New Phytologist

Research

A transgenic strategy for controlling plant bugs (*Adelphocoris suturalis*) through expression of double-stranded RNA homologous to fatty acyl-coenzyme A reductase in cotton

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Received: *22 February 2017*
Accepted: *2 May 2017*

New Phytologist (2017) **215:** 1173–1185
doi: 10.1111/nph.14636

Key words: double-stranded RNA, fatty acyl-coenzyme A reductase (FAR), pest control, plant bugs (*Adelphocoris suturalis*), RNA interference, transgenic cotton.

Summary

- Plant bugs (Miridae species), which are sap-sucking insects, have emerged as major pests of cotton in China. Most Miridae species are not sensitive to commercial *Bacillus thuringiensis* (Bt) cotton, resulting in significant economic losses and an increased application of insecticide, which eventually may compromise the future of Bt cotton.
- We demonstrate that *FATTY ACYL-COA REDUCTASE* (*AsFAR*) plays an essential role in the reproduction of the bug *Adelphocoris suturalis*. Down-regulation of *AsFAR* expression by injection of double-stranded RNA suppresses ovarian development and female fertility, resulting in females producing few viable offspring.
- To determine the viability of an RNA interference approach to limit *FAR* expression and reproductive ability in *A. suturalis*, a dsRNA targeting the *AsFAR* gene (*dsAsFAR*) of *A. suturalis* was expressed in transgenic cotton plants. *AsFAR* transcription levels were significantly downregulated in *A. suturalis* feeding on the transgenic plants. In contained field trials, the transgenic cotton lines significantly suppressed the development of *A. suturalis* populations and were resistant to damage caused by plant bug infestation.
- These results suggest a new strategy for the management of plant bug pests of cotton.

Introduction

The development and commercial cultivation of transgenic crops has revolutionized agriculture worldwide. In 2015, > 179.7 million hectares of transgenic crops were planted in 28 countries (James, 2015). For example, farmers have adopted crops that produce *Bacillus thuringiensis* (Bt) insecticidal proteins to prevent crop yield losses caused by herbivorous field pests. These crops effectively limit insect infestation, including by lepidopteran and coleopteran pests, and vastly reduce the application of broad-spectrum insecticides (Wu *et al.*, 2008; James, 2015). Nevertheless, some long-term ecological effects of Bt crops on non-target pests, such as hemipterans, have emerged. In Bt cotton, subsequent to substantial reduction of the use of broad-spectrum insecticides, mirid bugs have emerged as important economic pests of cotton in major cotton production countries including the USA, India and China (Lu *et al.*, 2008a, 2010; Musser *et al.*, 2009; Mallapur *et al.*, 2015). In China, two Miridae species, *Adelphocoris suturalis* and *Apolygus lucorum*, are emerging as the two most destructive pests in major cotton growing regions. These mirid bugs, as highly polyphagous insect species, can attack a broad range of cultivated crops, such as cotton, beans, alfalfas, vegetables and fruit crops. In cotton, both nymphs and adults feed on cotton flower buds, tender shoots and buds, causing damage by sucking plant sap, resulting in abscission, wilting, abnormal growth, and eventually losses in yield and quality (Jiang *et al.*, 2015). Currently, *A. suturalis* and *A. lucorum* have become the major pests in regions of Bt cotton cultivation in China, and the adoption of broad-spectrum insecticides is currently the preferred method for managing these mirid bugs (Lu & Wu, 2008); these mirid bugs may eventually compromise the future of Bt cotton. Hence, developing new strategies for controlling mirid bugs is a desirable objective for cotton.

*These authors contributed equally to this study.

Plant-mediated RNA interference (RNAi) technology, to suppress critical gene(s) in insects feeding on transgenic plant tissues, has been developed as a new approach to pest control. The technology provides high specificity and stable resistance, and other benefits include convenience, low cost and environmental friendliness. Since 2007, the technology has been successfully applied for the control of cotton insect pests by using transgenic plants expressing double-stranded RNAs (dsRNAs) to knock down specific target insect genes (Mao *et al.*, 2007). For example, resistance to insects was significantly improved in transgenic tobacco plants expressing dsRNA from whiteflies (Thakur *et al.*, 2014). Jin *et al.* (2015) successfully expressed dsRNAs of the *CYTOCHROME P450 MONOOXYGENASE*, *V-ATPase* and *CHITIN SYNTHASE* genes using chloroplast transformation, and these dsRNAs disrupted target insect larval development and pupation. Recently, this group developed transgenic cotton plants expressing dsRNA of a *3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE* (*HMGR*) gene and these plants showed increased resistance to cotton bollworm (Tian *et al.*, 2015). These findings strongly suggest that plant-mediated RNAi is a feasible and effective strategy for crop protection with potentially greater safety and specificity than currently available pesticides or Bt toxin.

FATTY ACYL-COA REDUCTASES (FARs) belong to the NAD(P)H-dependent oxidoreductase family of proteins, and catalyze the reduction of fatty acyl-CoA precursors into fatty alcohols, and play a variety of biological roles in the vast majority of living organisms. Previous reports showed that FARs are involved in metabolizing energy storage reserves in microorganisms (Teerawanichpan & Qiu, 2010a), biosynthesis of surface wax esters in plants and birds as a protective barrier against water loss, UV light and pathogens (Rowland *et al.*, 2006; Biester *et al.*, 2012), and biosynthesis of both ether lipids and wax esters in mammals (Cheng & Russell, 2004; Honsho *et al.*, 2010). In insects (e.g. moths and Hymenoptera), FARs act as the key enzymes required for the production of oxygenated functional groups in the biosynthesis pheromones (Liénard *et al.*, 2010; Teerawanichpan *et al.*, 2010b; Lassance *et al.*, 2013). Recently, we identified a putative *FAR* gene from *A. suturalis*, and named it *A. suturalis FAR* (*AsFAR*). Due to the structural similarities of pheromones in *A. suturalis* and moths, and as the *AsFAR* was expressed at a relatively high level in female metathoracic scent glands (MTGs) at the calling period, we initially proposed this gene as a candidate pheromone biosynthetic gene (Luo *et al.*, 2014). However, in subsequent experiments, we unexpectedly found that silencing *AsFAR* expression by injection of dsRNA of *AsFAR* (*dsAsFAR*) into *A. suturalis* had no effect on pheromone production, but severely suppressed ovarian development. Therefore, we hypothesize that *AsFAR* is involved in *A. suturalis* reproduction.

In this study, we demonstrate that *AsFAR* plays an essential role in the development of ovary and female fertility. Down-regulation of *AsFAR* expression by injection of dsRNA clearly suppressed ovarian development and female fertility, suggesting it as a promising target for *A. suturalis* control via plant-mediated RNAi. We correspondingly show that transgenic plants expressing *dsAsFAR* exhibit strong resistance to *A. suturalis*, providing a new strategy for the control of plant bug pests.

Materials and Methods

Insect rearing and plant materials

Plant bugs (*A. suturalis*) used in this study were initially collected in the field at Wuhan (Hubei Province, China) in August 2015, and were maintained in climate chambers (75 ± 5% relative humidity, 26 ± 2°C temperature and a 16 h : 8 h, light : dark cycle) and fed with green beans and 5% sugar solution (Lu *et al.*, 2008b). Newly emerged adults were separated daily and considered to be 0 d post-eclosion (PE). *Gossypium hirsutum* cv Jin668 was used for *Agrobacterium*-mediated genetic transformation.

Isolation of the cDNA of *AsFAR* from *A. suturalis*

A cDNA library from 10 d PE *A. suturalis* females was used as a template for open reading frame (ORF) amplification of *AsFAR* with the corresponding primers (Supporting Information Table S1). An expected band of 1939 bp was gel-purified (Promega), ligated into the T vector using the pEASY-T1 Simple Cloning Kit (TransGen, Beijing, China) and subjected to Sanger DNA sequencing. The ExPASy Translate tool (http://web.expasy.org/translate/) was used to deduce the amino acid sequence. SMART software (http://smart.embl.de/) was used to predict the protein functional domains. Molecular Evolutionary Genetics Analysis (MEGA) 7.1 software was used to construct the unrooted phylogenic tree by the neighbor-joining method, and the implemented JTT model was used as a substitution model for amino acids. The protein sequence alignments were performed with DNAMAN 6.0 using the ClustalX color scheme. The nucleotide and amino acid sequences of *AsFAR* were obtained from GenBank (*AsFAR*, KY274178).

