was relatively low (LD_{15}) (Fig. 5). However, the induction status and level in
395 malathion-treated *L. migratoria manilensis* were relatively less consistent than those in
396 deltamethrin-treated insects among the different treatments. These inconsistencies are
397 probably due to relatively low levels of the induction in malathion-treated *L. migratoria*
398 *manilensis*. In contrast, carbaryl appeared to repress the expressions of *CYP409A1* and
399 *CYP408B1* at high concentration (LD_{50}) (Fig. 4). Generally, however, carbaryl at low
400 concentration (LD_{15}) did not affect the expression of *CYP409A1* and *CYP408B1* (Fig. 5).
401 Although our results were based on studies with three commonly used insecticides
402 which belong to three different major classes of insecticides, we should not make any
403 assumption that other insecticides within the same class have similar abilities to induce the
404 orthologs of these cytochrome P450 genes in other insect species. Furthermore, although it
405 has been suggested that the induction profiling of insect detoxification enzymes could serve
406 as a means to identifying the major enzymes involved in insecticide detoxification (Poupardin
407 et al., 2008), the results based on our induction studies can not pinpoint whether or not these
408 two cytochrome P450 genes are involved in insecticide metabolism, as experienced by other

409 researchers. This is mainly due to the diversity, rapid evolution, and little information about

410 the substrate specificity of cytochrome P450 enzymes (Willoughby et al., 2006).

411 To clarify whether or not the two deltamethrin-inducible genes *CYP409A1* and

412 *CYP408B1* are involved in the detoxification of deltamethrin, we performed RNAi to silence

413 each of the two genes by injecting sequence-specific dsRNA to second-instar locust nymphs

414 followed by deltamethrin bioassay. The mortalities of the locusts injected with nuclease-free

415 water (control), dsRNA of *CYP409A1* and dsRNA of *CYP408B1* after exposed to

416 deltamethrin at the dose of 2.0 ng per gram of body weight were 22.9, 44.0 and 39.6%,

417 respectively. Thus, our results showed increased mortality of the locusts after each of the two

418 cytochrome P450 genes was silenced by RNAi. These two new cytochrome P450 genes are

419 likely to be involved in deltamethrin detoxification in the locust.

420 In summary, we identified and characterized two new cytochrome P450 genes, which

421 belong to two novel families of the cytochrome P450 gene superfamily, from a major

422 agricultural insect pest *L. migratoria maniensis*. *CYP409A1* belongs to the CYP409 family

423 whereas *CYP408B1* belongs to the CYP408 family. Our findings of these highly evolved

424 detoxification genes in *L. migratoria manilensis* suggest that there may be more extensive

425 diversification within the cytochrome P450 superfamily in organisms (Sasabe et al., 2004).

426 We further demonstrated that both *CYP408B1* and *CYP409A1* were involved in deltamethrin

427 detoxification in the locust by using RNAi for each of the two genes followed by

428 deltamethrin bioassay. Our study may help us better understand functions of the insect

429 cytochrome P450 genes and their interactions with pesticides at molecular levels and provide

430 researchers with very much needed genetic information to assess potential consequences of

431 insecticide exposures in insects and other organism.

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441 manuscript preparation.

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560 **Figure Legends:**

561

562 **Fig. 1.** Nucleotide and deduced amino acid sequences of the two cytochrome P450 cDNAs of

563 *L. migratorria manilensis*. The start codon (ATG) and stop codon (TGA) are highlighted in

564 black. The two cytochrome P450 signature motifs (W×××R, E××R××P and P××F×PERF) are

565 highlighted in blue, and the heme signature regions F××G×R×C×G and FGLAGSNYAN are

566 underlined in black. The sequences were deposited in the GenBank (accession numbers:

567 HM153425 and HM153426).

568

569 **Fig. 2.** Comparison of amino acid sequences of *CYP409A1* (HM153425), *CYP408B1*

570 (HM153426) and *CYP6H1* (AF115777) from *L. migratorria manilensis* and *CYP408A1*

571 (GQ911996.1) of *Nilaparvata lugens* using GENEDOC. Several conserved motifs of these

572 cytochrome P450 proteins are boxed.

573

574 **Fig. 3.** Expression profiles of *L. migratoria manilensis CYP409A1* and *CYP408B1* genes in

575 different tissues of the fifth-instar nymphs and different developmental stages in the whole

576 body as evaluated by semi-quantitative PCR. (A) Their expression patterns were examined in

577 seven different tissues including foregut (FG), midgut (MG), gastric caecum (GC), hindgut

578 (HG), Malpighian tubules (MT), fatbody (FB), muscles (MC), ovary (OV) and testis (TE). (B)

579 Their expression patterns were examined in seven different developmental stages including

580 egg (EG); first-instar (N1), second-instar (N2), third-instar (N3), fourth-instar (N4) and

581 fifth-instar (N5) nymphs; and adult (AD). *β -actin* was used as an internal reference gene.

