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Silencing of *CYP6AS160* in *Solenopsis invicta* Buren by RNA interference enhances worker susceptibility to fipronil

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Research Paper

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Abstract

Cytochrome P450 monooxygenases play a key role in pest resistance to insecticides by detoxification. Four new P450 genes, *CYP6AS160*, *CYP6AS161*, *CYP4AB73* and *CYP4G232* were identified from *Solenopsis invicta*. *CYP6AS160* was highly expressed in the abdomen and its expression could be induced significantly with exposure to fipronil, whereas *CYP4AB73* was not highly expressed in the abdomen and its expression could not be significantly induced following exposure to fipronil. Expression levels of *CYP6AS160* and *CYP4AB73* in workers were significantly higher than that in queens. RNA interference-mediated gene silencing by feeding on double-stranded RNA (dsRNA) found that the levels of this transcript decreased (by maximum to 64.6%) when they fed on *CYP6AS160*-specific dsRNA. Workers fed ds*CYP6AS160* had significantly higher mortality after 24 h of exposure to fipronil compared to controls. Workers fed ds*CYP6AS160* had reduced total P450 activity of microsomal preparations toward model substrates *p*-nitroanisole. However, the knockdown of a non-overexpressed P450 gene, *CYP4AB73* did not lead to an increase of mortality or a decrease of total P450 activity. The knockdown effects of *CYP6AS160* on worker susceptibility to fipronil, combined with our other findings, indicate that *CYP6AS160* is responsible for detoxification of fipronil. Feeding insects dsRNA may be a general strategy to trigger RNA interference and may find applications in entomological research and in the control of insect pests in the field.

Introduction

Many insects have evolved various mechanisms for metabolizing toxic chemicals in their environment (Mazumdar-Leighton and Broadway, 2001; Gatehouse, 2002; Wittstock *et al.*, 2004). Similar to most other insects, social insects such as red imported fire ant (RIFA; *Solenopsis invicta*, Hymenoptera, Formicidae), and the honey bee, *Apis mellifera*, rely in part on a suite of detoxification enzymes to metabolize naturally occurring xenobiotic compounds and pesticides. Chief among these enzymes are the cytochrome P450 monooxygenases (P450) (Ffrench-Constant *et al.*, 2004; Mao *et al.*, 2009), especially the members of the CYP4 and CYP6 subfamily, which have been studied extensively in insect species (Liu *et al.*, 2015; Zhang *et al.*, 2020). Specifically, P450s play a role in the detoxification of phytochemicals and synthetic pesticides (Feyereisen, 2005). For example, compounds including tau-fluvalinate, a pyrethroid acaricide used to control Varroa mites (Johnson *et al.*, 2006; Mao *et al.*, 2011), several pyrethroids including lambda-cyhalothrin (Pilling *et al.*, 1995), and organophosphates such as coumaphos (Johnson *et al.*, 2009; Mao *et al.*, 2011) are known to be metabolized by P450s.

The RIFA is recognized as the most invasive and destructive of alien species because of the complexity of its diet and its ferocious habits, rapid reproduction and strong competitive ability (Vinson, 1997). Many methods have been employed in attempts to control the fire ant. Chemical insecticides are still currently the most effective measures for control of the fire ant until a more effective control agent is found (Wang *et al.*, 2013). Fipronil, a phenylpyrazole insecticide, exhibits neurotoxic activity by blocking the GABA-regulated chloride channels of neurons. It is useful for the control of many domestic and agricultural insect pests, especially since its mechanism of toxicity is different from traditional insecticides to which resistance has been developed. Fipronil that is formulated into granules or bait has been shown to be effective against of the fire ant (Greenberg *et al.*, 2003; Marr *et al.*, 2003). In a study that evaluated broadcast application treatments with various contact insecticides, fipronil was the only insecticide to show a statistically significant reduction in the proportion of mounds that contained brood of the fire ant (Loftin *et al.*, 2003). Some success in killing the fire ant has been achieved in small areas, but eradication remains rare (Hoffmann *et al.*, 2016). Thus, new approaches to control the fire ant are needed.

RNA interference (RNAi) has been developed as an effective tool for both basic and applied applications in plants and animals (Fire *et al*., 1998; Tabara *et al*., 1999; Aravin *et al*., 2001; Wesley *et al*., 2001). For example, gene silencing can inhibit virus replication (Tenllado and Dıaz-Ruız, 2001) and so plants engineered to produce artificial microRNAs targeting virus genes can resist viral infection (Niu *et al*., 2006). Currently, many RNAi-based studies with various insects are in progress, and several reviews on their results have already been published. RNAi-based gene regulation has been reported in different insect orders including Lepidoptera, Hemiptera, Coleoptera, Diptera and Hymenoptera (Gordon and Waterhouse, 2007; Huvenne and Smagghe, 2010). In particular, Bellés (2010) comprehensively reviewed RNAi-based studies on insects, covering some 30 species representing nine orders (Orthoptera, Dictyoptera, Isoptera, Hemiptera, Coleoptera, Neuroptera, Hymenoptera, Lepidoptera and Diptera). However, because RNAi approaches are still relatively new, this field will continue to expand rapidly as the number of insect genome projects increases and as RNAi methods are used ever more extensively in functional genomics.