Tissue distribution and temporal analysis of *AsFAR* expression in *A. suturalis*

The transcription pattern of *AsFAR* in different tissues and developmental stages of *A. suturalis* were examined by quantitative real time polymerase chain reaction (qRT-PCR). Head, MTG, midgut, ovary and fat body were collected separately from 10 d PE females of *A. suturalis* (calling period) (Zhang *et al.*, 2011) to determine the RNA distribution profile. The ovary and fat body of 0, 3, 4, 5 and 10 d PE females (chosen according to ovarian development stage) and the eggs from stage I, II, III and IV were collected separately. Total RNA was extracted using an SV total RNA isolation system with a DNase purification step (Promega) following the manufacturer's instructions. In total, 1 μg RNA was reverse transcribed using the PrimeScript™ RT Master Mix (perfect real time) (Takara, Shiga, Japan). For insect tissues, qRT-PCR was performed with a Bio-Rad iQ2 Real-time PCR Detection System (Bio-Rad) using SYBR® Premix ExTaq™ II (Takara) in a volume of 10 μl. The PCR was performed under the following conditions: 95°C for 30 s, followed by 40 cycles of

95°C for 5 s and 64°C for 30 s. All qRT-PCR tests were performed in 96-well blocks following the MIQE (Minimum Information for publication of Quantitative real time PCR Experiments) guidelines (Bustin *et al.*, 2010). The primers used for qRT-PCR are listed in Table S1. *RIBOSOMAL PROTEIN S15* (*RPS15*) and *ELONGATION FACTOR-1γ* (*EF1γ*) were used as reference genes for gene expression normalization in the tissue- and stage-dependent transcription pattern analyses, respectively. qRT-PCR data were collected from three independent biological replicates and at least three technical replicates and analyzed via the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008). Values are expressed as means ± SEM. Statistical significance of the differences was calculated using one-way ANOVA followed by Tukey's honest significant difference (HSD) multiple comparison, and statistical differences are shown as different letters.

The developmental stages of eggs were determined as described previously (Chen *et al.*, 2010). Ovarian development was divided into five stages as described for *A. lucorum* (Yuan *et al.*, 2013). A total of 250–270 females from different ages (0–24 d PE) were dissected to observe ovarian development. Images of the ovaries were collected using a stereo microscope fitted with a Nikon D5100 digital camera (Nikon, Tokyo, Japan).

RNAi in *A. suturalis* by injection of *in vitro* synthesized *dsAsFAR*

A 418 bp fragment of the *AsFAR* genes of *A. suturalis* was amplified by PCR using the corresponding primers (Table S1) and used to synthesize dsRNA as described previously (Liu *et al.*, 2016). dsRNA against *GREEN FLUORESCENT PROTEIN* (*GFP*) was synthesized (*dsGFP*) and used as a control. Using a micro-injector (World Precision Instruments, Sarasota, FL, USA), 1 μg of dsRNA in 100 nl was injected into female *A. suturalis* at 0 d PE. Total RNA was extracted from ovary and fat body ($n = 20$) at 5 and 10 d post-injection (PI) to determine the RNAi efficiency by qRT-PCR, and was monitored in whole bodies ($n = 3$) of mated females until 18 d PI (equal to 18 d PE). *EF1γ* was used as the reference gene for gene expression normalization. Three independent biological replicates were performed and the statistical significance of the differences was calculated using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Ovarian development and fertility assay

To analyze the effects of *in vitro* synthesized *dsAsFAR* on ovarian development in *A. suturalis*, >20 unmated females from each treatment were dissected to observe ovarian development at 10 d PI. Numbers of oocytes per ovary pair were counted. Estimates for the dry weight of single ovary pairs were obtained from 20 samples after drying at 90°C for 24 h (Reading, 1986), using a sensitive electrobalance (Mettler, Greifensee, Switzerland).

To test whether the down-regulation of *AsFAR* expression has negative effects on female fertility of *A. suturalis*, a single newly emerged male and dsRNA treatment virgin female were placed in a test tube (5 × 7 cm) for mating and reared under the conditions described above. Fresh green beans, as a food and oviposition substrate, were provided daily. Once the mated male died, another sexually mature virgin male was substituted. The egg output of individual treatments was recorded daily until the mated females were dead. More than 40 pairs of adults of each treatment were tested per biological replicate, and three biological replicates were carried out. Pre-oviposition period (POP), lifetime fecundity, adult longevity and egg hatch rate were used to evaluate changes in reproduction in response to dsRNA treatments. All these parameters except egg hatch rate were determined following the methods described previously (Luo & Li, 1993; Zhang *et al.*, 2006; Saastamoinen, 2007). For determination of egg hatch rate, eggs of *A. suturalis* from *dsAsFAR* and *dsGFP* treatments (before 18 d PI) were collected on four layers of moist filter paper and placed separately in Petri dishes (9 × 1.5 cm). Egg hatch rate was calculated as the number of newly hatched nymphs per number of eggs observed for the adult pair. More than 500 eggs were observed and this test was performed three times. Student's *t*-test was used to analyze statistical significance.

RNAi vector construction and cotton genetic transformation

The target fragment (*AsFAR*, 432 bp) from the conserved domain of the *AsFAR* gene was chosen for RNAi (Fig. 1a). *AsFAR* fragments were amplified by one pair of primers with attB1 and attB2 adaptors (Table S1) as described previously (Helliwell *et al.*, 2002). Purified PCR products were inserted into pHellsgate4 by BP recombination to generate the *AsFAR* RNAi vector according to the manufacturer's recommendations. The expression construct was used to transform cotton by *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation as described previously (Jin *et al.*, 2012, 2015).

Molecular analysis for the transgenic cotton plants

Transgenic cotton plants were identified by PCR and Southern blot. Genomic DNA was extracted from young leaves of putative transgenic and null plants (negative offspring derived from genetic segregation of a positive transgenic plant) using a Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China), and then used for PCR analysis. For Southern blot, 20 μg DNA was digested with *Hind* III-HF for 60 h and electrophoresed on a 0.8% agarose gel. Separated DNA fragments were blotted onto a Hybond N+ nylon membrane. Southern hybridization was performed using the DIG-High Prime DNA Labelling and Detection Starter Kit II (Roche), according to the manufacturer's instruction. The *npt* II gene probe was used for detecting transgene copy number.

For transcription analysis of *dsAsFAR*, total RNA was extracted from leaves of T1-positive transgenic and null plants using the modified guanidine thiocyanate method as previously described (Liu *et al.*, 2006), and 3 μg of total RNA was reverse-transcribed to cDNA with SuperScript III reverse transcriptase (Invitrogen).

