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The effect of silencing arginine kinase by RNAi on the larval development of *Helicoverpa armigera*

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Abstract

Arginine kinase (AK) is an important regulation factor of energy metabolism in invertebrate. An arginine kinase gene, named *HaAK*, was identified to be differentially expressed between Cry1Ac-susceptible (96S) and Cry1Ac-resistant (Bt-R) *Helicoverpa armigera* larvae using cDNA-amplification fragment length polymorphism analysis. The full-length open reading frame sequence of *HaAK* gene with 1068 bp was isolated from *H. armigera*. Quantitative reverse transcription polymerase chain reaction assay revealed that *HaAK* gene is specifically expressed in multiple tissues and at larval developmental stages. The peak expression level of *HaAK* was detected in the midgut of the fifth-instar larvae. Moreover, the expression of *HaAK* was obviously down-regulated in Bt-R larvae. We further constructed a dsRNA vector directly targeting *HaAK* and employed RNAi technology to control the larvae. The feeding bioassays showed that minute quantities of dsRNA could greatly increase the larval mortality and delay the larval pupation. Silencing of *HaAK* significantly retarded the larval development, indicating that *HaAK* is a potential target for RNA interference-based pest management.

Keywords: *Helicoverpa armigera*, arginine kinase, genes expression, RNA interference, pest control

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Introduction

The cotton bollworm, *Helicoverpa* (*Heliothis*) *armigera* (Hübner) (Lepidoptera: Noctuidae), is one of the most serious insect pests of agriculture and widespread in central and southern Europe, temperate Asia, Africa, Australia, Oceania, and Brazil (Tay *et al.*, 2013). Larvae of *H. armigera* are foliar feeders, causing a drastic yield loss estimated to be greater than US$2 billion annually (Jamal *et al.*, 2014). Unfortunately, *H. armigera* has been hard to be controlled because of the high level of resistance, since the wide-spread use of chemical insecticides and biological pesticides (Kranthi *et al.*, 2002). So the need is urgent to develop a new approach to manage *H. armigera*.

RNA interference (RNAi) is a specific and efficient method used to silence genes and has been a useful method for the control of pest insects. The successful feeding of TPS (trehalose-6-phosphate synthase for the synthesis of trehalose, main sugar reserve in haemolymph) dsRNA solutions to silence this gene was reported as a useful pest control agent (Chen *et al.*, 2010). Feeding insects plant material expressing dsRNA specific to cytochrome P450 gene retarded larval growth of *H. armigera*, and this method could be applied in entomological research and field control of insect pests (Mao *et al.*, 2007). The expression of the corresponding dsRNA of a corticotropin-releasing factor-like binding receptor in tobacco plants could

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significantly decrease the receptor activity *in vivo* through RNAi (Adeyemi & Whiteley, 2014).

In previous study, differentially expressed genes (DEGs) in Cry1Ac-susceptible (96S) and Cry1Ac-resistant (Bt-R) *H. armigera* were identified using cDNA-amplification fragment length polymorphism (cDNA-AFLP) (Zhang *et al.*, 2009; Guo *et al.*, 2014). One sequence, isolated based on cDNA-AFLP assay, was found to be homologous to the published arginine kinase (AK). Arginine kinase (ATP: L-arginine phosphotransferase EC 2.7.3.3), a central regulator of temporal and spatial ATP buffers, catalyses the reversible phosphorylation of L-arginine to form phosphoarginine (Pereira *et al.*, 1999; Lipskaya, 2001). It is directly associated with muscle contraction, ATP regeneration and energy metabolism in several invertebrates (Ellington, 2001; Dawson & Storey, 2011). High expression levels were often observed in tissues and stages for energy consumption and production. In *Litopenaeus vannamei*, the highest expression level of *AK* gene was found in the muscle and the lowest in the skin (Yao *et al.*, 2009). A broad expression of *MrAK-1* was found in *Macrobrachium rosenbergii*, with the highest expression in the muscle and the lowest in the eyestalk (Arockiaraj *et al.*, 2011). In *Toxocara canis*, *Toxocara vitulorum*, and *Ascaris lumbricoides*, variable expression levels of *AK* were found at different stages of the life cycle (eggs, larvae, and adult worms), highly concentrated in cellular and metabolically active parts (Kulathunga *et al.*, 2012).

In this study, the full-length arginine kinase gene (*HaAK*) was obtained from *H. armigera*, and a dsRNA directly targeting *HaAK* was constructed to silence the expression of *HaAK* gene via RNAi technology. Silencing of *HaAK* largely impaired the larval development, indicating the possibility to control *H. armigera* using RNAi technology.

Materials and methods

Insect

The original *H. armigera* strain was collected from a cotton field in Xinxiang County, Henan Province, China, in 1996 and reared on an artificial diet without exposure to any chemical insecticide or *Bacillus thuringiensis* (Bt) toxins in the laboratory. This strain was named 96S. The Bt-resistant strain (Bt-R) was fed on artificial diet with the Cry1Ac protoxin (provided by the Biotechnology Group in Institute of Plant Protection, Chinese Academy of Agricultural Sciences). Only about 20% of the neonates selected from each generation pupated successfully (Liang *et al.*, 2000, 2003). Except for the resistance trait between 96S and Bt-R strain, the Bt-R strain was crossed with the 96S strain in the 27th, 49th, 69th, and 87th generations to minimize other differences (Liang *et al.*, 2008). Up to date, Bt-R strain resistance had approximately increased 2971-fold. *H. armigera* was cultured at 27°C and 75% relative humidity with a photoperiod of 14:10 h (light: dark).

RNA extraction and reverse transcription

Total RNA was isolated from the whole body of *H.armigera* at different life stages and tissues of the fifth-instar larvae by the acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi, 1987). RNA quantity was determined using a NanoDrop (ND)-2000 spectrophotometer (Thermo, USA). First-strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase (Toyobo, Japan) following the manufacturer's protocol with Oligo $(dT)_{20}$.

Sequence analysis of HaAK *cDNA fragment*

HaAK primers were designed based on the cDNA sequences of the *H. armigera AK* gene (GenBank Accession No. EF600057). The primer sequences and polymerase chain reaction (PCR) programs are listed in table 1. The amplified PCR product was subcloned into the pMD18-T vector (Takara, Dalian, China) and subsequently sequenced (Sunbiotech Company, Beijing, China). The amino-acid sequences were aligned by DNAMAN software (Version 6.0).

