Spatiotemporal expression profiling of the farnesyl diphosphate synthase genes in aphids and analysis of their associations with the biosynthesis of alarm pheromone

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Abstract

The alarm behavior plays a key role in the ecology of aphids, but the site and molecular mechanism for the biosynthesis of aphid alarm pheromone are largely unknown. Farnesyl diphosphate synthase (FPPS) catalyzes the synthesis of FPP, providing the precursor for the alarm pheromone (E)-β-farnesene (EβF), and we speculate that FPPS is closely associated with the biosynthetic pathway of EβF. We firstly analyzed the spatiotemporal expression of FPPS genes by using quantitative reverse transcription-polymerase chain reaction, showing that they were expressed uninterruptedly from the embryonic stage to adult stage, with an obvious increasing trend from embryo to 4th-instar in the green peach aphid *Myzus persicae*, but *FPPS1* had an overall significantly higher expression level than *FPPS2*; both *FPPS1* and *FPPS2* exhibited the highest expression in the cornicle area. This expression pattern was verified in *Acyrthosiphon pisum*, suggesting that *FPPS1* may play a more important role in aphids and the cornicle area is most likely the site for EβF biosynthesis. We thus conducted a quantitative measurement of EβF in *M. persicae* by gas chromatography-mass spectrometry. The data obtained were used to perform an association analysis with the expression data, revealing that the content of EβF per aphid was significantly correlated with the mean weight per aphid ($r = 0.8534$, $P = 0.0307$) and the expression level of *FPPS1* ($r = 0.9134$, $P = 0.0109$), but not with that of *FPPS2* ($r = 0.4113$, $P = 0.4179$); the concentration of EβF per milligram of aphid was not correlated with the mean weight per aphid or the expression level of FPPS genes. These data suggest that *FPPS1* may play a key role in the biosynthesis of aphid alarm pheromone.

Keywords: farnesyl diphosphate synthase, spatiotemporal expression, aphid alarm pheromone, (E)-β-farnesene, *Myzus persicae*, *Acyrthosiphon pisum*

Introduction

The green peach aphid *Myzus persicae* and the pea aphid *Acyrthosiphon pisum* are the cosmopolitan agricultural pests and the major component of alarm pheromone released by them is (E)-β-farnesene (EβF) (Griffiths & Pickett, 1980;

Francis *et al.*, 2005), which plays important roles in mediating interactions among aphids, host plants and aphid natural enemies. EβF widely exists in plants (Kunert *et al.*, 2005), and EβF synthase genes have been isolated from different plants and some microorganisms (Picaud *et al.*, 2005; Zhao *et al.*, 2009) and researchers have been working on the use of EβF as an aphid repellent in the push-pull strategy (Cook *et al.*, 2007). The EβF synthase has been heterologously expressed in plants by transgenic technology and the EβF released by the transgenic plants repelled aphids and even attracted natural enemies (Beale *et al.*, 2006; Yu *et al.*, 2012; Bruce *et al.*, 2015). Nevertheless, as of now, the site and

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molecular mechanism of EβF biosynthesis in aphids are still largely unknown.

The aphid alarm pheromone EβF as a sesquiterpene is secreted from the cornicles of aphids, but the source of EβF is still unclear. Theoretically, there are three possible sources for the EβF released by aphids: the host plant, endosymbionts and the aphid itself. Yet, we have recently eliminated the possibility of the host plant and endosymbionts as the sources of EβF in aphids by rearing aphids on non-plant diets and antibiotic-treatment (Sun & Li, 2017). It is, therefore, most likely that the aphid itself is responsible for EβF synthesis. However, no sequences homologous to plant sesquiterpene synthase have been identified so far, although the genome sequence of several aphid species have been available, including *M. persicae* and *A. pisum* (Legeai *et al.*, 2010; The International Aphid Genomics Consortium, 2010).

Farnesyl diphosphate (FPP) is the precursor for biosynthesis of juvenile hormone (JH) in insects, and the site for JH biosynthesis is known to be the corpora allata (CA); FPP was also the precursor for the biosynthesis of EβF in plants (Sallaud *et al.*, 2009). The biosynthesis of FPP is catalyzed by the farnesyl diphosphate synthase (FPPS). The first insect FPPS was reported in the moth *Agrotis ipsilon* (Castillo-Gracia & Couillaud, 1999). Since then, many FPPS genes have been identified in other insects, including aphid species (Cusson *et al.*, 2006; Sen *et al.*, 2007; Lewis *et al.*, 2008; Taban *et al.*, 2009; Bomtorin *et al.*, 2014; Huang *et al.*, 2015). In our laboratory, we have worked on aphid FPPS genes for many years, including *MpFPPS1* and *MpFPPS2* in *M. persicae* (Zhang & Li, 2008, 2012), *AgFPPS* in the cotton aphid *Aphis gossypii* (Ma *et al.*, 2010; Zhang & Li, 2013, 2014; Sun & Li, 2017) and *RpFPPS1* and *RpFPPS2* in the bird cherry-oat aphid *Rhopalosiphum padi* (Sun & Li, 2012). *In vitro* analysis of enzymatic activity with recombinant proteins showed that aphid FPPSs displayed dual geranyl/FPPS activity, and a linked enzymatic assay using the aphid FPPS and peppermint sesquiterpene synthase produced EβF (Lewis *et al.*, 2008), indicating the potential involvement of aphid FPPS in the biosynthesis of alarm pheromone. Therefore, we suppose that FPP should also be the precursor for EβF biosynthesis in aphids if it is originated from the aphid itself. Moreover, the plasticity of FPPS and other isoprenoid synthases also prompted us to speculate that they might have a moonlighting synthase activity responsible for the biosynthesis of EβF in aphids. For instance, the geranyl diphosphate synthase (GPPS) in the bark beetle *Ips pini* showed both GPPS and monoterpene synthase activity (Gilg *et al.*, 2009); the α-farnesene synthase from apple displayed both sesquiterpene synthase and prenyltransferase activity (Green *et al.*, 2007), and the albaflavenone synthase (CYP170A1) in the soil bacterium, *Streptomyces coelicolor*, also exhibited the activity of a farnesene isomerase (Zhao *et al.*, 2009).