Delivery of double-stranded RNA (dsRNA) through feeding has also been shown to effectively induce RNAi of target genes. Examples of this include dsRNA-diet incorporated feeding to *Diabrotica punctate* and *D. undecimpunctata howardi*, and *Leptinotarsa decemlineata* to silence genes involved in growth and survival (Baum *et al*., 2007); plants expressing short hairpin dsRNA to feeding *Helicoverpa armigera* (Mao *et al*., 2007) to silence a P450 gene (*CYP6AE14*); droplet feeding to *Epiphyas postvittana* (Turner *et al*., 2006) to silence a gut carboxylesterase and a pheromone binding protein gene; feeding dsRNA to *Plutella xylostella* (Bautista *et al*., 2007) to silence a P450 gene (*CYP6BG1*) involved in permethrin tolerance; feeding dsRNA to *H. armigera* (Asokan *et al*., 2014) to silence five target genes related to the digestion of proteins and the detoxification host allelochemicals; and feeding dsRNA to *Sitobion avenae* (Zhang *et al*., 2020) to silence an aphid carboxylesterase gene related to tolerance to phoxim insecticides.

Our previous study indicated that *CYP6AS160* (12H3) could be involved in detoxification of fipronil because the expression of *CYP6AS160* in workers was induced strongly by fipronil (Zhang *et al*., 2016). Here, we further explore the function of *CYP6AS160* in workers, through RNAi-mediated gene silencing of *CYP6AS160* transcripts via voluntary feeding bioassays to determine the effects of knockdown of *CYP6AS160* on susceptibility to fipronil and total P450 activity in workers. Furthermore, we also determine the effect of silencing a non-overexpressed P450 gene (*CYP4AB73*), whose expression cannot be induced significantly by fipronil, to obtain further insight into the potential role of the overexpressed *CYP6AS160* (table 1).

Table 1. Fipronil toxicity to workers fed with dsRNA (ds*CYP6AS160* and ds*CYP4AB73*) vs. ds*GFP*

Time after dsRNA-feeding (h)	Controls (mortality) (%)	Treatments (mortality) (%)	
	ds*GFP*	ds*CYP6AS160*	ds*CYP4AB73*
12	11.11 ± 0.02a (1.00)	35.56 ± 0.03b (3.20)	6.70 ± 0.02a (0.60)
24	20.00 ± 0.03a (1.00)	77.78 ± 0.02b (3.89)	15.57 ± 0.02a (0.78)
36	51.11 ± 0.02a (1.00)	84.45 ± 0.02b (1.65)	31.21 ± 0.05b (0.63)
48	77.78 ± 0.02a (1.00)	95.56 ± 0.02b (1.23)	64.45 ± 0.02b (0.83)
60	84.45 ± 0.02a (1.00)	97.78 ± 0.02b (1.16)	80.00 ± 0.06a (0.95)

Notes: Different letters within the same time point indicate significant differences ($P < 0.05$). The data below the activity are the relative ratio of treatments/controls.

Materials and methods

Insect cultures

RIFAs were collected in Wushan (Coordinates, N23.16 E113.23) and Zengcheng (Coordinates, N23.3 E113.8) in Guangdong Province in China. The methods of collection and transport were based on Kuriachan and Vinson (2000). Rearing conditions were 27 ± 1°C, with 70–90% relative humidity and a 16:8h light: dark photoperiod.

The samples of different castes and tissues of *S. invicta* were collected as follows: the two castes were minor workers ($n = 5$) and dealate queens ($N = 3$) from polygyne nests. The different tissues were taken from minor workers ($N = 10$), each sample was repeated three times. All samples were snapped frozen in a liquid nitrogen flash freezer and then stored at −80°C for RNA extraction.

