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

Jing Luo[1,2,§], Sijia Liang[1§], Jianying Li, Zhongping Xu, Lun Li, Bangqin Zhu[2], Zhe Li[2], Chaoliang Lei[2], Keith Lindsey[3], Lizhen Chen[1,2]*, Shuangxia Jin[1]*, Xianlong Zhang[1]

[1] National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, 430070, China.

[2] Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, Huazhong Agricultural University, Wuhan, 430070, China.

[3] Department of Biosciences, Durham University, United Kingdom

[§] These authors contributed equally to this work

*Corresponding author: lzchen@mail.hzau.edu.cn or jsx@mail.hzau.edu.cn

Tel: 027-87283955

Fax: 027-8728001

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*is 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 expessing *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 16:8 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 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 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 $2^{-\Delta\Delta Ct}$ 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

176 *et al.*, 2002). Purified PCR products were inserted into pHellsgate4 by BP
177 recombination to generate the *AsFRA* RNAi vector according to the manufacturer's
178 recommendations. The expression construct was used to transform cotton by
179 *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation as described
180 previously (Jin *et al.*, 2005; Jin *et al.*, 2012).
181

182 ## Molecular analysis for the transgenic cotton plants

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

193 For transcription analysis of *dsAsFAR*, total RNA was extracted from leaves of
194 T1 positive transgenic and null plants using the modified guanidine thiocyanate
195 method as previously described (Liu *et al.*, 2006), and 3 μg of total RNA was
196 reverse-transcribed to cDNA with SuperScript III reverse transcriptase (Invitrogen,
197 Carlsbad, CA, USA). cDNA was used to determine the *AsFAR* gene expression level
198 via RT-PCR, and qRT-PCR using the ABI Prism 7500 system (Applied Biosystems,
199 Foster City, CA, USA). Expression of *dsAsFAR* in different transgenic cotton tissues
200 was determined by qRT-PCR. The *UBQ7* gene (GenBank accession no. DQ116441.1)
201 was selected as an internal control to normalize target genes expression values. Three
202 technical replicates and three independent biological replicates were performed for
203 each experiment. Primers are listed in Supporting Information Table 1.
204

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 linesconfirmed 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 wwere 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

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

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

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

430 High reproductive capability is not only reflected in oocyte quantity but also
431 quality. Oocyte quality or developmental competence affects embryonic development
432 and the health of the offspring (Eppig & O'Brien, 1998). Our results showed that the
433 down-regulation of *AsFAR* expression led to a lower egg hatchability, and embryo
434 development was blocked by *AsFAR* depletion at the primary stage. This result is
435 consistent with the tissue distribution and temporal expression pattern of *AsFAR*,
436 which was found to be highly expressed in ovary and increased during ovary
437 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 produciton 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 the grands of National Special Key Project for Transgenic Breeding（2016ZX08011002-006 and 2016ZX08010001-006）and Open funding from the State Key Laboratory for Biology of Plant Diseases and Insect Pests (grant SKLOF201415)

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

Biester EM, Hellenbrand J, Gruber J, Hamberg M, Frentzen M. 2012. Identification of avian wax synthases. *BMC Biochem.* **13**: 4.

Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FSB, Olsvik PA, Penning LC, Toegel S. 2010. MIQE précis: practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology* **11**(1): 1-5.

Chen PY, Feng HQ, Li GP, Guo XR. 2010. Morphology of diapause and non-diapause eggs of *Adelphocoris suturalis*. *Plant Protection* **36**(2): 80-82.

Cheng JB, Russell DW. 2004. Mammalian wax biosynthesis. I. Identification of two fatty acyl-Coenzyme A reductases with different substrate specificities and tissue distributions.

Journal of Biological Chemmistry **279**(36): 37789-37797.

Eppig JJ, O'Brien MJ. 1998. Comparison of preimplantation developmental competence after mouse oocyte growth and development in vitro and in vivo. *Theriogenology* **49**(2): 415-422.

Helliwell CA, Wesley SV, Wieloplska AJ, Waterhouse PM. 2002. High-throughput vectors for efficient gene silencing in plants. *Function Plant Biology* **29**: 1217-1225.

Honsho M, Asaoku S, Fujiki Y. 2010. Posttranslational regulation of fatty acyl-CoA reductase 1, Far1, controls ether glycerophospholipid synthesis. *Journal of Biological Chemmistry* **285**(12): 8537-8542.

Hughes CL, Kaufman TC. 2000. RNAi analysis of Deformed, *proboscipediaand Sex combs* reducedin themilkweed bug *Oncopeltus fasciatus*: novel roles for Hox genes in the Hemipteran head. *Development* **127**: 3683-3694.

James C. 2015. 20th Anniversary (1996-2015) of the Global Commercialization of Biotech Crops and Biotech Crop Highlights in 2015. *ISAAA Brief* **NO. 51**.

Jiang YY, Lu YH, Zeng j 2015. *Forecast and management of mirid bugs in multiplie agroecosystems of China*. Beijing: China Agriculture Press.

Jin SX, Liu GZ, Zhu HG, Yang XY, Zhang XL. 2012. Transformation of Upland Cotton (*Gossypium hirsutum* L.) with *gfp* Gene as a Visual Marker. *Journal of Integrative Agriculture* **11**(6): 910-919.

Jin SX, Singh ND, Li L, Zhang XL, Daniell H. 2015. Engineered chloroplast dsRNA silences *cytochrome p450 monooxygenase*, *V-ATPase* and *chitin synthase* genes in the insect gut and disrupts *Helicoverpa armigera* larval development and pupation. *Plant Biotechnology Journal* **13**(3): 435-446.

Jin SX, Zhang XL, liang SG, Nie YC, Guo XP, Huang C. 2005. Factors affecting transformation efficiency of embryogenic callus of Upland cotton (*Gossypium hirsutum*) with *Agrobacterium tumefaciens*. *Plant Cell Tissue and Organ Culture* **81**(2): 229-237.

Krisher RL. 2004. The effect of oocyte quality on development. *Journal of Animal Science* **82**: E14-E23.

Lassance JM, Liénard MA, Antony B, Qian S, Fujii T, Tabata J, Ishikawa Y, Löfstedt C. 2013. Functional consequences of sequence variation in the pheromone biosynthetic gene pgFAR for Ostrinia moths. *Proceedings of the National Academy of Sciences, USA* **110**(10): 3967-3972.

Liénard MA, Hagström ÅK, Lassance JM, Löfstedt C. 2010. Evolution of multicomponent pheromone signals in small ermine moths involves a single fatty-acyl reductase gene. *Proceedings of the National Academy of Sciences, USA* **107**(24): 10955-10960.

Liu D, Zhang X, Tu L, Zhu L, Guo X. 2006. Isolation by suppression-subtractive hybridization of genes preferentially expressed during early and late fiber development stages in cotton. *Molecular Biology* **40**(5): 741-749.

Liu F, Wang XD, Zhao YY, Li YJ, Liu YC, Sun J. 2015. Silencing the *HaAK* Gene by Transgenic Plant-Mediated RNAi Impairs Larval Growth of *Helicoverpa armigera*. *International Journal of Biological Sciences* **11**(1): 67-74.

Liu W, Li Y, Zhu L, Zhu F, Lei CL, Wang XP. 2016. Juvenile hormone facilitates the antagonism between adult reproduction and diapause through the methoprene-tolerant gene in the female *Colaphellus bowringi*. *Insect Biochemistry and Molecular Biology* **74**: 50-60.

Lu YH, Qiu F, Feng HQ, Li HB, Yang ZC, Wyckhuys KAG, Wu KM. 2008a. Species composition and seasonal abundance of pestiferous plant bugs (Hemiptera: Miridae) on Bt Cotton in China. *Crop Protection* **27**(3-5): 465-472.

Lu YH, Wu KM 2008b. In: Lu YH, Wu KM eds. *Cotton Mirid Bugs And Its Prevention.* Beijing: Golden Shield Press, 18-19.

