Journal of Insect Science (*2016*) 16(1): 49; 1–7
doi: 10.1093/jisesa/iew038
Research article

Molecular Characterization of Two Fatty Acyl-CoA Reductase Genes From *Phenacoccus solenopsis* (Hemiptera: Pseudococcidae)

Xiaolong Li,[1,2] Tianxiang Zheng,[1,2] Xiaowen Zheng,[2,3] Na Han,[2,3] Xuexin Chen,[4] and Dayu Zhang[1,2,3,5]

[1]School of Forestry and Biotechnology, Zhejiang A&F University, Linan, Zhejiang, China (190226808@qq.com; 490731301@qq.com; zhangdayu@zafu.edu.cn), [2]Collaborative Innovation Center of Green Pesticide, Zhejiang Province, Zhejiang A&F University, Linan, Zhejiang, China (190226808@qq.com; 490731301@qq.com; 554669561@qq.com;hnahaq@163.com; zhangdayu@zafu.edu.cn), [3]The Key Laboratory for Quality Improvement of Agricultural Products of Zhejiang Province, College of Agricultural and Food Science, Zhejiang A&F University, Linan, Zhejiang, China (554669561@qq.com; hnahaq@163.com; zhangdayu@zafu.edu.cn), [4]Institute of Insect Sciences, Zhejiang University, Hangzhou, Zhejiang, China (xxchen@zju.edu.cn) and [5]Corresponding author, email: zhangdayu@zafu.edu.cn

Subject Editor: Dr. Yu-Cheng Zhu

Received 11 January 2016; Accepted 5 May 2016

Abstract

Fatty acyl-CoA reductases (FARs) are key enzymes involved in fatty alcohol synthesis. Here, we cloned and characterized full-length cDNAs of two FAR genes from the cotton mealybug, *Phenacoccus solenopsis*. The results showed PsFAR I and PsFAR II cDNAs were 1,584 bp and 1,515 bp in length respectively. Both PsFAR I and PsFAR II were predicted to be located in the endoplasmic reticulum by Euk-mPLoc 2.0 approach. Both of them had a Rossmann folding region and a FAR_C region. Two conservative motifs were discovered in Rossmann folding region by sequence alignment including a NADPH combining motif, TGXXGG, and an active site motif, YXXXK. A phylogenetic tree made using MEGA 6.06 indicated that PsFAR I and PsFAR II were placed in two different branches. Gene expression analysis performed at different developmental stages showed that the expression of *PsFar I* is significantly higher than that of *PsFar II* in first and second instar nymphs and in male adults. Spirotetramat treatment at 125 mg/liter significantly increased the expression of *PsFar I* in third instar nymphs, but there was no effect in the expression of *PsFar II*. Our results indicated these two FAR genes showed different expression patterns during insect development and after pesticide treatment, suggesting they play different roles in insect development and detoxification against pesticides.

Key words: *Phenacoccus solenopsis*, fatty acyl-CoA reductase, spirotetramat, gene expression

The cotton mealybug (*Phenacoccus solenopsis* Tinsley) is a polyphagous, invasive insect belonging to Coccidae. It has caused grievous damages to crops and plants in many countries. *P. solenopsis* was first found in New Mexico, United States (Tinsley 1898). Since then, the cotton mealybug has been reported in many regions, including North America, South America, Asia, Africa, and Australia (Fuchs et al. 1991, Larraín 2002, Granara de willink 2003, Abbas et al. 2005, Culik and Gullan 2005, Akintola and Ande 2008, Hodgson et al. 2008, Muniappan et al. 2009, Wu and Zhang 2009). More than 150 plant species including cotton have been documented as host plants to *P. solenopsis*, (Arif et al. 2009). Wang et al.(2010) have reported that the increasingly serious damages caused by *P. solenopsis* threaten Asia's cotton industry. Other reports also showed that India and Pakistan have suffered great cotton loss (Nagrare et al. 2009). By using temperature-driven phenology model combined with geographic information system, Fand et al.(2014) predicted that the economic losses caused by *P. solenopsis* would become worse than climate change.

