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Transgenic tobacco expressing dsRNA of the arginine kinase gene exhibits enhanced resistance against *Helicoverpa armigera*

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Abstract

Helicoverpa armigera Hubner (cotton bollworm) affects commercial value crops and can cause major economic losses. The wide application of insecticides and *Bacillus thuringiensis* cotton (*Bt*-cotton) has led to the enhanced resistance of cotton bollworm along with insecticide pollution that affects food production and the environment. Transgenic plants that produce double-stranded RNA (dsRNA) to target insect genes are being developed as a pest control strategy. In this study, we used Agrobacterium-mediated transformation to produce transgenic tobacco plants expressing dsRNA to silence the *H. armigera* arginine kinase (*HaAK*) gene. Arginine kinase is a phosphotransferase that plays a critical role in cellular energy metabolism in invertebrates. Two different *HaAK* fragments, AK1 (containing the conserved domain) and AK2, were selected based on our previous experiment. When *H. armigera* second instar larvae were fed transgenic plants expressing *HaAK1* and *HaAK2* dsRNA for 2 days, *HaAK* transcript levels were significantly reduced by 86% and 74%, respectively. In addition, the ingestion of transgenic plants significantly delayed larval growth and development. An antifeedant bioassay showed that transgenic plant leaves had a significant antifeedant effect on *H. armigera* larvae based on the ecological interaction between the transgenic plants and the cotton bollworm. These results demonstrate the potential of plant-mediated RNAi for pest control.

Key words: *Helicoverpa armigera*, RNA interference, arginine kinase, transgenic tobacco, growth and development, antifeedant effect.

Introduction

Helicoverpa armigera Hubner (Lepidoptera Noctuidae) is a notorious polyphagous pest that causes severe yield loss in many important crops such as cotton, tobacco, chickpea, pigeon pea, eggplant, okra, and tomato (Razmjou *et al*., 2014). Management of this pest is a daunting task because of its increased resistance against current management strategies including chemical insecticides and transgenic crops expressing *Bacillus thuringiensis* Cry proteins (Du *et al*., 2012; Cao *et al*., 2014; Asokan *et al*., 2014). Spraying chemical pesticides continues to be the major approach for controlling *H. armigera* (Li *et al*., 2016), but it is a not very appropriate strategy in terms of insecticide resistance along with environmental and food pollution. Therefore, alternative management strategies are clearly needed.

RNA interference (RNAi) is a naturally existing defense mechanism in which long double-stranded RNA (dsRNA) is diced into small interfering RNA (siRNA) approximately 21-23 nucleotides in length by the RNase III enzyme Dicer (Eaton *et al*., 2002; Kumar *et al*., 2009; Xiong *et al*., 2016). Because siRNAs cleave their target mRNA in a sequence-specific manner, RNAi technology can be used to repress target genes with high specificity and selectivity, and it therefore has great potential for insect pest management (Mao *et al*., 2011; Xiong *et al*., 2013; Zhang *et al*., 2015; Jin *et al*., 2015). Critical for transgenic RNAi in plants is the identification of a target gene that would seriously impact the target pest but would be safe for its natural enemies, the environment, and human beings, necessitating a method to screen candidate genes from the pest gene pool. Pest control by dsRNA induction on the basis of feeding transgenic plants has been successfully tested in Lepidoptera, Hymenoptera, Diptera and Coleoptera (Gordon and Waterhouse, 2007; Price and Gatehouse, 2008). When cotton bollworm feeds on cotton expressing *CYP6AE14* dsRNA, the expression level of *CYP6AE14* in the body will be significantly reduced, which hinders the development of larvae. Furthermore *Arabidopsis thaliana* expressing *BtGSTs5* dsRNA significantly impedes the development of *Bemisia tabaci* (Gennadius) (Galit *et al*., 2018).

Arginine kinase (AK, L-arginine N-phosphotransferase, EC 2.7.3.3) is a phosphotransferase with a critical role in cellular energy metabolism in invertebrates including insects, crustaceans, and some unicellular organisms (Suzuki *et al*., 2009; Pereira, 2014). AK is not present in vertebrates, and both its biosynthetic pathway and phosphoarginine are completely different from those in mammalian tissues, making AK a potentially useful target in the control of pests (Liu *et al*., 2015). Most of the previous research on AK has focused on elucidating its catalytic mechanism and identifying its substrate analog inhibitors. In a previous study, Qi *et al*. (2015) obtained the full-length AK gene from *H. armigera* (*HaAK*) and generated constructs to express dsRNA targeting *HaAK*. They successfully used RNAi to silence *HaAK* expression, which largely impaired larval development. However, for future applications of RNAi to control *H. armigera* in the field, the potential harm to other insects also needs to be considered because the gene may be sufficiently conserved in other organisms including beneficial insects.

In this study, the full-length *HaAK* gene was truncated

to produce two interference fragments, *HaAK1* and *HaAK2* (jointly referred to as *HaAK1/2*). Agrobacterium-mediated transformation was used to generate transgenic tobacco plants expressing *HaAK1/2* dsRNA. The results of a breeding bioassay showed that the transgenic plants had significantly reduced *HaAK* expression levels, resulting in increased resistance to the cotton bollworm. Thus, full-length gene truncation was used to clarify the effects of plant-mediated AK gene silencing in *H. armigera*.