Lu YH, Wu KM, Cai XM, Liu YQ. 2008c. A rearing method for mirids using the green bean, *Phaseolus vulgaris* in the laboratory. *Acta Phytophysiologica Sinica* **35**: 251-269.

Lu YH, Wu KM, Jiang YY, Xia B, Li P, Feng HQ, Wyckhuys KAG, Guo YY. 2010. Mirid bug outbreaks in multiple crops correlated with wide-scale adoption of Bt cotton in China. *Science* **328**(5982): 1151-1154.

Luo J, Liu XY, Liu L, Zhang PY, Chen LJ, Gao Q, Ma WH, Chen LH, Lei CL. 2014. De novo analysis of the *Adelphocoris suturalis* Jakovlev metathoracic scent glands transcriptome and expression patterns of pheromone biosynthesis-related genes. *Gene* **551**(2): 271-278.

Luo LZ, Li GB. 1993. Effects of temperature on oviposition and longevity of adult meadow moth (*Loxostege sticticalis L.*). *Acta Entomologica Sinica* **36**: 459-464.

Mallapur CP, Sugandi R, Balikai RA. 2015. Mirid bug, Poppiocapsidea (=Creontiodes) biseratence (Distant) (Miridae: Hemiptera) - bio-ecology and management in BT cotton. *Journal of Experimental Zoology India* **18**(1): 1-10.

Mao YB, Cai WJ, Wang JW, Hong GJ, Tao XY, Wang LJ, Huang YP, Chen XY. 2007. Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nature Biotechnology* **25**(11): 1307-1313.

Moto K, Yoshiga T, Yamamoto M, Takahashi S, Okano K, Ando T, Nakata T, Matsumoto S. 2003. Pheromone gland-specific fatty-acyl reductase of the silkmoth, *Bombyx mori*. *Proceedings of the National Academy of Sciences, USA* **100**(16): 9156-9161.

Musser FR, Catchot AL, Stewart SD, Bagwell RD, Lorenz GM, Tindall KV, Studebaker GE, Rogers Leonard B, Scott Akin D, Cook DR, et al. 2009. Tarnished Plant Bug (Hemiptera: Miridae) Thresholds and Sampling Comparisons for Flowering Cotton in the Midsouthern United States. *Journal of Economic Entomology* **102**(5): 1827.

Pitino M, Coleman AD, Maffei ME, Ridout CJ, Hogenhout SA. 2011. Silencing of Aphid Genes by dsRNA Feeding from Plants. *PLoS One* **6**(10): e25709.

Pollard MR, McKeon T, Gupta LM, Stumpf PK. 1979. Studies on biosynthesis of waxes by developing jojoba seed. II. the demonstration of wax biosynthesis by cell-free homogenates. *Lipids* **14**(7): 651-662.

Reading CJ. 1986. Egg production in the Common toad, *Bufo bufo*. *Journal of Zoology* **208**(1): 99-107.

Rowland O, Zheng H, Hepworth SR, Lam P, Jetter R, Kunst L. 2006. CER4 encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis. *Plant physiology* **142**(3): 866-877.

Saastamoinen M. 2007. Mobility and lifetime fecundity in new versus old populations of the Glanville fritillary butterfly. *Oecologia* **153**(3): 569-578.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols* **3**(6): 1101-1108.

Tabashnik BE, Gassmann AJ, Crowder DW, Carriere Y. 2008. Insect resistance to *Bt* crops: evidence versus theory. *Nature Biotechnology* **26**(2): 199-202.

Teerawanichpan P, Qiu X. 2010a. Fatty Acyl-CoA Reductase and Wax Synthase from *Euglena gracilis* in the Biosynthesis of Medium-Chain Wax Esters. *Lipids* **45**(3): 263-273.

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

alcohols. *Insect Biochemistry and Molecular Biology* **40**(9): 641-649.

Thakur N, Upadhyay SK, Verma PC, Chandrashekar K, Tuli R, Singh PK. 2014. Enhanced Whitefly Resistance in Transgenic Tobacco Plants Expressing Double Stranded RNA of *v-ATPase* A Gene. *PLoS One* **9**(3): e87235.

Tian G, Cheng L, Qi X, Ge Z, Niu C, Zhang X, Jin S. 2015. Transgenic Cotton Plants Expressing Double-stranded RNAs Target HMG-CoA Reductase (HMGR) Gene Inhibits the Growth, Development and Survival of Cotton Bollworms. *International Journal of Biological Sciences* **11**(11): 1296-1305.

Wu KM, Lu YH, Feng HQ, Jiang YY, Zhao JZ. 2008. Suppression of cotton bollworm in multiple crops in China in areas with Bt toxin-containing cotton. *Science* **321**(5896): 1676-1678.

Wuriyanghan H, Falk BW. 2013. RNA Interference towards the Potato Psyllid, *Bactericera cockerelli*, Is Induced in Plants Infected with Recombinant *Tobacco mosaic virus* (TMV). *PLoS One* **8**(6): e66050.

Yuan W, Li WJ, Li YH, Wu KM. 2013. Combination of Plant and Insect Eggs as Food Sources Facilitates Ovarian Development in an Omnivorous Bug *Apolygus lucorum* (Hemiptera: Miridae). *Journal of Economic Entomology* **106**(3): 1200-1208.

Zhang L, Luo LZ, jiang XF, Hu Y. 2006. Influences of starvation on the first day after emergence on ovarian development and flight potential in adults of the oriental armyworm, *Mythimna separata* (Walker) (Lepidopterea: Noctuidae). *Acta Entomologica Sinica* **49**(6): 895-902.

Zhang WN, Liu B, Liang GM, Lu YH. 2016. Flowers promote ovarian development and vitellogenin gene expression in *Apolygus lucorum* (Heteroptera: Miridae). *Arthropod Plant Interactions* **10**(2): 113-119.

Zhang ZL, Luo J, Lu C, Zhao B, Meng J, Chen LZ, Lei CL. 2011. Evidence of female-produced sex pheromone of *Adelphocoris suturalis* (Hemiptera: Miridae): effect of age and time of day. *Journal of Economic Entomology* **104**(4): 1189-1194.

Zhu JQ, Liu SM, Ma Y, Zhang JQ, Qi HS, Wei ZJ, Yao Q, Zhang WQ, Li S. 2012. Improvement of Pest Resistance in Transgenic Tobacco Plants Expressing dsRNA of an Insect-Associated Gene *EcR*. *PLoS One* **7**(6): e38572.

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

625 eukaryotic organisms: Amel, *A. mellifera* (GenBank accession no. ADJ56408); Hsap,
626 *H. sapiens* (GenBank accession no. AAT42129); Dmel, *D. melanogaster* (GenBank
627 accession no. NP_651652); Bmor, *B. mori* (GenBank accession no. BAC79426); Atha,
628 *Arabidopsis thaliana* (GenBank accession no. NP567936). Identical amino acid
629 residues and conservative substitutions are indicated in black or gray, respectively.
630 The FAR structural elements include an N-terminal Rossmann-fold NAD-binding
631 domain (black box), the GXXGXX(G/A) NADPH-binding motif (blue double
632 underline), the active site motif YXXXK (red double underline) and a Sterile protein
633 domain (thick black line).
634
635 **Fig. 2** Tissue- and stage-dependent transcription pattern analysis of *AsFAR* and the
636 development of ovary and egg in *A. suturalis*. Ovary development of *A. suturalis* was
637 classified into five stages, namely Stage I (follicles at the stage of previtellogenesis,
638 no deposition of yolk protein; a and b); stage II (start of vitellogenesis, follicles have
639 some yolk protein deposition, no mature chorionated follicles; c); stage III (start of
640 oogenesis, large amount of yolk protein deposited in follicles and mature chorionated
641 eggs present; d); stage IV (presence of mature eggs, each ovariole contains at least 1–
642 2 mature eggs; e); and stage V (ovarioles begin to shrink, few mature follicles
643 observed; f). Egg development of *A. suturalis* was classified into four stages, namely
644 stage I (newly produced eggs; g); stage II (pale yellow eggs; h); stage III (red
645 compound eye period; i); and stage IV (Preincubation period; j). The tissue
646 distribution (k) and temporal analysis (l) of *AsFAR* transcripts were monitored by
647 qRT-PCR. The values are expressed as the means ± SEM based on three independent
648 biological replicates. Different letters shows significant differences ($P < 0.05$,
649 one-way ANOVA followed by Tukey's HSD Multiple Comparison).
650
651 **Fig. 3** Downregulation of *AsFAR* suppresses ovarian development. At the 0 days PE,
652 females were microinjected with *dsAsFAR* or *dsGFP* (control), and *AsFAR* gene
653 transcription level in ovary (a) and fat body (b) was determined at 5 and 10 days PI.
654 The silencing effect of *AsFAR* was monitored in whole insect body (c) until 18 days PI