582

583 **Fig. 4.** Effect of three insecticides (deltamethrin, malathion and carbaryl) on the expression of

584 *CYP409A1* and *CYP408B1* after the locust nymphs treated with each insecticide at three

585 different doses and analyzed at 24 h by qPCR. Three doses (LD_{10}, LD_{30} and LD_{50}) used for
586 each insecticide are: 1.2, 2.4 and 3.6 ng g^{-1} (nanogram per gram of body weight) for
587 deltamethrin, 60, 120 and 180 ng g^{-1} for malathion, and 300, 600 and 900 ng g^{-1} for carbaryl.
588 The relative level of gene expression shown in Y axis is the ratio of the gene expression in
589 each treatment in comparison with that of control in which acetone alone was used to treat
590 insects. The dashed line shows the relative activity for the control. Vertical bars indicate
591 standard errors of the mean (n=3). One or two asterisks on the standard error bar indicate
592 significant difference between the mean of the treatments with a particular insecticide for 24
593 h and the mean of the control at $P<0.05$ or $P<0.01$, respectively, based on unpaired Student's
594 *t*-test. Because the same control was used for the comparisons, it was normalized as 1.0
595 (shown by the dash line). Different letters on the bars indicate that the means are significantly
596 different among the three exposure doses of the same insecticide based on Fisher's LSD
597 multiple comparison test ($P< 0.05$).
598
599 **Fig. 5.** Effect of three insecticides (deltamethrin, malathion and carbaryl) on the expression of
600 *CYP409A1* and *CYP408B1* after the locust nymphs treated with each insecticide at the LD_{15}
601 concentration and analyzed at four different time points (6, 12, 24 and 48 h) using qPCR. *β*
602 *-actin* was used as an internal reference gene. Vertical bars indicate standard errors of the
603 mean (n=3). One or two asterisks on the standard error bar indicate significant difference
604 between the mean of the treatment with a particular insecticide and the mean of the control at
605 $P<0.05$ or $P<0.01$, respectively, within the same exposure time point based on unpaired
606 Student's *t*-test. Different letters on the bars indicate that the means are significantly different
607 among the four exposure times of the same insecticide treatment or the control based on
608 Fisher's LSD multiple comparison test ($p < 0.05$).
609

610 **Fig. 6.** Changes in the transcript levels of *CYP409A1* and *CYP408B1* after the locust nymphs
611 were injected with their corresponding dsRNA. The 2nd-instar nymphs of 2-day old were
612 used for injection experiments. RNA was extracted and quantified by semi-quantitative PCR
613 and qPCR at 12, 24 and 48h. Control nymphs were injected with equivalent volumes of
614 nuclease-free water. Different letters next to the standard deviation bars indicate statistically
615 significant differences in gene transcript levels between dsRNA treated and control nymphs
616 (t-test, $P<0.05$). (A and B) The transcript of *CYP409A1*/*CYP408B1* was examined by both
617 semi-quantitative PCR and qPCR. (C) Changes in the susceptibility of the locusts to
618 deltamethrin after the injection of *CYP409A1*/*CYP408B1* dsRNA in 2nd-instar nymphs. The
619 control locusts were injected with the same volumes of deionized water. Deltamethrin
620 bioassays were conducted 24 h after the injections by topical application. The mortalities of
621 the locusts were assessed 24 h after the deltamethrin treatments at the dose of 1.0 ng per gram
622 of body weight. Results are mean and standard errors of three biological replications ($n = 3$).
623 An asterisk next to the standard deviation bars indicate significant differences in the
624 mortalities among the control, *CYP409A1* or *CYP408B1* dsRNA based on LSD multiple
625 comparison test ($P<0.05$).
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637 Fig. 1.

