Fatty acyl-CoA reductase influences wax biosynthesis in the cotton mealybug, *Phenacoccus solenopsis* Tinsley

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Mealybugs are highly aggressive to a diversity of plants. The waxy layer covering the outermost part of the integument is an important protective defense of these pests. However, the molecular mechanisms underlying wax biosynthesis in mealybugs remain largely unknown. Here, we analyzed multi-omics data on wax biosynthesis by the cotton mealybug, *Phenacoccus solenopsis* Tinsley, and found that a fatty acyl-CoA reductase (*PsFAR*) gene, which was highly expressed in the fat bodies of female mealybugs, contributed to wax biosynthesis by regulating the production of the dominant chemical components of wax, cuticular hydrocarbons (CHCs). RNA interference (RNAi) against *PsFAR* by dsRNA micro-injection and allowing mealybugs to feed on transgenic tobacco expressing target dsRNA resulted in a reduction of CHC contents in the waxy layer, and an increase in mealybug mortality under desiccation and deltamethrin treatments. In conclusion, *PsFAR* plays crucial roles in the wax biosynthesis of mealybugs, thereby contributing to their adaptation to water loss and insecticide stress.
Mealybugs (Hemiptera: Coccoidea: Pseudococcidae) are globally distributed, highly aggressive pests of many commercial, non-commercial, and ornamental plants\textsuperscript{1,2}. They are a diverse group of sap-sucking insects, with at least 32 genera and around 105 species\textsuperscript{3}. Mealybugs can attack over 300 plant species, with host records extending to 76 families and over 300 genera\textsuperscript{4–7}. Both adults and crawlers suck the cell sap from the phloem of leaves, stems, twigs, flower buds, and young bulbs, causing withering and yellowing of leaves, dieback, and even death of the whole plant. Over the years, mealybugs have caused significant economic loss worldwide, with reported crop losses reaching 52\% in India resulting from infestation by the tracheal cuticle sublayer, termed the envelope, in embryos\textsuperscript{8}. Since the early 1990s\textsuperscript{9,10}, the pathways can be briefly divided into four steps: formation of fatty acid precursors, elongation into long-chain fatty acyl-CoAs, conversion to alcohols, and oxidation to aldehydes, which are respectively catalyzed by fatty acid synthase (FAS), fatty acid elongases (ELO), fatty acyl-CoA reductase (FAR), and cytochromes P450\textsuperscript{11}. Among these enzymes, FARs have been identified in several insect species but they exhibit varying biological functions. In Drosophila, one FAR gene—waterproof (DmWP)—plays a key role in long-chain fatty acid metabolism that ultimately affects the formation of the outermost tracheal cuticle sublayer, termed the envelope, in embryos\textsuperscript{12}. A total of 17 FAR genes (NIFARs) were identified in Nilaparvata lugens Stål by Li et al.\textsuperscript{20,21} by injection with targeted double-stranded RNAs (dsRNA): four had crucial roles in cuticle shedding, two were involved in the production of the CHCs required for waterproofing, and eight were essential for female fertility. Additionally, the AmFAR1 gene in Apis mellifera L.\textsuperscript{22} and FARs in some lepidopterans\textsuperscript{23–26} were reported to primarily affect pheromone biosynthesis by regulating the output of acetate or aldehyde derivatives. In scale insects (Hemiptera: Coccoidea), Hu et al.\textsuperscript{27} found the EpFAR gene of Euceris pela Chavannes to be highly expressed in the cuticle and functioned by converting acyl CoA to its corresponding alcohol. In the cotton mealybug, Pseudococcus solenopsis, Li et al.\textsuperscript{28} identified two FARs (PsFARs), of which only one (PsFAR 1) experienced increased relative expression levels after spirotetratam treatment; however, the functions of FARs in this mealybug remain undetermined due to a lack of direct functional assays. 

P. solenopsis is an invasive pest in Asia and beyond\textsuperscript{29}, possessing almost all of the features unique to mealybugs (Fig. 1a). It causes serious economic losses to over 150 crop and horticultural plant species and can be especially detrimental to cotton production\textsuperscript{30,31}. It is in this mealybug species that we chose to study the molecular mechanisms of wax biosynthesis. We first obtained the wax metabolome, integumentary transcriptome, and proteome data of cotton mealybugs. Using combined analyses of these omics data, a new FAR gene, PsFAR, was successfully identified. This gene was highly expressed in third-instar nymphs and adult females. Knocking down PsFAR with both micro-injection and transgenic tobacco-mediated RNA interference (RNAi), we observed reduced wax production associated with changes in CHC synthesis, which lead to lower water retention and higher mortality from insecticide treatments in the cotton mealybug.