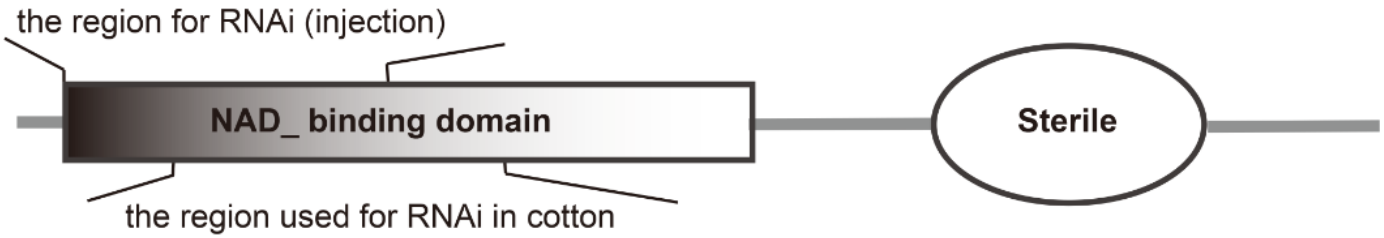
655 to determine whether *AsFAR* was stably suppressed throughout the reproductive phase.
656 *dsGFP* (d) or *dsAsFAR* (e) treatment ovaries were imaged at 10 days PI using a stereo
657 microscope. Oocyte numbers per ovary pair were counted (f) and the dry weight of
658 single ovary pairs was estimated (g) to quantify ovarian development. Values are
659 expressed as means ± SEM based on three independent biological replicates. Values
660 are expressed as means ± SEM based on three independent biological replicates.
661 Asterisks indicate statistical significance (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.01$
662 Student's t-test).
663
664 **Fig. 4** Downregulation of *AsFAR* suppresses female fertility. At the 0 days PE,
665 females were microinjected with *dsAsFAR* or *dsGFP* (control), and four reproductive
666 parameters, including lifetime fecundity (a), egg hatch rate (b), adult longevity (c) and
667 POP (d), were used to evaluate changes in female fertility in response to *dsAsFAR* and
668 *dsGFP* treatments. The development of eggs was observed at stage I (newly produced
669 eggs, a) stage IV (Preincubation period, b), and at 30 days (c), and imaged using a
670 stereo microscope. Values are expressed as means ± SEM based on three independent
671 biological replicates. Asterisks indicate statistical significance (* $P < 0.05$, ** $P < 0.01$
672 and *** $P < 0.01$ Student's t-test).
673
674 **Fig. 5** The plasmid vector, genetic transformation and plant regeneration of cotton. (a)
675 *AsFAR* gene expression cassette (T-DNA region) used for *Agrobacterium*-mediated
676 transformation. (b, c) Callus induction on selective media containing kanamycin. (d)
677 Somatic embryogenesis (e, f) Plant regeneration (g, h) Regenerated plants transferred
678 to the soil.
679
680 **Fig. 6** Molecular analysis of transgenic cotton plants. (a) PCR analysis of putative
681 transgenic cotton lines. M: Marker; P: Positive control; N: Negative control. (b)
682 Southern blot analysis of 8 lines from T1 transgenic plant populations. M: DNA
683 molecular weight marker (0.12-23.1 kb) (Roche, Germany). Numbers marked under
684 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.

(a)


the region for RNAi (injection)
NAD_ binding domain
Sterile
the region used for RNAi in cotton

(b)