The expression analysis of FPPS genes has mainly been focused on their association with JH biosynthesis. The highest expression of FPPS gene was detected in CA in the insects such as *Adhemarius ypsilon* (Castillo-Gracia & Couillaud, 1999), *Diploptera punctata* (Huang *et al.*, 2015) and *Leptinotarsa decemineata* (Li *et al.*, 2016). In the silkworm *Bombyx mori*, the gene *BmFPPS2* was highly expressed in CA, while *BmFPPS1* expressed in various tissues, suggesting that *BmFPPS2* played a key role in the biosynthesis of JH (Cusson *et al.*, 2006; Kinjoh *et al.*, 2007; Cheng *et al.*, 2014). Interestingly, four FPPS genes were identified in the cotton bollworm *Helicoverpa armigera*, and tissue expression showed that *HaFPPS1* and *HaFPPS2* were mainly expressed in the head,

and the expression level of *HaFPPS3* was very low in all tissues examined, but the transcripts of *HaFPPS4* were highly enriched in the head, epidermis and fat body (Zhang *et al.*, 2017). Subsequent injection of ds*FPPS4* significantly reduced the mRNA level of *HaFPPS4* in the head, which was accompanied by a decrease of JH titer *in vivo* and an effect on the normal molting of larvae. On the other hand, the results of temporal expression analysis of FPPS genes were more complicated, as they were influenced by various factors, including sex, molting, mating, stadium and feeding status (Kinjoh *et al.*, 2007; Bomtorin *et al.*, 2014; Cheng *et al.*, 2014; Li *et al.*, 2016; Zhang *et al.*, 2017). For example, the expression patterns of FPPS gene in the blister beetle *Mylabris cichorii* (emitting cantharidin as a defensive terpene) were different during different adult phases (Zha *et al.*, 2017). There was only one case in which the expression of an aphid FPPS gene was analyzed, indicating that the single FPPS gene in *Aphis gossypii* was highly expressed in CA at adult stage (Ma *et al.*, 2010). In the present study, we firstly conducted a spatiotemporal expression analysis of the two FPPS genes (*FPPS1* and *FPPS2*) in *M. persicae* and *A. pisum*; secondly, the amounts of EβF were quantitatively measured by gas chromatography-mass spectrometry (GC-MS), and finally, an association analysis was performed using the expression data and the amounts of EβF. Our results suggested that the cornicle area may be the site for EβF biosynthesis, and *FPPS1* gene may play a key role in the biosynthesis of aphid alarm pheromone.

Method

Sample acquisition

M. persicae and *A. pisum* were reared on pepper and broad bean plants, respectively, and both aphid species were maintained separately in our laboratory (20 ± 1 °C; L16:D8; relative humidity 65–75%). Apterous adult aphids were transferred to host plants and removed after 12 h to acquire developmentally consistent aphid offspring. The aphids of each developmental stage were collected, including the embryo, 1st-, 2nd-, 3rd-, 4th-instar nymphs, apterous and alate adults (supplementary fig. 1). For nymphs and adults, the antennae of aphids were gently touched with a soft brush, and when their stylets were pulled out from plant leaves, the aphids were then collected into a 1.5-ml microcentrifuge tube, ensuring the cornicle droplets were not released from aphids; the embryos were dissected directly from the adults in a plastic petri dish. The aphids at different stages were directly put into liquid nitrogen for RNA extraction. For tissue-specific expression analysis, the apterous adult aphids were selected for the convenience of tissue dissection, which were dissected in 0.1% DEPC water under a stereomicroscope; the CA, cornicle (cut off from the 5th- or 6th-tergite) and midgut were isolated and immediately transferred to a centrifuge tube containing TRIzol reagent (Aidlab, Beijing, China) (Bogaert *et al.*, 2015). Total RNA was extracted following the instructions, and the first-strand cDNA was synthesized with 500 ng RNA by using the PrimeScript™ RT reagent Kit (TaKaRa, Beijing, China), which was diluted five times with double-distilled water for further quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis.

Spatiotemporal expression analysis of FPPS1/2 by using qRT-PCR

A total of seven pairs of primers were designed based on the cDNA sequences of *MpFPPS1/2* of *M. persicae* (EU

334430, EU334431) and *ApFPPS1/2* of *A. pisum* (AY96858, XM_001950388), with four pairs of primers as reference primers accordingly (Mutti *et al.*, 2006; Ma *et al.*, 2010; Yang *et al.*, 2014; Kang *et al.*, 2017) (table 1). The specificity of primers was verified by PCR in a total reaction volume of 25 μl containing 2 μl cDNA template, 1 μl of each primer (10 μM), 2.5 μl 10 × PCR buffer (TransGen Biotech, Beijing, China), 2 μl dNTP (2.5 mM), 0.5 μl Taq DNA polymerase (5.0 U μl^{-1}) and 16 μl ddH$_2$O. The reaction conditions were 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final 10 min extension at 72 °C. PCR products were analyzed by 1% agarose gel electrophoresis.