Currently, chemical control methods are the most important and most widely used ways of addressing *P. solenopsis* infestations, like organophosphates, pyrethroids, and carbamates pesticides (Saddiq et al. 2014). However, because of the extensive use of pesticides, *P. solenopsis* has developed broad and intense resistance to chlopyrifos, profenofos, acetamiprids, and pyrethroids (Saddiq et al. 2014, Afzal et al. 2015), which reduces their effectiveness in pest control, new pest control techniques, and studies are needed. Recently, a series of pesticides acting on acetyl-CoA carboxylases (ACCs), which are the key enzymes of lipid synthesis and metabolism have been produced for controlling pest insects including scale insects (Cheng et al. 2013, Zu et al. 2013). These include spirotetramat, spirodiclofen, and spiromesifen (Cheng et al. 2013). An investigation performed by C. McJenna showed that the ratio of damage to vines due

to scale insects infestations decreased from 49% to only 7.5% after 12 mo of spirotetramat spraying, (McKenna et al. 2013). Spirotetramat is a new type of spirocyclic tetromic acid pesticide invented. Because spirotetramat has high efficiency, low toxicity, and long-lasting persistence, it is considered to be an ideal pesticide for integrated pest management (Crozier and Cutler 2014, Garzón et al. 2015).

Spirotetramat can resolve into enol-spirotetramat in plants (Lümmen et al. 2014). Once enol-spirotetramat was taken up by insects, it acts on the ACC and then interferes lipid synthesis in insects, which leads to insect cuticular deficiency (Xi et al. 2015) and death (Mohapatra et al. 2012). During the process of lipid synthesis, enzymes such as ACC and fatty acyl-CoA reductase (FAR) are involved. The carboxylation of acetyl-CoA to malonyl-CoA is the first step in the biosynthesis of fatty acids, which is catalyzed by ACCs (Zu et al. 2013). FAR is the key enzyme involved in long-chain primary fatty alcohol biosynthesis, which catalyzes the transformation of fatty acids into fatty alcohols with NADPH (Yang et al. 2012). Fatty alcohols and its derivatives wax-easter participate in insect cuticular formation (Nguyen et al. 2014). The spirotetramat acts on ACC and may affect enzymes in lipid biosynthese such as FARs.

Fatty alcohols are not only the precursors of sex pheromone components but also the precursors of wax-ester in insects (Teerawanichpan et al. 2010, Yang et al. 2012, Carot-Sans et al. 2015). Wax-ester is found throughout the insect epidermis and plays an important role in reducing water evaporation and enhancing defense against micro-organisms and environmental suitability (Jackson and Baker 1970, Cheng and Russell 2004). At present, only a few FAR genes in insects have been identified and characterized (Teerawanichpan et al. 2010, Yang et al. 2012, Jaspers et al. 2014), and no FAR genes of *P. solenopsis* have even been reported. Like ACCs, FARs may be a potential target for pest control. To facilitate understanding the characterization of FARs and relationship to ACCs, two full-length cDNA of PsFAR I and PsFAR II were cloned from *P. solenopsis*, a comparison analysis of sequences features was performed. The relative expression levels during different developmental stages of *P. solenopsis* and the effects of spirotetramat treatment were analyzed.

Materials and Methods

Insects

A population of the cotton mealybug *P. solenopsis* was collected from field and raised in a laboratory for 3 years without exposure to any pesticides. All insects were maintained on pumpkins in artificial climate chambers under the conditions of 28 ± 1°C, 80 ± 5% relative humidity, 24,000 LX illumination intensity, and a photoperiod of 14:10 (L:D) h. The specimens of first instar nymphs, third instar nymphs, male and female adults, and female adults during coviposition were then collected and stored at −80°C before RNA extraction.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from prepared insect specimens (20 mg at least) using RNAiso Plus (TAKARA, Dalian, China) according to the manufacture's protocol. The concentration and quality of total RNA was measured with spectrophotometer (BioDrop μLite, Cambridge, UK) and electrophoresis was carried out to verify the integrity of RNA, Only the total RNA with super quality and integrity was selected for cDNA synthesis by using a PrimeScript 1st Strand cDNA Synthesis Kit (TAKARA, Dalian, China).

Gene Cloning

A transcriptome analysis of *P. solenopsis* has been conducted (Data not shown) using de novo sequencing techniques. Some fragments coding *PsFar I* and *PsFar II* were found based on transcriptome analysis. *Far* sequences are incomplete due to the loss of their 3′ end sequence data. To obtain the complete coding sequence of the *PsFar I* and *PsFar II*, full-length cloning was conducted by rapid amplification of cDNA ends (RACE) using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). The 3′ cDNA ends were amplified in a nested polymerase chain reaction (PCR) system with rTaq using kit primers and gene-specific primers (Table 1).

The products of amplification were purified with an E.Z.N.A. gel extraction kit (Omega Bio-Tek, Norcross, GA) and linked with plasmid pMD19-T vector (TAKARA, Dalian, China) to form a recombinant plasmid, and then transferred into *DH5α* for sequencing using the sequence-specific primersunder the following cycling conditions: 95°C for 3 min, followed by 40 amplification cycles of 15 s at 95°C and 30 s at 55°C.