Materials and methods

Construction of hairpin RNAi vectors with partial *HaAK* (AK1, AK2) sequences

Two different *HaAK* fragments, AK1 (containing the conserved domain, 426 bp) and AK2 (379 bp), were selected based on our previous experiment and the identification of regions with the least sequence homology to non-target organisms to minimize the off-target effects of RNAi. To express *HaAK1/2* dsRNA in a plant system, the middle vector pUCCRNAi was used as an intermediate carrier. pUCCRNAi contains a 199 bp potato intron with one multiple cloning site on both sides of the intron, and the restriction sites within the multiple cloning sites are isocaudomers. We firstly inserted *HaAK1* and *HaAK2* into pUCCRNAi middle vector respectively, and then used *Xho*I and *Bgl*II isozyme to digest the pUCCRNAi-*AK1*/*AK2* vector, and then inserted *HaAK1* and *HaAK2* into the pUCCRNAi-*AK1*/*AK2* vector again to obtain pUCCRNAi-2*AK1*/2*AK2* vector respectively, so that the sequence of the clone insertion is reversed. The desired fragment was cleaved by *Pst*I for ligation into pCAMBIA2300, which contains a constitutive double enhancer 35S CaMV promoter and the NOS terminator. The two *HaAK*-RNAi constructs were separately introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation.

Preparation of transgenic tobacco plants

The NC89 tobacco seeds were plated onto sterilized MS medium and transplanted into sterile nutrient after 7 days. The plants were cultured at 26-28 °C under a 16:8 h (L:D) photoperiod. Tobacco plants at the four- to five-leaf-stage were selected for infiltration with *A. tumefaciens* containing the *HaAK*-RNAi constructs. After co-cultivation, three screening and differentiation steps, rooting culture, and 7 days of refining, the acclimatized plants were transferred to 12-inch earthen pots containing potting mix (1:1:3 ratio of perlite:vermiculite:black soil) and grown in a biosafety house for further analysis. Transgenic tobacco seeds of T0 generation were collected, and then planted on MS medium containing 250 mg/L Kana to select normal plants. The genomic DNA of the selected tobacco seedlings was extracted, and PCR-tested using the genome as a template to select T1 transgenic tobacco plants.

PCR and Southern blot analysis of RNAi tobacco

The genome of transgenic tobacco and wild-type tobacco was extracted with the New Plant Genome Extraction Kit and identified by genomic PCR using the following procedure, 94 °C, 5 minutes; 94 °C, 30 seconds; 61 °C, 30 seconds; 72 °C, 40 seconds; 35 cycles; 72 °C, 10 minutes. *HaAK1* sequence primers (5′-3′) F: CGGGATCCAAAAGTTGGAGGCTGGTT; R: GCGTCGACTCTCTTCCATCTCCTTGT; *HaAK2* sequence primers (5′-3′) F: CGGGATCCAG-CAGCTCATCGATGACC; R: GCGTCGACTGG-TACTTGGATG. After genomic PCR confirmation of the transgenic positive plants, we performed Southern blot analysis to confirm the stable integration of the transgenes in the tobacco genome. Genomic DNA was isolated using the Plant Genome Extraction Kit. The target fragment amplified by PCR was labeled with digoxigenin according to the manufacturer's protocol (Roche Diagnostics, USA), tested for efficiency, and used as a probe. About 10 μg of genomic DNA was digested with *Eco-R*I or *Sal*I then isolated on a 1% agarose gel. DNA was transferred to a positively charged nylon membrane (Roche Diagnostics, USA) using 20× SSC citric acid salt buffer. Prehybridization and hybridization were performed at 48 °C for 1 hour and overnight, respectively, and the subsequent steps were performed per the manufacturer's instructions. Chemiluminescent detection was performed using CSPD as substrate for alkaline phosphatase, and visualization and documentation were performed using the chemiluminescence gel documentation system (Fusion SL, Vilbur Lourmat, France).

Insect rearing

The laboratory strain of *H. armigera* was reared on an artificial diet (Zhang *et al*., 2013) in a conditioned room maintained at 25 ± 1 °C, 70 ± 5% relative humidity, and a 14:10 hours (L:D) photoperiod. Adults were maintained under the same conditions and supplied with a 10% sugar solution.

Effect of ingestion of the *HaAK*-RNAi tobacco line on *HaAK* expression by quantitative real-time PCR and immunohistochemistry

To study the silencing efficiency of the transgenic tobacco, the expression level of the target gene was detected in *H. armigera* second instar larvae (second day) after they had been fed tobacco leaf for 24 hours and 48 hours.

Total RNA from *H. armigera* larvae was extracted using the ISOLATE II RNA mini kit (Bioline, USA) for assessment of silencing. cDNA was synthesized by reverse transcription in a 20 μL reaction volume according to the manufacturer's instructions. qRT-PCR of each cDNA sample and template-free sample was performed in triplicate in a 20 μL reaction volume containing 10 μL SYBR Green Reaction Mix, 0.5 μL forward and reverse primers (5 mM each), 1 μL cDNA template, and 10.5 μL nuclease-free water. The cycling conditions were as follows: 95 °C for 4 minutes, followed by 40 cycles of 95 °C for 30 seconds and 58 °C for 30 seconds. The relative expression level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated with three independently isolated mRNA samples (biological replicates), and each reac-

tion was performed in triplicate to minimize intra-experiment variation (technical replicates). The qPCR primers for *HaAK* and *β-actin* are listed in table 1.

HaAK protein levels in the midgut were detected in larvae that had fed on the genetically engineered bacteria. Immunohistochemistry analysis (Zhao *et al*., 2016a) was performed on transverse sections of cotton bollworm larvae after fixation, dehydration, embedding, sectioning, dewaxing, the primary antibody was diluted with 2.5% Bovine Serum Albumin (BSA) at a ratio of 1:100, and 200 μL of the mixture was added to the sections for incubation. After the primary antibody was discarded and washed, the goat anti-mouse secondary antibody was diluted with 2.5% BSA at a ratio of 1:2000, and 200 μL of the mixture was added to the sections for incubation, and DAB staining. Photographs were taken with a Nikon NIS-Elements D (Nikon ECLIPSE Ti, Tokyo, Japan).