For qRT-PCR, the cDNA was successively diluted for 4^0, 4^1, 4^2, 4^3 and 4^4 times as templates, and the quality of primers was judged by the standard curve and melting curve. A technically qualified pair of primers for relative quantification should ensure that both the correlation coefficient and amplification efficiency of the standard curve are within the reference range ($R^2 > 0.99$; $90\% < E < 110\%$) and the melting curve is unimodal (Livak & Schmittgen, 2001; Pfaffl, 2001). The reaction was performed in a total volume of 20 μl containing 10 μl AceQ$^®$ qPCR SYBR$^®$ Green Master Mix (Vazyme, Nanjing, China), 0.4 μl of each primer (10 μM), 1 μl cDNA and 8.2 μl ddH$_2$O. Because the expression level of *ApFPPS2* was excessively low, the amount of cDNA was increased to 4 μl. The thermocycling program was 50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s and the conditions for melting curve stage were 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. All samples were assayed in triplicate on an ABI 7500 ver. 2.0.6. The relative expressions of target genes were calculated by the formula $2^{-\Delta\Delta C_t}$ (Livak & Schmittgen, 2001), where $\Delta\Delta C_t = (C_{t_{target}} - C_{t_{reference}})_{treatment} - (C_{t_{target}} - C_{t_{reference}})_{control}$. Three biological repetitions were conducted.

Quantitative analysis of EβF in M. persicae

M. persicae aphids of different developmental stages were carefully transferred with a soft brush from pepper plants to 1.5 ml centrifuge tubes for counting or weighing before analysis of EβF. A total of 350, 350, 350, 150, 50, 36 and 30 individuals for the embryo, 1st- to 4th-instar and adults were used, respectively. The individuals of similar size were selected on the 2nd day of each stage to eliminate the bias caused by body size. The sampling method was modified slightly from van Emden *et al.* (2014). Briefly, hexane (50 μl, with 0.005 μl undecane as the internal standard) was added to a 1.5 ml centrifuge tube and the aphids were fully crushed with a sealed plastic pipette tip, which was then centrifuged at 12 000 rpm for 5 min. Prior to analysis, the samples were stored at −20 °C for no more than 3 days. For GC-MS analysis, 30 μl supernatant without residues was transferred to a 2 ml glass chromatography vial with a 250 μl polypropylene intubation with polyurethane foot. A synthetic farnesene solution was diluted 200 times with hexane and used as the standard. The solvent extract (2 μl) and farnesene standard (2 μl) were analyzed, respectively, by Agilent 7890B/7200 (HP-5MS, 30 m × 0.25 mm inner diameter × 0.25 μm film thickness) with a low-pole column under the conditions reported by van Emden *et al.* (2014). The carrier gas was helium with a split ratio of 2:1 and mass spectra were obtained in the EI mode at 70 *eV*. The volatile substance was analyzed by Varian Saturn 2100D using NIST spectrum database and compared with farnesene standard. Three biological repetitions were conducted for each developmental stage. The measured amounts of EβF were corrected by the number of individuals or the mean weight per aphid (mg).

Data analysis

The gene expression in different tissues and EβF measurement data were sorted on Microsoft Excel and analyzed by using the Least Significant Difference (LSD) multiple comparison methods of one-way ANOVA and independent sample *t* test at 5% and 1% levels on SPSS v.21.0 software; the expression levels during developmental stages were analyzed by using Tukey's test, with the expression level of *MpFPPS1* at apterous adult stage as the control. Association analyses were performed by linear regression on Excel.

Results

Optimization of qRT-PCR conditions and spatiotemporal expression profiling of Mp*FPPS1/2 in* M. persicae

Totally seven pairs of primers (four FPPS genes and three reference genes) were checked. According to gel electrophoresis, the standard and melting curves, correlation coefficients (R^2) and amplification efficiencies (*Eff* %) (table 1), the primers *Mp*FPPS1-110, *Mp*FPPS2-119, Actin and 18S were selected for expression analysis of *Mp*FPPS genes. Similarly, four pairs of primers (three FPPS genes and one reference gene) were checked for *A. pisum*, and the primers *Ap*FPPS1-167 and *Ap*FPPS2-207 were selected for expression analysis of *Ap*FPPS genes. The reference gene as an internal control is very important for the quantitative analysis of the target gene. A qualified reference gene should be stably expressed in all tissues or at different developmental stages (Livak & Schmittgen, 2001). The *actin* gene is commonly used as an internal standard for qRT-PCR analysis (Ma *et al.*, 2010), and our analysis showed that it was expressed steadily during different developmental stages of *M. persicae*. We thus used *actin* as the reference gene for temporal expression analysis of *MpFPPS1/2*. The results showed that both *MpFPPS1* and *MpFPPS2* were expressed across the whole developmental period (fig. 1). Specifically, they were expressed uninterruptedly from the embryonic stage to adult stage, but *MpFPPS1* exhibited an overall significantly higher expression than *MpFPPS2* (*P* = 0.007). The expression levels of *MpFPPS1* were higher than those of *MpFPPS2* by 4.3-, 3.8-, 3.1-, 0.8-, 0.7-, 2.8- and 3.2-fold at the embryo, 1st- to 4th-instar, wingless and winged adult stages, respectively, reaching the highest at apterous adult stage, significantly higher than all other stages (*P* = 0.032) (table 2). In contrast, the expression of *MpFPPS2* showed an increasing trend from the embryo and the 1st-instar to the 3rd-instar, and reached the peak at the 4th-instar stage, significantly higher than all other stages (*P* = 0.026); thereafter, its expression dramatically dropped at the adult stage (fig. 1; table 2).