HaAK *gene promoter cloning*

Genomic DNA was extracted from the third-instar 96S larvae with a TIANamp Genomic DNA Kit (TIANGEN, China) following the manufacturer's protocol. DNA quantity was determined using a ND-2000 spectrophotometer (Thermo, USA).

Primers were designed based on the 5′ untranslated regions (UTR) sequences of the *H. armigera AK* gene (GenBank Accession No. EF600057). PCR conditions were designed with a Genome Walking Kit following the manufacturer's protocol (Takara, Japan). Resulting products were electrophoresed on 1% agarose gel. The bands linked to the cloning vector were subsequently sequenced. New primers were designed again based on the obtained sequences. Finally, the primers and program designed to detect the acquired sequences are listed in table 1. The promoter and transcription factors binding sites were predicted by Promoter Scan (http://www-bimas.cit.nih.gov/molbio/proscan/) and Transcriptional Factor Search (TFSEARCH, http://www.cbrc.jp/research/db/TFSEARCH.html), respectively.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of HaAK *expression*

HaAK expression was analysed at different developmental stages (second-, third-, fourth-, and fifth-instar larvae) and tissues (midgut, epidermis, and muscle) from fifth-instar larvae of the 96S and Bt-R strains.

The primers qRT-*HaAK* and qRT-*EF-1a* (housekeeping gene *EF-1a*) were designed based on the alignment of *H. armigera* cDNA sequences to two standard curves (Yu *et al.*, 2007; Guo *et al.*, 2014). They were cloned into the pMD18-T vector and subsequently sequenced. Recombinant plasmids were digested with *Hin*d III and serially diluted (10^3–10^9 copies μl^{-1}) to make the standard curve.

qRT-PCR was carried out in a Rad-IQ5 Real-time Detection System (Bio-Rad Company, USA). To maintain consistency, the baseline was automatically set by the software. The two standard curves were used to analyse *HaAK* expression levels. All data were given in terms of relative mRNA expressed as means. The target and reference standard curve met the experimental requirements ($R^2 > 0.99$, $E > 90\%$). The data were analysed by *T* test. *P* values < 0.05 were considered significant differences. *P* values < 0.01 were considered highly significant differences. qRT-PCR informations are listed in table 1.

Analysis of HaAK enzyme activity

The *HaAK* enzyme was extracted from the midgut and body (without midgut) of fifth-instar larvae. The sample was

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Table 1. Primer sequences used in this study.

Primer names		Usage	Sequences (5′→3′)	Program
HaAK		Cloning *HaAK*'s ORF	For: ATGGTGGACGCCGCAACAATCG Rev: TTACAGCGACTTCTCAATTTTGATCAG	94°C, 5 min; 94°C, 1 min; 57°C, 1 min; 72°C, 1 min; 30 cycles; 72°C, 10 min
Probe-HaAK		FISH probe	For: TTCGGCTCCACCCTCTTGGATTG Rev: TTGACGGCGTCGTACTCGGTGA	94°C, 5 min; 94°C, 45 s; 56°C, 45 s; 72°C, 45 s; 32 cycles; 72°C, 10 min
RNAi-HaAK		for *HaAK*'s RNAi construct	For: AGC***AAGCTT***CTTCACCCGTGTG Rev: TCA***GGTACC***AAGGGAATGCGC	94°C, 5 min; 94°C, 45 s; 55°C, 45 s; 72°C, 45 s; 30 cycles; 72°C, 10 min
First amplification primers	SP1 SP2 SP3	GenomeWalking	ATTGCACCAGACCAAGAAGG GCTGAAACCAGCCTCCAAC ATCAATGTATCGGAGCCGGG	
Second amplification primers	SP1 SP2 SP3	GenomeWalking	GCGCTTGTACAACCGTCAGAGTCG CGCGAGCCAGTTTAGCTTCTAGTCGTG CAGGGGGGTAGGTCACAAGGTCATG	
Third amplification primers	SP1 SP2 SP3	GenomeWalking	GTGCTCGCAACGCTCGCTCTC TGCTGTATCTCATGCAGGGCCGTC GCCGCACCGTCTCGTGATACTGAC	
HaAK gene		Cloning *HaAK* gene	For: GCCTACGACACAAAAGCACC Rev: GGTCCTCCTCATTGCACC	94°C, 5 min; 94°C, 1 min; 58°C, 1 min; 72°C, 4 min; 30 cycles; 72°C, 10 min
qRT-*HaAK*		Specific primer for the qRT-PCR	For: CGTCGTCTTCACCCGTGTG Rev: GCTGCTGGGTTTCCTTGGAC	94°C 3 min; 94°C 30 s; 60°C 30 s; 72°C 45 s; 40 cycles
qRT-*EF-1*		Specific primer for the qRT-PCR	For: GACAAACGTACCATCGAGAAG Rev: GATACCAGCCTCGAACTCAC	94°C 3 min; 94°C 30 s; 60°C 30 s; 72°C 45 s; 40 cycles

Restriction enzyme sites are marked by bold face and underlining.

grinded and supplemented with the leaching buffer (1 mM EDTA, 14 mM C_2H_6OS, 50 mM Tris–HCl, pH 8.0) at 4°C. The crude protein concentration was determined by the Bradford method with bovine serum albumin as the standard by measuring the absorbance of the protein solution at 595 nm (Bradford, 1976). Then the enzyme activity was carried out by the Phosphorus method with modification (Seals & Grossman, 1988). The crude enzyme was added into reaction buffer (100 mM Tris–HCl (pH 8.0), 2 mM ATP-Na, 5 mM $C_2H_{11}MgO_6$, 10 mM L-Arg, and 10 mM C_2H_6OS). After 10 min at 30°C, the reaction was terminated by 10% CCl_3COOH. The reaction solution was heated at 100°C for 1 min and immediately transferred on ice for 1 min. The dye buffer (1%$(NH_4)_6Mo_7O_{24}$, 5%$FeSO_4$ and 1 M H_2SO_4) was sufficiently mixed and incubated at 30°C for 10 min. In the end, the absorbance of the reaction solution at 660 nm was determined. One unit of the enzyme was defined as 1.0 μmol of L-arginine and ATP converted to N-phospho-L-arginine and ADP per min at a pH of 8.0 and temperature of 30°C.

Fluorescence in situ *hybridization (FISH) detection*

The hybridization protocol was followed as previously described (Pinkel *et al*., 1986; Amann *et al*., 1990) with minor modifications. The primers and amplification program for probe are listed in table 1. Purified PCR product was labelled with the Alex-488-dUTP with exonuclease-free Klenow for 30 min. After mixing the probe with herring sperm DNA, probe efficiency was measured.