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                                                                         ACGCGGGAGTGGTGTGGGGACGTCCGCCGGCTGGTACA     38
CGCTGTTGCTATCGCTACTGCTGCGAGACTGTCCATTACCTCATCACTGCCTGGGACCGCAGGGCCAGTTGTCTACGCCTGTCAGGCAGAAGGGCGTGGGAAACAGTCCATCCATCCATC    158
ATGGCGGTCGACTGGTATACCACGGGCGCCCTGGTTGTGTTAGCTGCGTGGCTACTATGGAAGTACCTGTCATGGAACTACGGCTACTGGCAACGTTTGGGAGTGCCCTGTATCGAACCT    278
M   A   V   D   W   Y   T   T   G   A   L   V   V   L   A   A   W   L   L   W   K   Y   L   S   W   N   Y   G   Y   W   Q   R   L   G   V   P   C   I   E   P
TCGGTGCCTTTCGGCAATTTCAAGGACTTGTTTCTTGGAAACAAATCTCCATTAGAATCATTCGAAGAGATTCATCGCCGGTTTGATGGTGAGCCCTTCTGCGGCGTCTATCGCTTACGA    398
S   V   P   F   G   N   F   K   D   L   F   L   G   N   K   S   P   L   E   S   F   E   E   I   H   R   R   F   D   G   E   P   F   C   G   V   Y   R   L   R
CAAGCAGCGCTGTTCGTGAGAGACGCCGAGCTTTTGCGCCAGATGATGATCAGCGACTTTGCTTCCTTCCACGACAACAACAACTACGTCAACGAAGACCAAGATCCTATATTCGCAAGG    518
Q   A   A   L   F   V   R   D   A   E   L   L   R   Q   M   M   I   S   D   F   A   S   F   H   D   N   N   N   Y   V   N   E   D   Q   D   P   I   F   A   R
AATCCGTTCTTTGTAAAAGGTACAAGGTGGAAGAAAAGTCGCTCCCGGCTGACTCCGTCATTCACGGCAAGCCGCATGAAGCCCATGTTCGTGTTGATGAAGGAAGTCTGCGACACCCTG    638
N   P   F   F   V   K   G   T   R   W   K   K   S   R   S   R   L   T   P   S   F   T   A   S   R   M   K   P   M   F   V   L   M   K   E   V   C   D   T   L
GTACGAGTACTGGAACAGGAAGGACCAGAAGCGATGCCAGACGGGCTGGAGGCATGGCGCCTGTGCATGCGGTACACAACGGACGTGGTGTCGTCCTGCGCGCTGGGCGTCACGGGTCGA    758
V   R   V   L   E   Q   E   G   P   E   A   M   P   D   G   L   E   A   W   R   L   C   M   R   Y   T   T   D   V   V   S   S   C   A   L   G   V   T   G   R
ACTCTGGAGGACAAGGATTCTGTTCTGGCGGACATGTGTCGTAGGCTTCTTGCTCCAACCTTCATGACCAATTTGAAGATCGCCGTGGCTTTTACATCACCAACTTTAGCTGATGTTTTG    878
T   L   E   D   K   D   S   V   L   A   D   M   C   R   R   L   L   A   P   T   F   M   T   N   L   K   I   A   V   A   F   T   S   P   T   L   A   D   V   L
CGTATTAGGATCATGCCTCTGGACGTGCACAACTTCGTGTACAAATGGGTGACGGAGACGGTCGCGCAGAGAGAACAGGGGAACGTGCAGCGCAAGGACTACCTGCAGCTGCTGGTGGAG    998
R   I   R   I   M   P   L   D   V   H   N   F   V   Y   K   W   V   T   E   T   V   A   Q   R   E   Q   G   N   V   Q   R   K   D   Y   L   Q   L   L   V   E
CTCAAGGCCAGAGGCTACCTGGACACCGAGGGACAGGGCCCAGTCGAGAAGTCCACCTTCACGGACATGGACGTAGTGGCGCAGGTGGTAACTTTCATGTTGGATGGGACCCACACGACC   1118
L   K   A   R   G   Y   L   D   T   E   G   Q   G   P   V   E   K   S   T   F   T   D   M   D   V   V   A   Q   V   V   T   F   M   L   D   G   T   H   T   T
GCCACGGCTATGTCGTTCGCTCTTTACGAACTGGCCCTGCATCCGGACATCCAGCATCGTTTACGAGAGAACCTCCGAGAAGCAGTGGACAAGCATGGCGGCCAACTGGGATATGACTCC   1238
A   T   A   M   S   F   A   L   Y   E   L   A   L   H   P   D   I   Q   H   R   L   R   E   N   L   R   E   A   V   D   K   H   G   G   Q   L   G   Y   D   S