Insect feeding bioassays

Feeding bioassay with isolated tobacco leaves

We assessed the silencing effect of the transgenic tobacco by analysing its impact on the growth and development of *H. armigera*. According to the results of Southern blot, the line 2 of the transgenic dsHaAK1 (dsHaAK1-2) and the line 3 of dsHaAK2 (dsHaAK2-3) were selected for the bioassay. A 12-well plate was used as a feeding vessel, and 1.5 ml of 1.5% agar solution was added to each well. Filter paper the same size as the hole was used as a cover to minimize the water loss of the leaves. Two pieces of transgenic and control leaves were placed in each well. Second instar larvae on the second day were sorted for size and weight uniformity. The larvae were released into each well-containing hole, with 24 insects per line. The plates were maintained at room temperature (25-27 °C), and the leaves were exchanged with fresh leaves every day. The length and body weight of five randomly selected larvae were measured for the different treatments every 12 hours, and both the larval morphology and feeding were recorded. The experiment was repeated three times.

To study the ecological interaction between the transgenic plants and cotton bollworm larvae, both selective and non-selective antifeedant experiments were performed. For the selective antifeedant experiment, a 10 × 10 cm plastic dish with a 1.5% agar solution was used for feeding, and filter paper was cut to cover the solidified agar to minimize leaf water loss. The leaves were rounded to a diameter of 2.7 cm then weighed, and wild-type and transgenic tobacco leaves and transgenic leaves were placed diagonally. Second instar larvae were placed at the centre of the dish (40 per dish), and the dish was sealed with plastic wrap to prevent the larvae from escaping. The petri dishes were maintained at room temperature (25-27 °C), and the wraps were lifted every few hours to ensure sufficient ventilation for the larvae. The residual leaves were taken out and the feeding area of the transgenic leaves and the wild-type leaves were measured with the coordinate paper method to evaluate the antifeedant effects of the transgenic tobacco. The bioassay was performed with three biological replicates, and the following formula was used for the analysis:

$$S = [(S_w - S_t) / (S_w + S_t)] \times 100$$

where S is the selective antifeedant rate (%), S_w is wild-type tobacco leaf area eaten by larvae and S_t is transgenic tobacco leaf area eaten by larvae.

The positive plants were repeated 3 groups. S stands the area of tobacco leaf are eaten by *H. armigera* larvae.

Feeding bioassay with whole tobacco plants

To simulate the real ecological interaction between the transgenic tobacco and *H. armigera*, three transgenic tobacco plants (expressing dsHaAK1 and dsHaAK2) and three wild-type tobacco plants with uniform height were selected. Each group was placed in a tray with sufficient water to prevent plant drought. Synchronous third instar larvae were selected and randomly released on top of three tobacco leaves, with ten individuals per leaf. The pots were placed on a plant culture rack and maintained at 24-25 °C. The leaf-feeding profiles were visually assessed and recorded.

Based on the above antifeedant effects of transgenic tobacco plants on *H. armigera*, we next simulated the field interaction between transgenic plants and *H. armigera*. First, synchronous transgenic (expressing dsHaAK1 and dsHaAK2) and wild-type tobacco plants (two each) were selected. A4 paper was cut with a hole in the centre to surround the tobacco stalk to cover the pot. Synchronous second instar larvae were randomly released on top of four tobacco leaves, with 20 individuals per leaf. Finally, the pot was placed on a table covered by white paper to detect the larvae, and the temperature was maintained at 24-25 °C. The feeding status was recorded every 24 hours, and the number of survivors, escaped larvae, and deaths were counted for each tobacco plant.

Statistical analysis

All data were analysed for statistical differences by one-way ANOVA using SPSS version 19 for Windows. Data for more than two groups were analysed with one-way ANOVA followed by the Tukey test. Data for two groups were analysed with a t-test, and discrete data were analysed using a χ^2 test.

Table 1. The qRT-PCR primers of *AK* and *β-actin*.

Primer Name	Primer Sequence
β-actin-forward	5'-ATCATCGACGCTCCCGGACA-3'
β-actin-reverse	5'-TAGCTGCTTGACTCCGAGGGTG-3'
AK-forward	5'-TGGAGACCCTCGGCAACCTGGAC-3'
AK -reverse	5'-CTGCTGCTGGGTTTCCTTGGAC-3'

Results

Generation of transgenic tobacco expressing dsRNA

Transgenic tobacco seeds of T0 generation were collected, and then planted on MS medium containing 250 mg/L Kana to select normal plants. The genomic DNA of the selected tobacco seedlings was extracted, and PCR-tested using the genome as a template to select T1 transgenic tobacco plants before Southern blot analysis. We identified seven dsHaAK1 and ten dsHaAK2 T1 generation transgenic tobacco plants by genomic PCR (figure 1a), and three dsHaAK1 and six dsHaAK2 T1 positive plants were selected for Southern blot analysis. For dsHaAK1, all of the selected lines had three bands compared with wild-type tobacco, indicating that the dsHaAK1 transgenic tobacco lines were multi-copy lines. For dsHaAK2, line 3 had two bands, and lines 2, 4, 5, and 6 had one band (figure 1b). Thus, the Southern blot confirmed that both the dsHaAK1 and dsHaAK2 transgenic tobacco lines could be used for the subsequent bio-feeding analysis.