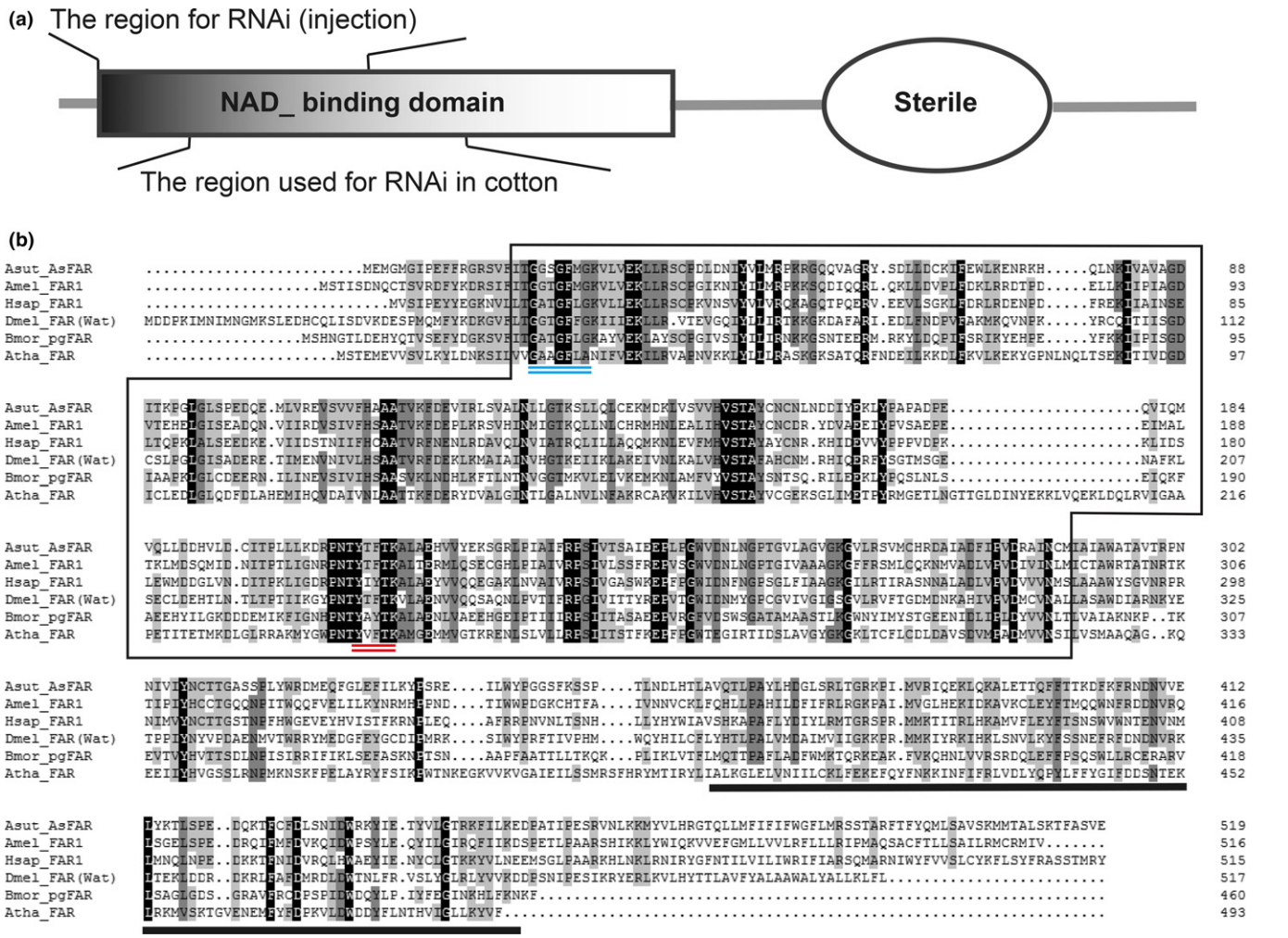

Fig. 1 Structural domains and protein sequence alignment of *Adelphocoris suturalis* fatty acyl-CoA reductase (AsFAR). (a) Schematic diagram illustrating the functional domains of AsFAR. (b) Alignment of the AsFAR from *A. suturalis* (GenBank accession no. KY274178) and other FAR proteins from eukaryotic organisms: Amel, *Apis mellifera* (GenBank accession no. ADJ56408); Hsap, *Homo sapiens* (GenBank accession no. AAT42129); Dmel, *Drosophila melanogaster* (GenBank accession no. NP_651652); Bmor, *Bombyx mori* (GenBank accession no. BAC79426); Atha, *Arabidopsis thaliana* (GenBank accession no. NP567936). Identical amino acid residues and conservative substitutions are indicated in black or gray, respectively. The FAR structural elements include an N-terminal Rossmann-fold NAD-binding domain (black box), the GXXGXX(G/A) NADPH-binding motif (blue double underline), the active site motif YXXXK (red double underline) and a Sterile protein domain (thick black line).

cDNA was used to determine the *AsFAR* gene expression level via RT-PCR, and qRT-PCR using the ABI Prism 7500 system (Applied Biosystems). Expression of *dsAsFAR* in different transgenic cotton tissues was determined by qRT-PCR. The *Gossypium hirsutum ubiquitin 7* (*GhUB7*) gene (GenBank accession no. DQ116441.1) was selected as an internal control to normalize target gene expression values. Three technical replicates and three independent biological replicates were performed for each experiment. Primers are listed in Table S1.

Insect bioassays on transgenic cotton plants in the field

Eight independent transgenic lines were used for insect bioassays under field conditions. Field evaluation experiments were conducted in two experimental plots (10.5 × 4.5 m) located on the campus of Huazhong Agricultural University. Plots were covered with a 60-dot mesh and each line ($n = 16$; two rows of eight plants) was separated by the mesh to prevent the escape of *A. suturalis* (Fig. S1). Field management followed standard agricultural practice, but without insecticide spray and no topping for the whole growing season. To prevent the invasion of other pests, insect trapping was performed when other cotton pests emerged, mainly *Helicoverpa armigera*, *Sylepta derogate* and *Spodoptera litura*. Control plants were cultivated under the same conditions. Field evaluations were performed in two successive years.

In the 2015 growing season (from June to October), all eight T1 lines and control plants were challenged with 3rd instar nymphs of *A. suturalis* (three bugs per plant) and plant phenotype was examined 1 month later. A total of 15 plants of each line were randomly selected to measure plant height, damage holes and branch numbers per plant. Damage hole count was recorded from second to sixth leaves from the top. In 2016 (from June to October), Lines 3 and 4 were selected to repeat the insect

bioassay, due to the higher and stable expression of *dsAsFAR*. All experimental plots were challenged with 3rd instar nymphs of *A. suturalis* (three bugs per plant). In addition to examining signs of infestation, the total number of adult progeny from different cages was counted. Several representative photographs of *A. suturalis* damage and field layout are shown in Fig. S2. All data were statistically analyzed by Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Quantification of *AsFAR* expression by qRT-PCR in *A. suturalis* after feeding on transgenic plants

To analyze the transcription inhibition of *AsFAR* in the target plant bugs, newly emerged females were reared on transgenic cotton flower buds and control plants. Three days later, they were collected and total RNA was extracted from whole insects and analyzed by qRT-PCR as already described.

Bioassay for non-target insects

To test possible effects of *dsAsFAR* on non-target insects, transgenic cotton plants (Line 3) were challenged with two major insect species for cotton plants, *H. armigera* and *Aphis gossypii*. Fully expanded leaves (third to fifth from the top) from transgenic and control plants were excised and challenged with 3rd instar larvae of *H. armigera* as a representative chewing pest. Leaves were placed in Petri dishes with wet filter paper and replaced every day. After 4 d of feeding, larva weight was recorded and the bioassay was performed for three biological replicates. Student's *t*-test was used to perform statistical analysis of the data. As a representative of sucking insect pests, 30 aphids were released on each transgenic and control plant and their population sizes were monitored on days 7 and 15.

Results

Identification of *FAR* in *A. suturalis*

Based on the *A. suturalis* transcriptome data (Luo *et al.*, 2014), we isolated a full-length cDNA corresponding to the *FAR* gene, designated as *A. suturalis FAR* (*AsFAR*). The cloned full-length cDNA transcript is 1939 bp, encompassing an ORF of 1563 bp that encodes a protein of 520 amino acid residues. Protein domain searches against the Pfam database revealed that the AsFAR has domains characteristic of eukaryotic FARs, including a Rossmann-fold NAD_binding domain between amino acid positions 18 and 289, and a Sterile domain located in the C-terminal residues 360–452 (Fig. 1a). The conserved NAD(P)H-binding motif GXXGXX(G/A) and the active site motif YXXXK found in other FARs were also present in the Rossmann-fold domain of AsFAR. A query of the public database with the deduced AsFAR amino acid sequence using BLAST revealed that the AsFAR protein shared 44% sequence similarity with the *Apis mellifera* FAR1 responsible for the biosynthesis of aliphatic alcohols in honey bees (Teerawanichpan *et al.*, 2010b); 28% similarity to the *Bombyx mori* pheromone-gland-specific FAR (Moto *et al.*, 2003); and 39% sequence similarity to the *Homo sapiens* FAR1 related to the synthesis of the precursors of wax monoesters and ether lipids (Cheng & Russell, 2004) (Fig. 1b). A neighbor-joining tree was constructed using the AsFAR protein sequences and different FAR proteins from various organisms. The results showed that AsFAR was clustered with other Hemiptera FARs and *A. mellifera* FAR, which were distantly related to *Euglena* FAR and plant FARs (Fig. S3). Gene identification and sequence analyses indicated that AsFAR may have a role similar to that of *A. mellifera* FAR1.