ATCAACGAGTGTACCTACCTCGACATGGTCCTCTCAGAGGTGCTGCGGCTGCACCCGCCCATCGGACACCTGGAGAAGAAGTGCACGGCGGCCTACCCCATGACCACCGCCTCCGGTCGC   1358
I   N   E   C   T   Y   L   D   M   V   L   S   E   V   L   R   L   H   P   P   I   G   H   L   E   K   K   C   T   A   A   Y   P   M   T   T   A   S   G   R
GCCTTCACCGTGCAGCCGGGCACCGCCGTCGTCTTCTCCATCGCAGGCATACACAGAGACCCGCGATACTTCCGCAACCCGGACGTCTTCGACCCCGAACGCTTCTCGCCCGACAACAAG   1478
A   F   T   V   Q   P   G   T   A   V   V   F   S   I   A   G   I   H   R   D   P   R   Y   F   R   N   P   D   V   F   D   P   E   R   F   S   P   D   N   K
GACCCCACCTCGATGGTGGCTTACATGCCTTTTGGATTGGGCGCTCGTACATGCTTGGGACAACGGTTCGCGCTGTCGCAGGTGAAGATGGGCGTCGCGTGCCTGGTGCTCAACTTCAGG   1598
D   P   T   S   M   V   A   Y   M   P   F   G   L   G   A   R   T   C   L   G   Q   R   F   A   L   S   Q   V   K   M   G   V   A   C   L   V   L   N   F   R
GTCTGCACCACGCCCCGGACCCCGATGCCCCTCGAGGTGGACCCCAACTCCTTCGCGAAGGCCGCCAAAGGGGGCATGTGGCTGGGCTTCCAACCCCTGGTCCACCTCTGACCGCGCAGA   1718
V   C   T   T   P   R   T   P   M   P   L   E   V   D   P   N   S   F   A   K   A   A   K   G   G   M   W   L   G   F   Q   P   L   V   H   L   *
ACAGTGCGGTTGTACTCCCGTGTAAAACAACCCAGACCTCCAGGAACATACACACTGTGCTGAGATCGAAACAACATAGCGTGTTATAATGAATGTGGTATTTCACGAATGACAGTTCGT   1838
GACAAACACTCCTAATAAAGACATTTTTCTGTAAAAAAAAAAAAAAAAAAAAAAAAAA                                                                1895
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                                                           ACGCGGGGACCGCACTACAAACGGAACCGACTGCAGCTACAGCTACAACTACAGCGGCACC     61
ATGGTGGAACTGCTCACCGCGGCCTGCGTCCTCGTGGCGCTGGTGGCCGTCGCCCTCGCTGCTGTCTACAGATACCTGACTGCGACCTTCGACTACTTCGAGAAGAGAGGCATCGTGGGC    181
M   V   E   L   L   T   A   A   C   V   L   V   A   L   V   A   V   A   L   A   A   V   Y   R   Y   L   T   A   T   F   D   Y   F   E   K   R   G   I   V   G
CCTAAGCCGCAGCCGCTCTTTGGGAACTACTACAAGCTGTGGAACAAGGTGTTCGGAGAGGAAGATGTGAAGAACGTCCACCAGTATGGAAAGGTTTTCGGGACGTTCGACGGCCGCACG    301
P   K   P   Q   P   L   F   G   N   Y   Y   K   L   W   N   K   V   F   G   E   E   D   V   K   N   V   H   Q   Y   G   K   V   F   G   T   F   D   G   R   T
CCCAACCTGGTTGTGGCGGACGCAGACCTGGCCAAGGCCATTCTGGACAAGGAGCGCGACCACTTCCGCAACAGGAGGTCTGCATCTGTCAAGAATCCTCTGGTGCAGAAGAGTCTTTCT    421
P   N   L   V   V   A   D   A   D   L   A   K   A   I   L   D   K   E   R   D   H   F   R   N   R   R   S   A   S   V   K   N   P   L   V   Q   K   S   L   S
ATTCTGGCAGGAGAAGTAAAAGGATCACCTGATGTAGTGGTAGCCCTCAGCCGTGAGAAACTTAGGAAGCTTACTCCAAGGATTCTGAAGTCACTGGAGTTTCTCACACAAAATCTTAAG    541
I   L   A   G   E   V   K   G   S   P   D   V   V   V   A   L   S   R   E   K   L   R   K   L   T   P   R   I   L   K   S   L   E   F   L   T   Q   N   L   K
AAATCTCTCGAAGCATCTGAACCAAGCATTGACATTACATACGCTGTCCGTAACTTCCTCGCTCATTCATTGGCTTTGACTCTTCTTGACAAGGATTCACAGGCAGAAGAGCCTAAGGTA    661