RNAi efficiency analysis of transgenic tobacco

To determine whether ingestion of the transgenic plants triggered gene-specific silencing, the target gene transcript levels in cotton bollworm larvae were detected by qRT-PCR after larval ingestion of the dsHaAK1-2 and dsHaAK2-3 tobacco lines. *HaAK* transcript levels were reduced at 24 hours relative to wild type and decreased gradually with time, with a relative reduction of 86% and 74% at 48 hours for dsHaAK1-2 and dsHaAK2-3, respectively (figure 2a). To further confirm the silencing effect, *HaAK* protein levels were detected by immunohistochemistry. The colour fades with time, the expression of the *HaAK* protein were obviously reduced after feeding with transgenic tobacco for 48 hours. But the content of the *HaAK* protein fed with wild type tobacco had not changed. The results were consistent with the qRT-PCR analysis (figure 2b).

Effects of transgenic tobacco on larval growth and development

To follow up on the molecular analysis that indicated a silencing effect, we studied the effect of the transgenic tobacco on the growth and development of cotton bollworm. To avoid mutual harm between larvae, we adopted a separate feeding method. Initially, second instar larvae on the first day were selected but often died on the following day, probably because the insects were too young. Therefore, second instar larvae on the second day were selected for the feeding bioassays. The body length and body weight of the larvae were measured every 12 hours. For larvae fed dsHaAK1-2 and dsHaAK2-3 transgenic tobacco leaves, both body length and weight were significantly lower than the control over time (figure 3a, 3b). Additionally, the larvae in the treatment groups were visible smaller in size (figure 3c, 3d).

Antifeedant effect of transgenic tobacco on larvae

To investigate whether the transgenic tobacco had an antifeeding effect on the cotton bollworm, a selective antifeedant experiment was conducted. Both dsHaAK1-3 and dsHaAK2-2 transgenic plants had antifeeding effects on *H. armigera* larvae, and the selective antifeedant rates were 75% and 42% at 24 hours, respectively (figure 4a). The average weight of larvae ingesting dsHaAK1-3 leaves and dsHaAK2-2 leaves was 29.9 mg and 43.13 mg, respectively, which was obviously less

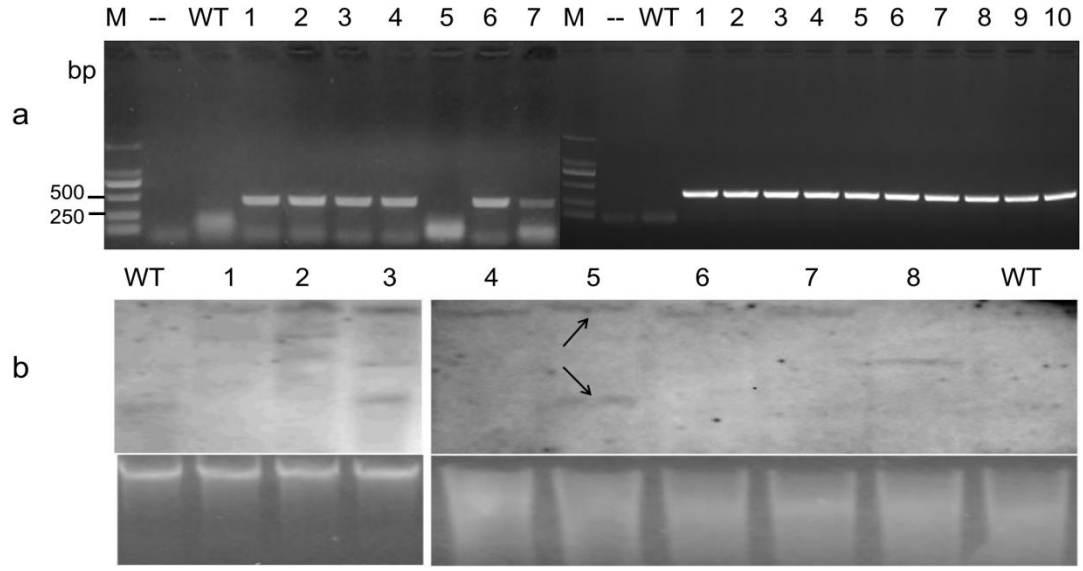

Figure 1. Molecular analysis of transgenic plants. **(a)** Detection of *HaAK1/2* in the non-transformed control and transgenic plants by PCR. Lane M: DNA marker DL2000; --: negative control; WT: wild-type tobacco control; lanes 1-7: *HaAK1* transformants; lanes 1-10: *HaAK2* transformants. **(b)** Southern blot analysis of dsHaAK1/2 transgenic tobacco plants. WT: wild-type tobacco control; lanes 1-8: transformants (dsHaAK1-1, dsHaAK1-2, dsHaAK1-3, dsHaAK2-2, dsHaAK2-3, dsHaAK2-4, dsHaAK2-5, and dsHaAK2-6). Positive double strands was indicated by arrows.