The third-instar 96S larvae were transversely sectioned. After incubation for 1 h at 60°C, the tissue was de-waxed three times for 5 min in xylene and twice for 5 min in absolute alcohol. The slide was tilted and air-dried. It was then incubated in 30% sodium bisulfite (2 × saline sodium citrate (SSC)) for 20 min at 43°C and washed twice for 5 min in 2 × SSC. Following a Proteinase K digestion (0.25 mg ml^{-1}) at 37°C 10 min, the slide was washed twice for 5 min in 2 × SSC. The slide was dehydrated for 5 min each in 70, 85, and 100% ethanol and then air-dried.

The cover slip was applied on 10 μl of hybrid liquid and immediately covered. Hybridization was carried out at 37°C in a wet box (2 × SSC) overnight. After carefully removing the cover slip, the slide was washed three times for 5 min at 43° C in 50% deionized formamide (2 × SSC). Then it was washed twice for 5 min in 2 × SSC and 0.5 × SSC at room temperature. DAPI was counterstained for 10 min at 37°C. The slide was washed with PBS twice and the sections were sealed with glycerol. The hybridization signal was detected by laser scanning confocal microscope (Bio-Rad MRC-1024, USA).

Construction of RNAi recombinant plasmid and dsRNA preparation

To construct a recombinant plasmid (RNAi-*HaAK*) that produces a HaAK-specific dsRNA, primers (table 1) with *Hin*d III and *Kpn* I restriction sites were designed to amplify a 414 bp fragment based on the *HaAK* gene sequence (GenBank Accession No. GU396008). The target fragment was inserted into the pMD18-T vector and then sequenced. The partial *HaAK* gene was cloned into the RNAi vector L4440, obtained from Addgene (Timmons & Fire, 1998; Timmons *et al*., 2001). The recombination plasmid was named L4440-*HaAK* and sequenced again. Subsequently, the L4440-*HaAK* recombination plasmid was used to transform into *Escherechia coli* HT115 (DE3) strain (Yang & Han, 2014). Cultures were grown at 37°C overnight by inoculating 100 ml of 2 × YT medium (yeast extract, 5 g l^{-1}; tryptone, 8 g l^{-1}; NaCl, 5 g l^{-1}) containing the appropriate antibiotic (Amp 100 μg ml^{-1}, Tet 12.5 μg ml^{-1}) with 1 ml culture. Incubation continued until the culture reached an OD_{595} of approximately 0.4. IPTG was then added to a final concentration of 0.4 mM. The recombinant bacteria were collected after 4 h and resuspended in sterile water (250×), and then used for *H. armigera* feeding bioassays (Tian *et al*., 2009; Guo *et al*., 2014).

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*Ds*HaAK *feeding bioassays*

A randomized block design was used for this feeding experiment. The artificial diet was cut into small pieces, which was overlaid with a 50 µl suspension of one of the following: bacterial culture containing bacteria expressing dsRNA for *HaAK* (RNAi-*HaAK*), plasmid L4440 (RNAi-CK), or ddH_2O (CK). All diets were replaced daily. All experimental groups were consisting of the first day of the first-instar 96S larvae, respectively. The larva was fed in an individual chamber as groups in the same conditions as described above. Each group, 30 insect, was replicated three times. The survival amount and pupation amount were recorded daily until 12th day. Then, the pupation ratio was calculated (Pupation ratio = pupation amount/survival amount × 100%). HaAK enzyme activity was determined at 10th day. The data were analysed by SAS. When treatment effects were detected, Duncan's test was used to determine whether significant differences exist among the groups.

qRT-PCR analysis of HaAK *silencing*

To detect the *HaAK* transcription levels after dsHaAK ingested by larvae, RNA was isolated from the treated and untreated control larvae after continuous feeding. First strand cDNA was prepared and qRT-PCR was conducted according to the protocols described above.

Results

Cloning and sequence analysis of the HaAK *gene*

The full-length open reading frame (ORF) sequence of *HaAK* gene was successfully amplified from the 96S and Bt-R larvae by PCR. There was no difference between the sequences from two strains. It contained 1068 bp fragment and encoded 355 amino-acid residues (GenBank Accession No. GU396008) (fig. 1a). *HaAK* shared the sequence characteristic of AK, including CPTNLGT residues located in the C-terminus. In addition, four conserved residues among AK, C270, L112, T272, and R329, were also observed in amino-acid sequence of HaAK.

Phylogenetic analysis was performed to decipher the relationship between HaAK and related phosphokinase sequences in other vertebrate and invertebrate animals. Using MEGA 5.0, 18 available phosphokinase sequences from different animal species were constructed to a neighbour-joining phylogenetic tree, which consisted of four different subgroups: AK, creatine kinase, tyrosine-protein kinase, and cAMP-dependent protein kinase. HaAK, AmAK, BmAK, FcAK, PxAK, SaAK, and SlAK were clustered into the clade of AK (fig. 2a). Moreover, the amino-acid sequence of HaAK showed 87, 88, 82, 94, 94, and 97% identity with *Schistocerca Americana* AK (SaAK, AAC47830), *Apis mellifera* AK (AmAK, NP_001011603), *Fenneropenaeus chinensis* AK (FcAK, AAV83993), *Bombyx mori* AK (BmAK, ABB88514.1), *Spodoptera litura* AK (SlAK, ADW94627.1), and *Papilio xuthus* AK (PxAK, BAM17790.1), respectively (fig. 2b). The highest identity was observed with PxAK.

Intron and promoter region analysis of HaAK

The promoter region of the *HaAK* gene was cloned using the technique of genome walking. The sequence with 2 kb was obtained from the upstream region of the *AK* gene 5′ UTR (GenBank Accession No. EF600057) (fig. 3a, b). The sequence was deposited in the GenBank database as JN185455. The alignment analysis showed that there were two introns and two exons in the *HaAK* gene (fig. 3c). The first intron was located in the 5′UTR, while the second one was removed to form the ORF. Only the second intron was consistent with the GT–AG rule. The typical TATA box was not found by the Promoter Scan. Several different types of potential transcriptional factor binding sites, including Bicoid (Bcd), Deformed (Dfd), Hunchback (Hb), AP-1 binding sie (AP-1), Heat Shock Factor (HSF), Chorion Factor 1 (CF1), Tramtrack 69 K (Ttk 69 K), Broad-Complex Z4 (BR-C Z4), Dorsal (Dl), Hairy, and Crocodile regulator of head development (Croc) were identified in the promoter of *HaAK* gene using TFSEARCH online program (fig. 3d and table 2).