Sequence Analysis

Full-length cDNA nucleotide sequences of *PsFar I* and *PsFar II* were translated into amino acid sequences and physicochemical properties were then analyzed using Protparam (http://web.expasy.org/protparam/). Signal peptide cleavage sites were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Transmembrane structures were calculated using TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Subcellular locations were predicted using Euk-mPLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/). NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/) was applied to forecast phosphorylated site and SOMPA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl? page=npsa_sopma.html) was used for the prediction of protein secondary structure. To clear the evolutionary relationships of PsFAR I and PsFAR II with other species, multiple sequence alignment was conducted using blastp and then a phylogenetic tree was constructed and analyzed using MEGA 6.06 (neighbor joining).

Pesticide Treatment

A leaf dip bioassay method was used for spirotetramat (Bayer Crop Science AG, Germany) treatment to different developmental stages of *P. solenopsis* (Nauen et al. 2008). Three concentrations of spirotetramat solutions, 31.2, 62.5, and 125.0 mg/liter, were diluted with distilled water containing 0.1% Tween-80 (Sigma-Aldrich, Shanghai China), and the control were treated with 0.1% Tween-80 solution only. Fresh Chinese cabbage leaf discs were cut and dipped in each solution for 15 s and allowed to dry before being placed to Petri dishes (5.0 cm in diameter). A total of 10 third-instar nymphs were used in each dish, and three replicates were performed. All the treatments were kept under standard conditions as described above. After 7 d of treatment, the rate of mortality was recorded and the living insects were collected for RNA isolation and quantitative real-time-PCR (qRT-PCR).

Quantitative Real-Time PCR

The cDNA products of first instar nymphs, third instar nymphs, female adults, male adults, and female adults during oviposition were diluted to 500 ng per microcentrifuge tube. The special primers (Table 1) were designed according to the *PsFar I* and *PsFar II*

Table 1. Sequence of primers used

Purpose	Primer name	Primer sequence (5′-3′)
3′RACE	3′RACE *PsFar I* forward outer primer	TGTCGGCTTAATGCGGTC
	3′RACE *PsFar I* forward inside primer	GCCTTCCTCCATGTCGGT
	3′RACE *PsFar II* forward outer primer	AAGGGATGATAACAGCAGCT
	3′RACE *PsFar II* forward inside primer	TCAGCGGTCAACAGATTA
cDNA cloning	*PsFar I -BamHI-* forward primer	CGGGATCCATGGAGGGCGGCGATAAC
	PsFar I - XhoI - reverse primer	CCGCTCGAGCTTAATTTACACTAAATGCG
	PsFar II -BamHI- forward primer	CGGGATCCATGGAAGAATCGATCGAG
	PsFar II - XhoI - reverse primer	CCGCTCGAGTTAAATAGCGGGCTGGGT
qRT-PCR	qRT-PCR- *PsFar I* - forward primer	TGCATTGCGTTCGATTAGC
	qRT-PCR- *PsFar I* - reverse primer	ATCGCAGCAACTTGGAGA
	qRT-PCR- *PsFar II* - forward primer	CGCTGATGAACTCAAAGA
	qRT-PCR- *PsFar II* - reverse primer	TTCGACGATTAACTTCCTAT
	qRT-PCR-*β-tubulin*- forward primer	CCGTACCTGAACTCACGCAACA
	qRT-PCR-*β-tubulin*- reverse primer	GAATACGGCGGCGACGGTTAA