K   S   L   E   A   S   E   P   S   I   D   I   T   Y   A   V   R   N   F   L   A   H   S   L   A   L   T   L   L   D   K   D   S   Q   A   E   E   P   K   V
GACCAGAATGCCTTTTCTGGAACACTAGAACAAGTTCTGAAAGTTGACAATCCAACTTATCCAGTTGCATCTTTCCCATTTGTTCTTCCTGCATTTTACTCTCAAGACTGCTTTGTTCTG    781
D   Q   N   A   F   S   G   T   L   E   Q   V   L   K   V   D   N   P   T   Y   P   V   A   S   F   P   F   V   L   P   A   F   Y   S   Q   D   C   F   V   L
CGAAACAGTGCTGCCAGGTACCTTATTCCTTTAATCCATTCCAGTGTTAAGGAAAAAATCAGTGCAGAAAAGTCATCGGACGAGAAGAAAGTTCCTGATCTGGTAGACATACTGCTCGAA    901
R   N   S   A   A   R   Y   L   I   P   L   I   H   S   S   V   K   E   K   I   S   A   E   K   S   S   D   E   K   K   V   P   D   L   V   D   I   L   L   E
ACTGTTTCTGAACAGAAGCAGACAACAAAAGATGAGGGCAAAGAGGTGGAAGGTGCTTCTGCTGTGAACGAAGAAGCTCTTGTAGCTCAGTCACTAGCTGTTCTTCTGAATACAGCACAA   1021
T   V   S   E   Q   K   Q   T   T   K   D   E   G   K   E   V   E   G   A   S   A   V   N   E   E   A   L   V   A   Q   S   L   A   V   L   L   N   T   A   Q
GCAACAAAGTCGACAATTGCACTGTCTGTTGCCACATTGGCTTCCAAACCAGAGATTCAGGATAAGCTGCATTCTGAACTTAACAAACAGTTACAGACATCTAATGAGATATCCTTCGAG   1141
A   T   K   S   T   I   A   L   S   V   A   T   L   A   S   K   P   E   I   Q   D   K   L   H   S   E   L   N   K   Q   L   Q   T   S   N   E   I   S   F   E
GCAGTACAGAATGCACAGTACCTTGACATGTTCCTCAATGAGGTTCTTCGTATGTATCCATCTGAATACAGGATTGAACACGAGTGCCTGGAAAACACGGAGTTTGAGAACCTGAAAATT   1261
A   V   Q   N   A   Q   Y   L   D   M   F   L   N   E   V   L   R   M   Y   P   S   E   Y   R   I   E   H   E   C   L   E   N   T   E   F   E   N   L   K   I
GAGAAGGGAACTATAGTATCAATTCCACTGTATGCACTTCATCGTTTGGAAGACTACTATCCAGAGCCACATACTTTCAATCCAGACAGATTTTCTCCAGCCATAGCAGAAAAGCGTCAC   1381
E   K   G   T   I   V   S   I   P   L   Y   A   L   H   R   L   E   D   Y   Y   P   E   P   H   T   F   N   P   D   R   F   S   P   A   I   A   E   K   R   H
CCATACACATACCTACCATTTGGTTTGGCTGGTTCGAATACGGCTAATGTCAGTGTTCAATATGGAATGCTGGTTACTAAGTTGACTGTTGCAACACTGATAAAGAACTTTAAGTTTGTG   1501
P   Y   T   Y   L   P   F   G   L   A   G   S   N   T   A   N   V   S   V   Q   Y   G   M   L   V   T   K   L   T   V   A   T   L   I   K   N   F   K   F   V
CCAGCCGAGGAGACTCAGATACCACCACCTTTGGAAGAGGGAATCACAGGAGTCATGAAGCCAAAACCAGTGAAAATTAGTGTGGAACTTCGCAAATGAGCACGTATGAGATCACAAGGC   1621
P   A   E   E   T   Q   I   P   P   P   L   E   E   G   I   T   G   V   M   K   P   K   P   V   K   I   S   V   E   L   R   K   *
TGAAAGGAACACAGTGTATTTTTGACACCAACTAAATTAAATTTTGTATGTCATTGAGCCATTAATGTTAATACTGATATTTGTGTTTACACATTTCTAGTTTAATTTTATTTTTAATAC   1741
CACTTTTGTATTATCACATTCAAGACTTTTCAAGACTCAGTTTGCAGTTCTGATAAAATGGGAGAAATGTTTCAAGACTTTTGAAGTACAGTGTAATTATTGGTTTGACTAGATCAATTA   1861
CTTTTAAGAAGTGCCTATATTACTGAACATTCATAGAAGACATTCCACATTGCAGA                                                                  1917
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647 Fig. 2.