Chemicals

Fipronil (95% technical powder) was obtained from Wuxi Ruize Pesticide Co., Ltd. Chlorpyrifos (96% technical oil) was obtained from the Tianjin Jingjin Pesticide Factory. Nicotinamide adenine dinucleotide phosphate (NADPH), diethylpyrocarbonate and phenylmethanesulphonyl fluoride (PMSF) were obtained from Sigma-Aldrich (USA); dithiothreitol (DTT) and Tris base were purchased from Promega (USA). Ethylenediaminetetraacetate acid (EDTA) and bovine serum albumin (BSA) were purchased from Beijing Tongzheng Biological Company; *p*-nitroanisole (*P*-NA) and *p*-nitrophenol were purchased from Beijing Chemical Reagents; TRIzol reagent was purchased from Invitrogen (USA). Taq DNA polymerase and DNA Marker DL 2000 were purchased from Sangon Company (Shanghai, China). Agarose, DNase I and SYBR Green I were purchased from TaKaRa (Dalian, China). The MEGAscript® RNAi kit was purchased from Ambion (USA). All other chemicals used were of reagent grade.

Enzyme preparations

Workers (dsRNA-fed) samples were homogenized in 0.1 M phosphate buffer (pH 7.5), containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10% glycerol in an ice-cold mortar. The homogenate was centrifuged at 10,800×***g***, at 4°C, for 20 min. The supernatant was collected, filtered through cotton and either used immediately for the P450 assays or stored at −80°C.

The o-demethylase activity of total P450

The *o*-demethylase activity of total P450 was measured using methods described by Rose *et al*. (1995). Reactions were carried out in 1.5 ml centrifuge tubes in a water bath for 30 min at

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Table 2. *o*-Demethylase activity of total P450 in workers treated with dsRNA (ds*CYP6AS160* and ds*CYP4AB73*) vs. controls (ds*GFP*)

Time (h) after dsRNA-feeding	The *o*-demethylase activity of total cytochrome P450		
	Controls (ds*GFP*)	Treatments (ds*CYP6AS160*)	Treatments (ds*CYP4AB73*)
24	5.85 ± 0.18ab (1.00)	4.97 ± 0.28b (0.85)	7.14 ± 0.43a (1.22)
48	4.09 ± 0.25b (1.00)	2.30 ± 0.11c (0.56)	6.165 ± 5.03a (1.50)
72	4.17 ± 0.32a (1.00)	1.38 ± 0.26b (0.33)	5.83 ± 0.45a (1.48)
96	3.93 ± 0.31b (1.00)	1.77 ± 0.60c (0.45)	5.20 ± 0.20a (1.32)

Notes: Different letters within the same time point indicate significant differences ($P < 0.05$). The *o*-demethylase activity of total cytochrome P450 is here reported as μmol mg^{-1} protein/30 min; the data below the activity are the ratio of treatments/controls.

30°C. About 345 μl of enzyme preparation was added to initiate the reactions; the initial reaction solutions contained 30 μl of 9.6 mmol l^{-1} NADPH, 375 μl of 2 μmol l^{-1} *P*-NA (dissolved in 0.1 mol l^{-1} PBS, pH 7.8). The final volume of the reaction system was 750 μl. The control samples used 1 mol l^{-1} PBS (pH 7.8) instead of the enzyme preparation. For analysis, we added 200 μl of reaction solution (three replicates per reaction) to 96-well microtitre plates, then used a vmax microplate reader and at a wavelength of 405 nm to record spectrophotometric values. Protein content was determined by the method of Bradford (1976), using BSA as a standard. The *o*-demethylase activity of total P450 was expressed as μmol mg^{-1} protein/30 min (table 2).

Primer design

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis primers were designed using primer3 (http://primer3.ut.ee/). The primers were designed on the basis of the following sequences published in NCBI: *CYP6AS160* (accession no. MT482289), *CYP6AS161* (accession no. MT482313), *CYP4AB73* (accession no. MT482314) and *CYP4G232* (accession no. MT482315) (personal communication by Professor Nelson, D.R., University of Tennessee, Memphis, TN, USA), *CYP4AB2* (accession no. AY345971), *RPL18* (accession no. EH413666) and *EF1-beta* (accession no. EH413796). *RPL18* and *EF1-beta* were chosen as an internal control according to Cheng *et al.* (2013). The primers used in this study are detailed in table 3.