Expression profiles of the HaAK *gene between 96S and Bt-R strains*

To monitor the tissue and stage-dependent expression profiles of the *HaAK* gene in *H. armigera*, qRT-PCR was performed to investigate expression levels of the *HaAK* gene in different tissues and at different development stages of 96S and Bt-R. R^2 of the standard curve was 0.9983 and E was 97.65%, meeting the experimental requirements ($R^2 > 0.999$, $E > 90\%$).

As shown in fig. 4a, b, *HaAK* was extensively and differentially expressed at different larval stages and in various tissue types. The level of *HaAK* transcripts gradually increased from second to fifth-instar larval stages. *HaAK* gene expression at the fifth-instar stage was highest in the midgut, and lowest in the epidermis. *HaAK* expression in Bt-R has the same trend as well. Meanwhile, compared with 96S, the *HaAK* expression maintained significantly lower in Bt-R. Moreover, the HaAK enzyme activity was analysed in the body (without midgut) and midgut of fifth larvae. The HaAK activity was highest in midgut of 96S (fig. 4c).

FISH analysis of HaAK *expression in 96S*

To further investigate the expression of *HaAK* in detail, FISH assay was conducted to analyse the expression of *HaAK* gene in tissues of the third larvae using the probe derived from ORF-specific fragment of *HaAK* gene. The 884 bp amplified fragment corresponding to 133–1016 nucleotides of *HaAK* ORF was labelled by Alex-488 and was used as the probe. The fluorescence intensity of probe was detected, indicating successful combination with Alex-488. As shown in fig. 5, *HaAK* was expressed in epidermis, muscle, midgut of the 96S larvae, which is consistent with the qRT-PCR results above.

RNAi-mediated suppression of HaAK *expression in 96S larvae*

To dissect the physiological function of *HaAK* in *H. armigera*, RNAi was employed to suppress the expression of endogenous *HaAK* (fig. 6a). In both control (CK and RNAi-CK) groups, the expression of *HaAK* increased from 2nd to 10th day post ingestion (dpi), and then decreased at 12 dpi. In RNAi-*HaAK* groups, although the expression pattern of *HaAK* was similar to that of CK and RNAi-CK, the expression level was largely reduced at 2nd, 4th, 6th, 8th, and 10th dpi compared with that of CK and RNAi-CK groups. These results

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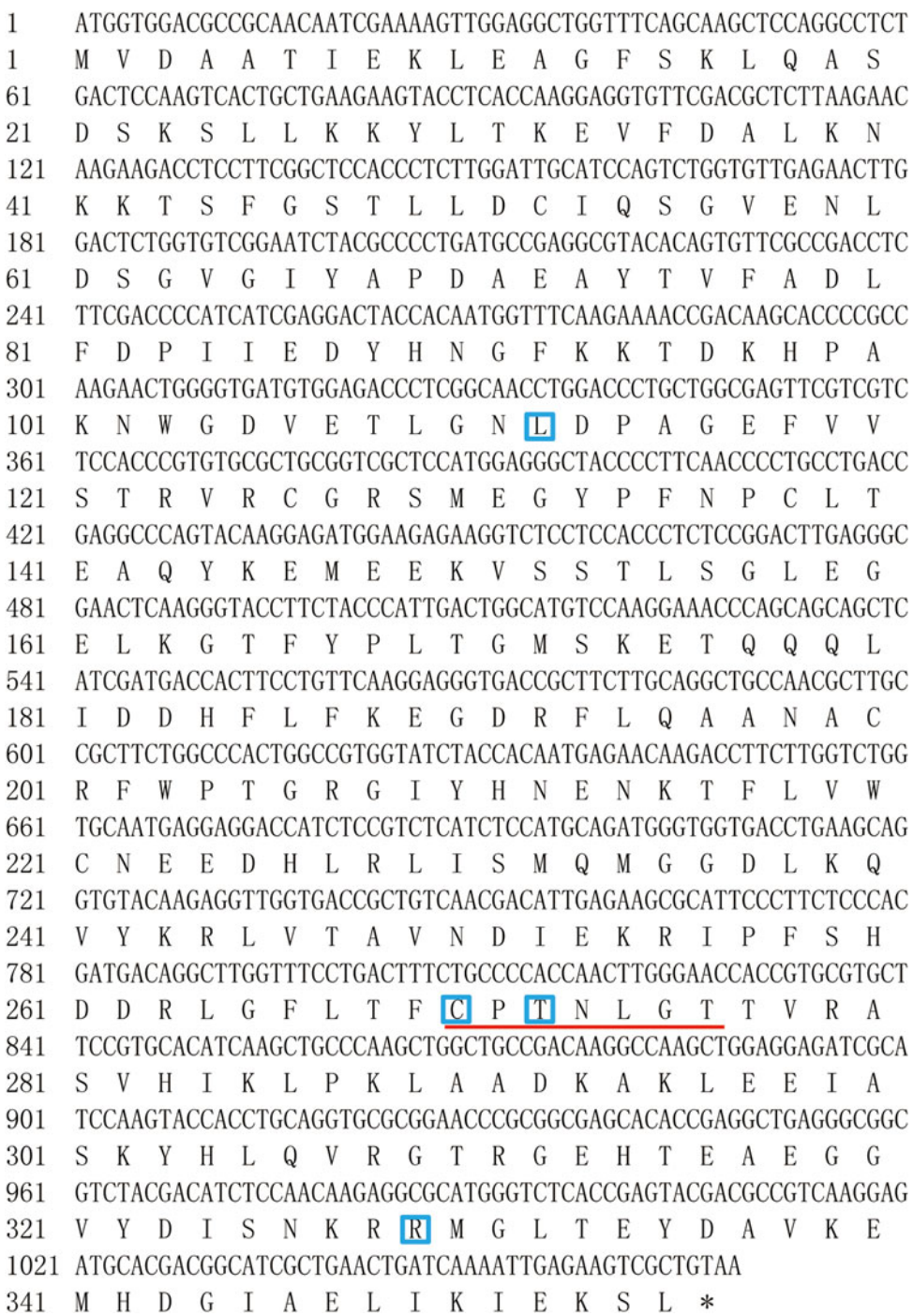

Fig. 1. The ORF sequence of the *HaAK* gene. The ORF and deduced amino-acid sequences of *HaAK*. Nucleotide and deduced amino-acid residues are numbered on the left. * indicates the stop codon. The active sequences are underlined and the active sites are marked with boxed, respectively.

directly demonstrated that the *HaAK* transcripts were silenced after feeding of bacterially expressed dsRNA in *H. armigera*.

