A transgenic strategy for controlling plant bugs

(*Adelphocoris suturalis*) through expression of double-stranded RNA (dsRNA) homologous to Fatty acyl-CoA reductase (FAR) in cotton

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Introduction

The development and commercial cultivation of transgenic crops has revolutionized agriculture worldwide. In 2015, more than 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 nontarget pests, such as hemipteran pests, have emerged. In Bt cotton, subsequent to substantial reduction of the use of broad-spectrum insecticides, true bugs have emerged as important economic pests of cotton in major cotton production countries including USA, India and China (Lu et al., 2008a; Musser et al., 2009; Lu et al., 2010; 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 a 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 leading to 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, 2008b); development of resistance in 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.

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 the the control of cotton insect pests by using transgenic plants expressing 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). In 2015, Jin and colleagues 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 (Jin et al., 2015). Recently, this group developed transgenic cotton plants expressing dsRNA of a 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE (HMGR) gene and 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 pathogen (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 (MTG) 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 ºC temperature and a 16:8 h light:dark cycle) and fed with green beans and 5% sugar solution (Lu et al., 2008c). Newly emerged adults were separated daily and considered to be 0 days 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 days PE A. suturalis females was used as a template for open reading frame (ORF) amplification of AsFAR with the corresponding primers (Table S1). An expected band of 1,939 bp was gel-purified (Promega, Madison, Wisconsin, USA), ligated into the T vector using the pEASY-T1 Simple Cloning Kit (TransGen, 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 phylogenetic 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 the 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 days 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 days 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 a SV total RNA isolation system with a DNase purification step (Promega, Madison, Wisconsin, USA) following the manufacturer’s instructions. 1µg RNA was reverse transcribed using the PrimeScript™ RT Master Mix (perfect real time) (Takara, Japan). For insect tissues, qRT-PCR was performed with a Bio-Rad iQ2 Real-time PCR Detection System (Bio-Rad, Hercules, California, USA) using SYBR® Premix ExTaq™ II (Takara, Japan) 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 were listed in Supporting Information Table 1. 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−ΔΔCt method (Schmittgen & Livak, 2008). Values were expressed as the means ± standard error mean (SEM). Statistical significance of the differences was calculated using one-way ANOVA followed by Tukey's 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 to 24 days PE) were dissected to observe ovarian development. The images of the ovaries were collected using a stereo microscope fitted with a Nikon D5100 digital camera (Nikon, Tokyo, Japan). The images of different developmental stages ovary and egg are shown in Fig. 2.

**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, USA), 1 μg of dsRNA in 100 nl was injected into female A. suturalis at 0 days PE. Total RNA was extracted from ovary and fat body at 5 and 10 days post-injection (PI) to determine the RNAi efficiency by qRT-PCR, and was monitored in whole bodies of mated females until 18 days PI (equal to 18 days PE). EF1γ was used as the reference gene for gene expression normalization. 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*, more than 20 unmated females from each treatment were dissected to observe the ovarian development at 10 days PI. Numbers of oocytes per ovary pair were counted. Estimates for the dry weight of single ovary pair were obtained from 20 samples after drying at 90 °C for 24 h (Reading, 1986), using a sensitive electrobalance (Mettler, 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 previously (REF). Fresh green beans, as a food and oviposition substrates, 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 adult 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 egg hatch rate determination, eggs of *A. suturalis* from *dsAsFAR* and *dsGFP* treatments (before 18 days PI) were collected on 4 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 nymph per number of eggs observed for the adult pair. More than 500 eggs were observed and this test was performed for three times. The Student's t-test was used to analyze statistical significance.

**RNAi vector construction and cotton genetic transformation**

The target fragment (*AsFAR*, 432 base pairs) from the conserved domain of *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 AsFRA 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., 2005; Jin et al., 2012).