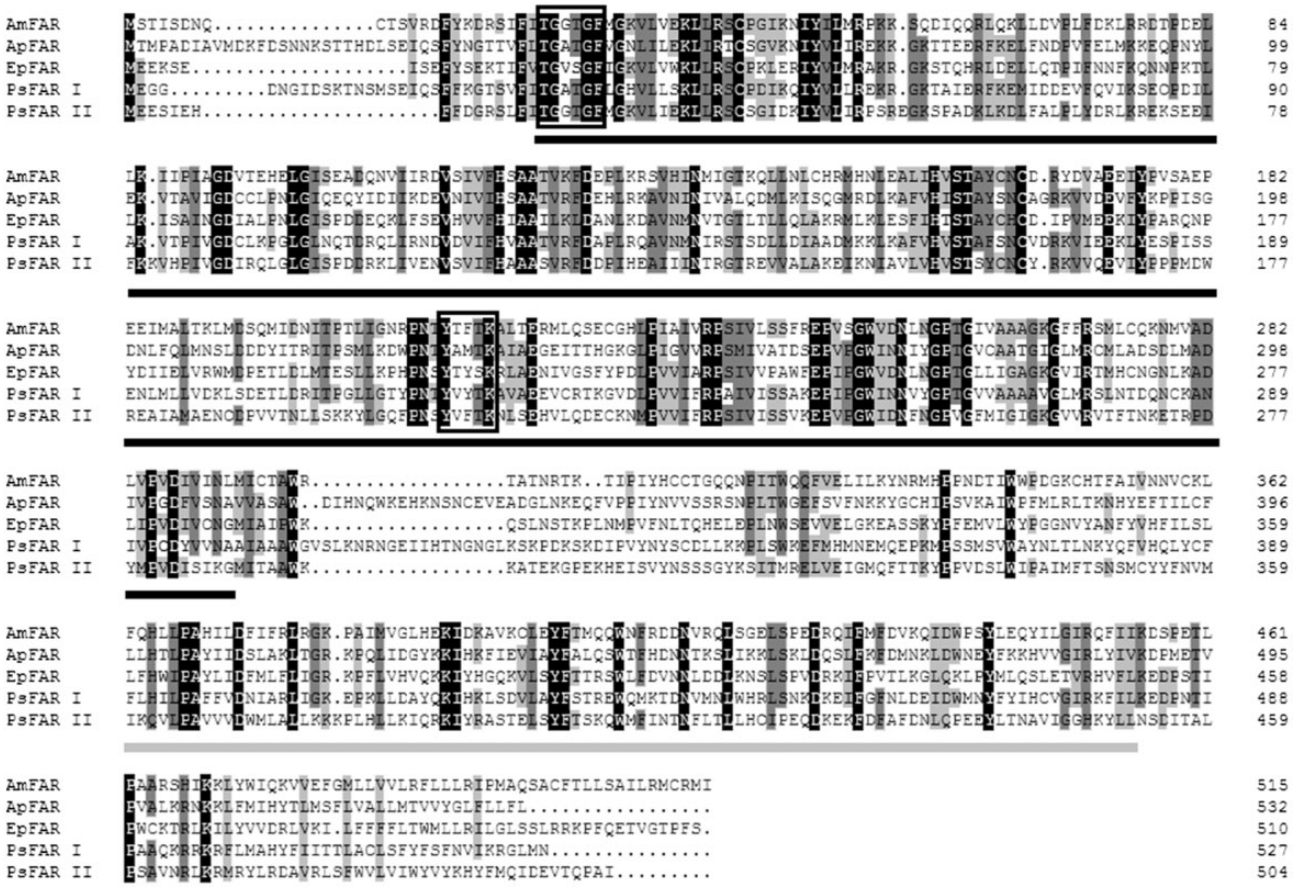

Fig. 1. Multisequencing alignment of FARs of *P. solenopsis* and other insects including *Apis mellifera* (AmFAR, Genebank accession number: ADJ56408.1), *Acyrthosiphon pisum* (ApFAR, Genebank accession number: XP_003243174.1), and *Ericerus pela* (EpFAR,Genebank accession number: AGK27745.1). Rossmann folding region and FAR_C region were indicated in black underline and gray underline, respectively. Two motifs TGXXGG and YXXXK were indicated in black box.

sequences. The qRT-PCR reaction consisted of 20 µl including 10 µl Platinum SYBR Green qPCR SuperMix-DUG (Invitrogen, CA), 1 µl of 10 µM forward and reverse primer separately, 1µl of diluted cDNA and ddH_2O. *β-tubulin* was selected as reference gene. Two-step RT-PCR was conducted on ABI-7300 system (Applied Biosystems, Foster City, CA) under the procedures as follows: 95 °C for 3 min, followed by 40 amplification cycles of 15 s at 95 °C and 30 s at 55 °C. Then melting curves were used to verify the specificity of amplifications. Three biological replicates were carried out per treatment. Relative quantification analysis was then calculated according to $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen 2001).