AsFAR is highly transcribed in the *A. suturalis* ovary

Since an analysis of expression pattern might inform an understanding of gene function, monitoring of *AsFAR* transcription in different tissues and developmental stages of *A. suturalis* was performed by qRT-PCR. The results showed that *AsFAR* exhibits highest levels of transcription in ovary, with high levels also detected in the fat body, while negligible expression was observed in other tissues (Fig. 2k).

Transcription of *AsFAR* in ovary and egg at different developmental stages was monitored to determine whether *AsFAR* expression was correlated with oocyte and embryo development. Ovarian development in *A. suturalis* was classified into five stages following the methods described for *A. lucorum* (Yuan *et al.*, 2013), as follows. Stage I (0–4 d PE): follicles at the stage of previtellogenesis, when no deposition of yolk protein is observed (Fig. 2a,b). Stage II (4–5 d PE): start of vitellogenesis, in which follicles had some yolk protein deposition, and no mature chorionic follicles were observed (Fig. 2c). Stage III (5–6 d PE): start of oogenesis, when large amounts of yolk protein are deposited in follicles and mature chorionic follicles are observed (Fig. 2d). Stage IV (6–20 d PE): presence of mature eggs, with each ovariole containing at least one mature egg (Fig. 2e). Stage V (21 d PE): ovarioles begin to shrink and few mature follicles are observed (Fig. 2f). The *A. suturalis* ovary from 0 d (the first day of stage I), 3 d (the day before stage II), 4 d (stage II), 5 d (stage III) and 10 d (stage IV) PE and the egg from stage I (newly produced eggs; Fig. 2g), stage II (pale yellow eggs; Fig. 2h), stage III (red compound eye period; Fig. 2i) and stage IV (pre-incubation period; Fig. 2j) were collected separately for transcriptional analysis. The results show that *AsFAR* exhibited peak expression in eggs at stage I, whereas a low level of *AsFAR* transcripts was detected in subsequent embryonic developmental stages (egg stages II–IV). A higher level of *AsFAR* transcription was detected at all stages of ovarian development and showed a rising trend of expression with the development of ovary (Fig. 2l). These results indicate that *AsFAR* may play a more important role in ovarian development than in embryonic development. Since abundant *AsFAR* transcripts were detected in the fat body, which is involved in energy storage, metabolism and regulation in the life cycle of insects, we carried out a more detailed analysis of *AsFAR* transcription levels in the fat body at 0, 3, 4, 5 and 10 d PE. The result shows that *AsFAR* is expressed in all the stages of fat body development, and there was no significant difference between them (Fig. 2l).

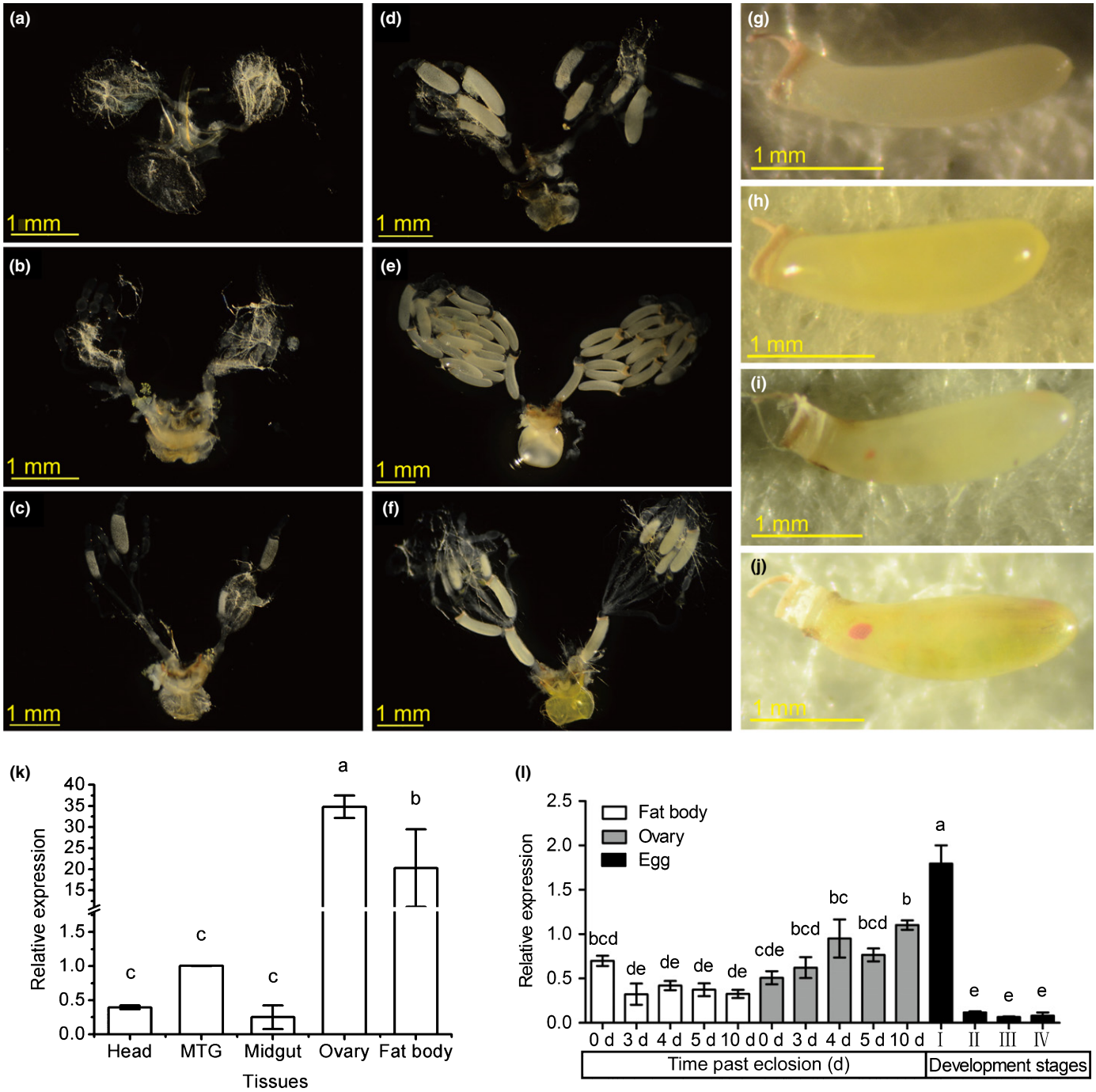

Fig. 2 Tissue- and stage-dependent transcription pattern analysis of *AsFAR* and the development of ovary and egg in *Adelphocoris suturalis*. Ovary development of *A. suturalis* was classified into five stages, namely Stage I (follicles at the stage of previtellogenesis, no deposition of yolk protein; a and b); stage II (start of vitellogenesis, follicles have some yolk protein deposition, no mature chorionated follicles; c); stage III (start of oogenesis, large amount of yolk protein deposited in follicles and mature chorionated eggs present; d); stage IV (presence of mature eggs, each ovariole contains at least one to two mature eggs; e); and stage V (ovarioles begin to shrink, few mature follicles observed; f). Egg development of *A. suturalis* was classified into four stages, namely stage I (newly produced eggs; g); stage II (pale yellow eggs; h); stage III (red compound eye period; i); and stage IV (preincubation period; j). The tissue distribution (k) and temporal analysis (l) of *AsFAR* transcripts were monitored by qRT-PCR. Values are expressed as means ± SEM based on three independent biological replicates. Different letters shows significant differences ($P < 0.05$, one-way ANOVA followed by Tukey's HSD multiple comparison). MTG, metathoracic scent glands.