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 positive transgenic plant) using Plant Genomic DNA Kit (Tiangen Biotech, China), and then used for PCR analysis. For Southern blot, 20 µg DNA was digested with Hind III-HF for 60 hours and electrophoresed on 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, Mannheim, Germany), 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, Carlsbad, CA, USA). cDNA was used to determine the AsFAR gene expression level via RT-PCR, and qRT-PCR using the ABI Prism 7500 system (Applied Biosystems, Foster City, CA, USA). Expression of dsAsFAR in different transgenic cotton tissues was determined by qRT-PCR. The UBQ7 gene (GenBank accession no. DQ116441.1) was selected as an internal control to normalize target genes expression values. Three technical replicates and three independent biological replicates were performed for each experiment. Primers are listed in Supporting Information Table 1.
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 mesh of 60-dot and each line (n = 16; 2 rows of 8 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. In order to prevent the invasion of other pests, insects trapping was performed when other cotton pests emerged, mainly *H. 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* (3 bugs per plant) and plant phenotype was examined a 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* (3 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

For analyzing 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 described above.

Bioassay for nontarget insects

To test possible effects of dsAsFAR on nontarget insects, transgenic cotton plants (Line 3) were challenged with two major insect species for cotton plant *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 larvae of *H. armigera* as a representative chewing pest. Leaves were placed in Petri dishes with wet filter paper and replaced every day. After four days of feeding, larva weight was recorded and the bioassay was performed for three biological replicates. The Student’s t-test was used to perform statistical analysis of the data. As a representative of sucking insect pests, thirty aphids were released on each transgenic and control plants and their population sizes were monitored on day 7 and day 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 *FATTY ACYL-COA REDUCTASE (FAR)* gene, designated as *A. suturalis FAR (AsFAR)*. The cloned full-length cDNA transcript is 1,939 bp, encompassing an ORF of 1,563 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 data base 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 homology 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 tissue and developmental stages of A. suturalis was performed by qRT-PCR. 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 days PE): follicles at the stage of previtellogenesis, when no deposition of yolk protein is observed (Fig. 2a,b). Stage II (4-5 days 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 days PE): start of oogenesis, when large amount of yolk protein are deposited in follicles and mature chorionic follicles are observed (Fig. 2d). Stage IV (6-20 days PE): presence of mature eggs, with each ovariole containing at least 1–2 mature eggs (Fig. 2e). Stage V (21 days after PE): ovarioles begin to shrink and few mature follicles are observed (Fig. 2f). The A. suturalis ovary from 0 (the first day of stage I), 3 (the day before stage II), 4 (stage II),
5 (stage III) and 10 (stage IV) days 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 (preincubation 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 to 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 lifecycle of insects, we carried out a more detailed analysis of AsFAR transcription levels in the fat body at 0, 3, 4, 5 and 10 days 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).

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

In order 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 were chosen as an RNAi target (Fig. 1a). Firstly, 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 days PI in both fat body and ovary, with a reduction of 64-95% (Fig. 3a,b). In order 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 days PI (equal to 18 days 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 days 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%. In 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. In contrast, 89% eggs from 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 432bp, Fig. 1a) was chosen as the target sequence for RNAi, and two inverted repeats of this target fragments were driven by CaMV 35S promoter to transcribe the dsRNA. The T-DNA region of the Ti plasmid vector is shown in Fig. 5a. Agrobacterium-mediated genetic transformation was performed (Fig. 5). 30 regenerated T0 plants were obtained and they were transferred to pots for further growth in the greenhouse (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 (2 plants from each line) confirmed transformation (Fig. 6b). 3 out of the 8 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 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 of line 3. dsAsRNA was 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 the development of *A. suturalis* population. All transgenic plants were caged by mesh for the whole growing season. After release the plant bugs in the cage for one month, the bug population (progeny, nymphs and newly emerged adults) was measured. The results show that the development of *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 more than 20 per control plant (Fig. 7b). The 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, the transcription levels of endogenous *AsFAR* in *A. suturalis* adults were significantly suppressed at 3 days post-feeding, with a reduction of 36–51% (Fig. 7a).