Results
Hydrocarbons are the dominant chemical components in wax. The chemical components of mealybug cuticular wax vary by species\textsuperscript{11} and are speculated to influence the biological functions of the wax, thus it is of great importance to obtain a complete catalog of chemical components in the mealybug wax. Here, we used n-hexane and methanol independently to dissolve the powdery wax collected from the body surface of female cotton mealybugs, then tested it on polar and nonpolar chromatographic columns using GC–MS. At least eight groups of chemical components were identified: hydrocarbons, fatty acids, olefins, alcohols, aromatic derivatives, esters, ketones, and aldehydes. Their relative contents varied between the four dissolvent-column treatments (Fig. 1b and Supplementary Fig. 1), where the top three components of each of the four treatments were (1) hydrocarbons, fatty acids, and olefins (n-hexane, polar column), (2) hydrocarbons, alcohols and fatty acids (methanol, nonpolar column), (3) hydrocarbons, aromatic derivatives and esters (n-hexane, nonpolar column), and (4) hydrocarbons, alcohols and aromatic derivatives (methanol, nonpolar column). Among them, hydrocarbons (CHCs) made up 59–68\% of the chemical profiles of the four treatments and were mainly composed of C\textsubscript{13}–C\textsubscript{16} n-alkanes and C\textsubscript{12}–C\textsubscript{20} branched chemicals (Supplementary Data 1). These results revealed that CHCs are the dominant components of cotton mealybug wax.

Screening of the PsFAR gene in the CHC biosynthesis pathway. To identify genes playing crucial roles in CHC biosynthesis in cotton mealybugs, we first performed transcriptome sequencing of integument tissues, which contain adherent waxy glands and fat bodies, and the remaining tissues (without integument) of adult female cotton mealybugs (Supplementary Table 1 and Supplementary Data 2). In the integument, 527 upregulated and 34 downregulated differentially expressed genes (DEGs) were found (Supplementary Fig. 2 and Supplementary Data 2). Among the significantly upregulated DEGs, we focused on those that were annotated as the three key CHC synthesis pathway-related enzymes, i.e., FAS, ELO, and FAR. Six such DEGs in the integument were identified, including two FAS genes (gene i.d.: PSOL09676 and PSOL12170), two ELO genes (gene id: PSOL06704 and PSOL00600), and two FAR genes (gene id: PSOL02039 and PSOL06156) (Supplementary Table 2, Supplementary Data 2).

We next performed proteome sequencing of the integument from adult female mealybugs. When the six DEGs described above were mapped to the proteomics data, at least one protein sequence for each could be BLASTed with an e-value < 2E–92 (Supplementary Table 3), confirming the expression of these genes in the integument. In terms of wax biosynthesis, only one FAR gene (gene id: PSOL02039) was found expressed at a

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significantly high level in female nymphs and adults (Fig. 2 and Supplementary Fig. 3). These results suggest that this FAR gene (named PsFAR) has crucial functions in CHC biosynthesis in the cotton mealybug.

Fig. 1 Mealybug wax and its relative chemical composition in adult P. solenopsis females. a The wax covering on female P. solenopsis (top) appears filamentous under SEM (<4000, below). b Relative contents of identified chemical components based on GC–MS analysis. Wax, respectively, dissolved in n-hexane and methanol were analyzed on polar and nonpolar chromatographic columns, producing four test results. The solvent and column type are shown on top of each pie chart.

Fig. 2 Sequence analysis and expression profiles of PsFAR. a Multiple alignments of FARs from P. solenopsis and other organisms. Bm: Bombyx mori, Os: Ostrinia scapulalis, Ye: Yponomeuta evonymellus, Am: Apis mellifera, Nl: Nilaparvata lugens, Dm: Drosophila melanogaster. Identical residues are shaded black, and conservative substitutions are shaded gray. The FAR contains a characteristic N-terminal Rossmann fold NAD(P) (+)-binding domain (black underline), a sterile alpha motif protein domain (gray underline), a cofactor binding motif (TGXXGF), and an active site motif (YXXXK) (black boxes). b Phylogenetic tree of FARs from different species. The tree was constructed using amino acid sequences with the neighbor-joining algorithm in MEGA. Differently colored sectors of the circles represent three main branches of insects (blue), mammals (red), and plants (green). The protein sequence of PsFAR, marked by a blue circle, clusters with insect FARs. Branches marked with gray circles indicate a confidence value larger than 0.50. Detailed information on the species used here can be found in Supplementary Data 3. c Developmental (left) and tissue-specific (right) expression profiles of PsFAR in P. solenopsis. The relative expression level was normalized and visualized as mean ± SEM with four biological replicates. Different lower-case letters indicate a significant difference (P < 0.05) based on ANOVA followed by Tukey’s multiple comparison test.

Sequence analysis and expression profiles of PsFAR. Prior to verifying gene functions, the full-length sequence of PsFAR was obtained by rapid amplification of cDNA ends (RACE). This gene is composed of a 1671 bp ORF located downstream of a 177-bp 5'
UTR and upstream of a 78-bp 3’ UTR (GenBank accession number: OM371326). The ORF encodes 556 amino acid residues with a predicted molecular mass of 64.2 kDa and an isoelectric point of 8.6. Multiple sequence alignment showed that FAR sequences shared a characteristic N-terminal Rossmann fold NAD(P) (+)-binding domain, a sterile alpha motif protein domain, a cofactor binding motif (TGXXGF), and an active site motif (YXXXXK) (Fig. 2a). To investigate the relationship of various PsFAR genes, we constructed a phylogenetic tree for FARs from 40 other species (Fig. 2b). Three main branches, representing plant, mammal, and insect, FARs were clustered in the tree, and PsFAR clustered with other insects FARs as expected.