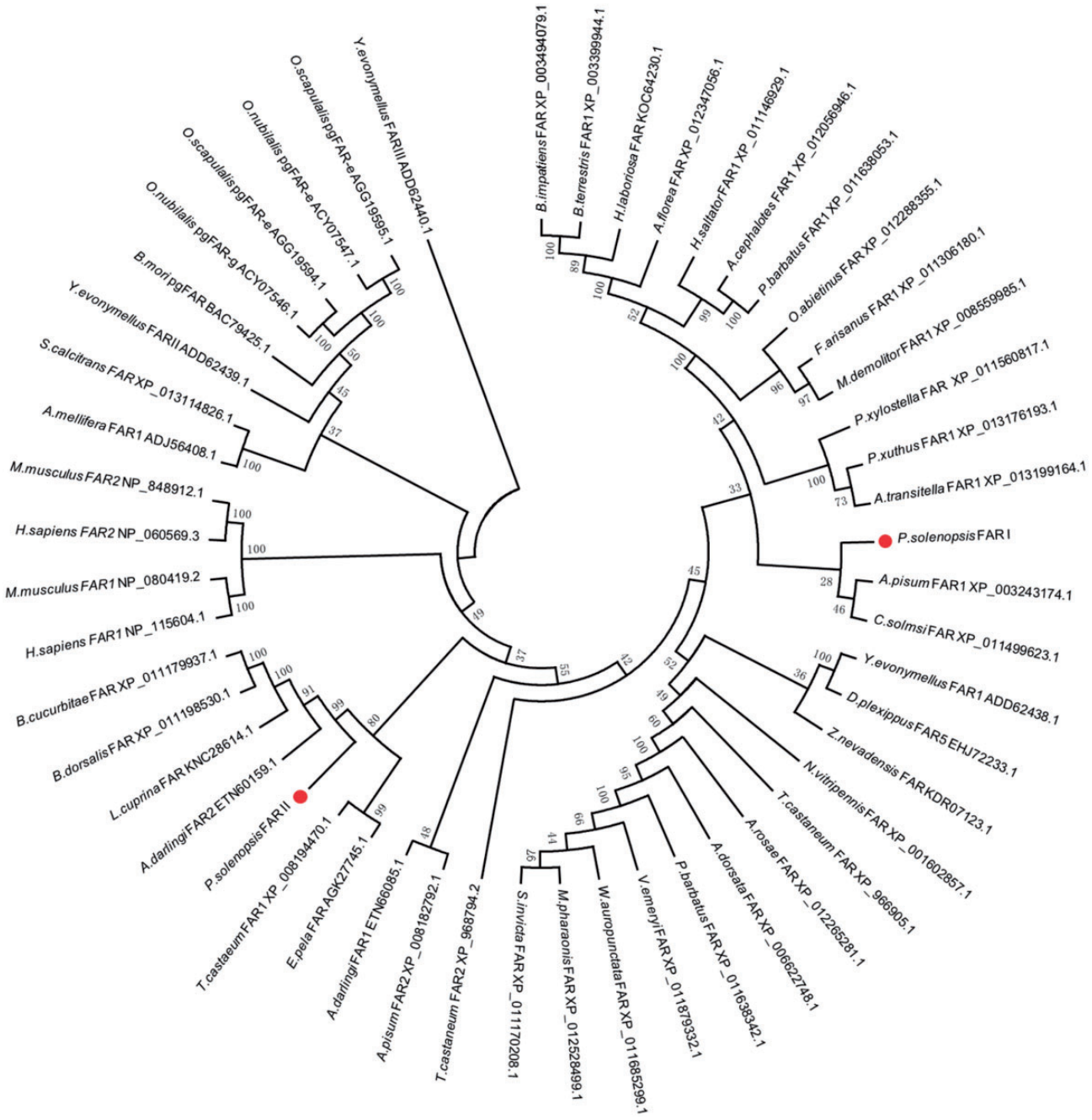

Fig. 2. The molecular phylogenetic tree of FARs from *P. solenopsis* and other organisms. The phylogenetic tree was constructed using Neighbor-Joining method with 1,000 bootstrap replicates. PsFAR I and PsFAR II are classified into different branches. Each species was followed by its accession number.

Results

Sequence Analysis of PsFAR I and PsFAR II

The opening reading frames (ORFs) of full-length *PsFar I* and *PsFar II* were identified using DNAStar, and the data indicated that two ORFs were 1,584 bp and 1,515 bp in length, encoding 527 and 504 amino acids, respectively (NCBI accession numbers: KU963289, KU963290). The protein physicochemical properties of PsFAR I and PsFAR II were analyzed and showed that both PsFAR I and PsFAR II contained 20 common amino acids, of which Leu and Lys were the most plentiful in PsFAR I, reaching 9.5% and 8.0%, respectively. Both PsFAR I and PsFAR II were nonsecretory proteins since no signal peptide was found by SignalP 4.1. Transmembrane domains of PsFAR I (amino acid site: 20–42, 499–521) and PsFAR II (amino acid site: 340–358, 496–504) were analyzed using TMHMM Server 2.0. Prediction of subcellular locations showed that both PsFAR I and PsFAR II were localized in the endoplasmic reticulum. The phosphorylated site predicted by NetPhos 2.0 Server also resulted in differences between PsFAR I and PsFAR II. Protein secondary structure prediction of PsFAR I showed the proportion of α-helixes, β-turns, random coils, and extended strands among total amino acids to be 49.34%, 7.02%, 27.89%, and 15.75%, respectively. The ratio in PsFAR II was 40.87% α-helixes, 8.33% β-turns, 28.77% random coils, and 22.02% extended strands.