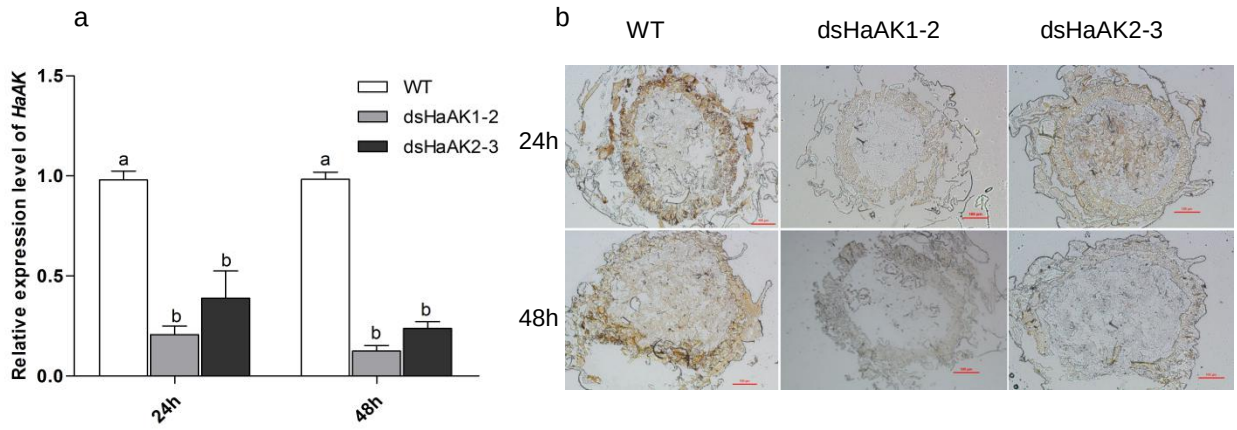

Figure 2. RNAi efficiency analysis of second instar *H. armigera* larvae feeding on transgenic tobacco. **(a)** *HaAK* expression analysis of *H. armigera* larvae fed transgenic tobacco leaves measured by qRT-PCR. *HaAK* mRNA transcript levels were suppressed in second instar larvae fed dsHaAK1-2 and dsHaAK2-3 plants. The actin gene was amplified as an internal control. Data from each time point were tested by One-way ANOVA, and different letters above the bars indicate a significant difference ($p < 0.05$). **(b)** Detection of *HaAK* protein in the second instar larvae of *H. armigera* fed wild type tobacco and transgenic tobacco leaves for 24 and 48 hours, respectively. WT: wild type tobacco; dsHaAK1-2: express *HaAK1* dsRNA transgenic tobacco; dsHaAK2-3: express *HaAK2* dsRNA transgenic tobacco.

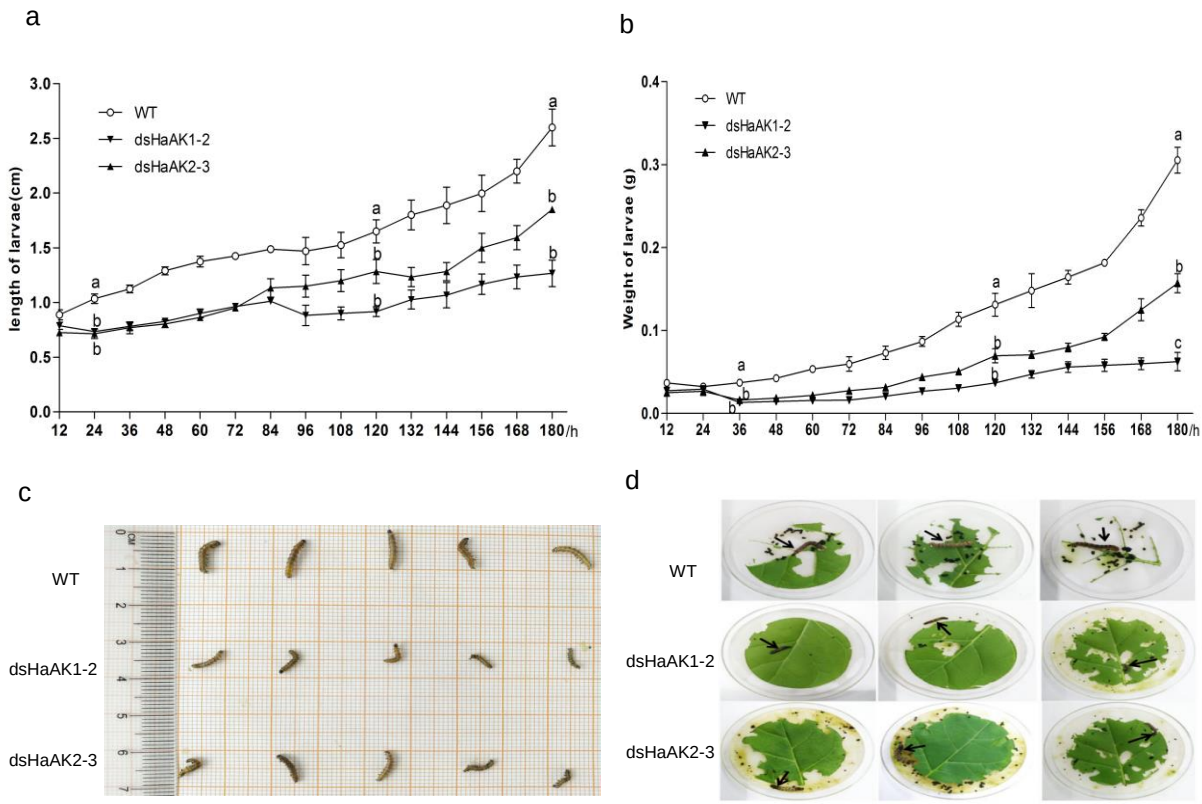

Figure 3. The effects of ingestion of transgenic tobacco on larval growth and development. **(a)** The reductions in body length of *H. armigera* larvae fed transgenic tobacco. **(b)** The reductions in body weight of *H. armigera* larvae fed transgenic tobacco. The error bars indicate the standard deviation from three biological replicates, and each repetition has 24 larvae. WT: wild type tobacco; dsHaAK1-2: express *HaAK1* dsRNA transgenic tobacco; dsHaAK2-3: express *HaAK2* dsRNA transgenic tobacco. Data from each time point were tested by One-way ANOVA, and different letters above the bars indicate a significant difference ($p < 0.05$). **(c)** The reductions in body size of *H. armigera* larvae fed transgenic plants at 3 days. **(d)** Phenotypic observation of *H. armigera* larvae fed wild type tobacco and transgenic tobacco. *H. armigera* larvae were indicated by arrows.