We determined the expression profiles of PsFAR at various developmental stages (first, second, and third-instar nymphs, pupa, adult males, and adult females) and tissues (integument, fat body, gut, and ovary) (Fig. 2c). Expression of this gene was highest in third-instar nymphs and adult females and lowest in adult males. It was significantly higher expressed in the fat body relative to the other three tissues in adult females, indicating that PsFAR plays important functions in the fat body of female cotton mealybugs.

RNAi knockdown of PsFAR by dsRNA microinjection and feeding on transgenic tobacco. We performed RNAi experiments to knockdown PsFAR expression using two strategies, conventional microinjection of PsFAR complementary dsRNA and feeding on transgenic tobacco expressing dsRNA. The dsPsFAR expression cassette pCAMBIA1301-dsPsFAR vector is shown in Fig. 3a. A total of 26 tobacco transformants (generation T0) were produced after Agrobacterium-mediated transformation, incubation, differentiation, and rooting (Fig. 3b). To identify positive transformants among the 26 T0 tobacco plants, a 557-bp fragment of the selective marker gene hygromycin B phosphotransferase (Hpt) were amplified using PCR, which suggested a transformation efficiency of 100% (Fig. 3c and Supplementary Fig. 4). To screen tobacco transformants with the highest expression of dsPsFAR, we determined the relative expression levels in nine randomly selected T0 genetically modified (GM) tobacco plants using RT-qPCR. Two transformants, #19 and #26, showed the highest dsPsFAR expression levels (Fig. 3d); the #19 GM tobacco line was selected for further analysis.

To determine the RNAi efficiency of dsRNA microinjection and GM tobacco feeding, we assessed the resulting PsFAR gene expression in cotton mealybugs under different treatments by RT-qPCR. The results showed that PsFAR expression was successfully suppressed 3 days after dsRNA microinjection (reduced to 54.8 ± 3.7%) and 5 days after feeding on GM tobacco (reduced to 43.0 ± 1.1%) (Fig. 4a). Meanwhile, no significant changes were detected in the expression levels of other PsFAR genes, including PsFAR (ID: PSOL06156), PsFAR I, and PsFAR II (Supplementary Fig. 5). Mortality also occurred in both RNAi treatments, with insects exhibiting abnormal coloration, reduced wax secretion, and unsuccessful molting after injection with dsPsFAR or feeding on dsPsFAR GM tobacco (Fig. 4b). Moreover, significant (P < 0.05) declines in survival rates were observed from day 2 (9.6%) to day 14 (20.2%) after dsRNA injection, and from day 5 (11.1%) to day 14 (25.6%) after feeding on GM tobacco (Fig. 4c), demonstrating some degree of lethality in cotton mealybugs after knocking down PsFAR expression.

PsFAR is required for CHC biosynthesis and wax output. To explore whether PsFAR is responsible for CHC biosynthesis in the cotton mealybug, we used GC–MS to determine the composition and contents of CHCs in mealybug wax 4 days after dsRNA injection and 9 days after feeding on GM tobacco. The results showed that the wax contained a mixture of several n-alkanes with different chain lengths (Fig. 5a). The same types and
Fig. 4 RNAi effects, lethality, and survival rate of *P. solenopsis* microinjected with dsRNA or fed on dsRNA expressing GM tobacco. 

**a** Validation of RNAi effects of dsRNA microinjection (left) and GM tobacco feeding (right) using RT-qPCR. Total RNA for *PsFAR* was extracted from cotton mealybugs 3 days post injection (*n*= 7) and after feeding on GM tobacco for 5 days (*n*= 10). 

**b** Lethality in treated insects: dsGFP and WT tobacco-treated cotton mealybugs were normal and covered with a heavy waxy layer around their body surface, while dsPsFAR treated and GM tobacco treated insects exhibited abnormal coloration, reduced wax secretion, and unsuccessful molting. 

**c** Dynamic analysis of the survival rate of *P. solenopsis* after dsRNA injection (left) and feeding on tobacco plants (right). WT: insects fed on wild-type plants, Transgenic: insects fed on GM tobacco plants. dsGFP and WT were respectively set as negative controls, *n*= 30 insects. Means ± SEM were calculated with results from three biological replicates. Asterisks denote a significant difference as determined by Student’s t-test; *P* < 0.05, **P** < 0.01.

Fig. 5 GC-MS and SEM analysis of wax. 

**a** Effects of the relative CHC contents of *P. solenopsis* wax following dsRNA injection (left) and tobacco feeding (right). The contents of total CHCs and *n*-alkanes of different lengths (*C*<sub>9</sub>–*C*<sub>37</sub>) were analyzed by GC-MS. The *n*-heneicosane (*C*<sub>21</sub>) was set as an internal standard. WT: insects fed on wild-type plants, Transgenic: insects fed on GM tobacco plants. Results are shown as nanograms per milligram of fresh body mass ± SEM from six biological replicates. * and ** respectively indicate significant differences at *P* < 0.05 and *P* < 0.01 between dsGFP and dsPsFAR, or WT and Transgenic (Student’s t-test). 