AsFAR is required for ovarian development and female fertility in *A. suturalis*

To determine whether the down-regulation of *AsFAR* expression could affect the reproduction of female *A. suturalis*, a 418 bp fragment in the conserved domain of *AsFAR* was chosen as an RNAi target (Fig. 1a). First, we examined the effect of RNAi treatment on *AsFAR* transcript abundance by qRT-PCR. Compared with the *dsGFP* control, the transcriptional levels of *AsFAR* were significantly suppressed at 5 and 10 d PI in both fat body

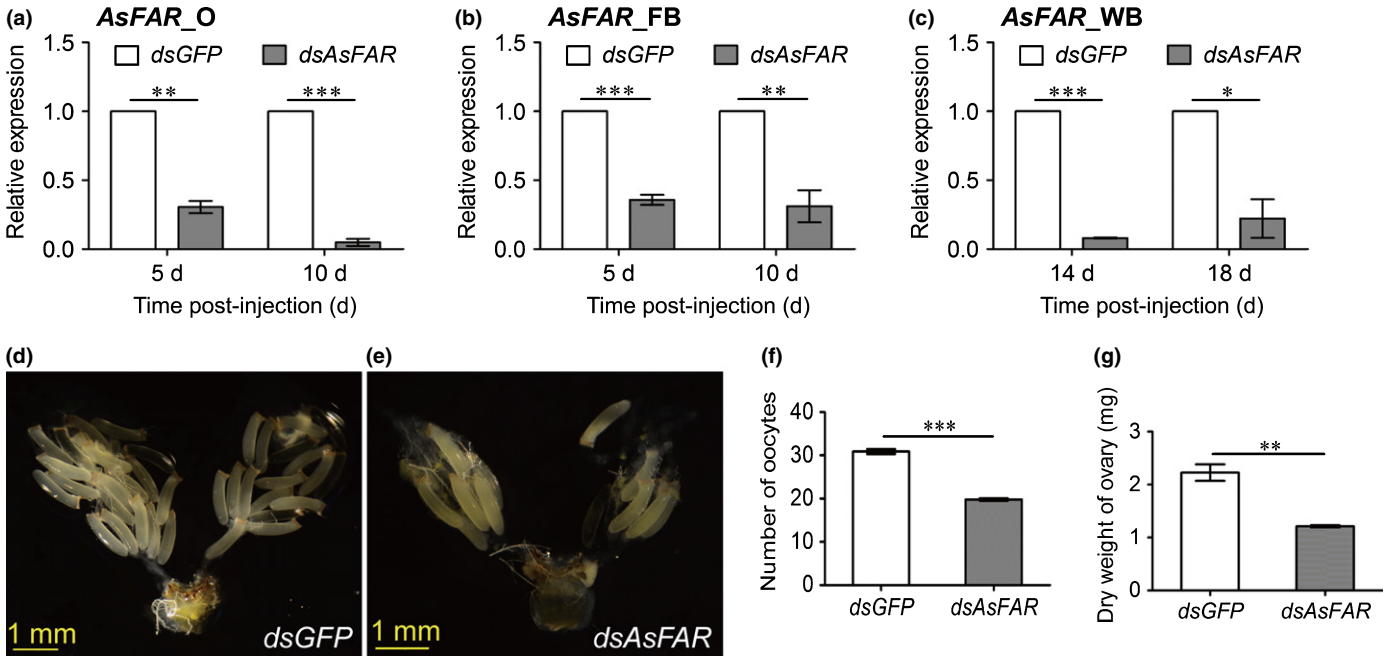

Fig. 3 Downregulation of *AsFAR* suppresses ovarian development of *Adelphocoris suturalis*. At 0 d post-eclosion (PE), females were microinjected with dsRNA of *AsFAR* (*dsAsFAR*) or dsRNA of *Green fluorescent protein* (*dsGFP*) (control), and *AsFAR* gene transcription level in ovary (a) and fat body (b) was determined at 5 and 10 d post-injection (PI). The silencing effect of *AsFAR* was monitored in whole insect body (c) until 18 d PI to determine whether *AsFAR* was stably suppressed throughout the reproductive phase. *dsGFP* (d) or *dsAsFAR* (e) treatment ovaries were imaged at 10 d PI using a stereo microscope. Oocyte numbers per ovary pair were counted (f) and the dry weight of single ovary pairs was estimated (g) to quantify ovarian development. Values are expressed as means ± SEM based on three independent biological replicates. Statistical significance (Student's *t*-test): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and ovary, with a reduction of 64–95% (Fig. 3a,b). To determine whether expression of *AsFAR* in females is stably suppressed throughout the reproductive phase, the silencing effect of *AsFAR* was monitored in whole bodies of mated females until 18 d PI (equal to 18 d PE), when the numbers of mature eggs in the ovarioles began to decrease rapidly and the ovary began to shrink. Results show that the transcription of *AsFAR* was significantly suppressed for 18 d PI by the injection of exogenous *dsAsFAR* (Fig. 3c).

Ovarian development and four reproductive parameters (POP, lifetime fecundity, adult longevity and egg hatch rate) were investigated to evaluate reproductive changes in response to RNAi treatments. Results show that injected synthetic *dsAsFAR* suppresses ovarian development (Fig. 3d,e). The numbers of oocytes and dry weight of per-ovary pair were reduced by 36 and 46%, respectively, compared with the *dsGFP* control treatment (Fig. 3f,g). *dsAsFAR*-treated females showed low fertility (Fig. 4). The lifetime fecundity of *dsAsFAR*-treated females was reduced by 52% compared to the *dsGFP* control (Fig. 4a). The egg hatch rate of the *dsAsFAR*-treated group was only 28%. By contrast, it was as high as 89% in the *dsGFP* control group (Fig. 4b). Monitoring of egg development in both groups revealed that exogenous *dsAsFAR* severely impaired the development of embryos, which remained at the primary stage. By contrast, 89% of eggs from the *dsGFP* group successfully completed embryonic development (Fig. 4e–g). The POP and adult longevity data did not exhibit significant differences between the *dsAsFAR* treatment and the *dsGFP* control (Fig. 4c,d). The results therefore show that knockdown of *AsFAR* in *A. suturalis* suppresses ovarian development and female fertility.

Transgenic cotton plants expressing *dsAsFAR* have a normal phenotype

In this study, a conserved domain (*AsFAR* 432 bp, Fig. 1a) was chosen as the target sequence for RNAi, and two inverted repeats of this target fragment were driven by the cauliflower mosaic virus (CaMV) 35S promoter to transcribe the dsRNA. The T-DNA region of the Ti plasmid vector is shown in Fig. 5(a). *Agrobacterium*-mediated genetic transformation was performed (Fig. 5). Thirty regenerated T0 plants were obtained and transferred to pots for further growth in the glasshouse (Fig. 5g,h). The majority of these regenerated plants exhibited a normal phenotype and were fertile. Eight independent T0 transgenic lines were confirmed by PCR analysis (Fig. 6a), and selected to generate the T1 populations for further analysis. Southern blotting of T1 transgenic lines (two plants from each line) confirmed transformation (Fig. 6b). Three out of the eight lines contained a single T-DNA copy, and the other lines have multiple T-DNA copies.

RT-PCR and qRT-PCR analysis of the T1 lines confirmed expression of the *dsAsFAR* (Fig. 6c,d). Lines 3 and 4, which carried a single copy and multiple copies, respectively (Fig. 6b), were selected for further study on the basis of relatively high *dsRNA* transcription levels, and normal agronomic performance. The expression pattern of *dsAsFAR* was also analyzed in various tissues