Based on the temporal expression analysis of *MpFPPS1/2* and for the convenience of tissue dissection, the apterous adults were used for tissue expression analysis. When *actin* was used as the internal control, the results showed that *MpFPPS1* had the highest expression in the midgut, followed by in the cornicle area, with the lowest in the CA. The transcript abundance of *MpFPPS1* was more than 100 times higher in the midgut than in the CA (supplementary fig. 2). In comparison, *MpFPPS2* showed approximately sevenfold higher expression in the cornicle area and midgut than in the CA.

Table 1. Primers sets for qRT-PCR.

Name of primer	Primer sequence (5′→3′)	Fragment size (bp)	R^2	Eff %
MpFPPS1-110	F: AAATTGGCACAGACATAGAAGAT	110	0.999	103.256
	R: ATCAATACCATAGTTGTCCTCCA			
MpFPPS1-228	F: AACATCAATGACCCAGAAATC	228	0.990	106.813
	R: ATCAATACCATAGTTGTCCTCCA			
MpFPPS2-119	F: TGGAAAATGTTCATGGTTGG	119	0.991	102.109
	R: AATCTTTGATGACTGCGACA			
MpFPPS2-151	F: ACAATGAAATCTGCTATGGGT	151	0.993	98.056
	R: CGAGACAAACAGGAAGAACAAA			
Actin-254	F: TCTTGGGAATGGAATCTTGC	254	0.999	106.369
	R: GGACAGAGAAGCCAAGATGG			
18S-80	F: TCAACACGGGAAACCTCACCA	80	0.992	102.458
	R: CACCACCCACCGAATCAAGAA			
L27-231	F: CCGAAAAGCTGTCATAATGAAGACC	231	—	—
	R: GGTGAAACCTTGTCTACTGTTACATCTTG			
ApFPPS1-167	F: ATGGGTCAGTGTCTTGATATG	167	0.994	105.700
	R: TCTGGGTCATTGATGTTTG			
ApFPPS2-207	F: TTCTTCCCGTGTTTCTCGC	207	0.992	99.980
	R: GCAAAGCCACTACAGCCAAC			
ApFPPS2-205	F: TCTTCCCGTGTTTCTCGC	205	—	—
	R: CAAAGCCACTACAGCCAAC			
SDHB-90	F: CTGAATTCCTGTGGACCTATGG	90	0.996	103.706
	R: ACGGCAAGAACGCCTAAA			

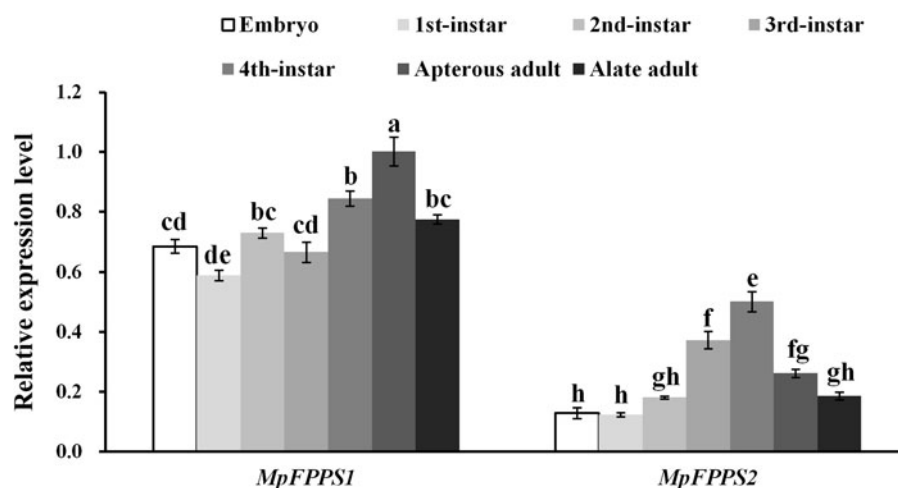R^2, correlation coefficient; Eff %, amplification efficiency.

Fig. 1. Expression analysis of *MpFPPS1* and *MpFPPS2* at different developmental stages of *M. persicae*. Different lowercase letters on the bars indicate a significant difference in the expression level of *MpFPPS1/2* between different stages at 5% level by using Tukey's multiple comparison methods. The alphabetical order corresponds to decreasing expression level. The same letters indicate no significant difference at 5% or 1% levels.

However, *actin* was found expressed inconsistently in different tissues (Supplementary fig. 3), implying that it is unsuitable for being used as an internal reference for tissue-specific expression analysis. Therefore, we tried the 18S gene reported by Kang *et al*. (2017) for *M. persicae*. The results demonstrated that *18S* was expressed steadily in different tissues (supplementary fig. 3). With *18S* as the internal control, expression analysis showed that both *MpFPPS1* and *MpFPPS2* had the highest expression level in the cornicle area, and the overall expression of *MpFPPS1* was remarkably higher than that of *MpFPPS2* (fig. 2). More specifically, there was a significant difference in the expression of *MpFPPS1* between different

tissues ($P = 0.003$), with the lowest observed in the CA. Similarly, *MpFPPS2* had approximately ninefold and 27-fold higher expression in the cornicle area than in the CA and midgut, respectively ($P < 0.001$), and there was no significant difference between CA and midgut (fig. 2).