**b** SEM analysis for dsRNA injection (left two) and tobacco rearing (right two) treated groups. 24 h post-emergence, live insects were collected for SEM. Images show the dorsal side of the cotton mealybug body from the third thoracic segment. Cotton mealybugs injected with dsGFP and fed on WT tobacco plants were used as negative controls.
numbers of n-alkanes were observed in both RNAi-treated groups (dsPsFAR and Transgenic) and the control groups (dsGFP and WT). As compared with the tobacco (WT and Transgenic) fed group, the microinjection (dsGFP and dsPsFAR) group lacked C_{23}, C_{25}, C_{27}, and C_{29}. In terms of CHC contents, the relative amounts of n-alkanes in RNAi-treated insects were significantly decreased, with the mean values of total n-alkanes per milligram of fresh body mass reduced by 6.86 and 6.3 ng/mg after dsPsFAR microinjection and GM tobacco feeding, respectively. This decrease likely resulted from reductions of different alkanes, particularly C_{16}, C_{18}, C_{28}, and C_{50} in the dsPsFAR group and C_{19} and C_{25} in the Transgenic group, each of which decreased dramatically (Fig. 5a and Supplementary Table 4). These results reveal the critical involvement of PsFAR in CHC biosynthesis in the cotton mealybug.

As CHCs were found to be the dominant chemical component in cotton mealybug wax, we speculated that knocking down PsFAR might further affect wax output. To this end, 24-h-old female adults from the microinjection and GM tobacco-fed groups were subjected to scanning electron microscopy (SEM) and transmission electron microscopy (TEM) examinations. SEM observations from the third thoracic segment showed that all the dsPsFAR-microinjected and WT tobacco-fed insects were covered with a waxy layer on the epicuticular surface, but this waxy layer was reduced in both dsPsFAR-injected and GM tobacco-fed mealybugs (Fig. 5b). Additionally, the envelope integrity did not change evidently after knockdown of PsFAR as revealed by TEM analysis (Supplementary Fig. 6). Collectively, these results demonstrate that PsFAR has direct effects on wax biosynthesis in the cotton mealybug.

Knockdown of PsFAR leads to reduced water retention. To further investigate the functional relationships of PsFAR with water retention and waterproofing in cotton mealybugs, we carried out desiccation and water spray assays. The desiccation assay showed no significant difference in survival days for dsPsFAR-treated groups under 75% humidity compared with corresponding controls (Fig. 6a and Supplementary Table 5). In contrast, the survival days decreased dramatically in both dsPsFAR injected and GM tobacco-fed groups when insects were exposed to <10% humidity. However, for the water spray assay, the survival rate did not differ significantly between the controls and PsFAR knockdown groups (Supplementary Fig. 7). These results indicate that PsFAR participates in water retention but not waterproofing in the cotton mealybug.

Knockdown of PsFAR increases the contact killing efficiency of insecticides. We also investigated whether PsFAR is functionally important in preventing cotton mealybugs from contact-killing insecticides. As the concentration of deltamethrin decreased, the survival rate increased significantly in both female adults sampled from tomato plants and those from WT tobacco plants (Supplementary Fig. 8). At a concentration of 25 mg/L deltamethrin, the two groups had a survival rate of 78.89 ± 2.94% and 72.22 ± 2.94%, respectively, at 48 h after spraying with the insecticide. This concentration was therefore used in further contact-killing assays.

For groups not treated with deltamethrin, the survival rate decreased significantly in both dsPsFAR and GM tobacco-treated insects when compared to controls (Fig. 6b and Supplementary Table 5), suggesting that both groups would suffer increased mortality when PsFAR expression is suppressed. When treated with 25 mg/L deltamethrin, a more remarkable decrease in survival rate was observed 48 h after insecticide spray (Fig. 6b), which was 16.56% for dsPsFAR microinjected insects and 22.22% for GM tobacco-fed insects. Our results suggest that PsFAR functions in protecting cotton mealybugs from insecticides.

Discussion

As a group of highly aggressive and invasive insect pests, the waxy layer is crucial for mealybugs to adapt to differing environments. By focusing on the molecular mechanism responsible for wax biosynthesis, we demonstrated that CHCs are the dominant chemical components in the waxy layer of cotton mealybugs, and the biosynthetic pathways of these chemicals are associated with six DEGs that were significantly up-regulated in the integument. Particularly, we identified one female-enriched FAR gene, PsFAR, which plays a crucial role in the generation of CHCs and waxy filaments in the cotton mealybug. PsFAR was also found to contribute to the protective functions of the waxy layer, including water retention and as a barrier against contact insecticides.

Insect wax is a lipid-based substance that contains a wide variety of chemical compounds. Here, the waxy filaments of cotton mealybug were determined to be a combination of hydrocarbons, fatty acids, olefins, alcohols, aromatic derivatives, esters, ketones, and aldehydes. CHCs are the main components of cotton mealybug wax, which contains hydrocarbons, esters, alcohols, ketones, and acids. As we used both polar (methanol) and nonpolar (hexane) solvents, as well as polar...
and nonpolar chromatographic columns in GC–MS, the chemical compounds of the waxy filaments of cotton mealybug, should have been well characterized, according to Chen et al.34. Thus, the data reported here could prove very informative for future metabolomic analyses of the waxy filaments of mealybugs.