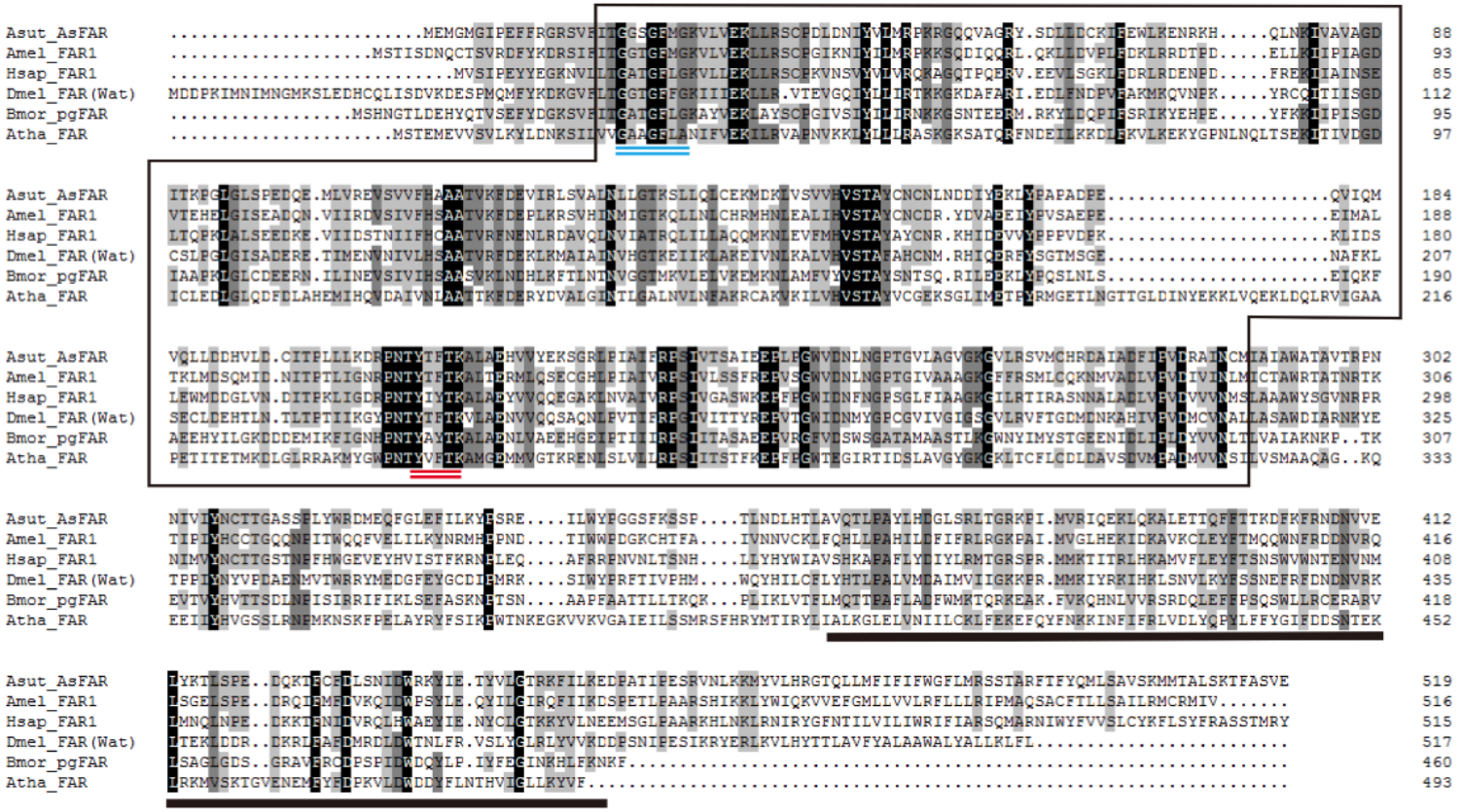

Asut_AsFAR
Amel_FAR1
Hsap_FAR1
Dmel_FAR(Wat)
Bmor_pgFAR
Atha_FAR

Fig.1

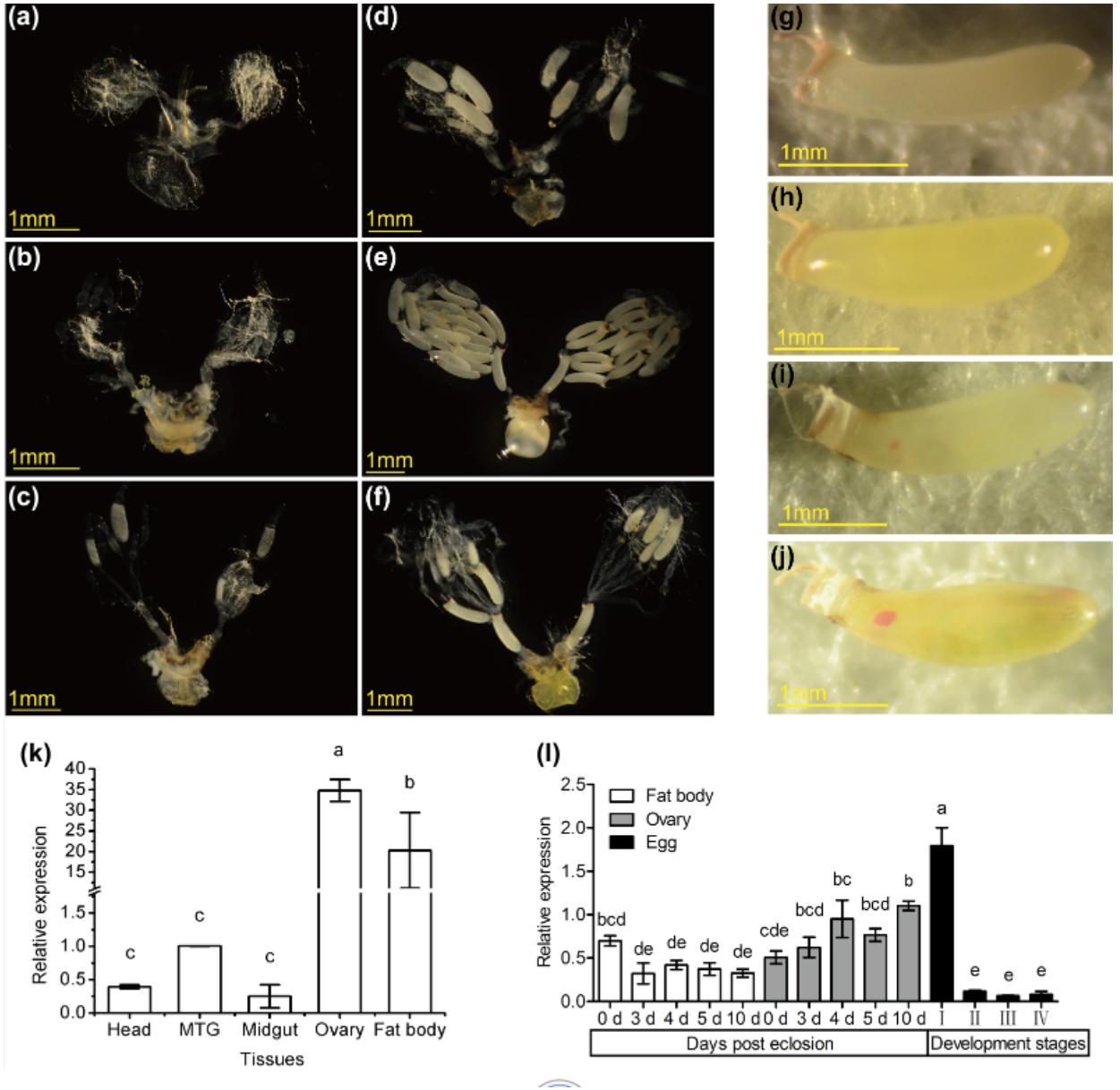

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
1mm
(k)
Relative expression
Head
MTG
Midgut
Ovary
Fat body
Tissues
(l)
Fat body
Ovary
Egg
Relative expression
0 d 3 d 4 d 5 d 10 d 0 d 3 d 4 d 5 d 10 d I II III IV
Days post eclosion
Development stages

Fig.2

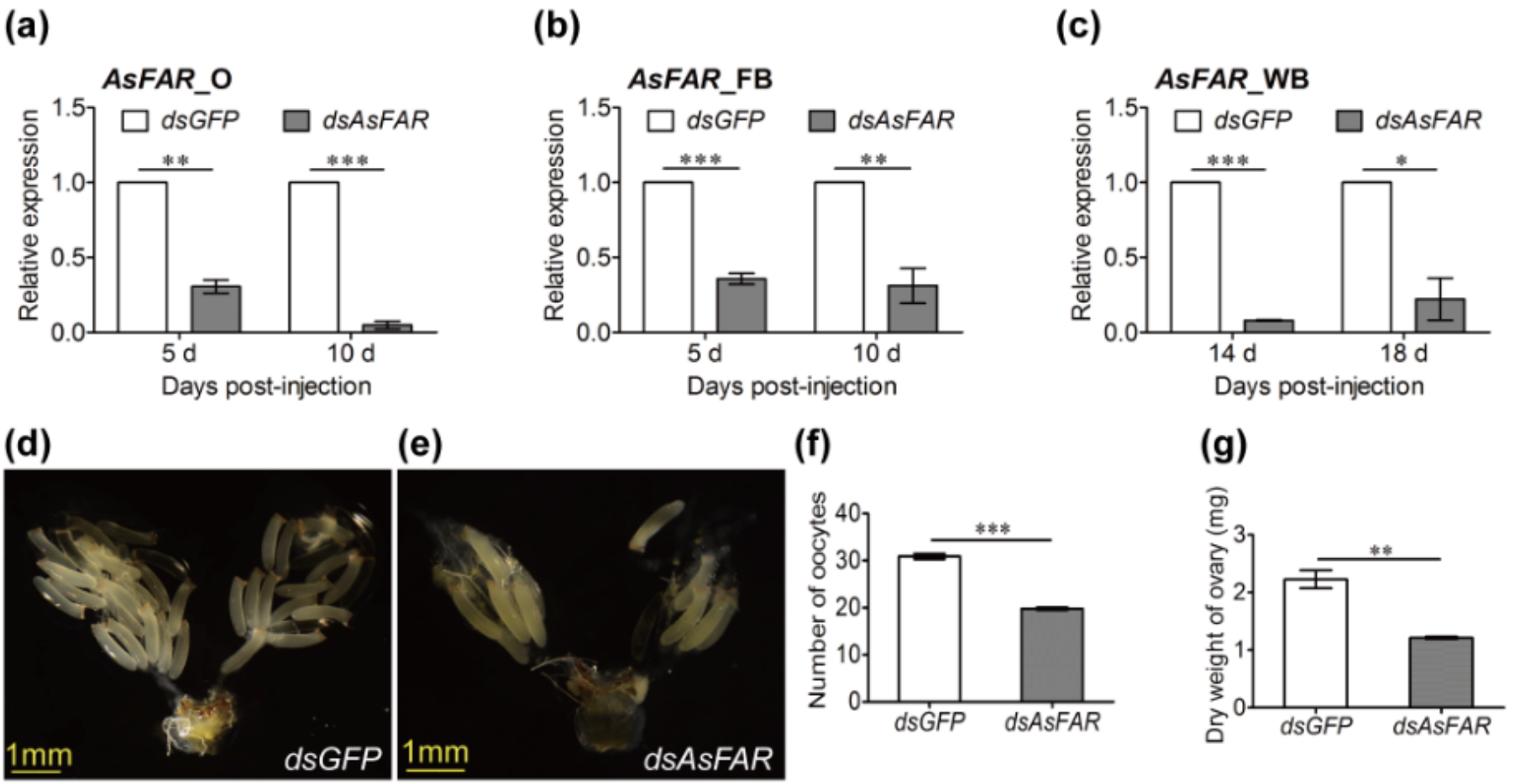

(a)
AsFAR_O
dsGFP
dsAsFAR
Relative expression
1.5
1.0
0.5
0.0
**
***
5 d
10 d
Days post-injection
(b)
AsFAR_FB
dsGFP
dsAsFAR
Relative expression
1.5
1.0
0.5
0.0
***
**
5 d
10 d
Days post-injection
(c)
AsFAR_WB
dsGFP
dsAsFAR
Relative expression
1.5
1.0
0.5
0.0
***
*
14 d
18 d
Days post-injection
(d)
1mm
dsGFP
(e)
1mm
dsAsFAR
(f)
Number of oocytes
40
30
20
10
0
***
dsGFP
dsAsFAR
(g)
Dry weight of ovary (mg)
3
2
1
0
**
dsGFP
dsAsFAR