Ecological analysis of HaAK-*silenced 96S larvae*

We further investigated the ecological parameters including survival amount and pupation ratio over time in *HaAK*-silenced *H. armigera*. As shown in fig. 6b, the survival amount was significantly lower in *HaAK*-silenced (RNAi-*HaAK*) groups than in control groups (CK and RNAi-CK) ($P < 0.05$). Compared with control groups, the pupation of RNAi-*HaAK* group was dramatically delayed ($P < 0.05$) (fig. 6c; Supplementary 1). The pupation ratio of RNAi-*HaAK* group was decreased by 3.15% at 9 dpi, 17.08% at 10 dpi,

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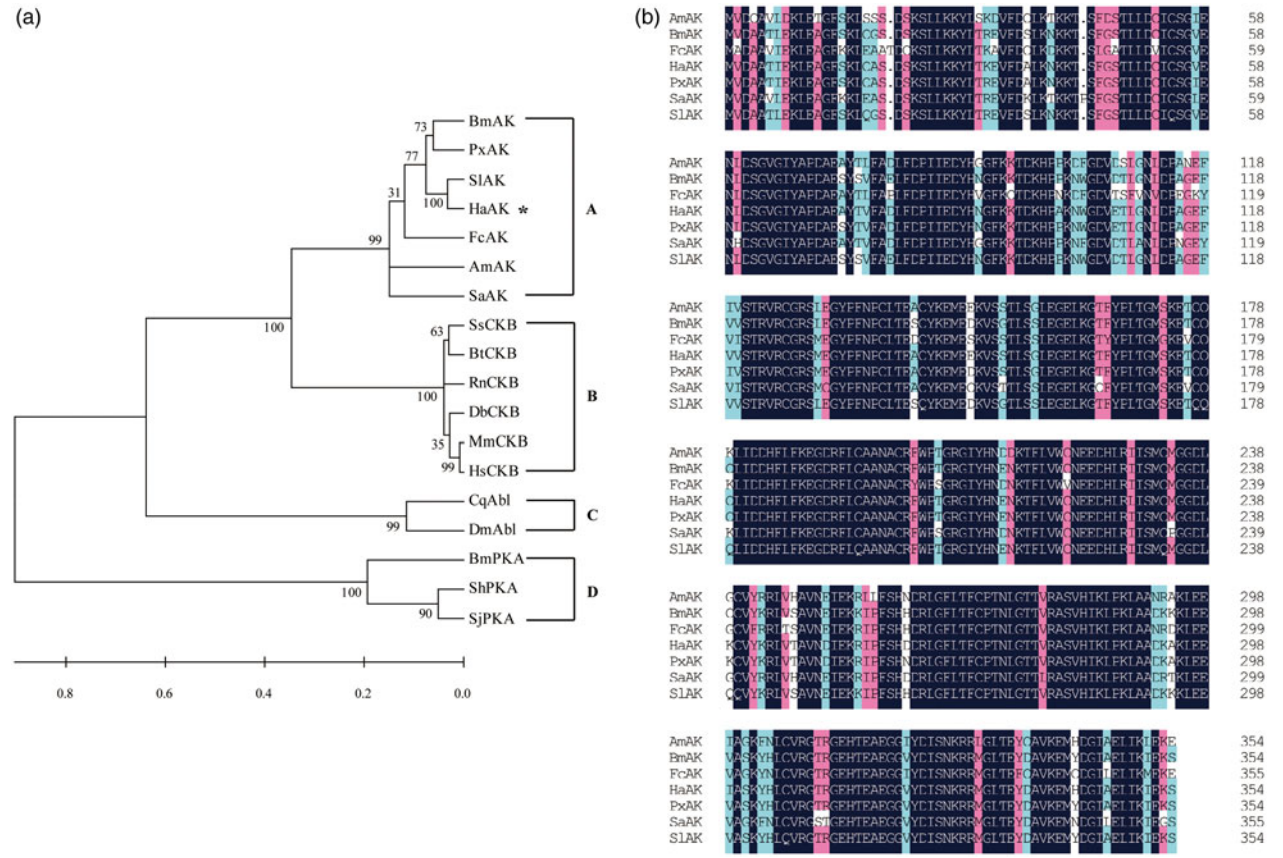

Fig. 2. The sequence analysis of the *HaAK* gene. (A) Phylogenetic analysis of the nucleotide sequences of *HaAK* and 17 additional phosphokinase sequences. Phylogenetic tree constructed by neighbour-joining algorithms of MEGA 5.0 software after the multiple phosphokinase sequences alignment using the CLUSTAL W program. Bootstrapping was performed 1000 times to obtain support values for each branch. Four groups of phosphokinase sequences, including AK, creatine kinase, tyrosine-protein kinase, and cAMP-dependent protein kinase, were represented by letters A, B, C, and D, respectively. The GenBankaccession numbers of phosphokinase sequences are as follows: *HaAK* (GU396008), *AmAK* (001011603.1), *BmAK* (FJ013046.1), *FcAK* (AY661542.1), *PxAK* (AK401168.1), *SaAK* (U77580.1), *SlAK* (HQ840714.1), *ShPKA* (GU116484.1), *BmPKA* (001099833.1), *SjPKA* (GU130533.1), *CqAbl* (001845615.1), *DmAbl* (NM_001275032.1), SsCKB (NM_001243575.1), *BtCKB* (NM_001015613.1), *DbCKB* (M13453.1), *MmCKB* (NM_001267031.1), *RnCKB* (NM_012529.3), and *HsCKB* (NM_001823.4). (B) Alignment of *HaAK* (GU396008), *SaAK* (AAC47830), *AmAK* (NP_001011603), *FcAK* (AAV83993), *BmAK* (ABB88514.1), *SlAK* (ADW94627.1), and *PxAK* (BAM17790.1). The predicted amino-acid sequences and alignment were performed using DNAMAN software (Version 6.0).

20.00% at 11 dpi, and 20.75% at 12 dpi, when compared with the RNAi-CK. The decreasing range from 9 and 12 dpi was 3.15–20.75%, and the mean was 15.35%. Likewise, the enzyme activity of HaAK was also measured in *HaAK*-silenced *H. armigera*. The significant effect of dsRNA on HaAK activity showed in fig. 6d indicates that the HaAK activity of RNAi-*HaAK* group was also impaired compared with CK and RNAi-CK groups.