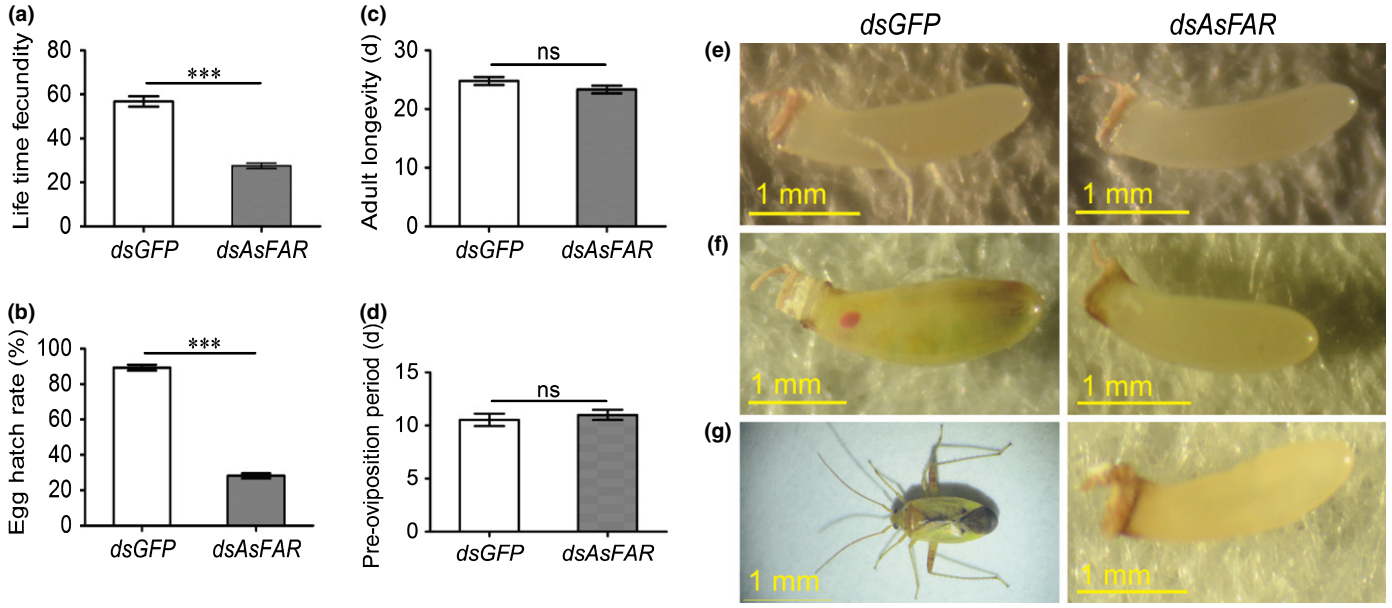

Fig. 4 Downregulation of *AsFAR* suppresses *Adelphocoris suturalis* female fertility. At 0 d post-eclosion (PE), females were microinjected with *dsAsFAR* or *dsGFP* (control), and four reproductive parameters, lifetime fecundity (a), egg hatch rate (b), adult longevity (c) and pre-oviposition period (POP) (d), were used to evaluate changes in female fertility in response to *dsAsFAR* and *dsGFP* treatments. The development of eggs was observed at stage I (newly produced eggs, e) stage IV (preincubation period, f) and at 30 d (g), and imaged using a stereo microscope. Values are expressed as means ± SEM based on three independent biological replicates. Statistical significance (Student's *t*-test): ***, $P < 0.001$; ns, not significant.

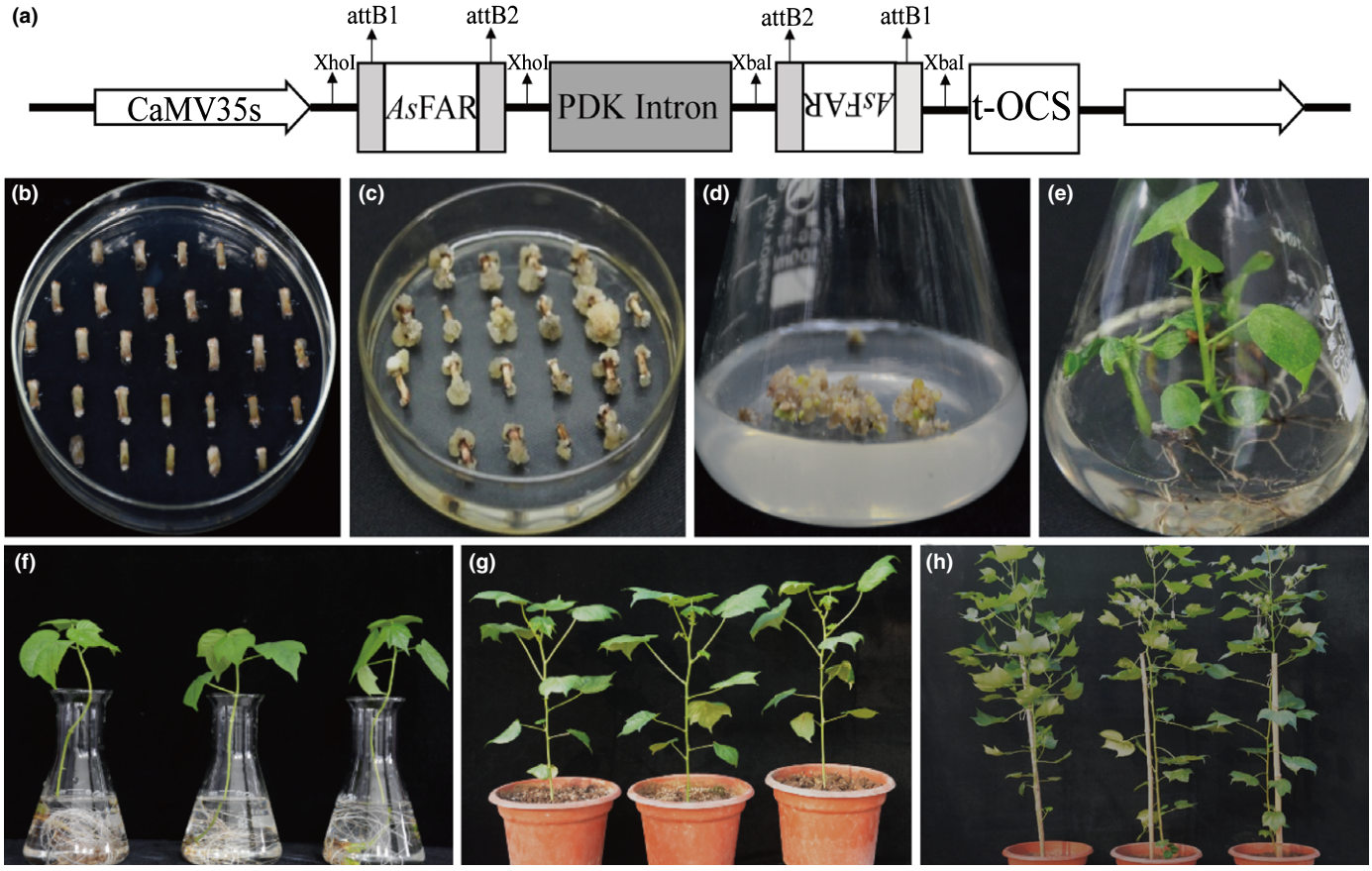

Fig. 5 The plasmid vector, genetic transformation and plant regeneration of cotton. (a) *AsFAR* gene expression cassette (T-DNA region) used for *Agrobacterium*-mediated transformation. (b, c) Callus induction on selective media containing kanamycin. (d) Somatic embryogenesis. (e, f) Plant regeneration. (g, h) Regenerated plants transferred to the soil.

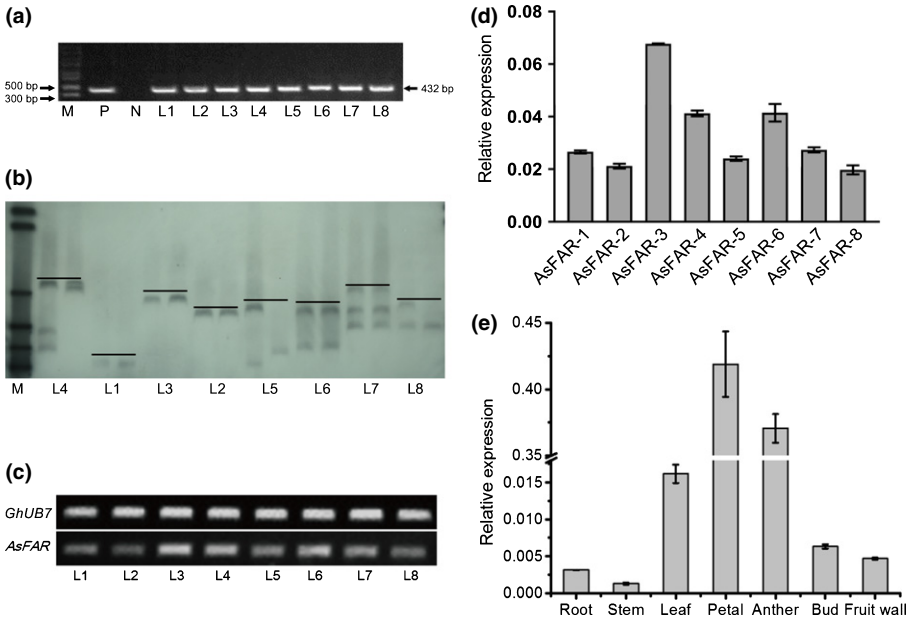

Fig. 6 Molecular analysis of transgenic cotton plants. (a) PCR analysis of putative transgenic cotton lines. M, marker; P, positive control; N, negative control. (b) Southern blot analysis of eight lines from T1 transgenic plant populations. M, DNA molecular weight marker (0.12–23.1 kb) (Roche). Numbers marked under the gel indicate corresponding lines. (c) RT-PCR analysis of *dsAsFAR* transcription levels in young leaves. *Gossypium hirsutum ubiquitin 7* (*GhUB7*) was used as an RNA loading control. (d) *dsAsFAR* relative transcription in T1 transgenic cotton plants was detected by qRT-PCR. (e) qRT-PCR analysis of *dsAsFAR* in different tissues of transgenic plants. Relative transcription of *dsAsFAR* was highest in petal and anther. The experiments were repeated three times, each time with three technical replicates per line; values are shown as means ± SEM.

of line 3 and *dsAsRNAs* were expressed at high levels in petals and anthers. Moderate expression levels were detected in leaf and bud, which were two primary feeding targets of plant bugs. Lower *dsAsFAR* was detected in the boll shell, root and stem (Fig. 6e).