Fig.3

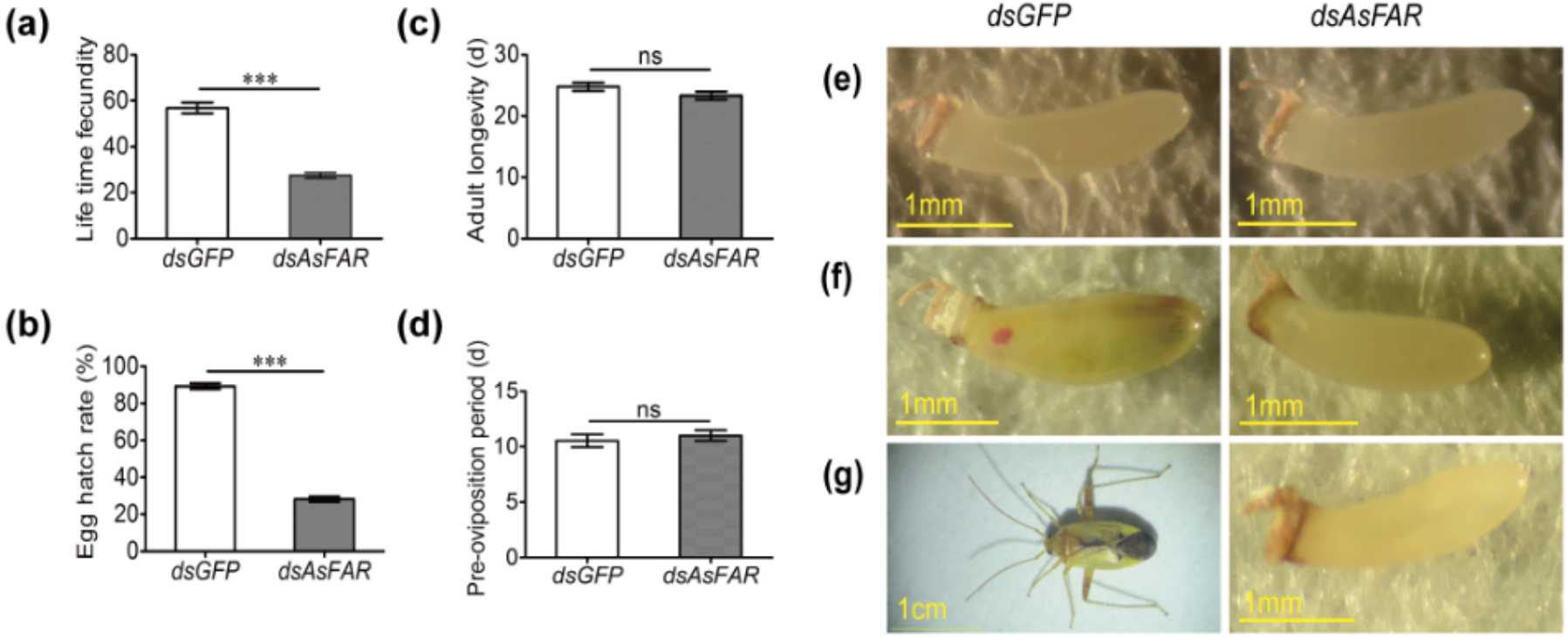

(a)
Life time fecundity
80
60
40
20
0
***
dsGFP
dsAsFAR
(b)
Egg hatch rate (%)
100
80
60
40
20
0
***
dsGFP
dsAsFAR
(c)
Adult longevity (d)
30
20
10
0
ns
dsGFP
dsAsFAR
(d)
Pre-oviposition period (d)
15
10
5
0
ns
dsGFP
dsAsFAR
dsGFP
dsAsFAR
(e)
1mm
1mm
(f)
1mm
1mm
(g)
1cm
1mm

Fig.4

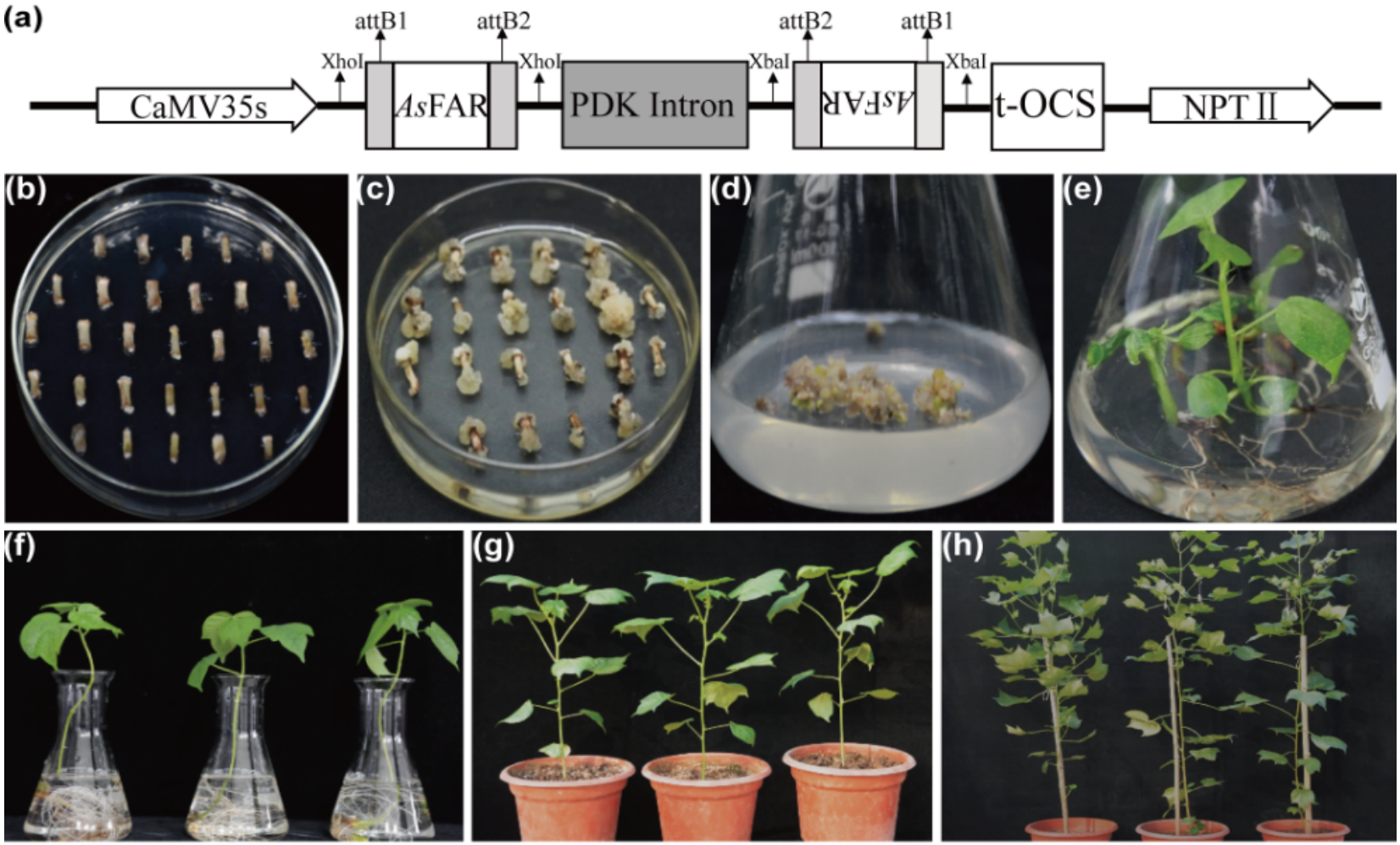

(a)
attB1
attB2
attB2
attB1
XhoI
XhoI
XbaI
XbaI
CaMV35s
AsFAR
PDK Intron
AsFAR
t-OCS
NPT II
(b)
(c)
(d)
(e)
(f)
(g)
(h)

