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RNA interference-mediated knockdown of some genes involved in digestion and development of *Helicoverpa armigera*

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Abstract

Helicoverpa armigera is a significant agricultural pest and particularly notorious for its resistance to many types of common insecticides. RNA interference (RNAi) is a mechanism of post-transcriptional gene silencing and trigged by double-strand RNA (dsRNA), has become a widely used reverse genetics and potent tool for insect pest control. In this study, the effect of ingestion and injection delivery methods of dsRNA related two important enzyme genes, α-amylase (*HaAMY48, Ha-AMY49*) and juvenile hormone esterase (*Ha-JHE*), were examined on growth and development of *H. armigera*. After 24, 48, 72 and 96 h of feeding bioassay, significant down regulation was observed about; 56, 68, 78, 80.75% for *HaAMY48*, 60, 70, 86.5 and 96.75%, for *Ha-AMY49* and 14, 27.5, 23 and 31.7% for *Ha-JHE*, respectively. The results for injection assay was 61.5, 71.5, 74 and 95.8% for *Ha-AMY48*; 70, 88, 91.5 and 97.7% for *Ha-AMY49* and 22, 61, 75 and 74% for *Ha-JHE* after 24, 48 and 72 h of last injecting, respectively. Larvae that treated with dsRNA, fed or injected, lost more than half of their weight. 50% mortality in treated larvae was observed in the case injection bioassay with ds*Ha-JHE* and 59% of larvae that fed of dsRNA-treated cubes survived. Ds*Ha-AMY48* and *49* have significant mortality, but mixing of them is more effective in both bioassays. Injection bioassay has a potent inhibitory effect on α-amylase-specific activity about more than 87% in treated larvae with mix of ds*Ha-AMY48* and *49*. Adult malformation percent was evaluated for feeding (28, 35.5 and 43% for *Ha-AMY48, 49* and *Ha-JHE*, respectively) and injection bioassay (23, 42 and 29% for *Ha-AMY48, 49* and *Ha-JHE*, respectively). All these finding suggest that *Ha-AMY48*, *Ha-AMY49* and *Ha-JHE* can be new candidates to scheming effective dsRNAs pesticide for *H. armigera* control.

Keywords: RNAi, dsRNA, α-amylase, juvenile hormone esterase, *Helicoverpa armigera*

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Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a highly serious and major lepidopteran insect pests that producing severe damages to many economically main crops universal (Challa *et al*., 2013). *H. armigera* attacks causes US$ 17.7 billion financial damages every year. Because of its polyphagous nature, more than 200 plant species are not safe from its damages and control of this

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pest is problematic due to high fecundity and fast adaptation to synthetic insecticides (Ghosh *et al.*, 2010; Devi *et al.*, 2011). On the other hand, wide applying of insecticides, cause this pest resistance to most of them (Scott & Wen, 2001; Heckel, 2012). Today the destructive effects resulting from the use of pesticide on human health by contaminating environments is clear completely (Dietz & van der Straaten, 1992; Chikate *et al.*, 2016). Maybe one of the proven ways for controlling of *H. armigera* is transgenic plants producing Bt toxins, but because of large cultivation and wide usage, the resistance process is likely (Chikate *et al.*, 2016). So this is necessary to develop and use of new strategies for control of this pest and another pests without damages to the environment, natural enemies and humans.

As a modern influential and accurate tool, RNA interference (RNAi) has been widely applied in many aspects and powerfully accelerates the progress of life knowledge (Fire *et al.*, 1998; Aravin *et al.*, 2001). Despite the fact that the RNAi pathways operate by highly conserved strategies in different organisms, they comprise different proteins and mechanisms. RNAi is a post-transcriptional regulator tool involving sequence-specific degradation of a target mRNA and now it is obvious that gene silencing is a conserved mechanism in numerous eukaryotes and it has been used in entomological investigation, too (Geley & Muller, 2004; Ghanim *et al.*, 2007). RNAi is triggered by double-strand RNA (dsRNA) structures and the specificity is based on the sequence of one strand of these structures consistent to partial or complete sequence of a specific gene transcript (Siomi & Siomi, 2009). This mechanism is interceded by the creation of small interfering RNAs (siRNAs) from the dsRNA. They are cleaved by dsRNA-specific endonucleases such as dicers (from the dicer gene) identified in *Drosophila melanogaster*. Wherein Dicer RNase III type enzymes bind and digest cytoplasmic dsRNAs and change them to siRNAs that have 21–23 nucleotides. These siRNA cleavage products then function as sequence-specific interfering RNA in transcript income, cleavage and translational inhibit (Yoo *et al.*, 2004).