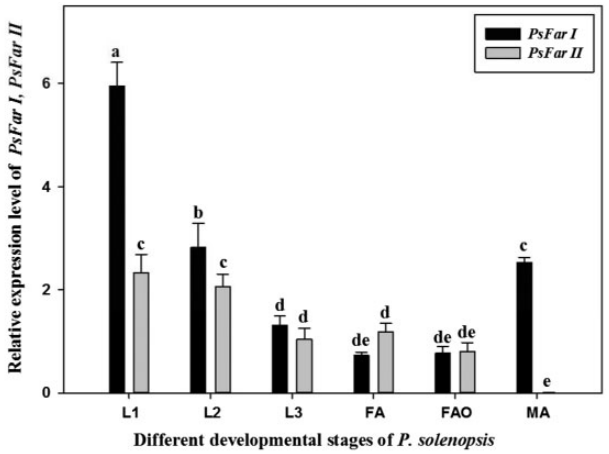

Fig. 3. Relative expression levels of *PsFar I* and *PsFar II* at different development stages of *P. solenopsis*. L1: first nymph, L2: second nymph, 3L: third nymph, FA: female adult, FAO: female adult during oviposition, MA: male adult. The transcript levels of *PsFar I* and *PsFAR II* at each developmental stage were conducted by qRT-PCR using *β-tubulin* as a reference gene. The relative expression of each gene at each developmental stage was calculated relative to the expression level of *PsFar II* in third nymph. Duncan's multiple range tests were used in data analysis. *y* axis were represented as the means ± standard error (SE) of relative expression levels; Lowercase letters (a, b, c, d, e) represent significant differences ($P < 0.05$).

The result of protein blast showed that both PsFAR I and PsFAR II have a Rossmann folding and a FAR_C region (Fig.1). The multisequencing alignment of FAR amino acid sequences of *P. solenopsis*, *Acyrthosiphon pisum*, *Ericerus pela*, and *Apis mellifera Ligustica* conducted using DNAMAN showed that the homogeneity reached 49.34% and that several highly conserved motifs were discovered as shown in Figure 1, including a NADPH combining motif, TGXXGG, and an active site motif, YXXXK.

A molecular phylogenetic tree containing human, mouse, and insect genes was then produced using the neighbor-joining method and MEGA 6.06 (Fig. 2). Results placed PsFAR I of *P. solenopsis*, FAR I of *Acyrthosiphon pisum*, and FAR of *Ceratosolen solmsi* on the same branch. PsFAR II of *P. solenopsis* was on the same branch as FAR II of *Anopheles darlingifa*, and FAR of *Lucilia cuprina*, *Bactrocera cucurbitae*, and *Bactrocera dorsalis*.

Gene Expression at Different Developmental Stages

The expression profiles of *PsFar I* and *PsFar II* at various developmental stages were generated using qRT-PCR (Fig. 3). The expression of *PsFar I* was detected at all tested stages including first, second, third instar nymph, female adults, female adults during oviposition, and male adults. The level of *PsFar I* was significantly higher in first instar nymphs than other developmental stages. With respect to *PsFar II*, the relative high expression was detected at first and second nymphs and very low expression was found at male adults. Between these two genes, the expression of *PsFar I* was higher than *PsFar II* in first instar, second instar nymphs, and male adults. No differences were found among third instar nymphs, female adults, and female adults during oviposition.

Gene Expression After Spirotetramat Treatment

To test whether the expression of FAR gene is inducible by spirotetramat, three concentrations of spirotetramat, 31.2, 62.5, and 125 mg/liter and one control were applied to treat *P. solenopsis* using a leaf dip bioassay. In 7 d treatment, we observed that the cuticle of *P. solenopsis* under spirotetramat treatment turned yellow and the wax on the cuticle reduced. We also observed that 10.7% of *P. solenopsis* died at the 125 mg/liter treatment, whereas 0.3% and 3.7% of *P. solenopsis* died at 31.2 and 62.5 mg/liter treatment respectively (Fig. 4).

The expression of two *PsFar* from the living insects after 7 d treatment was examined by qRT-PCR. The data were analyzed using SPSS 19.0. The expression level of *PsFar I* increased as the concentration of spirotetramat increased. Upregulation of expression of *PsFar I* by spirotetromat showed a dose dependant pattern (Fig. 4). One hundred and twenty-five milligram per liter treatment group showed a more pronounced increase (Fig. 4). The expression of *PsFar II* showed that there was no significant difference among the treatments at different concentrations of spirotetramat (Fig. 4).