Fig.5

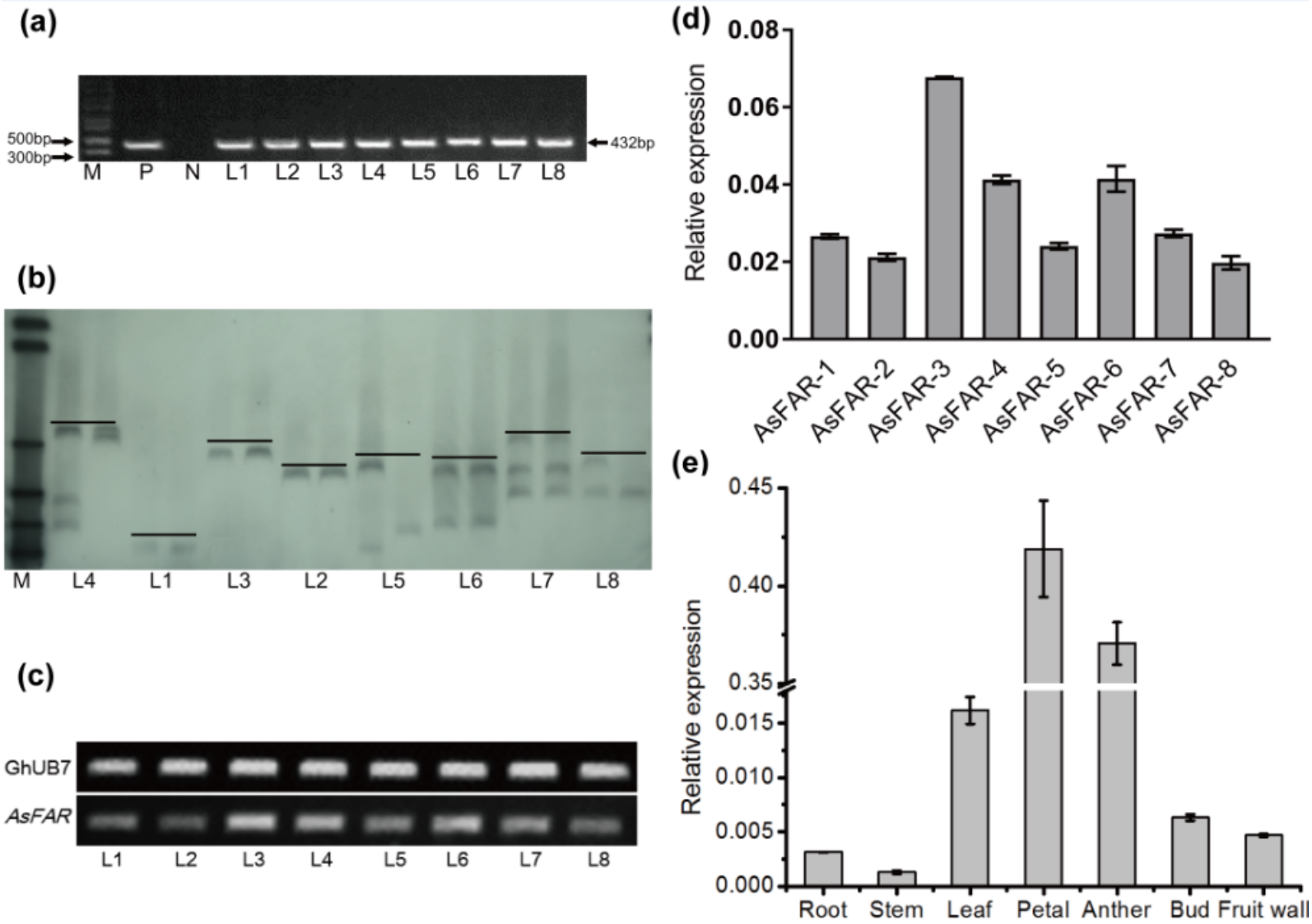

(a)
500bp
300bp
432bp
M P N L1 L2 L3 L4 L5 L6 L7 L8
(b)
M L4 L1 L3 L2 L5 L6 L7 L8
(c)
GhUB7
AsFAR
L1 L2 L3 L4 L5 L6 L7 L8
(d)
Relative expression
0.08
0.06
0.04
0.02
0.00
AsFAR-1 AsFAR-2 AsFAR-3 AsFAR-4 AsFAR-5 AsFAR-6 AsFAR-7 AsFAR-8
(e)
Relative expression
0.45
0.40
0.35
0.015
0.010
0.005
0.000
Root Stem Leaf Petal Anther Bud Fruit wall