Spatiotemporal expression analysis of FPPS genes in A. pisum

Phylogenetic analysis based on FPPS amino acid sequences showed that *A. pisum* and *M. persicae* are closely related aphid species (Ma *et al*., 2010). Here *A. pisum* was used to verify the FPPS expression patterns observed in *M. persicae*. Since

Table 2. Expression analysis of *MpFPPS1/2* at different developmental stages of *M. persicae*.

	MpFPPS1	*MpFPPS2*
Embryo	0.684 ± 0.023BCcDd	0.128 ± 0.019Dd
1st-instar	0.589 ± 0.018Dd	0.123 ± 0.007Dd
2nd-instar	0.729 ± 0.017BbCcD	0.18 ± 0.004CcDd
3rd-instar	0.665 ± 0.033CcDd	0.372 ± 0.029Bb
4th-instar	0.844 ± 0.025ABb	0.501 ± 0.034Aa
Apterous adult	1.002 ± 0.048Aa	0.261 ± 0.014BCc
Alate adult	0.774 ± 0.015BbCc	0.186 ± 0.013CcDd

Data are mean ± S.E. ($n = 3$). Different lower and uppercase letters within the same column indicate a significant difference in the expression level of *MpFPPS1/2* at 5% and 1% levels, respectively, by using Tukey's multiple comparison methods. The alphabetical order corresponds to decreasing expression level. The same letters indicate no significant difference at 5% or 1% levels.
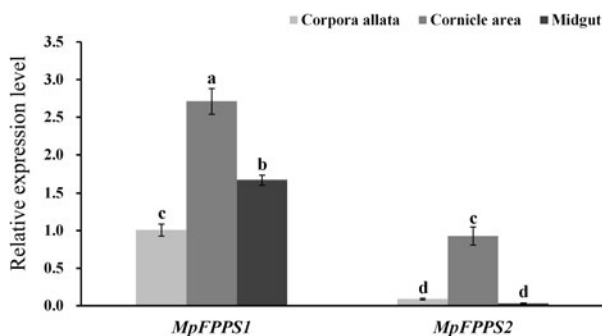

Fig. 2. Expression analysis of *MpFPPS1 and MpFPPS2* in different tissues of the apterous *M. persicae* adults with 18S gene as the internal control. Different lowercase letters on the bars indicate a significant difference in the expression level of *MpFPPS1/2* between different tissues at 5% level by using LSD multiple comparison methods. The alphabetical order corresponds to decreasing expression level. The same letters indicate no significant difference at 5% or 1% levels.

MpFPPS1 and *MpFPPS2* had the highest expression in the 4th-instar and apterous adult stages, these two stages were used for expression analysis of *ApFPPS1/2* in *A. pisum*. The SDHB gene was reported to be a suitable internal reference for *A. pisum* (Yang *et al.*, 2014) and we confirmed its validity (supplementary fig. 4). Our qRT-PCR results showed that *ApFPPS1* had a significant lower expression at the 4th-instar stage than at apterous adult stage ($P = 0.038$), but there was no significant difference in the expression of *ApFPPS2* between these two stages ($P = 0.146$). Like *MpFPPS1*, *ApFPPS1* showed significantly higher overall expression than *ApFPPS2* during the two developmental stages ($P < 0.001$) (fig. 3).

In tissue expression analysis, the CA, cornicle and midgut were dissected from apterous *A. pisum* adults. The results showed that both *ApFPPS1* and *ApFPPS2* were expressed significantly higher in the cornicle area. More specifically, the expression level of *ApFPPS1* was significantly different between the tissues ($P < 0.01$), with the least in the midgut, but *ApFPPS2* exhibited no significant expression between the CA and midgut ($P = 0.37$) (fig. 4).
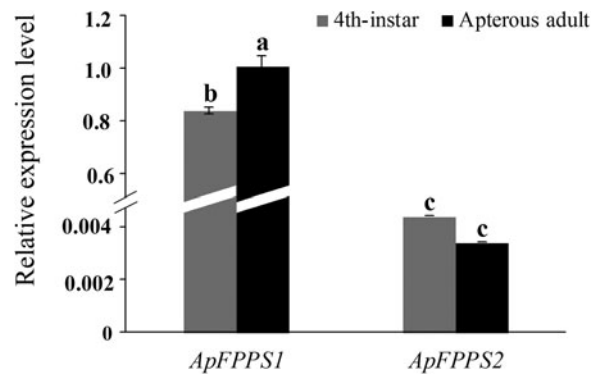

Fig. 3. Expression analysis of *ApFPPS1* and *ApFPPS2* at the 4th-instar and apterous adult stages of *A. pisum*. Different lowercase on the bars indicates a significant difference in the expression level of *ApFPPS1/2* between different stages at 5% level by using LSD multiple comparison methods. The alphabetical order corresponds to decreasing expression level. The same letters indicate no significant difference at 5% or 1% levels.
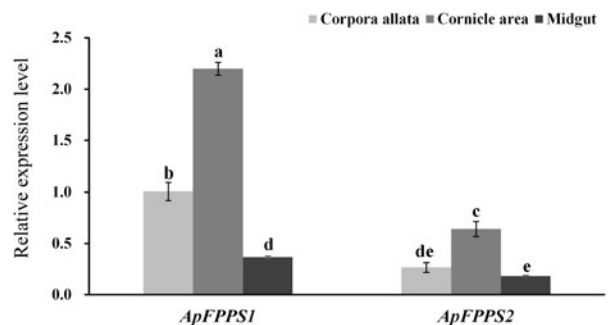

Fig. 4. Expression analysis of *ApFPPS1* and *ApFPPS2* in different tissues of *A. pisum*. Different lowercase letters on the bars indicate a significant difference in the expression level of *ApFPPS1/2* between different tissues at 5% level by using LSD multiple comparison methods. The alphabetical order corresponds to decreasing expression level. The same letters indicate no significant difference at 5% or 1% levels.