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 cause holes which 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 2015 and 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 branches numbers of transgenic plants were healthy and normal, while in contrast, the height of control plants was reduced by approximately 19~22% compared with transgenic plants (Fig. 7c,f), and the number of branches/plant of control plant was significantly increased by 62~68% (Fig. 7e,h). These results suggest that transgenic cotton expressing of *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 aphid 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 (REFs). 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 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 a wide range of aliphatic alcohols. Phylogenetic analysis shows a close phylogenetic relationship between *AsFAR* and *A. mellifera* FAR1, suggesting that *AsFAR* may have a similar role with *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 the 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 capability is not only reflected in oocyte quantity but also 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 previously reported that the oocyte quality, or developmental competence, was acquired during folliculogenesis as the oocyte grows, and during the period of oocyte maturation (Krisher, 2004). Therefore these results shows 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 (REF). However, the excessive spraying of chemical insecticides carries the risk of the emergence of pest resistance (Tabashnik *et al.*, 2008), 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 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 and was found to limit *A. suturalis* population growth and crop damage. Transgenic expression of *dsAsFAR* was high in petal, 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 was also found in transgenic *Arabidopsis* plants expressing dsRNA of *HaAK* (Liu *et al.*, 2015). Previous studies shown that *A. suturalis* prefer 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**

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**Author Contributions**

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

**References**


Teerawanichpan P, Robertson AJ, Qiu X. 2010b. A fatty acyl-CoA reductase highly expressed in the head of honey bee (Apis mellifera) involves biosynthesis of a wide range of aliphatic fatty


Supporting Information

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

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

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

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

**Table S1** Primers used in this study.

**Fig. 1** Structural domains and protein sequence alignment of 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, *A. mellifera* (GenBank accession no. ADJ56408); Hsap, *H. sapiens* (GenBank accession no. AAT42129); Dmel, *D. melanogaster* (GenBank accession no. NP_651652); Bmor, *B. 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) NADP-binding motif (blue double underline), the active site motif YXXXXK (red double underline) and a Sterile protein domain (thick black line).

**Fig. 2** Tissue- and stage-dependent transcription pattern analysis of *AsFAR* and the development of ovary and egg in *A. 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 1–2 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. The values are expressed as the 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).

**Fig. 3** Downregulation of *AsFAR* suppresses ovarian development. At the 0 days PE, females were microinjected with *dsAsFAR* or *dsGFP* (control), and *AsFAR* gene transcription level in ovary (a) and fat body (b) was determined at 5 and 10 days PI. The silencing effect of *AsFAR* was monitored in whole insect body (c) until 18 days PI.
to determine whether *AsFAR* was stably suppressed throughout the reproductive phase. *dsGFP* (d) or *dsAsFAR* (e) treatment ovaries were imaged at 10 days 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. Values are expressed as means ± SEM based on three independent biological replicates. Asterisks indicate statistical significance (* P < 0.05, ** P < 0.01 and *** P < 0.01 Student’s t-test).

**Fig. 4** Downregulation of *AsFAR* suppresses female fertility. At the 0 days PE, females were microinjected with *dsAsFAR* or *dsGFP* (control), and four reproductive parameters, including lifetime fecundity (a), egg hatch rate (b), adult longevity (c) and 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, a) stage IV (Preincubation period, b), and at 30 days (c), and imaged using a stereo microscope. Values are expressed as means ± SEM based on three independent biological replicates. Asterisks indicate statistical significance (* P < 0.05, ** P < 0.01 and *** P < 0.01 Student’s t-test).

**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 8 lines from T1 transgenic plant populations. M: DNA molecular weight marker (0.12-23.1 kb) (Roche, Germany). Numbers marked under the gel indicate corresponding lines. (c) RT-PCR analysis of *dsAsFAR* transcription
levels in young leaves. *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. The relative transcription *dsAsFAR* was highest in petal, anther and leaf. The experiments were repeated 3 times, each time with 3 technical replicates per line; error bars, means ± SEM.

**Fig. 7** Transgenic cotton plants exhibiting resistance to *A. 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), damage holes count (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. Asterisks indicate statistical significance (*P* < 0.05, **P** < 0.01 and ***P*** < 0.01 Student’s t-test). Scale bars, 1cm.