Fig.6

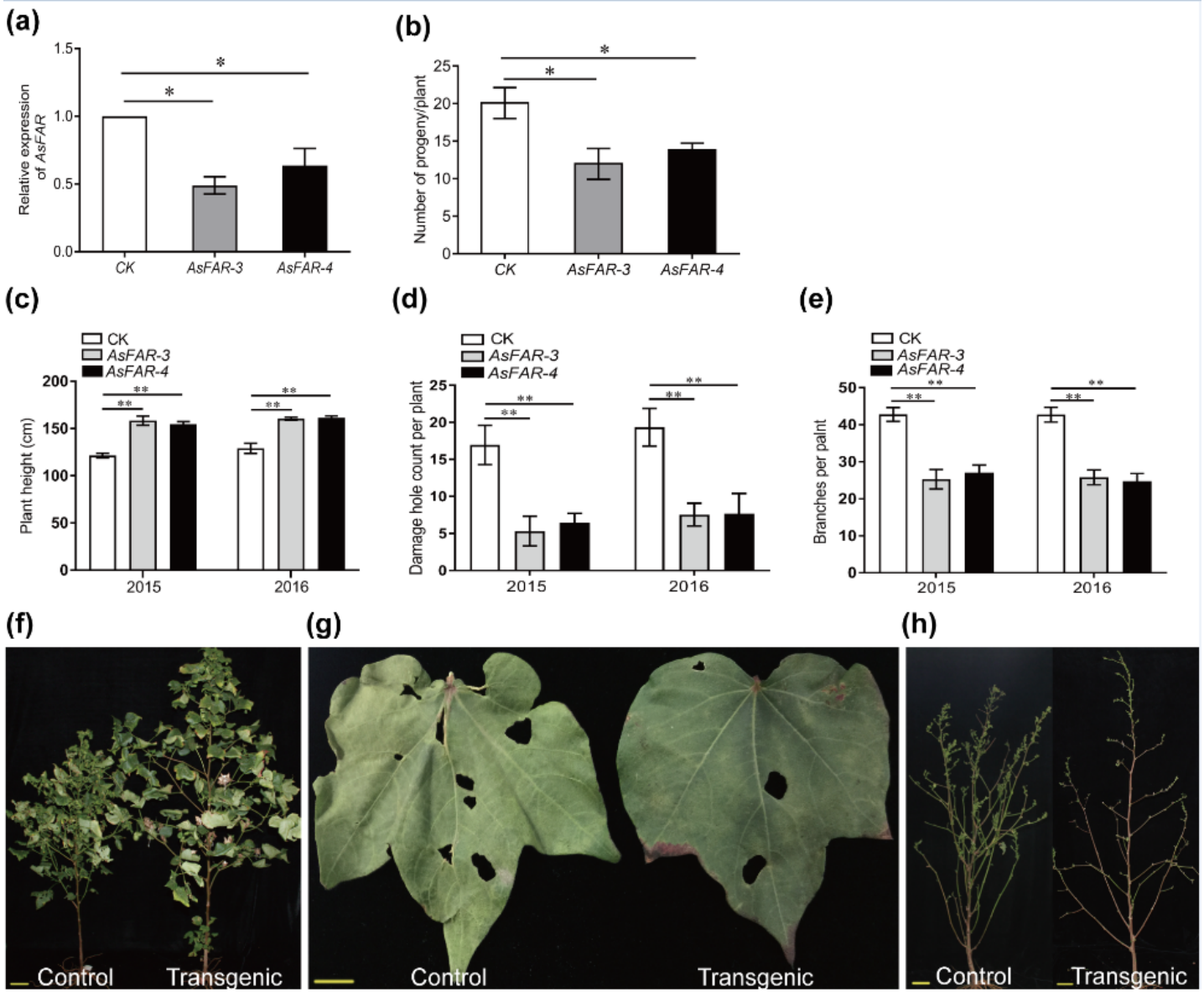

(a)
Relative expression of AsFAR
1.5
1.0
0.5
0.0
*
*
CK
AsFAR-3
AsFAR-4
(b)
Number of progeny/plant
25
20
15
10
5
0
*
*
CK
AsFAR-3
AsFAR-4
(c)
CK
AsFAR-3
AsFAR-4
Plant height (cm)
200
150
100
50
0
**
**
**
**
2015
2016
(d)
CK
AsFAR-3
AsFAR-4
Damage hole count per plant
25
20
15
10
5
0
**
**
**
**
2015
2016
(e)
CK
AsFAR-3
AsFAR-4
Branches per palnt
50
40
30
20
10
0
**
**
**
**
2015
2016
(f)
Control
Transgenic
(g)
Control
Transgenic
(h)
Control
Transgenic

Fig.7

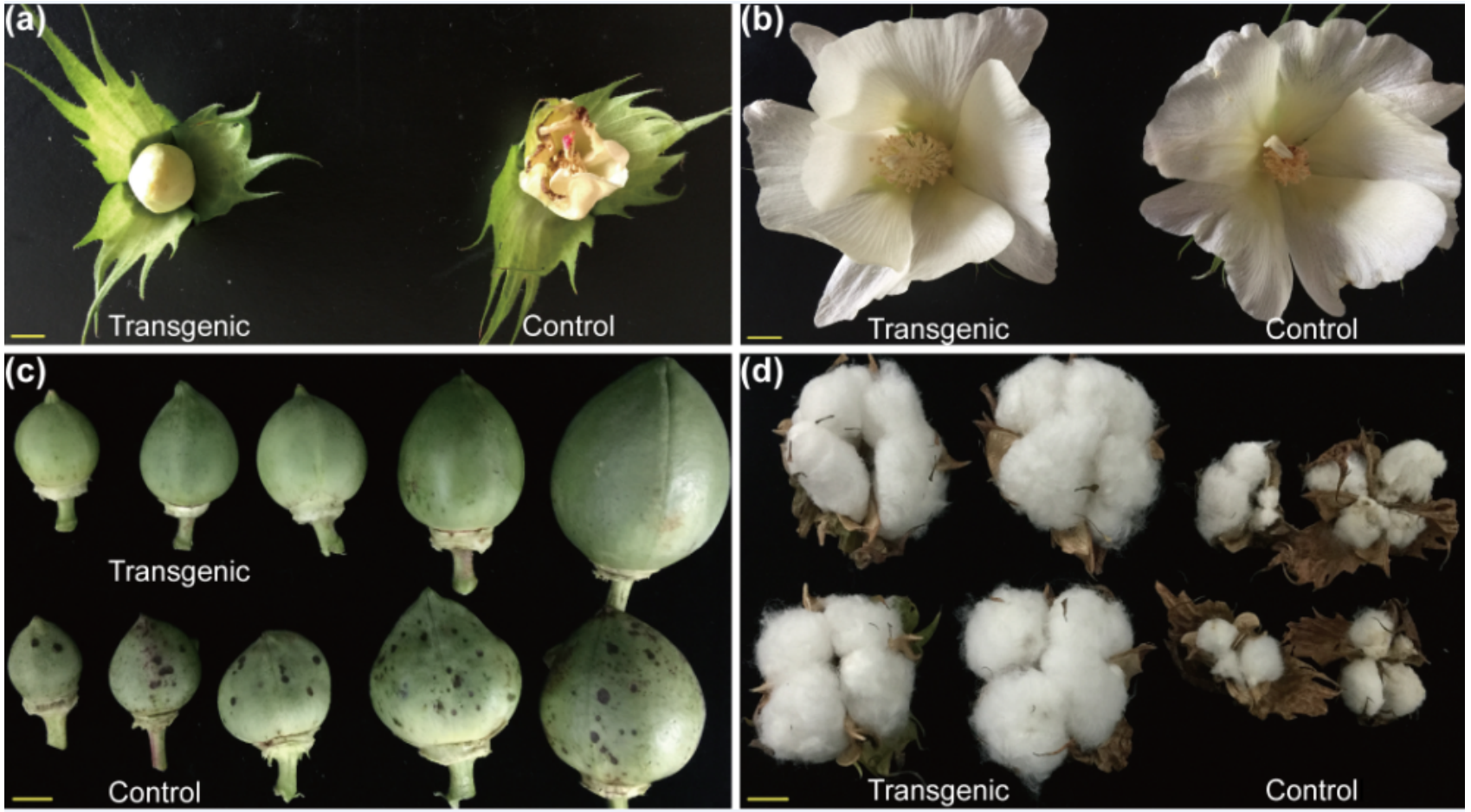

(a)
Transgenic
Control
(b)
Transgenic
Control
(c)
Transgenic
Control
(d)
Transgenic
Control

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 Luo[1,2,§], Sijia Liang[1§], Jianying Li, Zhongping Xu, Lun Li, Bangqin Zhu[2], Zhe Li[2], Keith Lindsey[3], Lizhen Chen[1,2,*], Shuangxia Jin[1]*, Chaoliang Lei[2], Xianlong Zhang[1]

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.

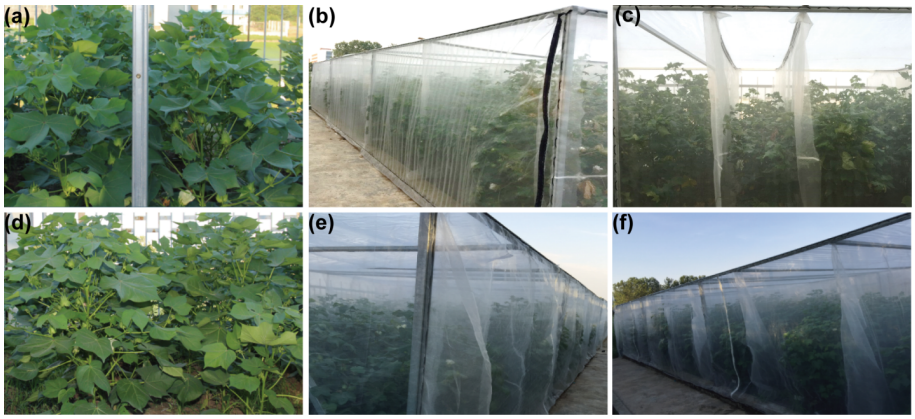


Fig. S1 Insect bioassay of T1 and T2 *AsFAR*-transgenic cotton plants in field conditions. (a-c) T1 generation transgenic cotton plants used for insect bioassays. (d-e) T2 generation transgenic cotton plants for insect bioassays.