qRT-PCR experiments

Total RNA was isolated from worker samples using TRIzol reagent. Sample RNA concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) at 260 nm. After total RNA (1.0 μg $sample^{-1}$) was treated with DNase I (Fermentas, USA) to remove possible genomic DNA contamination, first-strand cDNA was synthesized in a 20 μl reaction system using a First Strand cDNA Synthesis Kit (Fermentas, USA) with oligo (dT)18 as the primer. The relative expression levels of the six target P450 genes (*CYP6AS160*, *CYP6AS161*, *CYP4AB1*, *CYP4AB2*, *CYP4AB73* and *CYP4G232*) in the fire ant were examined using qRT-PCR. A total of 1 μg RNA was used as the template, three biological replicates were conducted; each sample was analyzed with two technical replicates and then immediately frozen in liquid nitrogen and stored at −80°C for total RNA isolation. qRT-PCR reactions were performed in a 20 μl mixture containing 1 μl of cDNA, 10 μl of SYBR Green qRT-PCR SuperMix-UDG, 0.15 μl of each primer and 8.7 μl of H_2O. The amplification efficiency of the target genes and housekeeping genes (*RPL18* and *EF1-beta*) was estimated using $E = 10^{-1/\text{slope}} - 1$, where the slope was derived from the plot of the cycle threshold (C_t) value vs. the log of the serially diluted template concentration. The optimized qRT-PCR program consisted of an initial step at 50°C for 2 min, 94°C or 2 min, followed by 50 cycles of 94°C for 15 s and 60°C for 30 s. After the cycling protocol, melting curves were obtained by increasing the temperature from 60 to 95°C (0.2°C s^{-1}) to denature the dsDNA. The qRT-PCR amplifications were carried out in 96-well plates. The assays were performed in an ABI 7500 system using SDS v.1.4 application software (Applied Biosystems). Quantification of the transcript levels of six P450 genes was performed using the comparative $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

CYP6AS160 *and* CYP4AB73 *gene silencing*

Synthesis of dsRNAs

dsRNAs were synthesized using an Ambion®MEGAscript® RNAi kit (USA) according to the manufacturer's instructions. The primers used to produce cDNA with T7 promoter sequences were designed based on the nucleotide sequences flanking the 391–726 positions of *CYP6AS160* and the nucleotide sequences flanking the 581–959 positions of *CYP4AB73* (table 4). As there is a high risk of cross-suppression or co-suppression in RNAi among closely related P450s, the primers targeted the sequences outside the signature motifs of P450s. The PCR products were examined on agarose gels prior to *in vitro* transcription to verify that the products showed a single band of the expected sizes. The two amplification products possessing a single T7 promoter sequence on opposite ends were combined (1 mg:2 mg). All dsRNA preparations were quantified spectrophotometrically and stored at −20°C until use.

Administration of dsRNA by feeding

RNAi-mediated gene silencing was accomplished using voluntary feeding according to the method of Zhou *et al.* (2008). Workers (minors) of the fire ant were prestarved for 12 h before use, and the dealate queens were used as samples of different castes to compared expression levels of P450s between workers (minors) and queens. Feeding dsRNA assays were performed by placing groups of 80 workers into disposable plastic cups containing a paper disc moist with honey water (5%) containing 400 ng μl^{-1} of dsRNA (ds*CYP6AS160* (336 bp) or ds*CYP4AB73* (379 bp)) or the control which was 400 ng μl^{-1} of ds*GFP* (green fluorescent protein gene), and paper disc moisture with honey water (5%) containing 400 ng μl^{-1} of dsRNA was monitored each day. Mortality owing to the ds*GFP*-feeding was less than 10%. Three replicates were used for four survival timeframes: 24, 48, 72 and 96 h. Survivors at the end of each timeframe were subjected to qRT-PCR to quantify the expression levels of the P450.

To assess the sensitivity of workers to fipronil after silencing the target genes by RNAi, at 12, 24, 36, 48, and 60 h of dsRNA feeding, the workers were transferred to fipronil at the sublethal dose (0.05 μg ml^{-1}) according to the method of the residual film in a disposable plastic cup (Hu *et al.*, 2005). One milliliter

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Table 3. Sequences of primers used for relative expression of the genes tested from the fire ant

Gene name	T_m (°C)	Sequence (5′–3′)	Efficiency (%)	Product length (bp)	R^2
CYP6AS160	58.94	Sense: TGACGGACATTACACGAAGC	101	102	0.998
	59.09	Anti-sense: CCAATTCGTACATTGCATGG			
CYP6AS161	59.03	Sense: CCATGTTCGCTGATAGAGGA	108.2	109	0.993
	59.15	Anti-sense: CACCTTGGTCCGCTACTTTC			
CYP4AB1	58.93	Sense: AGAACGTGGGCTTTCTTTGA	99.8	104	0.995
	58.95	Anti-sense: GCCATTTGGAACCTCCACTA			
CYP4AB2	58.99	Sense: GGCATTTACGGCGAAAGATA	95.6	100	0.990
	59.11	Anti-sense: AGTTCCCATGGCAGTTTCAC			
CYP4AB73	58.75	Sense: TTTTGTCAGCGTTCACCATC	98.6	90	0.991
	59.63	Anti-sense: AGCATCTTTCGCCTTTTGTG			
CYP4G232	58.96	Sense: GTCTGTCGCTGTCACCAAAA	103.3	106	0.996
	59.10	Anti-sense: ACTACGGCAGCTGGTTCAAG			
RPL18	60.18	Sense: GCATGATCGGAAAGTGCG	95.9	96	0.998
	59.13	Anti-sense: TTCAGCCACTTGACTGCG			
EF1-beta	60.12	Sense: TGAAGACCGATAAGGGCA	95.9	110	0.998
	60.55	Anti-sense: TCGTCCGAACCAAAGAGA			