In recent years, RNAi technique has been used to study gene function in various organisms and also exhibited remarkable changes in pest control (Hannon, 2002; Gordon & Waterhouse, 2007; Price & Gatehouse, 2008; Huvenne & Smagghe, 2010). However, successful RNAi is difficult to be achieved in some insects, especially for Lepidoptera (Terenius *et al.*, 2011), but in many studies acceptable outcome is achieved and maybe it need and this is a rationale reason for further study on this group of insects by this technique. In a new study, the effect of RNAi technique of selected candidate genes such as serine and cysteine proteases and another gene on growth and development of *H. armigera* was studied. The dsRNA-fed larvae showed significant reduction in the larval mass and abnormalities at the various stages of *H. armigera* development compared with their control diet (Chikate *et al.*, 2016). In another study, was showed that gene silence effect in insects with various dsRNA delivery methods consist mainly of injection, ingestion, soaking and feeding with transgenic plant, could be persuaded with most of these methods (Yu *et al.*, 2013). The data of a study on effects of period RNAi on V-ATPase expression and rhythmic pH changes in the vas deferens of *S. littoralis* demonstrate that the upper vas deferens molecular oscillator involving the period gene have an crucial role in the regulation of rhythmic V-ATPase activity and periodic acidification of the lumen (Kotwica-Rolinska *et al.*, 2013). Wang *et al.* (2013) screened *H. armigera* 3-hydroxy-3-methylglutaryl coenzyme A reductase and silenced it using systemic RNAi. This reduced the fecundity of the females, effectively inhibited oviposition and significantly reduced vitellogenin mRNA levels. Another studies can be referred that exhibited down regulation on the specific gene with dsRNA feeding such as silencing in chitin synthase in *Spodoptera exigua* (Chen *et al.*, 2008) production of a transgenic tobacco that expressed ecdysone receptor encoding (EcR) dsRNA from *H. armigera* (Zhu *et al.*, 2012) and RNAi on acetyl cholinesterase in *H. armigera* (Kumar *et al.*, 2009). The effects of siRNA on seven target genes on *Bombyx mori* embryos were determined. Results show that injection of siRNA is an operative reverse-genetics instrument for the study of embryogenesis *in vivo* in insects (Yamaguchi *et al.*, 2011). In a control pest study through RNAi on western corn rootworm *Diabrotica virgifera virgifera* was surveyed 290 possible target genes in by feeding of artificial diet (AD) with dsRNA and the results displayed that only 14 genes were established specific down regulation and caused in notable larval stunting and death (Baum *et al.*, 2007). A previous study in the Lepidopteran *Spodoptera litura* found that injection of dsRNA could competently down regulate the amino peptidase-N expression level in larval midgut, but feeding bioassay of dsRNA did not induce any RNAi effect (Rajagopal *et al.*, 2002).

Maybe the first step for a successful RNAi for control of a pest is to find a target gene (Jing & Zhao-jun, 2014). This gene can be an enzyme such as α-amylase and Juvenile hormone esterase (JHE). Alpha-amylases (α−1,4-glucan-4-glucanohydrolases; E.C. 3.2.1.1) belong to an important category of digestive enzymes, in some of substrate such as starch, glycogen and various other related carbohydrates catalyze the hydrolysis of α-D-(1,4)-glucan linkages (Franco *et al.*, 2000). At the end, they convert starch to oligosaccharides, which are supplementary hydrolyzed to glucose by α-glucosidase and make a resource full of energy (Zeng & Cohen, 2000). In insects, α-amylases improvement insect's digestive capability, help them to survive in varied situations and upsurge their fitness rate. Even throughout non-feeding phases such as pupa, amylases are also active that refers to the importance of these group of enzymes for development stages of insects (Zhu *et al.*, 2005).