Among the CHC biosynthesis pathway-related DEGs, only PsFAR was highly expressed in third-instar and adult females (Fig. 2c and Supplementary Fig. 3), suggesting that this gene performs crucial functions in wax biosynthesis, as only females are known to produce this thick waxy barrier. The importance of PsFAR in wax biosynthesis can also be demonstrated by its expression sites. According to previous studies, eooncetes are the site for CHC biosynthesis in insects18,35; and they exist in both the epidermis and fat body36; as such, genes responsible for wax biosynthesis would likely be highly expressed in these tissues. Consistently, in our study, the expression level of PsFAR in the fat body was significantly higher than in other tissues (Fig. 2c). It should be noted, however, that other FARs identified in cotton mealybugs may play very different roles, as indicated by their expression profiles. For example, PsFAR I and PsFAR II, reported by Li et al.28, are highly expressed in male adults (not covered by thick wax) and first- and second-instar nymphs, respectively. Such functional differences are supported by the phylogenetic tree provided in this study, where PsFAR, PsFAR I, and PsFAR II are clustered into three different branches (Fig. 2b).

To identify wax biosynthesis-related genes, we performed in vivo RNAi experiments to knock down PsFAR expression and observed reductions of CHCs and wax in RNAi-treated insects. This provided direct evidence not only for PsFAR involvement in wax biosynthesis but also for the significant contribution of CHCs content to the stability and function of mealybug wax. We paid careful attention to the potential effects of host plants on CHCs composition, as reported by Ahmad et al.10. Thus, we compared the difference in CHCs profiles between dsRNA microinjection groups that were reared on tomato or tobacco groups, where C23, C25, C27, and C29 were found to be absent in the former. These observations make searching for PsFAR function more feasible and validated the credibility of our results.

In terms of n-alkane amounts, four n-alkanes (C16, C18, C20, and C24) in the dsPsFAR group and two n-alkanes (C18 and C24) in the transgenic group decreased significantly (Fig. 5 and Supplementary Table 4). The types of n-alkanes we identified were different from the findings in N. lugens injected with dsNIFAR7 and dsNIFAR9 (decreased n-alkanes were C14, C20, C27, and C28, and C29, and C27 and C29, respectively). Overall, only a small number of n-alkanes experienced changes in accumulation after the knockdown of PsFAR expression; a potential reason for this is that PsFAR might only regulate n-alkanes of a specific length. Meanwhile, decreased n-alkanes were those located in waxy filaments, but not those in the integumentary envelope because no obvious changes were detected in the envelope integrity of dsPsFAR-treated insects by TEM analysis (Supplementary Fig. 6). Yet, in Drosophila, DmwP is essential for morphogenesis of the tracheal envelope by regulating the formation of long-chain fatty alcohols which can serve as substrates for downstream CHCs biosynthesis19. Therefore, CHCs in the waxy filaments and envelope may be regulated by different FAR genes; in the cotton mealybug PsFAR mainly regulates CHCs content in the waxy filaments rather than the envelope. Functions of other FAR genes in the cotton mealybug remain unclear. According to the genome data, there are 22 annotated FARs in the cotton mealybug37. Higher FARs diversities have also been found in other insects. For example, there are 17 such genes in N. lugens20, and 26 in E. pelle28. Besides FARs, other genes may also contribute to CHC biosyntheses, such as FAS, ELO, and cytochromes P450. Further research is needed to clarify the functions of these genes in cotton mealybugs and more deeply understand the biosynthetic mechanisms underlying cotton mealybug waxy filament production.

The waxy layer of the integument has been reported to reduce water loss through the cuticle40,48 and similar results were observed in this study. When RNAi-treated mealybugs were kept under 75% humidity, their survival days did not differ significantly from the control groups, but decreased significantly at <10% humidity (Fig. 6a). This is different from the report in N. lugens, in which mortality due to low humidity was not affected by knockdown of NIFARs20,21. Thus, as demonstrated, FARs can have very diverse functions depending on insect species, and in cotton, mealybug plays a crucial role in wax biosynthesis. When PsFAR is knocked down, the mealybug suffers more water loss through the cuticle due to integument instability. One interesting finding is that some individuals can survive for more than 2 weeks without eating (Fig. 6a), reflecting an extremely strong adaptation of cotton mealybugs to severe environmental conditions47,49.

After spraying with water, the survival rate of the PsFAR knockdown group did not change significantly as compared with the control groups (Supplementary Fig. 7), suggesting that PsFAR contributes little to waterproofing. This result is not strange, according to Blomquist and Ginzel18, the lipids on the insect body surface have a high hydrophobicity. As such, even a small amount of wax on the cotton mealybug body surface could provide sufficient waterproofing against water spraying, despite the reduction in wax. This, again, supports our finding described above, that PsFAR does not influence the body envelope, as changes to the envelope would lead to higher mortality in the tested mealybugs.