Discussion

In this study, two full-length *PsFar I* and *PsFar II* cDNA of *P. solenopsis* were successfully cloned and characterized. Both PsFAR I and PsFAR II were predicted to be located in the endoplasmic reticulum by EukmPLoc approach. Protein confirmation analysis of PsFAR I and PsFAR II indicated that both had a Rossmann folding region and a FAR_C region. In particular, two FAR conservative motifs were existed in Rossmann folding region by sequence alignment including a NADPH combining motif, TGXXGG, and an active site motif, YXXXK (Chacón et al. 2013). Recent studies reported that all the FAR genes identified in insects were predicted to be locating in the endoplasmic reticulum. FAR of *Heliothis virescens* was confirmed to be present in the endoplasmic reticulum by subcellular localization (Hagström et al. 2013).

A molecular phylogenetic tree constructed by neighbor-joining method placed PsFAR I and PsFAR II of *P. solenopsis* in two different branches. Different FARs even from same organism cannot be categorized to the same branch, which indicates that these FARs have different functions. It is speculated that the function of PsFAR I differs from that of PsFAR II. Therefore, *Far* expression was analyzed during various developmental stages of *P. solenopsis* and the effects of pesticide treatment.

The relative expression levels of *Far* genes in different developmental stages and insect tissues vary considerably (Teerawanichpan et al. 2010, Yang et al. 2012, Antony and Ishikawa 2013). Plenty of studies of Lepidoptera have reported that some *Fars* are only found in the sexual glands of insects, such as *Ostrinia scapulalis*, *Yponomeuta evonymellus*, *Spodoptera littoralis*, and *Bombyx mori* (Moto et al. 2003, Antony et al. 2009, Liénard et al. 2010, Hagström et al. 2012, Antony and Ishikawa 2013). Moto et al. (2003) indicated that *Far* of *B. mori* is only expressed in sexual glands and only before female adult eclosion. None was found in male adults. In this study, *PsFar I* was found to be highly expressed in nymphs and male adults of *P. solenopsis*, which indicated that *PsFar I* was not expressed solely in sexual glands. *PsFar II* was found pretty low expression in male adults, which is similar as *Far* from *B. mori*. For this reason, it was speculated that PsFAR I may be involved in wax-ester synthesis whereas PsFAR II may play roles in pheromone synthesis.

The results showed *PsFar I* and *PsFar II* of *P. solenopsis* have a high level of expression in first and second instar nymphs. High levels of expression suggested that the fatty alcohol synthesis was much more active during the first and second instar than during other periods. The various expression levels of *Far* at different developmental stages have also been reported in *Ericerus pela* (Yang et al. 2012). The substrates of different FARs vary. In mice, the enzyme of FAR I preferred unsaturated fatty acids with 16 or 18 carbons as substrates, whereas the preferential substrates of FAR II were