JHE is a carboxylesterases (COEs) that has got much attention for its crucial role in metamorphosis regulating in arthropods and insects specially. The key hormone, juvenile hormone (JH) is hydrolyzed by JHE and titer of it is controlled by JHE, too. Growth, development, metamorphosis, diapause and reproduction in insects are critical options that are controlled by JH (Denlinger, 1985; Riddiford *et al.*, 2003). Before the beginning of metamorphosis, a reduction in the biosynthesis of JH and an increase in JHE activity happens (Roe & Benkatesh, 1990). This then creates the step for the promotion of ecdysteroid level (Mizoguchi, 2001). At the time of elevation in ecdysteroid levels for larval molts, JH typically exists and guarantees larval molt and going them to next instar although, at the time of the last instar molt, JH fades and ecdysone induces metamorphosis (Riddiford, 1996). All of these proven information show that α-amylase and JHE are critical enzymes and can be the excellent targets for RNAi.

In this study, for the first time we focused on this two enzymes and selected them as a target for RNAi on *H. armigera* to elucidate the effect of the direct injection and feeding bioassay on this pest. The results indicated that feeding and injecting bioassay of both α-amylase and JHE dsRNA induced RNAi in *H. armigera*. Effects of RNAi also elucidated on the enzyme

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activity level of α-amylase and malformation and abnormality on pupa and adults, and on expression of the mRNA level.

Materials and methods

Insect culture

The cotton bowllworms, *H. armigera* were collected from tomato plants at Manujan, Kerman Province, Iran in 2015 (27°26′N, 57°30′E). *H. armigera* larvae were reared in controlled chambers at 26±°C at 75 ± 5% relative humidity under a photoperiod of 14/10-h light/dark using an AD (Kotkar *et al*., 2012). Before to the 3rd instar, every five larvae were placed on a piece of the diet in the same container, and subsequently because of cannibalism the larvae were reared separately.

Sequences and phylogenetic analysis

The nucleotide sequences of the α-amylase and JHE were detected from GeneBank database of National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Within Blast Program available on this website the sequences was blasted as blastn. Then amino acid residues were translated within Lasergene, EditSeq builder software. Sequences were aligned by using Clustal W method using the MegAlign Software and phylogenetic tree was drawn with MEGA 6.0 software.

RT–PCR of α-amylase and JHE genes

Total RNAs from whole body of 1st to 5th instar larvae, pupae, adult moth and midgut, epidermis, fat body, hemolymph of 5th instar larvae were extracted with GeneAll RibospinTM total RNA purification Kit (GeneAll). First strand cDNA was produced from 1 µg RNA using HyperScriptTM First strand Synthesis Kit (GeneAll) with oligo dT18 primer. The synthesized single-stranded cDNA was used as a template for PCR amplification with gene-specific primers (table 1) with 35 cycles under conditions of 1 min at 95°C for denaturation, 1 min at 54°C for annealing and 1 min at 72°C for extension. PCR of β-actin was used as a control with specific primers (table 1).