Quantitative analysis of EβF in the aphid

Since both MpFPPS and ApFPPS genes showed significantly higher expression in the cornicle area and the cornicle is the organ for secretion of aphid alarm pheromone, we thus speculated that FPPS genes should be closely related to the biosynthesis of EβF. Therefore, we conducted a quantitative measurement of EβF emitted by *M. persicae* by using GC-MS. We measured the content of EβF per aphid and the concentration of EβF per milligram of aphid. The retention time of EβF was 20.784 min and the characteristic ions were 69, 93, 105, 120, 133 and 161 (supplementary fig. 5). Quantitative analyses showed that, with the growth of *M. persicae*, the average weight of aphid significantly increased from 1st- to 4th-instar, but there were no significant differences between embryo and 1st-instar and between 4th-instar and apterous adult (table 3). Accordingly, the content of EβF per aphid showed an obvious increasing trend from embryo to adult: the content of EβF per aphid (*y*) was significantly correlated with the average weight

Table 3. Quantitative analysis of EβF in the green peach aphid *M. persicae* during different developmental stages.

	Mean weight per aphid (mg)	Amount of EβF per aphid (ng)	Amount of EβF per mg aphid (ng)
Embryo	0.020 ± 0.001 Dd	0.072 ± 0.001 F	3.638 ± 0.0183 F
1st-instar	0.021 ± 0.001 Dd	0.674 ± 0.006 E	32.781 ± 1.184 C
2nd-instar	0.057 ± 0.001 C	2.464 ± 0.004 D	43.236 ± 0.829 A
3rd-instar	0.149 ± 0.005 B	3.229 ± 0.019 C	21.697 ± 0.853 D
4th-instar	0.233 ± 0.003 Aa	4.029 ± 0.151 B	17.263 ± 0.443 E
Apterous adult	0.240 ± 0.028 Aa	9.339 ± 0.094 A	38.913 ± 0.150 B

Data are mean ± S.E. ($n = 3$). Different lower and uppercase letters within the same column indicate a significant difference in the average weight, amount of EβF per aphid or amount of EβF per mg of aphid at 5% and 1% levels, respectively, by using LSD multiple comparison methods. The alphabetical order corresponds to decreasing weight or amount. The same letters indicate no significant difference at 5% or 1% levels.

per aphid (x), with the linear regression equation as followed: $y = 27.82x - 0.037$ ($r = 0.8534$, $P = 0.0307$); the content of EβF had a dramatic leap from the lowest level ($0.072 ± 0.001$ ng) at embryonic stage to 1st-instar stage. Thereafter, the content of EβF per aphid rose steadily to the highest ($9.339 ± 0.094$ ng) at the adult stage. Nevertheless, when measured by weight, the concentration of EβF per mg of aphid fluctuated drastically during different stages ($r = 0.1212$, $P = 0.8191$) (fig. 5). Two peaks were seen at the 2nd-instar and adult stages, but a valley was observed at 4th-instar stage (table 3).

Association analysis

The expression data of MpFPPS genes (table 2) were used to perform an association analysis with the quantitative data of EβF per aphid during different developmental stages (table 3). The regression analyses revealed that there was a significant correlation between the amount of EβF per aphid (y) and the expression level of *MpFPPS1* (x), with the regression equation as followed: $y = 20.405x - 12.047$ ($r = 0.9134$, $P = 0.0109$) (fig. 6). In contrast, there was no significant correlation between the amount of EβF per aphid and the expression level of *MpFPPS2* ($r = 0.4113$, $P = 0.4179$). In addition, the concentration of EβF per mg of aphid was neither correlated with the mean weight per aphid ($r = 0.1212$, $P = 0.8191$) nor with the expression level of *MpFPPS1* ($r = 0.2553$, $P = 0.62532$) or *MpFPPS2* ($r = 0.1728$, $P = 0.7434$).

Discussion

The alarm behavior plays a key role in the ecology of aphids, but the site and molecular mechanism for the biosynthesis of aphid alarm pheromone (EβF) are largely unknown up to now. In the present study, the spatiotemporal expression analyses of the two FPPS genes in *M. persicae* and *A. pisum* suggested that *FPPS1* may play a more important role than *FPPS2* in aphids and the cornicle area is most likely the site for EβF biosynthesis; association analysis between the amounts of EβF and the expression levels of FPPS genes indicated that *FPPS1* is very likely involved in the biosynthesis of aphid alarm pheromone. Taken together, these data suggest that *FPPS1* may play a key role in the biosynthesis of EβF in the aphid.