RNAi-treated insects experienced a high mortality rate after spraying with a low concentration (25 mg/L) of deltamethrin (Fig. 6b; Supplementary Fig. 8), indicating that the waxy layer plays an important role in protecting cotton mealybugs from insecticide permeation. In other words, if the waxy filaments are reduced, such as by RNAi knockdown of PsFAR gene, the mortality of mealybugs would likely be in response to insecticide treatment. This further demonstrates the significance of PsFAR in wax biosynthesis in this mealybug.

RNAi has become a powerful tool for the study of gene functions, but off-target effects may occur in RNAi experiments50. In this study, to avoid off-target effects caused by dsRNA, two different dsRNA molecules targeting PsFAR were used in micro-injection and oral delivery by transgenic plants, as suggested by Jarosch and Moritz31. Both dsRNAs showed satisfactory RNAi efficiency (Fig. 4a), similar to those obtained via other RNAi methods used in the cotton mealybug, such as siRNA microinjection52 and direct dsRNA feeding53. Meanwhile, shared phenotypes produced by the two molecules further proved the same on-target effects for both of them. Additionally, the gene expression of PsFAR paralogs was not changed significantly before and after dsPsFAR treatment (Supplementary Fig. 5), and the two dsRNAs did not have matches when blasted against all other insect species, despite PsFAR clustering with other FARs in the phylogenetic tree (Fig. 2b); thus it is almost impossible for the two dsRNA molecules having off-target effects on paralogous and orthologous genes of PsFAR. Together, these results indicate that off-target effects are less likely to occur in this study. To our knowledge, this is the first report to utilize a dsRNA-expressing transgenic host plant to induce RNAi in mealybugs and demonstrate that this technique is quite effective, as reported for other phloem-feeding hemipterans54,55.

In summary, by performing multi-omics screening and functional validation, we identified an important FAR gene in the
cotton mealybug, *PsFAR*. Using two separate RNAi approaches, we showed that *PsFAR* plays a vital role in wax biosynthesis, which is essential for water retention and protects the insects from insecticide treatment. Our findings will hopefully deepen our understanding of the molecular mechanisms underlying wax biosynthesis. Further research is needed to identify new or interacting factors of FAR in mealybugs.

**Methods**

**Insect rearing.** The cotton mealybugs used in the study were originally collected from Baoji, Shaanxi, China (*Malvaceae*). In June 2016, they were maintained on fresh tomato plants (cv. Hezuo-903, Shanghai Changsheng Seeds Industry Co., Ltd, China) in a climatically controlled chamber maintained at 27 ± 1 °C, 75% relative humidity (RH), and a photoperiod of 14:10 (L:D). For detailed insect rearing and tomato cultivation methods see ref. 37.

**Scanning electron microscopy (SEM) of *P. solenopsis* wax.** SEM was used to observe changes in wax on the body surface of adult *P. solenopsis* females according to the methods of Huang et al.37. Briefly, collected insects were taped onto a stub and dried in an ion sputter (Hatchi, Tokyo, Japan) under a vacuum. After gold sputtering, the samples were observed using a TM-1000 SEM (Hatchi, Tokyo, Japan). Photos were scanned from the dorsal part of the third thoracic segment. Thirty insects were used for both RNAi-treated and control groups.

**Chemical composition analysis of mealybug wax.** A small soft brush was used to collect wax filaments from the body surface of *P. solenopsis* females. Prior to use, the brush was washed successively by 70% ethanol, sterile water, and 1× sterile phosphate-buffered saline (PBS, pH 7.4). The wax was collected into a clean chromatography vial for the following experiments. Two vials of wax, each collected from 100 adult female mealybugs, were dissolved in 1 ml of methanol and 1 ml of n-hexane, respectively. The vials were stirred gently for 3 min, kept at room temperature for 30 min, and then put into an S06H ultrasonic vibrator (Zealway, Tokyo, Japan). Photos were scanned from the dorsal part of the third thoracic segment. Thirty insects were used for both RNAi-treated and control groups.

**RNA extraction and RT-qPCR.** Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Multiple sequence alignments were conducted with ClustalX and GeneDoc. The phylogenetic tree was constructed in MEGA X62, and a photoperiod of 14:10 (L:D). For detailed insect rearing and tomato cultivation methods see ref. 37.

**Proteome analysis of the integument.** Integuments dissected from 300 adult *P. solenopsis* females were ground in liquid nitrogen using a mortar and pestle, then dissolved in 400 μl SDT lysis buffer (4% SDS, 100 mM Tris–HCl, 1 mM DTT, pH 7.6). After sonication (ten pulses of 10 s with 10 s intervals, 100 W) and 15 min boiling, samples were centrifuged at 13,000×g at 4 °C for 40 min. Proteins were quantified by the BCA method (Solarbio, Beijing, China). Protein bands were checked by SDS-PAGE and Coomassie blue staining. In total, 300 μg of proteins were used in this experiment. The 100 μM DTT was removed by repeated ultrafiltration (10 kD microseep) using 200 μl of UA buffer (8 M urea, 150 mM Tris–HCl, pH 8.0), then 100 μl of iodoacetamide (100 μM in UA) was added to block reduced cysteine residues, and the samples were incubated for 30 min in the darkness. Then, the filters were washed twice in 100 μl of UA buffer and twice in 100 μl of 25 μM NH4HCO3 buffer. Finally, the protein suspensions were digested with 6 μg trypsin (Promega) in 40 μl of 100 mM NH4HCO3 buffer for 16–18 h at 37 °C. The resulting peptides were desalted on C18 cartridges (Empore SPE Cartridges C18 (standard density), bed ID 7 mm, volume 3 ml) (Sigma-Aldrich, MO, USA), concentrated by vacuum centrifugation, and reconstituted in 40 μl of 0.1% (v/v) formic acid.