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LmCYP409A1 : ---MAVDWYTTGALVVLAAWLLWKYLSWNYGYWQRLGVPCIEPSVPP-GNFKDLFLGNKSPLESFEEIHR-RFDGEPECGVYRLRQAALFVRDAELLRQMMISDFA : 101
LmCYP6H1   : ---MA---EVTLALAGCALLALWVLVGWRQSYWRKRGVAC-PPGWPLLGHVLNLLLLRRSPGQFFRDLYN-QAPGEPLVGFYIFGKPAVLVRSPELLRSVLVKDFA :  98
LmCYP408B1 : MVELLTAACVLVALVAVALAAVYRYLTATFDYFEKRGIVGPKPQPLP-GNYYKLW--NKVFGE--EDVKNVHQYGKVF-GTFDGRTPNLVVADADLAKAILDKERD : 100
NlCYP408A1 : -MELQVLA-IATSVLTLALAYVYRFITSKENYFQKQGIDGPKPSFPP-GTWKGAW--KKNFGE--NDLNNVKTYGQTF-GSFDGRTPNLIVSDPHLIQKILVDEAD :  98

LmCYP409A1 : SFHDNNNYVNEDQDPIFARNPFFVKGTRWKKSRSRLTPSFTASRMKPMFVLMKEVCDTLVRVLEQEGPEAMPDG-LEAWRLCMRYTTDVVSSCALGVTGRTLEDK- : 205
LmCYP6H1   : AFADRYNISDERSDPLGTRNLFVIKGPEWKYLRAKLSPTFTSGKMRAMFPLVVR-CDQQMLDFMQSRLNARGSAELDIKEVTACCTTDVINTCAFGISSNSLANP- : 202
LmCYP408B1 : HFRNRRSASVK--NPLVQKSLSLL--AGEVKGSPDVVVALSREKLRKLTPRILKSLEFLTQNLKKS-LEA-SEPSIDITYAVRNELAHSLALTLLDKDSQAEEPKV : 200
NlCYP408A1 : KFHSNRAFRVK--QKPYMKCCALLRVVVDVEVNKLLSPALNAAKVKKLESRIAKTVDLFVEGFKKE--AA-PEKVINANEHVSREITDVLAFTLFSFDLAADN-QI : 198
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Helix C
WXXXR motif