Since the possibility of the host plant and endosymbionts as the sources of EβF in aphids has recently been eliminated (Sun & Li, 2017), it is thus postulated that the genes in the isoprenoid biosynthetic pathway are responsible for the synthesis of EβF in the aphid. Considering FPPS catalyzes the synthesis of FPP and theoretically, FPP is the precursor for

biosynthesis of EβF, the FPPS gene should be closely correlated to the pathway leading to the production of EβF in aphids. We thus examined the spatiotemporal expression of the two FPPS genes in two important agricultural aphid species. *M. persicae* and *A. pisum* are two phylogenetically close aphid species based on FPPS sequences, implying that the spatiotemporal expression data obtained from one species can be corroborated with the data achieved from the other. Our results clearly demonstrated that the overall expression level of *FPPS1* was significantly higher than that of *FPPS2*, although both of them were expressed extensively in all stages and tissues. Moreover, the expression patterns of the two genes were roughly similar during different developmental stages and in different tissues. According to these experimental results, it is speculated that the physiological functions of *FPPS1* and *FPPS2* may be similar, but the contributions of *FPPS1* is hopefully greater than *FPPS2*. A possible explanation for this phenomenon might be that *FPPS2* is undergoing an evolutionary degeneration as observed in the cotton aphid *A. gossypii* which contains only one FPPS gene in its genome (Ma *et al.*, 2010).

The only spatiotemporal expression analysis of FPPS gene that has been reported in the aphid was from *A. gossypii* (Ma *et al.*, 2010), where the stage-specific expression pattern of *AgFPPS* was generally consistent with that of *MpFPPS1* except the alate adult stage, but the tissue-specific expression patterns were different between the two aphids, as the expression level of *AgFPPS* was significantly higher in the CA than in other tissues. Similarly, the highest expression of FPPS gene was observed in CA in other insect species such as *A. ypsilon*, *Diploptera punctata* and *Leptinotarsa decemineata* (Castillo-Gracia & Couillaud, 1999; Huang *et al.*, 2015; Li *et al.*, 2016). This is obviously different from the tissue-specific expression pattern of *M. persicae* and *A. pisum* FPPS1/2 with far more expression in the cornicle area. This distinct variation might be resulted from at least two possible reasons: one is that the FPPS genes of the other examined insects are mainly responsible for providing the precursor for JH biosynthesis in CA; another reason may be that most aphids produce EβF as the major component of alarm pheromone in the cornicle area. In fact, the level of EβF in *A. gossypii* was very low (Byers, 2005). Our results also showed that the expression of *MpFPPS1/2* was higher in wingless adults than in winged adults. There were two possible explanations for this: (a) the reproduction of insects is regulated by JH (Truman & Riddiford, 2002) and winged adults have lower fecundity (Kaitala, 1991; Dixon & Agarwala, 1999), so the overall gene expression remains at a low level; (b) winged aphids are more active, making them easier to avoid predation and thus need less EβF.
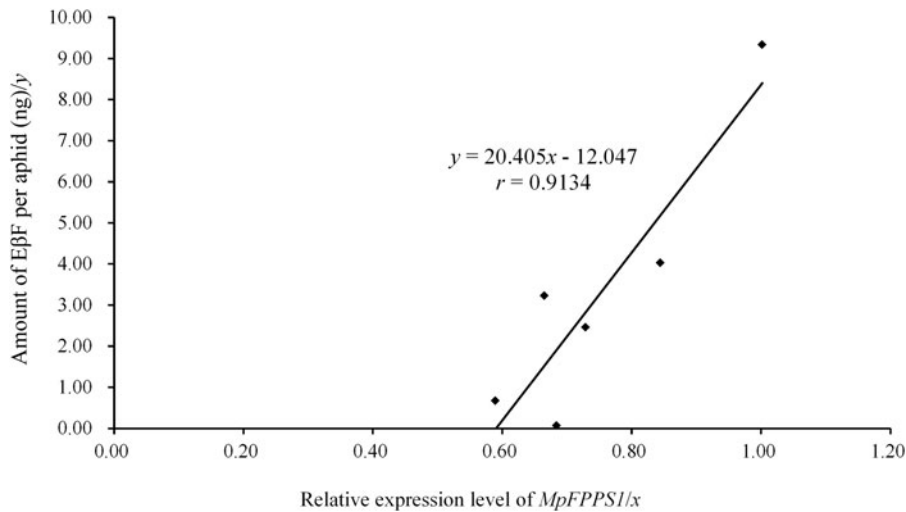
Fig. 5. Association analysis between the amount of EβF per aphid and the mean weight per aphid. Refer the data to table 3.
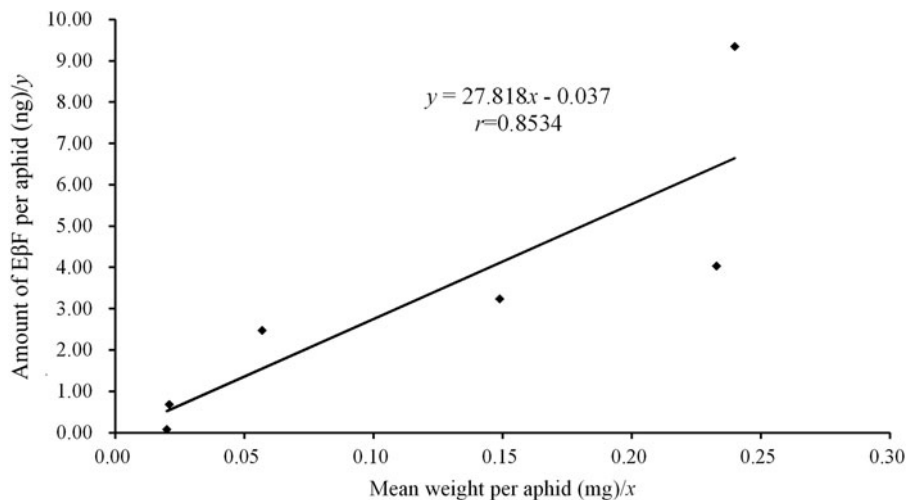

Fig. 6. Association analysis between the amount of EβF per aphid and the expression level of *MpFPPS1*. Refer the data to tables 2 and 3.