Discussion

Since the extensive use of biotic and chemical measures to control the *H. armigera*, increasing resistance against the management was discovered in laboratory and field (Tabashnik, 1994; Soberon *et al.*, 2009; Tabashnik *et al.*, 2013). Thus, the regulation factors involved in the development of *H. armigera* are considered to be of marked concern (Zhao *et al.*, 2004; Xiong *et al.*, 2013; Chen & Xu, 2014). In this study, *HaAK* was isolated from the 96S and Bt-R larvae. HaAK contained a CPTNLGT domain (residues 270–276) and four conserved residues (C270, L112, T272, and R329) of AK. The amino-acid residues CPTNLGT located at C-terminus formed a functional domain (Uda & Suzuki, 2004). The C270 was demonstrated to be involved in holding AK activity and constraining the orientation of the substrate arginine (Guo *et al.*, 2004), while L112, T272, and R329 played key roles in AK activity, substrate synergism, and structural stability, respectively (Li *et al.*, 2013; Wang *et al.*, 2013; Wu *et al.*, 2014). The *HaAK* expression analysis indicated that it may participate in the process of larval development. As the major tissue for food digestion and detoxification, the midgut requires high amount of energy, the increase in the expression level of *HaAK* can be due to this reason (Lauzon *et al.*, 2003; Citelli *et al.*, 2007; Xu *et al.*, 2014). The down-regulated *HaAK* expression was significantly discovered in different larval stages and tissues of Bt-R strain. This may be related to the increased fitness costs, including larval

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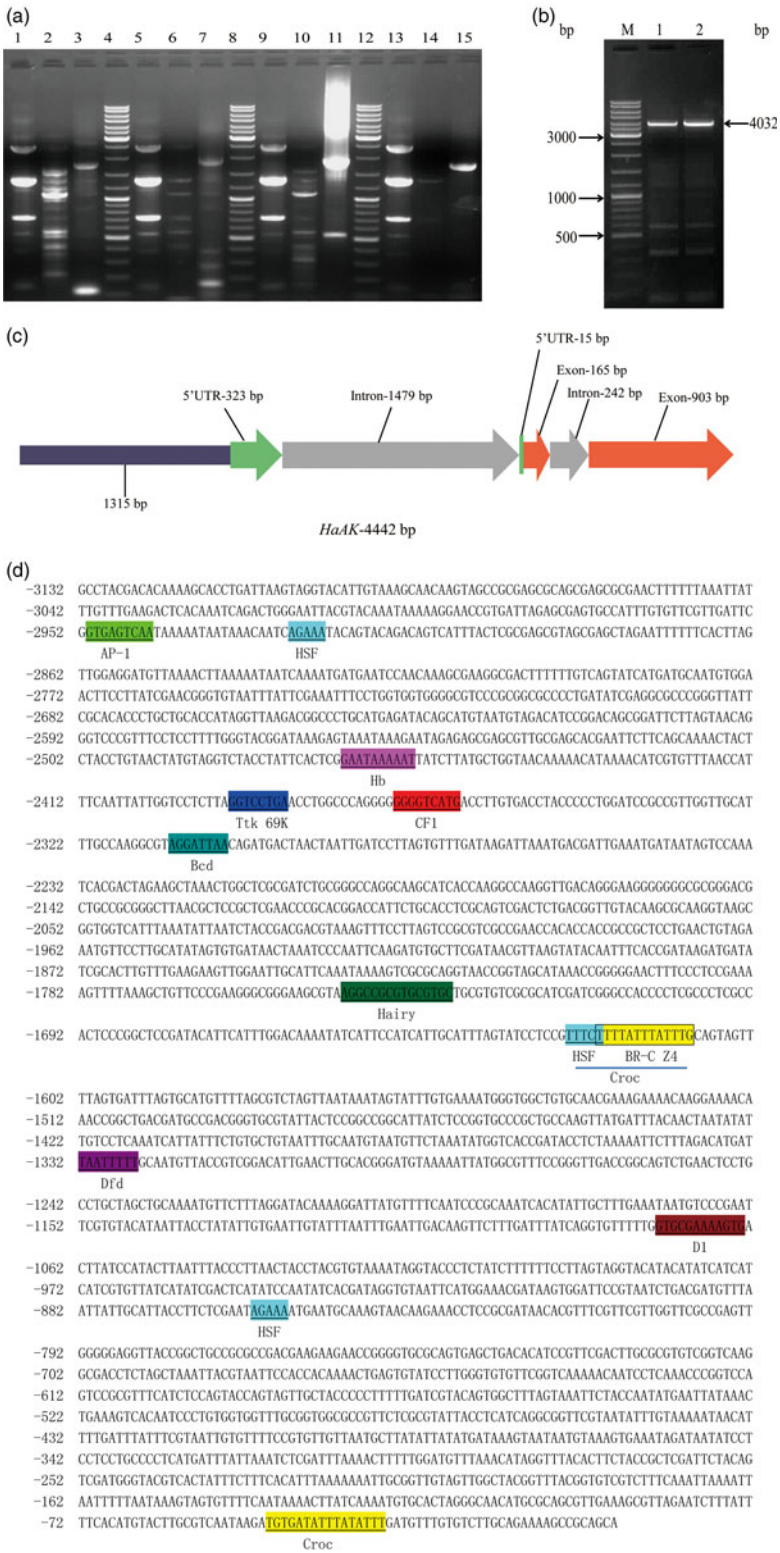

Fig. 3. Analysis of the promoter region of the *HaAK* gene. (A) Amplification of the *HaAK* promoter by Genome Walking. Lanes 1, 2, and 3: results of the first, second, and third AP1 primer; lanes 5, 6, and 7: results of the first, second, and third AP2 primer. Lanes 9, 10, and 11: results of the first, second, and third AP3 primer; lanes 13, 14, and 15: results of the first, second, and third AP4 primer; lanes 4, 8, and 12: DNA ladder (Fermentas, USA). (B) Detection of the *HaAK* gene promoter. M: DNA Marker; 1, 2: promoter amplification. (C) Prediction of the structure of the *HaAK* gene. (D) Transcriptional factor binding sites of *HaAK* gene at 5′ flanking region.