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LmCYP409A1 : -DSVLADMCRRLLAPTFMTNLKIAVAFTSPTLADVLRIRIMPLDVHNFVYKWVTETVAQREQ----GNVQRK-DYLQLLVELKARGYL----------DTEGQGP- : 294
LmCYP6H1   : -GGEFRGLGKKTFEYDIFRSLEVLVFFFTPYLSRLLGCVYMQKDTNDFLRRVFWETLTAREQSAAAGAPQRH-DLIDHLIQLKNQGAIQADAADADDADDEDAAPA : 306
LmCYP408B1 : DQNAFSGTLEQVLKVDNPTYPVASFPFVLPAFYS-QDCFVLRNSAARYLIPLIHSSVKEKISAEKSSDEKKVPDLVDILLETVSEQKQTT--------KDEG---- : 293
NlCYP408A1 : -RTKFERAIGKMFTVQYPDSIFSVTPEVFESLGS-LDNFILKPAYTRYLAGVAELVIDEKKKTSDVKPEGRALDIVDLLLVAAEEEKQAA-------AKEKS---- : 291

LmCYP409A1 : -----VEKST------FTDMDVVAQVVTFMIDGTHTTATAMSFALYELALHPDIQHRLRENLREAVDKHFGQLGYDSINECTYLDMVLSEVLRLHPPIGHLEKKCT : 389
LmCYP6H1   : LPVNDVDEDVLKRKFDFSGDDLLAQAAIFFVAGFETSSSTMTFCLYELALQPALQTRLRHEIRDGLQRAGGRFTYSMLHDMRYLDMVISETLRKYPILPFLDRTCL : 412
LmCYP408B1 : ---KEVEGASA-----VNEEALVAQSLAVLINTAQATKSTIALSVATLASKPEIQDKLHSELNKQLQTSN-EISFEAVQNAQYLDMFLNEVLRMYPSEYRIEHECL : 390
NlCYP408A1 : VDSDKLDSEGI-----LSKEQMINVWMQVLITVGGATKQTLAMSIYALANNTNVQDKLQAEVGKQLNRYE-EINYEFINESEYLDMFLGEILRIYPLEYRLERKTF : 391
```

P450 oxygen binding sequence
A/GGXD/ETT/S motif

Helix K
EXLR

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LmCYP409A1 : AAYPMTTASGRAFTVQPGTAVVFSIAGIHRDPRYFRNPDVFDPERFSPDNKDPTSMVAYMPFGL-GARTC-LGQRFALSQVKMGVACLVLNFRVCTTPRTPMPLEV : 493
LmCYP6H1   : RDYPVPGKPG--CVIERGTPVYVSLLGLHTDPNYFPHPDVFDPERFS--DKSAIAPFTYMPFGT-GPHNC-IGMRFGLMSAKAGVAHVLSQYEVSVAADTPVPLPM : 512
LmCYP408B1 : ENTEFEN-----LKIEKGTIVSIPLYALHRLEDYYPEPHTFNPDRFSPAIAEKRHPYTYLPFGLAGSNTANVSVQYGMLVTKLTVATLIKNEKFVPAEETQ--IPP : 489
NlCYP408A1 : EEMKLDE-----ITLTPGTMVSIPIYAVHHLEEFYPEPEKFIPERFSESNSDKRNPHTYLPFGQAGSNSSNIGVQLGVLIAKLAIVSLIKSVKLATAENPQNEVSS : 492
```