**LC–MS/MS analysis was performed on a Q Exactive Exact mass spectrometer (Thermo Fisher Scientific) coupled with Maxquant software (Thermo Fisher Scientific). Here, 2 μg of high pH resolved-phase peptide fragments were loaded onto a 2.1 mm × 50 mm reverse-phase column (Thermo Fisher Scientific Easy Column, 75-μm inner diameter, 10-cm-long, 3 μm resin) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (0.1% formic acid and 84% acetonitrile) at a flow rate of 300 nl/min. The eluted peptides were ionized, and the full MS spectrum (from 100 to 1,200 m/z) was acquired by a precursor ion scan using the Q-Exactive analyser with a resolution of r = 70,000 at m/z 200, followed by a 20 MS2 scan in the Q-Exactive analyser with a resolution of r = 17,500 at m/z 200. The MS raw files were translated into mgf files and searched against the integumentary transcriptome using Maxquant 1.3.6.5. Trypsin was defined as the cleavage enzyme allowing no more than two missed cleavages. Carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine was specified as a variable modification. Proteome extraction and sequencing were performed by Applied Protein Technology (Shanghai, China). To verify the existence of corresponding proteins expressed by insect transcriptome upregulated DEGs, 2BLG 2.120 was used to blast translated protein sequences of integumentary upregulated DEGs against the integumentary proteome data with the e-value set at 1E-5.

**Rapid amplification of cDNA ends of the *PsFAR* gene.** The SMART RACE cDNA Amplification Kit (Takara, Kyoto, Japan) was used to obtain full-length cDNAs of the *PsFAR* gene. 5′-UTR and 3′-UTR RACE cDNAs were synthesized from total RNA using SMARTscriptTM Reverse Transcriptase (Clontech), according to the manufacturer’s instructions. Specific primers (Supplementary Table 6) were designed using Primer-BLAST in NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Paired with the Universal Primer Mix supplied in the kit, one pair of forward and reverse gene-specific primers were, respectively, used in the 3′ and 5′ RACE first-step PCR reactions. PCR conditions were as follow: incubation at 94 °C for 3 min; five cycles at 72 °C for 2 min; five cycles at 94 °C for 40 s and 72 °C for 2 min; five cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min; and 25 cycles at 94 °C for 30 s, 66 °C for 30 s, 72 °C for 2 min. The final extension was 72 °C for 10 min. PCR products were purified using the FastPure Gel DNA Extraction Mini Kit (Yazhime Biotech Co., Ltd, Nanjing, China) and cloned using the pCla007 Simple Vector Kit (Tiangene Biotech, Beijing, China). Positive clones were selected and sequenced in Tsingke Biotech.

**Sequence analysis of *PsFAR*.** The amino acid sequences of FASs were derived from the corresponding cDNA sequence using ORFfinder in NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). Multiple sequence alignments were conducted with ClustalX and GeneDoc. The phylogenetic tree was constructed in MEGA X62, wherein neighbor-joining algorithm analysis using the JTT model for amino acids with 1500 bootstrap replicates was performed. Organisms and the GenBank accession numbers of sequences used here are shown in Supplementary Data 3.

**dsRNA synthesis and microinjection.** dsRNA was synthesized using the T7 High Yield RNA Transcription Kit (Invitrogen Biotech Co., Ltd, Nanjing, China) according to the manufacturer’s instructions. Briefly, the DNA template for dsRNA synthesis was amplified with primers containing the T7 RNA polymerase promoter at both 5′ ends (Supplementary Table 6). The purified DNA template (200 ng), a unique 435 bp fragment of *PsFAR*, was then used as template for dsRNA production. Synthetic dsRNAs were purified by isooamyl alcohol precipitation and re-suspended in nuclease-free water, and the concentration was quantified with a UV/Vis Spectrophotometer (Mettler-Toledo, Zurich, Switzerland).
Vector construction and tobacco transformation of dsPsFAR. To express dsPsFAR in tobacco, the 422 bp fragment of PsFAR was first amplified using a forward primer containing BamHI and SacI restriction sites and a reverse primer containing Apal restriction sites (Supplementary Table 2). The sense and antisense strands of the purified PsFAR fragment were respectively double digested with SacI and Apal and SacI + BamHI. Two digested strands were inserted into corresponding restriction sites of the plant expression vector pCAMBIA130163 one at a time. The resulting RNAi transformation vector pCAMBIA1301-dsPsFAR was validated by sequencer (Tsingke Biotech, Beijing, China).