Transgenic plants show resistance to plant bug infestation

The previous *in vitro* injection experiment shows that *dsAsFAR* suppresses female fertility and results in few viable offspring. We then tested whether the *dsAsRNA* generated by transgenic cotton plants has an impact on development of the *A. suturalis* population. All transgenic plants were caged by mesh for the whole growing season. After release of the plant bugs in the cage for 1 month, the bug population (progeny, nymphs and newly emerged adults) was measured. The results show that development of the *A. suturalis* population was significantly suppressed in transgenic plants ($P < 0.05$). There were on average 12–14 plant bugs per transgenic plant, compared with > 20 per control plant (Fig. 7a). Transcription levels of endogenous *AsFAR* in *A. suturalis* adults were investigated by qRT-PCR after feeding on transgenic plants expressing *dsAsFAR*. Compared with the control, transcription levels of endogenous *AsFAR* in *A. suturalis* adults were significantly suppressed at 3 d post-feeding, with a reduction of 36–51% (Fig. 7b).

Since cotton shoot tips, young leaves, squares, blooms and small bolls are the primary feeding targets of plant bugs (Jiang *et al.*, 2015), the damage phenotype of control and transgenic plants was recorded. As shown in Fig. 8, control plants exhibited curl petal, darkened anthers and damaged stigma (Fig. 8a,b), and scarring of the boll shell (Fig. 8c), which eventually led to a decline in yield and quality (Fig. 8d). Plant bugs feeding on plant shoot-tips also resulted in arbuscular branches and a dwarf plant phenotype, and feeding on young leaves caused holes that initially appeared as small black spots, but became larger, irregular holes as leaves grew. We recorded plant height, holes and branch numbers per plant to quantify the damage. We found that the transgenic plants showed a high level of resistance to *A. suturalis* during both the 2015 and the 2016 growing seasons. The number of damage holes on the transgenic plants was reduced by 60–64% (Fig. 7d,g) compared with the control plants. The plant height and branch numbers of transgenic plants were healthy and normal, whereas the height of control plants was reduced by *c.* 19–22% compared with transgenic plants (Fig. 7c,f), and the number of branches per plant of control plants was significantly increased by 62–68% (Fig. 7e,h). These results suggest that transgenic cotton expressing *dsAsFAR* shows a high level of resistance to *A. suturalis.*

Transgenic plants show no effects on non-target pests

The representative chewing pest *H. armigera* and sap-sucking insect pest aphids were selected to assess whether the transgenic plants had effects on non-target pests. The result show that the population size and growth of non-target pests were unaffected on transgenic plants (Fig. S4), suggesting that *AsFAR*-cotton has no adverse effect on *H. armigera* and aphid reproduction.

Discussion

FAR is reported as a key enzyme required for the production of pheromones in several insect pest species (Lassance *et al.*, 2010; Ding *et al.*, 2011; Antony *et al.*, 2016). In this study, we found that *AsFAR* plays an important role in the development and reproduction of *A. suturalis*, whereby silencing *AsFAR* expression suppresses ovarian development and female fertility. *AsFAR* was therefore chosen as a promising target for plant-mediated RNAi suppression, as a means of *A. suturalis* control. We successfully expressed *dsAsFAR* in upland cotton by genetic transformation. Several transgenic lines were obtained that had a relatively high level of dsRNA expression. Field evaluation

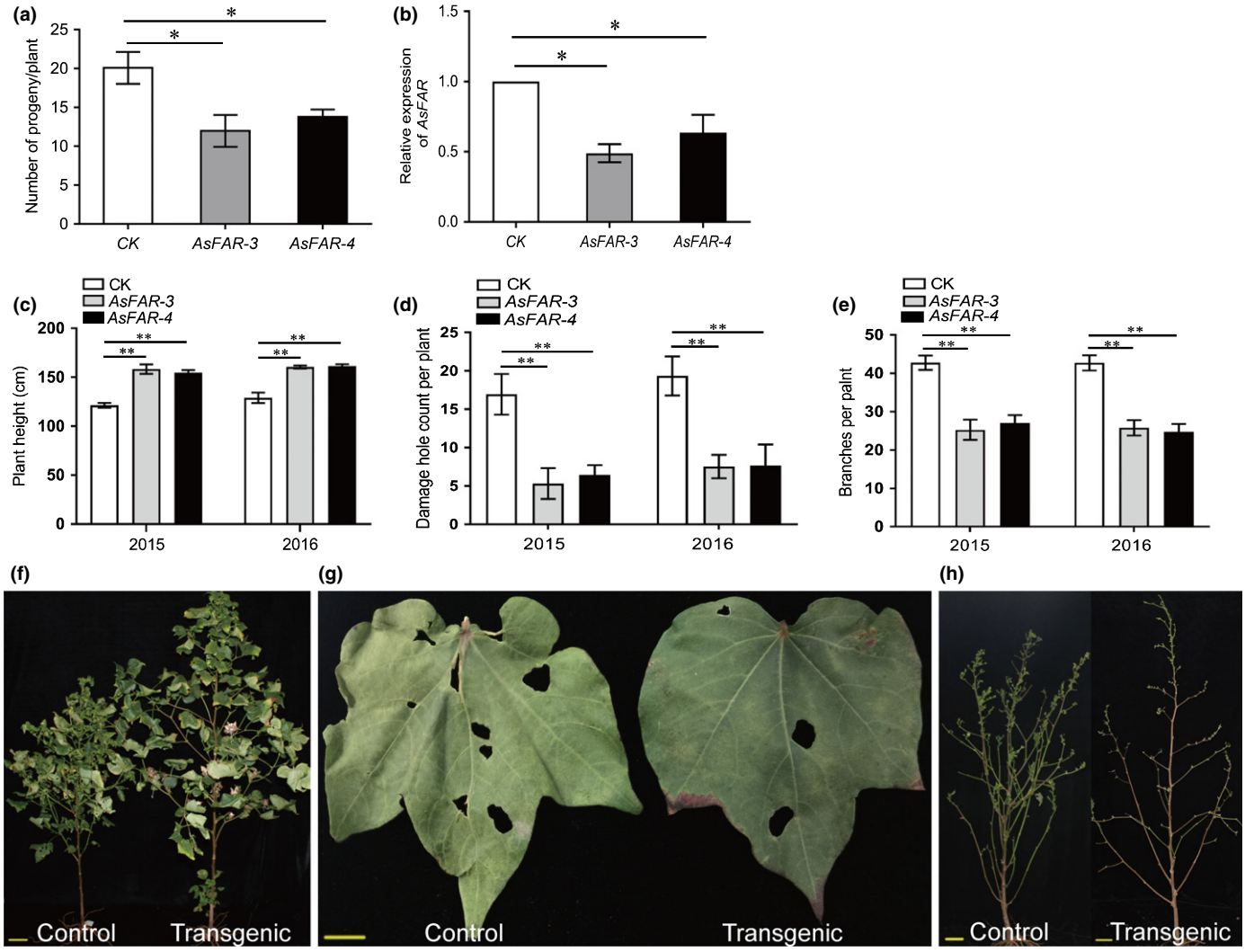

Fig. 7 Transgenic cotton plants exhibiting resistance to *Adelphocoris suturalis.* (a) Transcription of *AsFAR* gene revealed by qRT-PCR in *A. suturalis* after feeding on transgenic and control plants. (b) *A. suturalis* population size on transgenic lines 3 and 4 and control plants ($n = 16$ plants). The transgenic and control plants were challenged with *A. suturalis* and the damage phenotypes were investigated. Fifteen plants of each line were randomly selected and plant height (c, f), count of damage holes (d, g) and number of branches (e, h) were measured as infestation traits. Values are expressed as means ± SEM based on three independent biological replicates. Statistical significance (Student's *t*-test): *, $P < 0.05$; **, $P < 0.01$. Bars, 1 cm in (f)–(h).