Table 4. Primer sequences for production of dsRNA transcription templates

Gene name	T_m (°C)	Sequence (5′–3′)	Product length (bp)
Gfp(T7)-F	69.7	ggatcctaatacgactcactataggg TGACCACCCTGACCTAC	288
Gfp(T7)-R	68.8	ggatcctaatacgactcactataggg TTGATGCCGTTCTTCTGC	
CYP6AS160 (T7)-F	63.56	ggatcctaatacgactcactatagggg AATACGGTTGTCGCCTGTT	336
CYP6AS160 (T7)-R	62.88	ggatcctaatacgactcactataggg CGGTTTGAGGCAGGAAGTAG	
CYP4AB73 (T7)-F	66.68	ggatcctaatacgactcactat aggg TCCAGCACCAATATCGTGAA	379
CYP4AB73 (T7)-R	65.93	ggatcctaatacgactcactat agggg TATCGTGCCCTTCGAACAT	

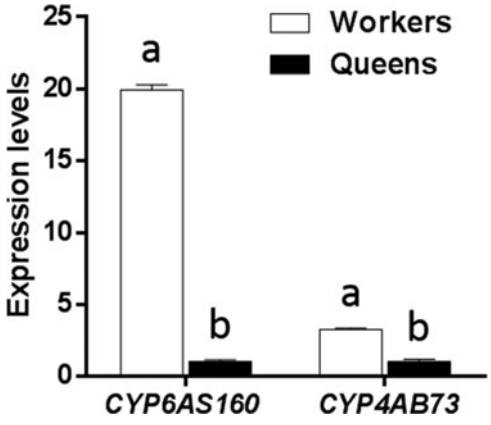

Figure 1. Transcript levels of *CYP6AS160* and *CYP4AB73* in tissues of multiple body parts combined from fire ant workers and queens. Different letters above the standard error bars indicate significant differences ($P < 0.01$).

of the liquid was pipetted into a disposable plastic cup (caliber, bottom diameter and height were 6.6, 4.4 and 6.8 cm, respectively) and the cup was shaken until the acetone completely evaporated to evenly coat the cup with fipronil. The caliber was then lined with talcum powder to prevent the workers climbing out. At least 30 workers were then placed into the cup and allowed to feed on honey water (5%) soaked into a small cotton ball. Workers with ds*GFP* feeding were used as the control. The mortality of workers was assessed at 24 h after insecticide exposure. Each treatment was replicated three times.

Data analysis

Relative transcript levels of *P450* in workers and queens, and time-dependent suppression of *P450* transcript expression in workers were analyzed with an unpaired *t*-test using GraphPad InStat 3.0 software (GraphPad Software, San Diego, CA, USA). Mortality of workers exposed to fipronil after feeding dsRNA, the *o*-demethylase activity of total P450 and relative transcript levels of *CYP6AS160* and *CYP4AB73* in different tissues of minor workers were analyzed by an analysis of variance followed by Tukey's multiple comparison test ($P < 0.05$) using software InStat v.3.0 (GraphPad Software, San Diego, CA, USA).

Results

Castes (workers and queens) and tissue-specific (minor workers) expression patterns of CYP6AS160 *and* CYP4AB73

The expression levels of *CYP6AS160* and *CYP4AB73* in workers were significantly higher than those in queens (19.9- and 3.2-fold, respectively) (fig. 1). The expression amount of

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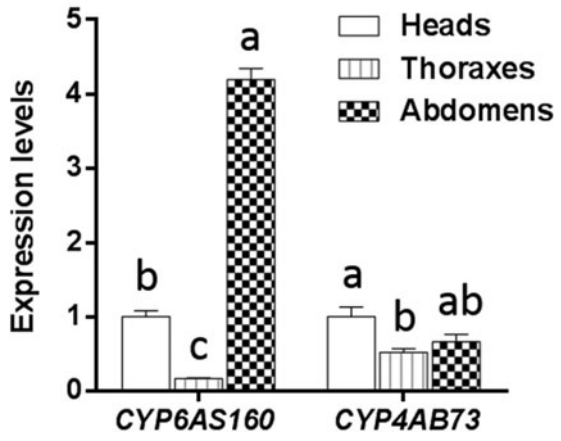

Figure 2. Transcript levels of *CYP6AS160* and *CYP4AB73* in tissues of multiple body parts from fire ant minor workers as determined by qRT-PCR. Different letters above the standard error bars indicate significant differences ($P < 0.05$).