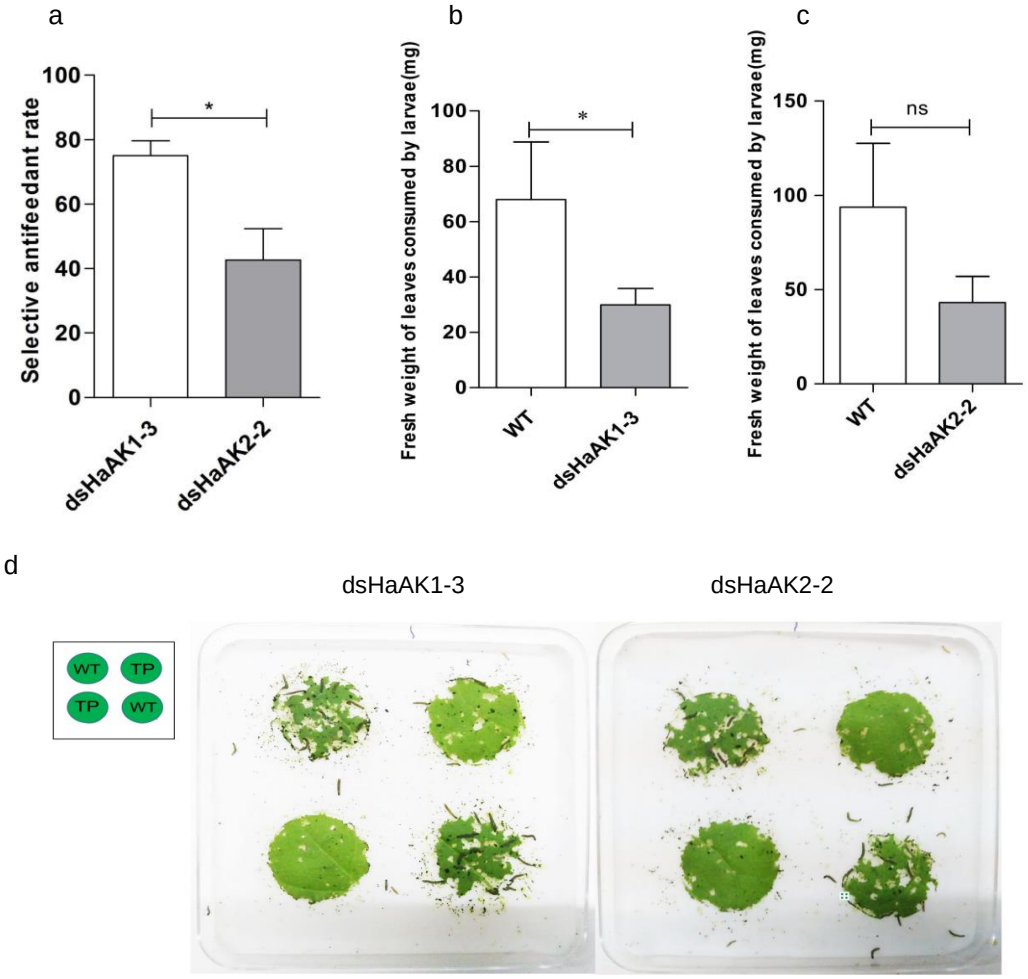

Figure 4. Antifeedant effect of transgenic tobacco leaves on *H. armigera* larvae. Transgenic and wild-type tobacco leaves were cropped into a circular shape with a diameter of 2.7 cm then arranged diagonally on filter paper as diagrammed in (**d**). Forty *H. armigera* second instar larvae (first day) were released at the centre of the plastic petri dishes. After 24 hours of feeding, the reduced damage on the transgenic tobacco leaves compared to the wild-type control indicated high antifeedant effects on *H. armigera*. The leaf area eaten by the larvae and the fresh weight of the leaves consumed by the larvae were quantified to evaluate the antifeedant effects of both the dsHaAK1-3 and dsHaAK2-2 transgenic tobacco lines. **(a)** Selective antifeedant rate of transgenic tobacco against *H. armigera* larvae. **(b-c)** The fresh weight of dsHaAK1-3 and dsHaAK2-2 tobacco leaves eaten by larvae at 24 hours. **(d)** Antifeeding effect of transgenic tobacco leaves on *H. armigera* larvae. WT: wild-type tobacco; TP: transgenic tobacco plant. The data were analysed by T test. The * represents the significant difference ($p < 0.05$).

than that of corresponding control (67.97 mg and 93.73 mg, respectively) at 24 hours. The fresh weight of dsHaAK1 and dsHaAK2 leaves consumed by larvae was less than that of wild-type tobacco (figure 4b-d), confirming the antifeeding effect of the transgenic tobacco on *H. armigera.*

Ecological interaction analysis

For a better approximation of the ecological interaction between *H. armigera* and transgenic tobacco in the field, cotton bollworm larvae were released onto whole tobacco plants. The results showed that the consumed leaf area of transgenic tobacco was significantly less than that of wild-type tobacco after 12 hours of feeding (figure 5), consistent with the findings from the feeding bioassays with isolated tobacco leaves.