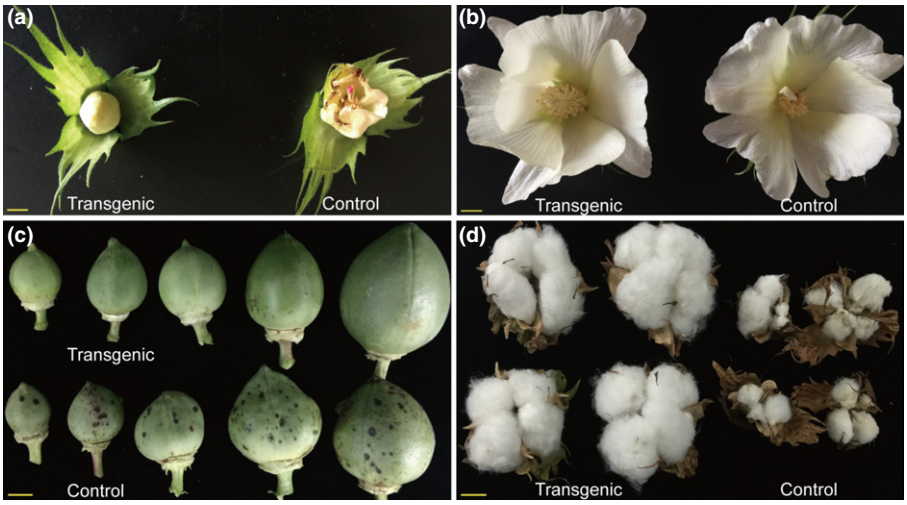

Fig. 8 Resistance phenotypes in tissues of transgenic plants expressing *dsAsFAR* and control genes, following *Adelphocoris suturalis* infestation. (a) *A. suturalis* infestation on control plants causes black spots and curling and thickening of petals. (b) Anther damage in control cotton plants. (c, d) Reduction in boll size and number, formation of black spots, developmental abnormality and cracking, leading to a decline in yield and quality. Bars, 1 cm.

results showed that the transgenics exhibited high levels of resistance to *A. suturalis*.

FARs catalyze the reduction of fatty acyl-CoA precursors into fatty alcohols using NAD(P)H as a reducing equivalent (Pollard *et al.*, 1979). In this study, we isolated a full-length cDNA of *AsFAR* from *A. suturalis* and found it plays an essential role during *A. suturalis* reproduction. AsFAR had protein domains characteristic of eukaryotic FARs, namely a Rossmann-fold NAD-binding domain and a Sterile domain. Sequence alignment comparison between AsFAR and other functionally characterized FARs showed that AsFARs have the highest sequence similarity (44% amino acid identity) to *A. mellifera* FAR1 (GenBank accession no. ADJ56408), which is responsible for the biosynthesis of a wide range of aliphatic alcohols. Phylogenetic analysis shows a close relationship between AsFAR and *A. mellifera* FAR1, suggesting that AsFAR may have a similar role to *A. mellifera* FAR1.

The role of *AsFAR* in *A. suturalis* reproduction was identified by RNAi suppression in the *A. suturalis* bug. Downregulation of *AsFAR* expression led to a significant decrease in the numbers of oocytes, dry weight of ovaries, lifetime fecundity and egg hatchability. Since oocytes take up a large proportion of the mature ovary, the loss of ovary dry weight is likely to be mainly caused by oocyte depletion. We cannot exclude the possibility that the reduction in dry matter accumulation results in a loss of ovary dry weight. The physiology of ovarian development is directly related to the individual's fecundity (Zhang *et al.*, 2016). The loss of female lifetime fecundity may be mainly caused by oocyte depletion, since the POP and adult longevity were not different between RNAi and control treatments.

High reproductive ability is not only reflected in oocyte quantity but also in quality. Oocyte quality or developmental competence affects embryonic development and the health of the offspring (Eppig & O'Brien, 1998). Our results showed that the down-regulation of *AsFAR* expression led to a lower egg hatchability, and embryo development was blocked by *AsFAR* depletion at the primary stage. This result is consistent with the tissue distribution and temporal expression pattern of *AsFAR*, which was found to be highly expressed in ovary and increased during ovary development, but showed low-level transcription in subsequent embryonic development. Krisher (2004) reported that oocyte quality, or developmental competence, was acquired during folliculogenesis as the oocyte grows, and during the period of oocyte maturation. Therefore, these results show that *AsFAR* plays a critical role in *A. suturalis* reproduction, being required for oocyte quality and quantity, and ultimately for viable offspring. *AsFAR* therefore represents a potentially valuable target for plant-mediated RNAi control of *A. suturalis*.

Insect pests pose a significant threat to crop yield and quality. The use of insecticides and the widespread adoption of Bt crops have effectively controlled pest infestation in recent years (Wu *et al.*, 2008). However, the excessive spraying of chemical insecticides carries the risk of the emergence of pest resistance, and may negatively impact the environment and human health. RNAi strategies offer higher specificity, stable resistance and a more environmentally friendly solution.

Many dsRNA delivery systems have been used successfully. Among these, injection remains the most used method due to its high efficiency and accuracy (Hughes & Kaufman, 2000), but is not suitable in a field situation. Plant-mediated RNAi suppression of insect gene(s) is an ideal system for dsRNA delivery to insects feeding on plant tissues, as the dsRNA can be expressed throughout the plant life cycle and successfully inhibit insect feeding or development (Pitino *et al.*, 2011; Zhu *et al.*, 2012; Wuriyanghan & Falk, 2013).

In this study, we successfully expressed *dsAsFAR* in transgenic cotton plants, which was found to limit *A. suturalis* population growth and crop damage. Transgenic expression of *dsAsFAR* was high in petal and anthers and lower in leaves, bud, fruit wall, root and stem, and the level of *dsAsFAR* expression in the crops was associated with the level of crop protection, a phenomenon also found in transgenic *Arabidopsis* plants expressing dsRNA of *HaAK* (Liu *et al.*, 2015). Previous studies have shown that *A. suturalis* prefers feeding on pollen and nectar (Jiang *et al.*, 2015). In the future, the production of transgenic cotton which has a high expression of dsRNA in anthers may have a greater potential for plant bug control.

Although transgenic cotton expressing the *dsAsFAR* described here did not cause detectable mortality in *A. suturalis*, it severely impaired population development, which was found to be important for pest damage limitation. Therefore, this study describes a new strategy for the control of *A. suturalis*, and our transgenic lines can be used as a germplasm resource to pyramid with existing Bt cotton to develop genetically modified crops with enhanced resistance to *A. suturalis* and other herbivorous pests.

Acknowledgements

This research was supported by grants of the National Special Key Project for Transgenic Breeding (2016ZX08010001-006) and Open funding from the State Key Laboratory for Biology of Plant Diseases and Insect Pests (grant SKLOF201415).

Author contributions

S.J., X.Z., L.C., C.L. and K.L. conceived and designed the experiments. J.L., S.L., J.L., Z.X., L.L., B.Z. and Z.L. performed experiments. J.L. and S.L. analyzed the data and wrote the manuscript. S.J. and K.L improved the manuscript. All authors read and approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Insect bioassay of T1 and T2 *AsFAR* transgenic cotton plants in field conditions.

Fig. S2 Typical damage characteristics caused by *Adelphocoris suturalis* on cotton plants.

Fig. S3 Phylogeny of *Adelphocoris suturalis* FAR (AsFAR) and other FARs.

Fig. S4 Effect of *AsFAR* transgenic cotton plants on *Helicoverpa armigera* and aphids.

Table S1 Primers used in this study

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