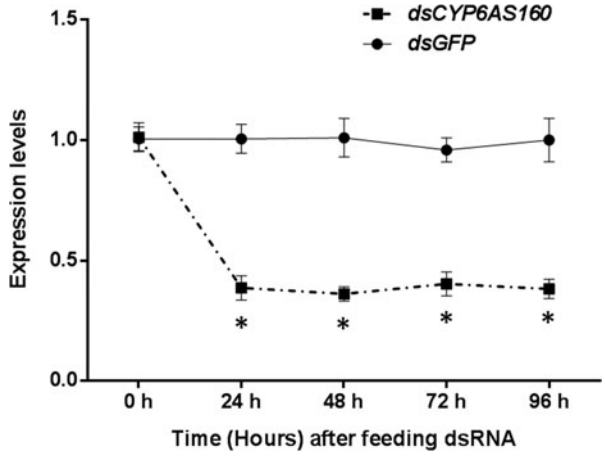

Figure 3. Time-dependent suppression of *CYP6AS160* transcript expression in workers of the fire ant fed with ds*CYP6AS160* and ds*GFP* as determined by qRT-PCR. *Indicates significant differences ($P < 0.05$).

CYP6AS160 was relatively more in the abdomen compared to that of the head (4.2-fold), whereas *CYP4AB73* was relatively more abundant in the head compared to the abdomen (1.6-fold) (fig. 2).

CYP6AS160 *silencing and specificity*

The expression of *CYP6AS160* was significantly reduced in ds*CYP6AS160*-fed workers by 61.8-, 64.6-, 60.3- and 61.8% in comparison with controls (ds*GFP*-fed workers) at 24, 48, 72 and 96 h, respectively (fig. 3). The efficiency of knockdown of *CYP6AS160* was relatively stable from 24 to 96 h.

To examine whether the knockdown was specific to *CYP6AS160* and did not affect the expression of other P450s belonging to the same CYP6, and CYP4 families, qRT-PCR analysis was also performed on similar samples with primer pairs specific for each of the P450s. The analysis revealed that the *CYP6AS160* transcript level was greatly reduced by dsRNA feeding, whereas the levels for *CYP4AB73* and *CYP4G232* remain unchanged (0.97- and 0.98-fold difference between ds*CYP6AS160*-fed and ds*GFP*-fed workers) (fig. 4). Slight

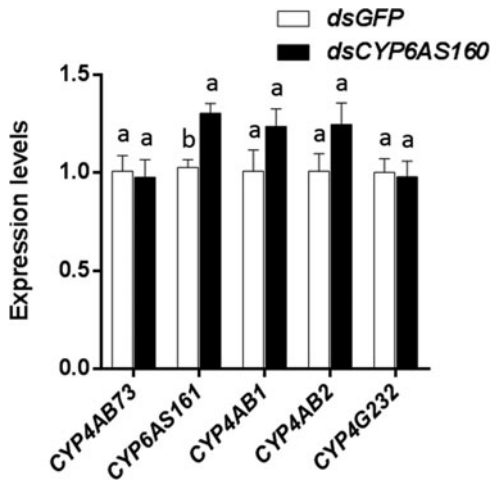

Figure 4. Transcript levels of *P450s* in tissues of multiple body parts of fire ant minor workers after exposure to ds*CYP6AS160* and dsGFP as determined by qRT-PCR analysis. Different letters above the standard error bars indicate significant differences ($P < 0.05$).

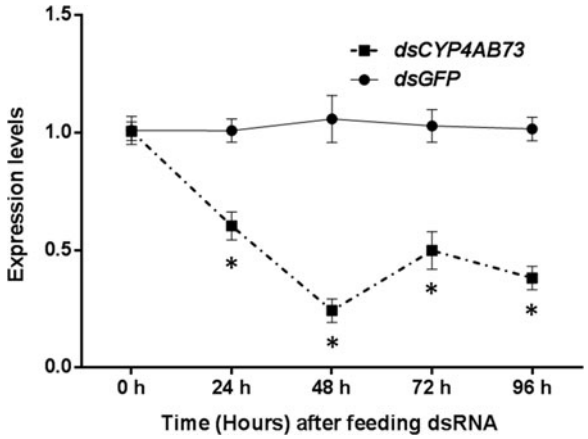

Figure 5. Time-dependent suppression of *CYP4AB73* transcript in fire ant workers fed with ds*CYP4AB73* and ds*GFP* as determined by qRT-PCR. *Below the standard error bars indicates significant differences ($P < 0.05$).

increases were observed for the expression of *CYP6AS161*, *CYP4AB1* and *CYP4AB2* (1.3-, 1.2- and 1.2-fold, respectively) in ds*CYP6AS160*-fed workers compared to controls (ds*GFP*-fed workers).