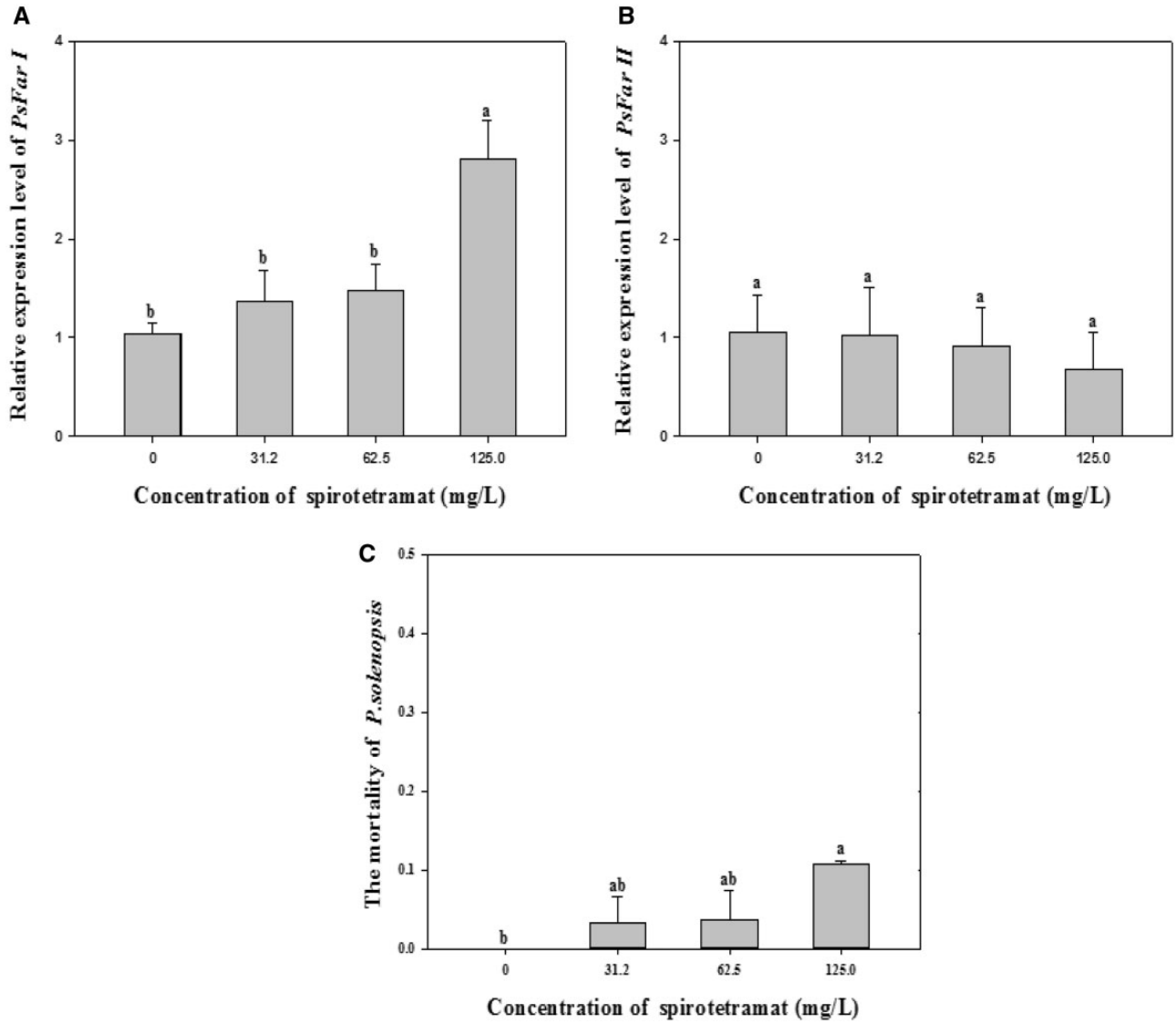

Fig. 4. Relative expression levels of *PsFar I* (A) and *PsFar II* (B) and mortality of *P. solenopsis* (C) after 7 d spirotetramat treatment. 0, 31.25, 62.5, and125 mg/liter of spirotetramat in 0.1% Tween-80 solution were used to treat third instar nymphs. Expression level of each gene was conducted using *β-tubulin* as a reference gene. Relative gene expression was calculated relative to the expression of control group. Duncan's multiple range tests were used in data analysis. Lowercase letters (a, b) represents significant differences ($P < 0.05$).

saturated fatty acids with 16 or 18 carbons (Cheng and Russell 2004). PsFAR I and PsFAR II of *P. solenopsis* may have different preferential substrates of enzyme, which needs further studies.

Spirotetramat interferes with fatty acids synthesis by competitively inhibiting the activity of acetyl-CoA (Lümmen et al. 2014). ACC is a rate-limiting enzyme in de novo fatty acid biosynthesis, in which acetyl-CoA can be catalyzed into malonyl CoA (Zu et al. 2013). Fatty acids are the substrates in fatty alcohol synthesis (Riendeau and Meighen 1985). Using Spirotetramat treatment to inhibit de novo fatty acid biosynthesis would affect fatty alcohol synthesis and may affect FAR gene expression level as well. In our study, we found *P. solenopsis* turned yellow and wax reduced after 7 d spirotetramat treatment, which was similar as cuticular deficiency observed in *Aphis gossypii* (Xi et al. 2015). The relative expression levels after spirotetramat treatment showed that *PsFarI* was upregulated and there was no effect on the expression of *PsFarII*, which indicated that PsFAR I and PsFAR II play different roles in responding to spirotetramat. Although the mechanism of fatty acid metabolism in detoxify spirotetramat is unclear, Xi et al. (2015) found fatty acid metabolism proteins were upregulated in resistant strain of *A. gossypii*, including ACC and faty acid synthase. This indicated that fatty acid metabolism may be involved in detoxifying spirotetramat. The upregulated expression of *PsFar I* may contribute to make up fatty alcohol and wax-ester in insect epicuticle. This needs further investigation.

Acknowledgments

The authors would like to thank for the financial support from the 973 Program of China (2013CB127603), Graduate Students Innovation Project of Priorities Forestry Discipline of Zhejiang Province (201520) and the Development Fund of Zhejiang A&F University (2012FR087).

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