Meander
PXXFXPXXF

Heme-binding domain
FXXGXXXCXG/A

```
LmCYP409A1 : DPNSFAKAAKGGMWLGFQ--PLVHL--------------------------- : 516
LmCYP6H1   : ATRGIVATTVGGVPLRFQHDPLVELVRPA----------------------- : 541
LmCYP408B1 : PLEEGLTGVMKPKPVKISVELRK----------------------------- : 512
NlCYP408A1 : PFVSGVTGVPQPKTIFVRADKKEE---------------------------- : 516
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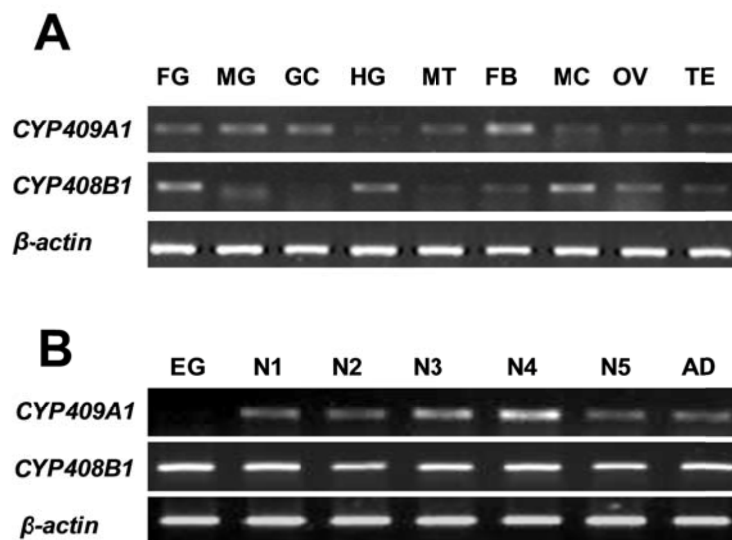
665 Fig. 3.



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685 Fig. 4.

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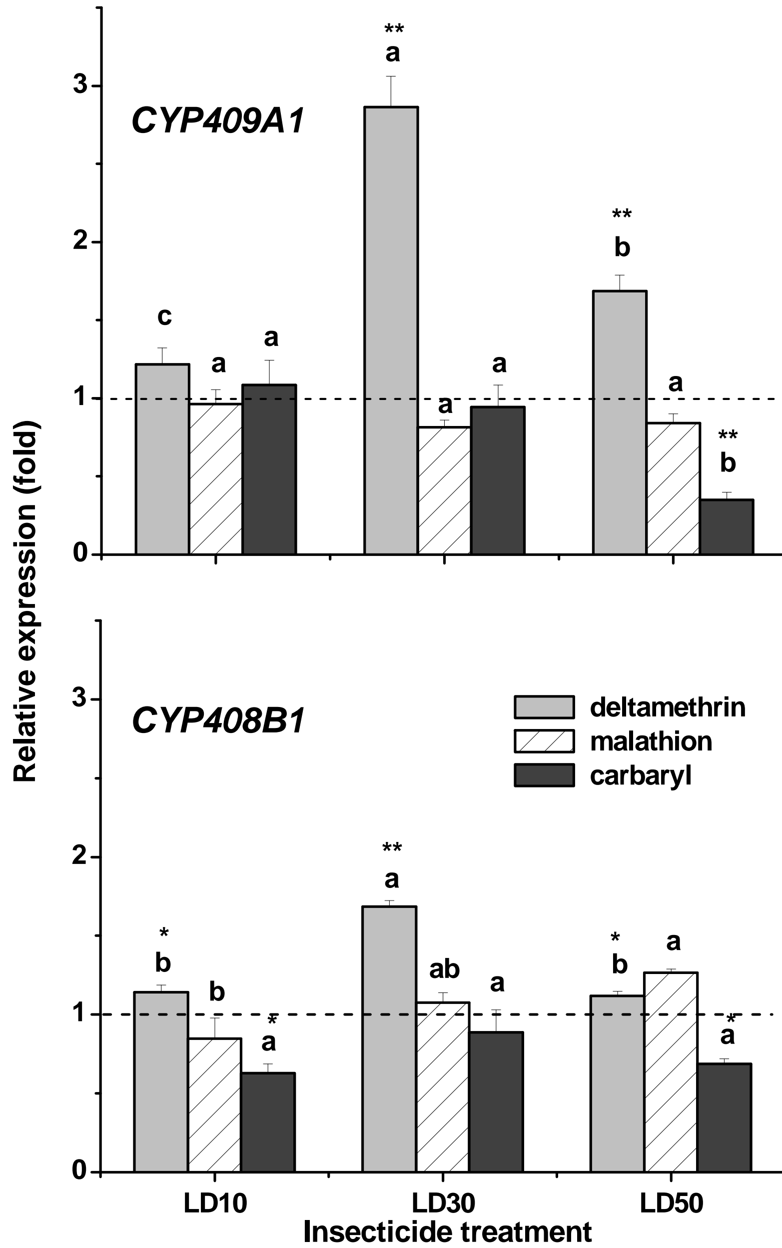

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691 Fig. 5.

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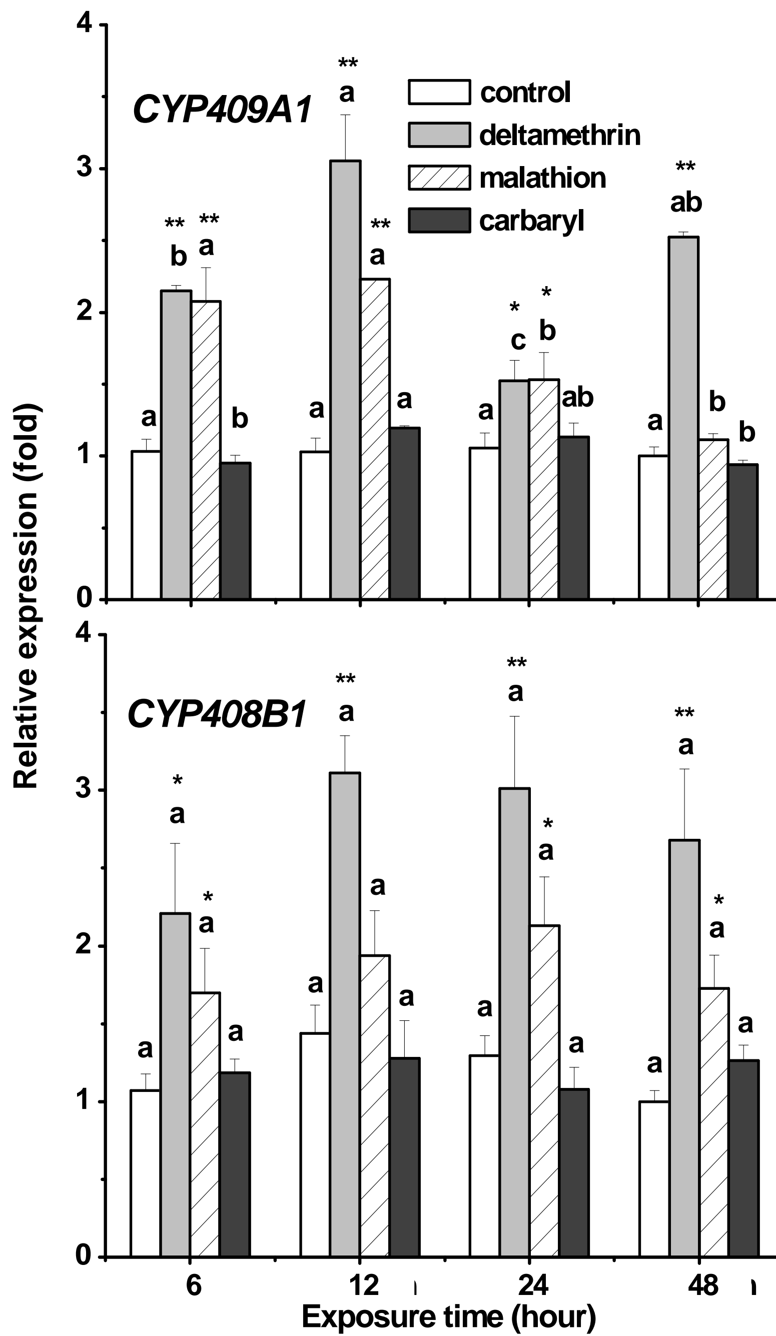

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695 Fig. 6.

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