We systematically analysed the ecological interaction between transgenic tobacco and *H. armigera*. Compared to wild-type tobacco, the transgenic tobacco expressing dsHaAK1 and dsHaAK2 had a significant repellent effect on *H. armigera* larvae at 24 and 48 hours according to the number of escaped larvae. After feeding the dsHaAK1 transgenic tobacco, the number of escaped larvae reached 43 at 24 hours and 76 at 48 hours, respectively; and feeding the dsHaAK2 transgenic tobacco, the number of escaped larvae reached 41 at 24 hours and 58 at 48 hours, respectively, and the number of escaped larvae was only 17 at 24 hours and 33 at 48 hours, respectively, in the wild-type tobacco. For the transgenic tobacco lines, the number of escaped larvae was significantly increased at 48 hours compared with 24 hours (figure 6a, 6b), showing that the interaction effect between transgenic tobacco and *H. armigera* increased over time.

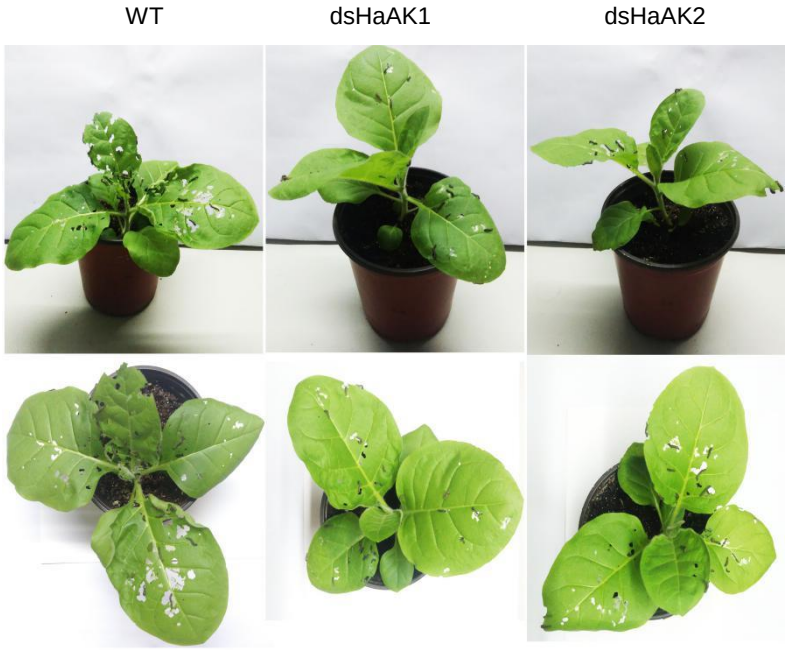

Figure 5. Resistance to *H. armigera* was improved in transgenic tobacco plants expressing *HaAK1/2* dsRNA. Tobacco plants were grown in nutrient-rich soil in individual 12 × 25 inch pots. Relative humidity was maintained at 60-70%. Twenty *H. armigera* larvae (second day of the third instar) were randomly released onto whole plants to evaluate the ecological interaction between transgenic tobacco and *H. armigera*. After 12 hours of feeding, the reduced damage to transgenic tobacco plants indicated greater resistance to *H. armigera* than that of the control. Wild-type tobacco plants were used as a control, and two different transgenic lines for dsHaAK1 and dsHaAK2 were used as the experimental groups.

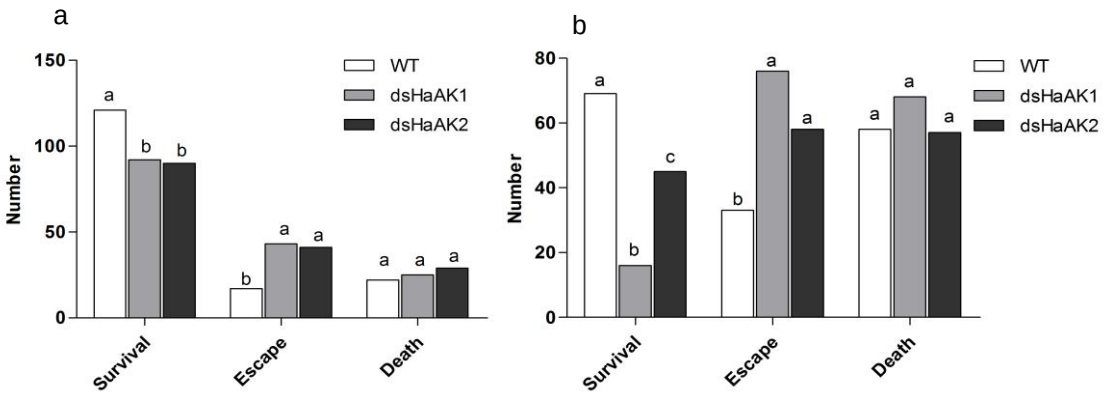

Figure 6. Ecological interaction analysis of transgenic tobacco and *H. armigera*. Tobacco plants were grown in nutrient-rich soil in individual 12 × 25 inch pots. Relative humidity was maintained at 60-70%. Eighty *H. armigera* larvae (first day of the second instar) were randomly released onto whole plants to systematically evaluate the interaction effects between transgenic tobacco and *H. armigera*. After 24 and 48 hours of feeding, transgenic tobacco plants exhibited higher evasion to *H. armigera* than the wild-type tobacco. **(a)** Ecological interaction analysis at 24 hours. The survival number of larvae on the different plant at 24 hours was analysed by χ^2 test, and different letters above the bars indicate a significant difference ($p < 0.05$). The analysis of both the escaped number and the death number of larvae on the different plant was the same as above. **(b)** Ecological interaction analysis at 48 hours. The data analysis was the same as above.