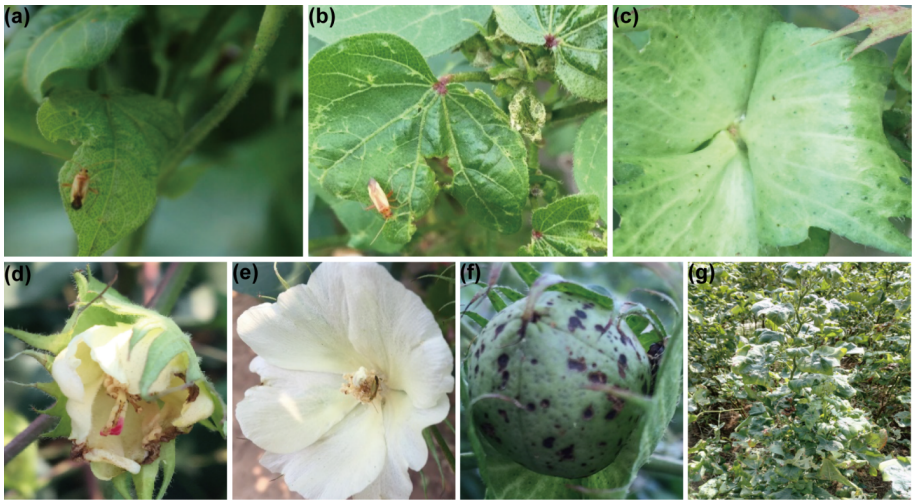


Fig. S2 T Typical damage characteristics caused by *A. suturalis* on cotton plants. (a) Irregular holes on cotton leaves resulting from feeding. Cotton plant shoot-tips (b) buds (c) petal (d) anther (e) and boll (f) after *A. suturalis* infestation. (g) Control plants showing decreased height, excessive branching and clustered phynotype after *A. suturalis* feeding.

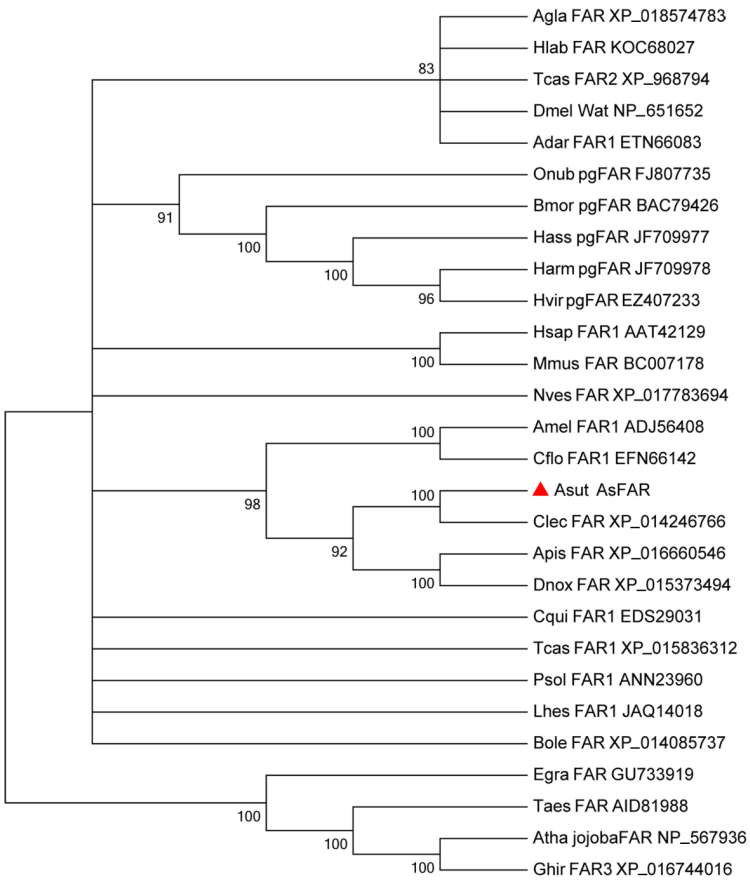


Fig. S3 Phylogeny of *A. suturalis* and other FARs. The Neighbor-joining algorithm analysis was computed using MEGA (v. 7.0) and the JTT model for amino acids, with confidence values at the edges derived from 1000 rapid bootstrap replicates. Sequence abbreviations correspond to species names as show 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.

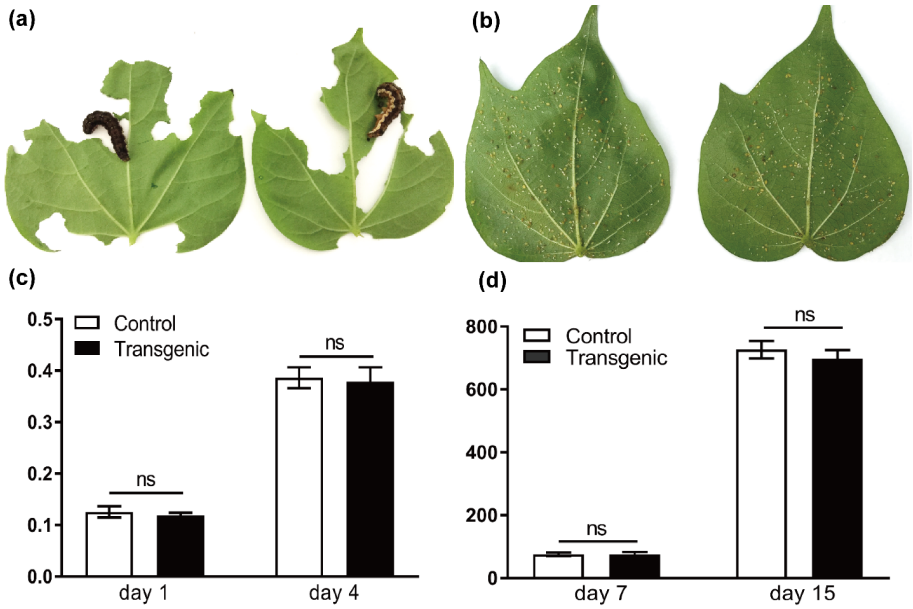


Fig. S4 Effect of on *AsFAR*-transgenic cotton plants *H. armigera* and aphids. (a) Leaves of transgenic and control cotton plants were challenged with 3rd instar larvae of *H. armigera.* (b) Representative photograph of transgenic and control leaves infested with aphids. (c) 3rd instar larvae of *H. 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 ($P > 0.05$).

Comment [Office1]: is this correct?

Table S1 Primers used in this study.

Primers	**Primer sequence**
For *AsFAR* ORF cloning	
AsFAR-F	GCTCGTGCCAAACCAGTCAG
AsFAR-R	ATCCACGTGGTTGGTGCTTG

For qRT-PCR in insect	
Q-*AsFAR*-F	CTGGATGGGTAGACAACCTCAACG
Q-*AsFAR*-R	TCGCGTCTCTATGGCACATCACAG
Q-*RPS15*-F	GCGCCTCCAAATGAAAAGCCCG
Q-*RPS15*-R	GGGCCTTCCGTGTTTGACAGGT
Q-*EF1γ*-F	GTTGGCCCTTGCTGCAGAACC
Q-*EF1γ*-R	TGAAATCATCCATCACCCAGGACCC
For exogenous dsRNA synthesis (injection)	
dsAsFAR-F	gcgtaatacgactcactatagg (T7 promoter) CAGGAGGCTCGGGGTTTATG
dsAsFAR-R	gcgtaatacgactcactatagg (T7 promoter) TATGCGGTGGAGACGTGAAC
dsGFP-F	gcgtaatacgactcactatagg (T7 promoter) TGGTCCCAATTCTCGTGGAAC
dsGFP-R	gcgtaatacgactcactatagg (T7 promoter) CTTGAAGTTGACCTTGATGCC
For RNAi vector construction (cotton)	
dsAsFAR-F	ggggacaagtttgtacaaaaaagcaggctca CGAATGGTTGAAAGAAAATAGG
dsAsFAR-R	ggggaccactttgtacaagaaagctgggta TTGGTGAAAGTGTAGGTGTTGG
For RT and qRT-PCR in cotton	
Q-*AsFAR*-F	CATCAACTGAACAAAATCGTGG
Q-*AsFAR*-R	TATGCGGTGGAGACGTGAAC