Validating dsPsFAR expression in GM tobacco plants. The T5 Direct PCR Kit (Plant) (Tsingke Biotech, Beijing, China) was used to identify positive T0/T1 dsPsFAR genetically modified (GM) tobacco plants. Briefly, leaves 1–2 mm in diameter of T0/T1 and wild-type (WT) N. tabacum plants were lysed in 50 μl of lysis buffer A, followed by 10 min of incubation at 95 °C. After fully shaking by hand for 30 s and a brief centrifuge, the supernatant was used for PCR amplification. The 50 μl PCR reaction contained 1 μl of template DNA (i.e., supernatant), 2 μl of each primer and 25 μl of 2×T5 Direct PCR Mix (Plant) and the following thermocycling conditions were used: initial denaturation at 98 °C for 3 min; 35 cycles at 98 °C for 10 s, 63 °C for 10 s, 72 °C for 15 s; and final extension at 72 °C for 5 min. Primers used here are listed in Supplementary Table 6; the RbcL gene was used as an internal control. Genomic DNA from WT plants and double-distilled water were both used as negative controls. PCR products were analyzed by agarose gel electrophoresis.

To select for GM plants with the highest dsPsFAR expression for subsequent bioassays, total RNA of leaves from 9 randomly selected T0 GM tobacco plants (50-day old) exhibiting similar growth were isolated. The relative expression levels of dsPsFAR were quantified by RT-qPCR, and the tobacco EF-1A gene was used as an internal control. All experiments were repeated in triplicate. T0 GM plants (50-day old) harvested from one T0 GM plant that had the highest dsPsFAR expression were used for rearing newly-emerged third-instar nymphs. The cultivation process used for T1 tobacco plants was the same as that used for tomato plants. Plants and mealybugs were reared under the same rearing conditions as described above.

Assessing RNAi knockdown of PsFAR in P. solenopsis. RT-qPCR was performed to assess the effects of RNAi. For the dsPsFAR and dsGFP microinjection groups, seven P. solenopsis individuals were collected 3 days after injection from each group and used for RT-qPCR. For the groups rearred on WT and GM tobacco plants, 10 third-instar nymphs already feeding on the plants for 5 days were collected for RT-qPCR analysis. Primers for PsFAR 1 and PsFAR 2 were cited from Li et al.28. Siblings were used for developmental observation and survival quantification. All experiments were performed in triplicate.

Extraction and quantification of CHCs in P. solenopsis wax. CHCs in P. solenopsis wax was extracted from adult females that were collected 4 days after injection, or after feeding on GM tobacco for 9 days, following a procedure modified from Li et al.30,31. Briefly, 50 females (approximately 15 mg) were placed in a clean chromatography vial and immersed in 200 μl n-hexane. After 3 min of sonication in an ultrasonic vibrator (S06H, Zealway, China), the solvent was drawn in a new clean chromatography vial. This procedure was repeated twice, and finally, 200 μl of hexane was used to rinse the nymphs and vial. All hexane extracts were combined, followed by 20 min of sonication. Undissolved impurities were pelleted by 10 min of centrifugation at 10,000 rpm, the supernatant was dried until all individuals died, and dead individuals were removed from the tube. Each treatment was performed in triplicate.

Bioassay. For microinjected mealybugs, 30 adult females 4 days post-injection were collected and maintained in a rearing box. 500 μl of water was sprayed 10 cm straight down using a mini-sprayer (1.4 cm in diameter and 11.5 cm in height) (Supplementary Fig. 9) daily for 10 days. For mealybugs fed on GM tobacco plants, water was sprayed on plants directly using the same spray method. Both adaxial and abaxial surfaces of leaves harboring mealybugs were sprayed with water. The number of surviving P. solenopsis was recorded daily. The assay was repeated in triplicate.

Injection of P. solenopsis was conducted with the Eppendorf InjectMan NI 2 microinjection system (Eppendorf, Hamburg, Germany). 4–5-day-old third-instar females were collected and pooled as a single biological replicate. After anaesthetizing with CO2 for 30 s, ~200 ng of dsRNA was injected into the ventral thorax between the mesocoxa and hind coxa. After injection, insects were kept in a plastic container (30 cm long, 10.5 cm wide, 5 cm high) containing tomato branch. Tomato branches were wrapped with moistened cotton at their base to provide water and renewed every 4 days.
PAS01729. The sequence data of the pCAMBIA1301-dsPsFAR plasmid has been deposited in Addgene (#190837). The source data underlying graphs, plots, and charts in the manuscript are presented in Supplementary Data 4.

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Author contributions

F.L. and M.J. conceived, directed, and supervised the project. F.L., M.J. S.W. and H.T. designed the studies. S.D. collected samples for chemical composition analysis and proteomic sequencing. H.T., Zih.L. Zic. L., and Yi.W. collected samples for RNA-seq. Zih.L. and H.T. analyzed the RNA-seq and proteomic data. Zih.L. constructed the recombinant expression vector for transgenic tobacco. H.T., Yu.W., M.A.A.O, and Zic.L. performed RNAi and bioassay experiments. H.T., F.L., and M.J. interpreted the data and wrote the manuscript. All authors have read and approved the manuscript submission.

Competing interests

The authors declare no competing interests.

Additional information

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