Silencing CYP4AB73 *and specificity*

The expression of *CYP4AB73* in ds*CYP4AB73*-fed workers was significantly reduced, by 40.2-, 77.1-, 51.6- and 62.4% in comparison with controls (ds*GFP*-fed workers) at 24, 48, 72 and 96 h, respectively (fig. 5). The knockdown efficiency of *CYP6AS160* was relatively stable from 24 to 96 h. The knockdown efficiency of *CYP4AB73* was not as effective as *CYP6AS160*, which suggests that knockdown efficiency may vary depending on the P450 gene target. In terms of knockdown efficiency of *CYP4AB73*, qRT-PCR analysis showed that the expression levels for *CYP6AS160*, *CYP4AB1* and *CYP4G232* remain unchanged (1.1-, 1.1- and 1.2-fold differences) between ds*CYP6AS160*-fed workers and controls (ds*GFP*-fed workers). A slight increase (1.3- and 1.9-fold) in

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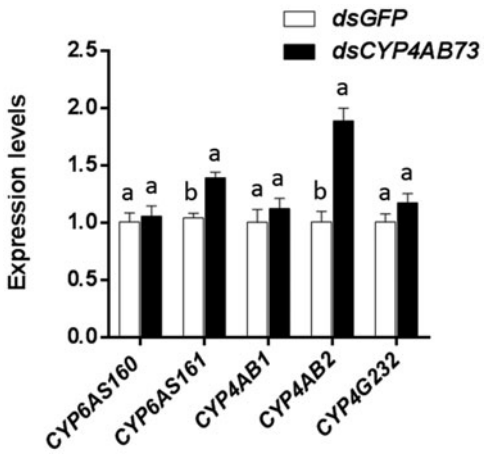

Figure 6. Transcript levels of *P450s* in tissues of multiple body parts of fire ant minor workers after exposure to ds*CYP4AB73* and dsGFP as determined by qRT-PCR analysis. Different letters above the standard error bars indicate significant differences ($P < 0.05$).

the expression of *CYP6AS161* and *CYP4AB1* was observed in ds*CYP4AB73*-fed workers compared to controls (ds*GFP*-fed workers) (fig. 6).

Susceptibility to fipronil and total P450 activity in workers fed with dsRNA

Mortality was significantly higher in ds*CYP6AS160*-fed workers (35.6, 77.8, 84.5, 95.6 and 97.8%, respectively) compared to controls (ds*GFP*-fed workers) (11.1, 20.0, 51.1, 77.8 and 84.5%, respectively at 24, 36, 48, 60 and 72 h) whereas no significant differences was observed in mortality between ds*CYP4AB73*-fed and controls (ds*GFP*-fed workers) (table 2).

The *o*-demethylase activity of total cytochrome P450 significantly decreased in ds*CYP6AS160*-fed workers (0.85, 0.56, 0.33 and 0.45-fold) compared to controls (ds*GFP*-fed workers) at 24, 48, 72 and 96 h respectively. Minimal *o*-demethylase activity of total cytochrome P450 were observed at 72 h (0.33–0.56 fold less than controls), whereas the *o*-demethylase activity of total P450 in workers treated with dsRNA (ds*CYP4AB73*) were slightly increased (1.22, 1.50, 1.48 and 1.32-fold) compared to controls (ds*GFP*-fed workers) at 24, 48, 72 and 96 h, respectively. Maximum of the *o*-demethylase activity of total cytochrome P450 was observed at 48 h (1.5-fold higher activity than the controls) (table 3).

Discussion

The midgut and fat body tissues in insects are generally considered to be the primary detoxification sites where most insect P450s related to detoxification are expressed (Liu and Scott, 1998). Furthermore, other tissues (the brain and nervous system) can also be important for P450 gene expression and response to insecticide resistance (Scott *et al.*, 1998). *CYP6AS160* was found to be more abundantly expressed in the abdomen compared to the head of workers, whereas *CYP4AB73* was not relatively more expressed in the abdomen compared to the head. The observed higher expression of *CYP6AS160* in the abdomen might reflect a role for *CYP6AS160* in the metabolism of insecticides. On the contrary, the observed higher expression of *CYP4AB73* in the head might not reflect a role for *CYP4AB73* in the metabolism of insecticides.