**Fig. 8** Resistance phenotypes in tissues of transgenic plants expressing *dsAsFAR* and control genes, following *A. 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 decline in yield and quality. Scale bars, 1cm.
Fig. 1

(a) the region for RNAi (injection)

NAD_binding_domain

Sterile

(b) the region used for RNAi in cotton

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<th>Amel_FAR1</th>
<th>Hsap_FAR1</th>
<th>Emel_FAR (Nat)</th>
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Fig.1
Fig. 2
Fig. 3

(a) AsFAR_O
(b) AsFAR_FB
(c) AsFAR_WB

Relative expression

Days post-injection

(d) Images of dsGFP and dsAsFAR

(e) Images of dsGFP and dsAsFAR

(f) Number of oocytes

(g) Dry weight of ovary (mg)
Fig. 6
Fig. 8
New Phytologist Supporting Information

Article title: A transgenic strategy for controlling plant bugs (*Adelphocoris suturalis*) through expression of double-stranded RNA (dsRNA) homologous to *Fatty acyl-CoA reductase* (*AsFAR*) in cotton

Authors: Jing Luo¹,², Sijia Liang¹, Jianying Li, Zhongping Xu, Lun Li, Bangqin Zhu², Zhe Li², Keith Lindsey³, Lizhen Chen¹, ², Shuangxia Jin¹, *, Chaoliang Lei², Xianlong Zhang¹

Article acceptance date: 07 May 2017

The following Supporting Information is available for this article:

**Fig. S1** Insect bioassay of T1 and T2 *AsFAR*-transgenic cotton plants in the Field condition.

**Fig. S2** The typical damage characters of *A. suturalis* on cotton plants.

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

**Fig. S4** Effect of on *AsFAR*-transgenic cotton plants *H. armigera* and aphid.

**Table S1** Primers used in this study.
**Fig. S1** Insect bioassay of T1 and T2 *AsFAR*-transgenic cotton plants in field conditions. (a-c) T1 generation transgenic cotton plants used for insect bioassays. (d-e) T2 generation transgenic cotton plants for insect bioassays.
Fig. S2 T Typical damage characteristics caused by *A. suturalis* on cotton plants. (a) Irregular holes on cotton leaves resulting from feeding. Cotton plant shoot-tips (b) buds (c) petal (d) anther (e) and boll (f) after *A. suturalis* infestation. (g) Control plants showing decreased height, excessive branching and clustered phenotype after *A. suturalis* feeding.
Fig. S3 Phylogeny of *A. suturalis* and other FARs. The Neighbor-joining algorithm analysis was computed using MEGA (v. 7.0) and the JTT model for amino acids, with confidence values at the edges derived from 1000 rapid bootstrap replicates. Sequence abbreviations correspond to species names as show above: Asut, *A. suturalis*; Tcas, *Tribolium castaneum*; Nves, *Nicrophorus vespilloides*; Agla, *Anoplophora glabripennis*; Amel, *Apis mellifera*; Hlab, *Habropoda laboriosa*; Lnig, *Lasius niger*; Cflo, *Camponotus floridanus*; Dmel, *Drosophila melanogaster*; Bole, *Bactrocera oleae*; Adar, *Anopheles darlingi*; Cqui, *Culex quinquefasciatus*; Bmor, *Bombyx mori*; Onub, *Ostrinia nubilalis*; Harm, *Helicoverpa armigera*; Hvir, *Heliothis virescens*; Hass,
Helicoverpa assulta; Hsap, Homo sapiens; Atha, Arabidopsis thaliana; Taes, Triticum aestivum; Egra, Euglena gracilis; Mmus, Mus musculus; Psol, Phenacoccus solenopsis; Lhes, Lygus hesperus; Clec, Cimex lectularius;Apis, Acyrthosiphon pisum; Dnox, Diuraphis noxia. Ghir, Gossypium hirsutum. The red triangles denote the A. suturalis Δ9-DES protein.
**Fig. S4** Effect of on AsFAR-transgenic cotton plants *H. armigera* and aphids. (a) Leaves of transgenic and control cotton plants were challenged with 3rd instar larvae of *H. armigera*. (b) Representative photograph of transgenic and control leaves infested with aphids. (c) 3rd instar larvae of *H. armigera* feeding on leaves of control and transgenic cotton for 96 h; weights were recorded at day 1 and 4, respectively. (d) Aphids were released on transgenic and control plants (30 per plant) and the population was recorded at day 7 and 15. Data shown as means ± SEM; n = 3. Statistical analyses were performed using Student’s *t* test. No significant difference in aphid population and *H. armigera* weight was observed between control and transgenic cotton plants (P >0.05).

**Table S1** Primers used in this study.

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For qRT-PCR in insect

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For exogenous dsRNA synthesis (injection)

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For RT and qRT-PCR in cotton

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