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Table 2. Transcription factor binding sites of *HaAK* gene at 5′ ‘flanking region’

Transcrption factor	Binding site	Location
Bcd	AGGATTAA	−2310—2302 (+)
Dfd	TAATTTTT	−1332—1324 (+)
Hb	GAATAAAAAT	−2467—2457 (+)
AP-1	GTGAGTCAA	−2951—2942 (+)
HSF	AGAAA	−2924—2919 (+); −859—854 (+)
HSF	TTTCT	−1627—1622 (−); −234—229 (−)
CF1	GGGGTCATG	−2370—2361 (+)
Ttk 69 K	GGTCCTGA	−2392—2384 (+)
BR-C Z4	TTTTATTTATTTG	−1623—1610 (−)
Dl	GTGCGAAAAGTG	−1075—1063 (+)
Hairy	AGGCCGCGTGCGTGC	−1747—1732 (−)
Croc	TGTGATATTTATATTT	−47—31 (−)
Croc	TTTCTTTTATTTATTTG	−1627—1610 (−)

The translation initiation site is numbered as 1.

weight, survival rate, life-history traits, etc. (Gassmann *et al.*, 2008; Cao *et al.*, 2014; Ahmad *et al.*, 2015).

Previous studies indicated the involvement of AK in the regulation of innate immune ability. It seemed to modulate the adaption of insects to adverse environments (Voncken *et al.*, 2013; Pereira, 2014). In this study, a series of putative transcriptional factor-binding sites predicted in promoter region have been reported to play important roles in larval development, resistance, and hormone responses. Bcd, Ttk 69 K, and BR-CZ4 were identified to be involved in a tissue-specific response to the steroid hormone ecdysone (Macdonald & Struhl, 1988; Read & Manley, 1992; von Kalm *et al.*, 1994). Dfd, Croc, CF1, Hb, Hairy, and AP-1 were found to negatively regulate the larval development (Regulski *et al.*, 1987; Perkins *et al.*, 1988; Stanojevic *et al.*, 1989; Christianson *et al.*, 1992; Fernandes *et al.*, 1994; Van Doren *et al.*, 1994; Hacker *et al.*, 1995). These indicated that the *HaAK* may be involved in hormone signalling pathway and larval development.

RNA interference (RNAi) has been proved as a powerful tool in functional genomic research of insects. Furthermore, it has considerable potential for the control of pest insects. To date, a number of successful RNAi experiments have been carried out in several different lepidopteran species (Quan *et al.*, 2002; Belles, 2010; Apone *et al.*, 2014). Many developmental indexes, including imaginal mortality, fecundity and fertility, were impaired following the ingestion of a dsRNA targeting *AK* in *Phyllotreta striolata* (Zhao *et al.*, 2008). After feeding *H. armigera* with transgenic *Arabidopsis* plants containing dsHaAK, expression of *HaAK* was found to be dramatically decreased. Mortality rate was much lower than the control group (Liu *et al.*, 2015). Considering the thick chitin in the skin of *H. armigera*, we employed feeding experiments to silence the *HaAK* gene. After *HaAK* expression silenced, the efficiency of energy metabolism was affected, which significantly reduced the larval development and survival ($P < 0.05$). Thus, the supression of *HaAK* gene and its impaired activity in this study suggests this gene to be an attractive target for *H. armigera* management.

In summary, we cloned the sequence of promoter and ORF of AK from *H. armigera*. Although typical TATA box was not discovered, massive binding sites of transcriptional factors were predicted, which suggested *HaAK* was involved in entomic

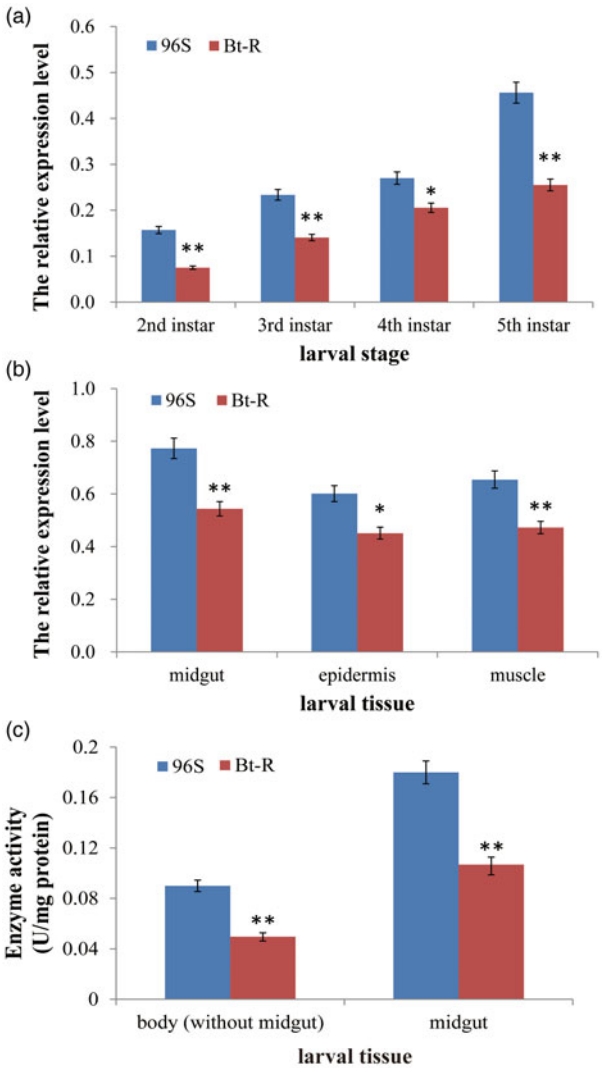

Fig. 4. Expression patterns of the *HaAK* gene in 96S and Bt-R larvae. (A) Relative expression levels of the *HaAK* gene at different life-stages. (B) Relative expression levels of the *HaAK* gene in different tissues of fifth-instar larvae. The relative level of *HaAK* gene expression as shown on the *Y*-axis is the ratio of gene expression in each sample compared with the housekeeping gene (*EF-1a*). (C) Enzyme activity of HaAK in the different tissues of fifth larvae. Bars represent mean ± SD ($n = 3$). (** showed $P < 0.01$, * showed $P < 0.05$, *T*-test.)

development and resistance. The expression profile of *HaAK* was evaluated by qRT-PCR. Its expression was widely detected and specially regulated in different tissues and at different developmental stages. Using dsRNA feeding methods, the function of *HaAK* gene was preliminarily explored. Silencing of *HaAK* could significantly retard the larval development. These results indicated that RNAi targeting *HaAK* may be an effective method for controlling this agricultural herbivorous pest.