Discussion

Genes involved in cell energy metabolism and its multifarious functions are potential targets for insect gene silencing by RNAi (Meister and Tuschl, 2004; Baum *et al*., 2007; Terenius *et al*., 2011; Zhi *et al*., 2016). Arginine kinase can directly regulate the balance of the ATP energy pool (Miranda *et al*., 2006; Zhao *et al*., 2008; Chen *et al*., 2015). Previous studies have shown that AK levels can be manipulated by RNAi by introducing dsRNAs targeting AK genes into insects (Wu *et al*., 2009). More broadly, transgenic plant-mediated RNAi targeting key insect genes can delay insect growth and development, thereby reducing the damage to plants (Zha *et al*., 2011; Yue *et al*., 2016). Since AK is not present in mammals and is the only phosphate kinase present in insects, AK is an ideal target candidate for the development of new and highly selective pest control.

We truncated the full-length AK gene to develop a safer and more effective pest management strategy in this study, the AK gene from *H. armigera* was truncated to produce two interference fragments (AK1, which contains the conserved domain of *HaAK*, and AK2) for the construction of dsRNA expression vectors, which were then introduced into tobacco plants. PCR and Southern blot analysis of transgenic tobacco plants expressing dsRNA of *HaAK1* and *HaAK2* revealed the presence of transgenes and their genomic integration in these plants. It has been reported that insect genes can be knocked out but not completely through the microinjection of dsRNA, uptake of exogenous dsRNA from an artificial diet, or through plant-mediated strategies (Tomoyasu and Denell, 2004; Mao *et al*., 2015). In this study, the ingestion of the dsHaAK1-2 and dsHaAK2-3 lines suppressed *HaAK* transcript levels by 86% and 74% after 2 days, respectively, and immunohistochemistry analysis was consistent with the above results. Liu *et al*. (2015) previously reported that ingestion of transgenic *Arabidopsis* plants suppressed *HaAK* expression by up to 52% over 5 days, and the efficiency of the plant-mediated RNAi reflected the concentration of complete dsRNA contained therein. In other words, higher dsRNA concentrations may lead to a stronger inhibitory effect of RNAi. To assess whether the transgenic tobacco in this study might similarly contain high concentrations of dsRNA, Southern blot analysis was performed, which revealed varying copy numbers in the different transgenic tobacco lines.

Body size is regulated by growth rate and duration (Mirth *et al*., 2014). In our experiment, the reduced larval weight and length were largely attributable to the reduced rate of growth after ingestion of transgenic leaves compared to the controls. Moreover, the larvae in the transgenic tobacco groups consistently exhibited slowness, curling, and decreased vitality. Larvae fed dsHaAK2 leaves developed blackening of the head and tail and whitening of the trunk at the fifth instar. Similarly, after silencing the AK of *Musca domestica* L., their larvae were significantly smaller than those of the control group. The tail of the larvae was often blackened and the activity of the larvae was poor. Even if the larvae survived to emerge into adult flies, the growth rate was much slower than that of the control group (Yu *et al*., 2015). Silencing the expression of the AK gene of *H. armigera* via artificial feeding of dsRNAs produced by engineering bacteria, the results showed that larval mortality increased significantly (Qi *et al*., 2015). As AK is associated with energy metabolism, its expression is spatiotemporally regulated. It is mainly concentrated in tissues that require energy (Kang *et al*., 2011) and is probably affected by ecdysone signaling pathway regulation (Zhang *et al*., 2017). Besides, our research group has found that there are significant changes of the arginine kinase at different developmental stage of *H. armigera* (Zhao *et al*., 2016b). In this study, the decrease in *HaAK* expression most likely affected the growth and development of insects, which would also be consistent with the reduced movement of *H. armigera* larvae that consumed dsHaAK1/2 tobacco.

In the feeding bioassay with isolated tobacco leaves, the consumed area of dsHaAK1/2 was significantly less than that of wild type. When insects come into contact with plants, they can sense the proportion of plant nutrients, dietary stimuli and other factors (Vet and Dicke, 1992). Interestingly, *H. armigera* larvae initially ingested some dsHaAK1/2 leaves then ran wildly onto wild-type tobacco leaves for feeding. So insects can sense the difference between resistant plants and non-resistant plants, and tend to be non-resistant plants. In the feeding bioassay with whole tobacco plants, we found that on wild-type tobacco, *H. armigera* larvae on the initially placed leaf migrated to the uppermost leaf to feed. In contrast, this phenomenon was not observed in transgenic tobacco. This difference may be attributable to the relatively higher expression level of dsRNA in young tissues. In the repellence assays, the number of escaped larvae on dsHaAK1/2 transgenic tobacco was significantly higher than that on wild-type tobacco. Thus, in addition to the findings on the antifeeding effects of the transgenic plants, we also conducted a comprehensive analysis of the interaction effects between transgenic plants and *H. armigera*. These findings may be of value for future transgenic cotton research and may provide new ideas for environmentally friendly and efficient pest control approaches.

We truncated the full-length AK gene to produce two interference fragments. The AK2 sequence corresponds to a non-conserved domain of *HaAK*, and the findings support the argument that transgenic plants producing *HaAK*-targeted dsRNA would specifically disturb the growth and development of the target insect while remaining safe for its natural enemies, the environment, and human beings. Although the overall pest resistance of dsHaAK2 plants was somewhat weaker than that of dsHaAK1 plants, dsHaAK2 plants nevertheless had significant insect resistance compared to the control group. To effectively control *H. armigera*, *HaAK*-RNAi could be combined with RNAi of other genes involved in insect growth and development to obtain bivalent or multivalent insect-resistant transgenic plants.

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