In social insects, queens produce pheromones to regulate the development, reproduction and sex ratios of their colonies (Passera *et al.*, 2001; Pennisi, 2001; Bloch *et al.*, 2009). Worker individuals perform the functions of defending and maintaining the colony (Robinson, 2002). Because previous studies have shown that P450 genes may be doubly important in caste differentiation and detoxification in social insects (Liu and Zhang, 2004; Cornette *et al.*, 2006; Mao *et al.*, 2009; Tarver *et al.*, 2012), we decided to investigate the role of P450 genes in these processes in the fire ant. Given that workers forage outside of mounds and can easily encounter insecticides, whereas queens would remain unexposed within the nest, it is reasonable to infer that the P450 genes from workers may ultimately contribute more to detoxification compared to queens. Thus, we measured the expression level of *CYP6AS160* and *CYP4AB73* in workers and queens. Indeed the expression levels of *CYP6AS160* and *CYP4AB73* in workers were significantly higher than those in queens. Furthermore, our previous study indicated that *CYP6AS160* was induced significantly following exposure to fipronil, whereas *CYP4AB73* was not induced significantly following exposure to fipronil (Zhang *et al.*, 2016). These findings strengthened the possibility that *CYP6AS160* plays a significant role in detoxification of insecticide in the fire ant, whereas *CYP4AB73* may not be involved in increased metabolism of insecticides.

In order to further clarify the function of *CYP6AS160* involved in detoxification of fipronil, we deemed workers as a more amenable caste for use in RNAi experiments, as previous studies into dsRNA acquisition by workers have suggested that they can rapidly acquire dsRNA through a combination of feeding and trophallaxis. Through the process of trophallaxis, worker termites readily share food and other resources with nestmates such as pheromones, semiochemicals and nest building materials (Hamilton, 1972; Zhou *et al.*, 2008). Trophallaxis can lead to the rapid transfer of materials throughout entire colonies in short periods of time (Cabrera and Rust, 1999; Buczkowski *et al.*, 2007). Our study shows that oral delivery is capable of inducing RNAi in social insect workers, as was also observed in *Reticulitermes flavipes* (Zhou *et al.*, 2008). The high specificity of dsRNA for *CYP6AS160* knockdown was confirmed by the unchanged levels of other P450 transcripts (i.e. *CYP4AB73* and *CYP4AB1*) between ds*CYP6AS160*-fed workers and controls (ds*GFP*-fed workers) and the slightly increased expression level of *CYP6AS161* in workers 24 h after ds*CYP6AS160* feeding. *CYP4AB73* knockdown also showed high specificity of dsRNA for *CYP4AB73*; this was confirmed by unchanged levels of other P450 transcripts (i.e. *CYP6AS160*, *CYP4AB1* and *CYP4G232*) between dsRNA (*CYP6AS160*)-fed and controls (ds*GFP*-fed workers) and the slightly increased expression level of *CYP6AS161* and *CYP4AB2* in workers 24 h after dsRNA (*CYP4AB73*) feeding. Although a small subset of P450s was analyzed, it is likely that the RNAi employed here was highly sequence-specific. Analysis of the knockdown effects of *CYP6AS160* on worker susceptibility to fipronil indicates that *CYP6AS160* is responsible for detoxification of fipronil.

In addition, the *o*-demethylase activity of total cytochrome P450 was significantly lower in ds*CYP6AS160*-fed workers than that in controls, whereas the *o*-demethylase activity of total cytochrome P450 was a little higher in ds*CYP4AB73*-fed workers than that in controls. Although there were slightly increased levels of *CYP6AS161* expression in workers, we observed significantly

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higher mortalities of the knockdown workers. Analysis of the knockdown effects of *CYP4AB73* on worker susceptibility to fipronil indicates that the gene *CYP4AB73* is not responsible for detoxification, although there was a significantly increased level of *CYP6AS161* expression in workers, lower mortalities of knockdown workers were observed. We think that *CYP6AS161* but not *CYP4AB2*, possibly be involved in insecticide detoxification as described above. These CYP450s that were upregulated may compensate for the silencing of *CYP6AS160* or *CYP4AB73*.

The use of RNAi is a promising tool for insect management, but currently remains problematic, especially for field applications. There have been some successful RNAi studies such as those performed on *Schistocerca gregaria* that targeted different genes in a wide variety of tissues and developmental stages (Badisco *et al.*, 2011; Ott *et al.*, 2012; Van Wielendaele *et al.*, 2012). However, there have also been many unsuccessful RNAi attempts, such as for feeding dsRNA to Lepidopteran pests (Shukla *et al.*, 2016). Our results demonstrate that RNAi is feasible by feeding dsRNA to worker ants, which is a base requirement for using RNAi as a pest management tool. Future studies should focus on demonstrating *CYP6AS160* metabolism of fipronil to determine a more precise definition of its role in detoxification. Knockdown of *CYP6AS160* in another larval caste of the fire ant would also be insightful.

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