Supplementary Material

The supplementary material for this article can be found at http://www.journals.cambridge.org/BER

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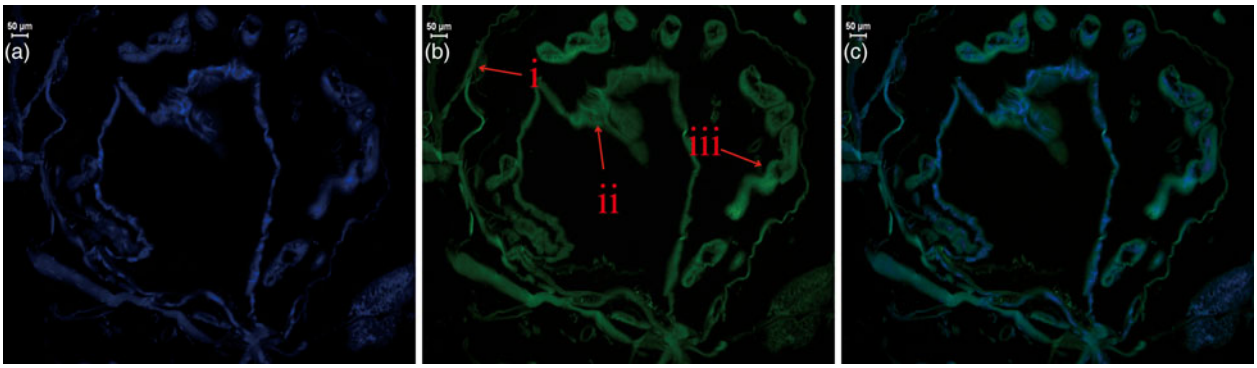

Fig. 5. FISH analysis of the expression of *HaAK* in the third-instar larvae of 96S. The sample was transected. The probe was labelled with the Alex-488-dUTP. Scale bars: 50 μm. i: epidermis; ii: muscle; iii: midgut. (a: Note the distribution of nucleated cells (blue); b: hybridized signal of the *HaAK* gene (green); c: a and b merged).

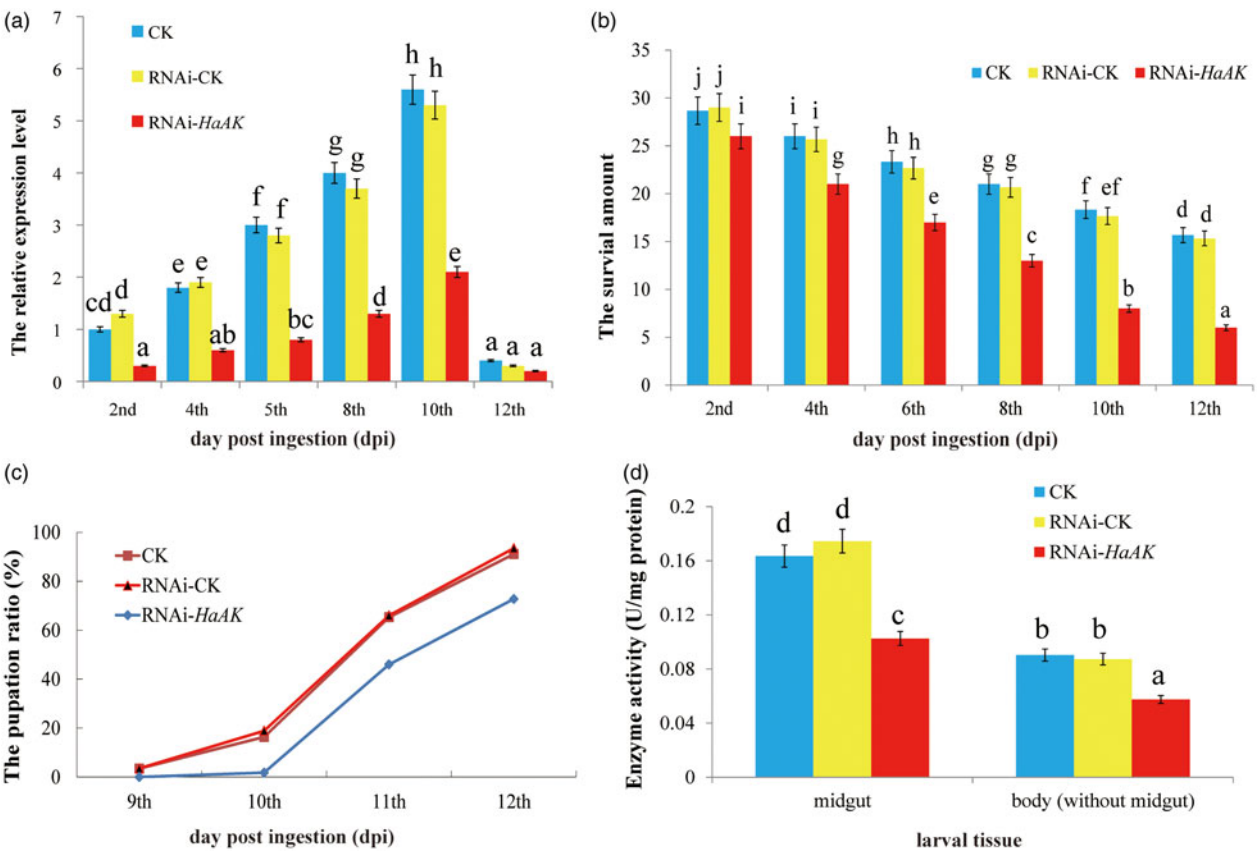

Fig. 6. Effects of ingesting bacterially expressed dsRNA on *HaAK* gene in 96S. (A) Silencing of *HaAK* in *H. armigera* by feeding bacterially expressed dsRNA. Total RNA was extracted from three groups larvae fed on varying diets and dsRNA. Expression levels of *HaAK* were detected by qRT-PCR. The housekeeping gene *EF-1a* was used as a reference. (B) Survival amount of *H. armigera* in CK, RNAi-CK, and RNAi-*HaAK* group. (C) Pupation ratio analysis. (D) HaAK enzyme activity analysis. The tissues were detached at tenth continuous feeding. The data were conducted in triplicate with the same results. Bars represent mean ± SD ($n = 3$; $P < 0.05$, Duncan's test). The data were analysed by SAS (SAS Institute 2001). The data with different letters are significantly different from each other ($P < 0.05$).

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