A detailed electron microscopic observations on the cornicles of the rose aphid *Macrosiphum rosae* was described more than four decades ago (Edwards, 1966; Chen & Edwards, 1972). The aphid was readily induced by mechanical stimuli to release waxy droplets containing triglyceride and volatile alarm pheromone (EβF) from a pore at the tip of the cornicle. It was proposed that the waxy droplet arises from the cornicle secretory cells (CSC) which may be modified oenocytes. The CSCs are in a separate compartment like a detached sac, distinct from fat body cells in location and structure (Chen & Edwards, 1972). Nevertheless, up to now, no biochemical and molecular evidences have been provided to support the proposition that EβF is biologically synthesized in the CSC sac. Our spatiotemporal expression analysis in two aphid species demonstrated that there was an excessively higher expression of both *FPPS1* and *FPPS2* in the cornicle area of the aphids, suggesting that FPPS genes are very likely involved in the biosynthesis of EβF. This is the first molecular evidence relating FPPS to the biosynthesis of aphid alarm pheromone.

The content of EβF per aphid increased steadily from embryo and 1st-instar to the adult stage with the growth of aphids, but when measured by weight, the concentration of EβF per mg of aphid fluctuated dramatically during different developmental stages (table 3). The amount of EβF per aphid represents the cumulative content of EβF in the body of aphid. The sharp increase in the content of EβF from embryo to 1st-instar stage suggests that the 1st-instar stage, as a turning point in aphid development, is a critical stage for EβF biosynthesis. Considering that the newly-born aphids have to face the dangerous environment independently, it is comprehensible that they will quickly synthesize a large amount of EβF to adapt to the environment. If not released or degraded, the EβF accumulated in aphids will naturally increase with the increase of aphid age and size. On the other hand, the amount of EβF per mg of aphid represents the concentration of EβF in the aphid body. Our studies found that, unlike the rising trend in the content of EβF during the whole developmental process, two peaks of concentration were observed at the 2nd-instar

and adult stages, and a valley appeared at the 4th-instar stage. This dramatic fluctuation in the concentration of EβF suggests that the biosynthesis of EβF in the aphid must have been affected by a variety of internal and external factors. Normally, when left undisturbed (without being attacked by predators), aphids keep releasing a small amount of EβF for detection of the population density (Almohamad *et al.*, 2009; Verheggen *et al.*, 2009); if attacked by predators, the aphids will suddenly release a large amount of EβF for defense purpose. Under the controlled laboratory conditions, the aphids will normally not disturbed by predators, and therefore the variation in the concentration of EβF may reflect the change in the aphid's physiological status. For instance, the sudden drop in the concentration of EβF at the 4th-instar stage may be caused by the need for saving more energy for embryonic development. Up to date, quantitative measurement of EβF as per weight or direct counting was only reported in *A. gossypii* (Byers, 2005), where the content of EβF per aphid increased with the weight of aphid and the content of EβF at the 1st-instar stage was much less than that in adults, which was consistent with our results; yet, the concentration of EβF was reported to decline with increasing weight. Obviously, the mechanism how the concentration of EβF is regulated in the aphid needs further studies.

The significant correlation between the amount of EβF per aphid and the expression level of FPPS1 gene indicates the close association of *FPPS1* with the biosynthesis of EβF, but the non-significant relation between the amount of EβF and the expression level of FPPS2 gene does not necessarily deny the involvement of *FPPS2* in the production of EβF, as the biosynthesis of EβF is inherently regulated by a complex of effectors, including the enzymes responsible for the synthesis and degradation of EβF, the effectors controlling the signaling pathway of alarm pheromone and the release of waxy droplets. Similarly, there is an inherent tissue-dependent bias in the association analysis between the amount of EβF per mg aphid (ng) and the expression level of MpFPPS1 gene during different developmental stages, since the expression levels were not measured specifically in the cornicle area (table 2).

FPPS catalyzes the synthesis of FPP, the precursor for EβF biosynthesis, and essentially, the FPPS genes are indispensable if the alarm pheromone is the product of the aphid's biosynthetic machinery. Moreover, FPPS also provides the precursor for the biosynthesis of JH, one of the key hormones in relation to the metamorphic development of aphids. Different from JH that plays a critical physiological role in the aphid, EβF is largely implicated in the ecological interactions, and therefore the release and thus the biosynthesis of EβF are to a great extent regulated by environmental factors. This might explain why the concentration of EβF in aphids fluctuates so drastically.

Conclusions

Based on the spatiotemporal expression data of the FPPS genes in *M. persicae* and *A. pisum*, it is postulated that: (i) *FPPS1* may play a greater role than *FPPS2* in aphids, and the latter seems to be undergoing an evolutionary process of degradation, while both *FPPS1* and *FPPS2* may have similar physiological functions; (ii) the cornicle area is most likely the site for EβF biosynthesis. Association analyses suggest that *FPPS1* may play a major role in the biosynthesis of aphid alarm pheromone, although the role of *FPPS2* cannot be eliminated. The biosynthetic pathway of EβF is inherently

regulated by a complex of effectors and further studies should be focused on *in vivo* functional characterization of FPPS genes and other genes directly involved in EβF biosynthesis. Transcriptome and functional analysis via RNA interference is useful in this line of work.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485318000706.

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