Synthesis of dsRNA

Total RNAs from whole body and midgut for JHE and α-amylase, respectively, were extracted with GeneAll RibospinTM total RNA purification Kit (GeneAll). First strand cDNA was produced from 1 µg RNA using HyperScriptTM First strand Synthesis Kit (GeneAll) with oligo dT_{18} primer. The dsRNAs were designed targeting at α-amylase and JHE genes, which both encoded vital functional enzyme for *H. armigera*. Sequence analyses were done by CLUSTAL W (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Green fluorescent protein (GFP) was applied as a negative control. Primers were designed by NCBI's primer-BLAST web and using gene-specific sequences that were adapted to T7 promoter sequence at 5′ end that the primer sequences and amplicon length for production of dsRNA of each mRNA are given in table 1. cDNAs synthesized from mRNA of treated larvae, were used for amplification of target regions. DsRNA was synthesized using the MEGAScript dsRNA synthesis kit (Ambion, Huntingdon, UK) according to the manufacturer's instructions. After produced, dsRNAs were purified and determined concentration for quality and quantity by Nanodrop 1000 spectrophotometer (Thermo scientific, Waltham, MA, USA) and agarose gel electrophoresis 1%. DsRNAs amplicon from this step were used for injecting and feeding bioassays of *H. armigera*.

Ingestion of dsRNA

To assess the insecticidal activity of the dsRNA specific on the growth and development of *H. armigera*, surface of AD cubes (∼1 cm) were covered with 60 µg dsRNA solution and after diffusing into cube it was delivered to 4^{th}-instar larvae for all genes. As non-dsRNA control, AD covered with DEPC-treated water was applied. DsRNA targeting GFP was used as negative control. DsRNA for all the target genes were fed larvae three times with interval of 24 h (larvae were allowed to feed for 72 h). After this time, rearing was continued on normal AD (without dsRNA) to observe for larval growth and development. 24, 48, 72 and 96 h post-feeding on normal AD, silencing effects were studied. Fifty insects were treated in each treatment, and the experiment was replicated for three times. After each time 20 larvae from each of groups were picked up randomly and were placed in liquid nitrogen for another experiments.

Injection of dsRNA

The 4th-instar larvae of cotton bollworms were ice anesthetized and 4 µl of the dsRNA solution was injected into larval hemocoel through the penultimate abdominal segments (Jing & Zhao-jun, 2014). Like the section '*Ingestion of dsRNA*' dsRNA for all the target genes were injected to larvae three times with interval of 24 h (each treated larvae received 10 µg dsRNA). In the two control groups, the same volume of DEPC and ds*gfp* were injected. After injection AD was delivered and rearing continuous was on normal AD to observe for larval growth and development. Fifty insects were treated in each treatment, and the experiment was replicated for three times.

Quantitative real-time PCR analysis

Ten larvae were selected from each group in digestion and injection bioassay. The RNAs from each group were extracted with GeneAll RibospinTM total RNA purification Kit (GeneAll) from whole body for *Ha-JHE* and midgut (for *Ha-AMY48* & *49*). For the elimination of genomic DNA contamination, the total RNA was treated with DNase I, RNase-free (Fermentas, Thermo Scientific). The quality and quantity of RNA were determined by spectrophotometric analysis using Nanodrop (Thermo Scientific) and agarose gel electrophoresis. First strand cDNA was produced from using HyperScriptTM First strand Synthesis Kit (GeneAll) according to the manufactures instruction. The relative transcript level of target genes was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The primers were designed by using of IDTDNA web, https://sg.idtdna.com/PrimerQuest (table 1). For normalization of relative expression was used of 18srRNA gene (*Ha18s*), GenBank: AJ577253.1.

Alpha-amylase-specific activity

The dsRNA treated and control larvae were cold-immobilized, dissected under a stereoscopic microscope, and their midguts

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Table 1. Nucleotide primers used for RT–PCR, to synthesize dsRNA, and to perform qRT–PCR.

Class	Used dsRNA	Gene accession no.	Gene function	Adults malformation% in injection bioassay	Adults malformation% in feeding bioassay
Green-fluorescent protein	*dsGFP*	L29345	Localization of protein in living cell	N.O.	N.O.
Carbohydrates	*dsHaAM49*	EF600049	Improvement insect's digestive capability	23.76	28.22
	dsHaAM48	EF600048		42.25	35.51
	Mix of dsHaAM49 and dsHaAM48	–		48.32	40.03
Esterases	*HaJHE*	HM588760	Metamorphosis regulating, Growth, development, metamorphosis, diapause and reproduction	49.05	43.41

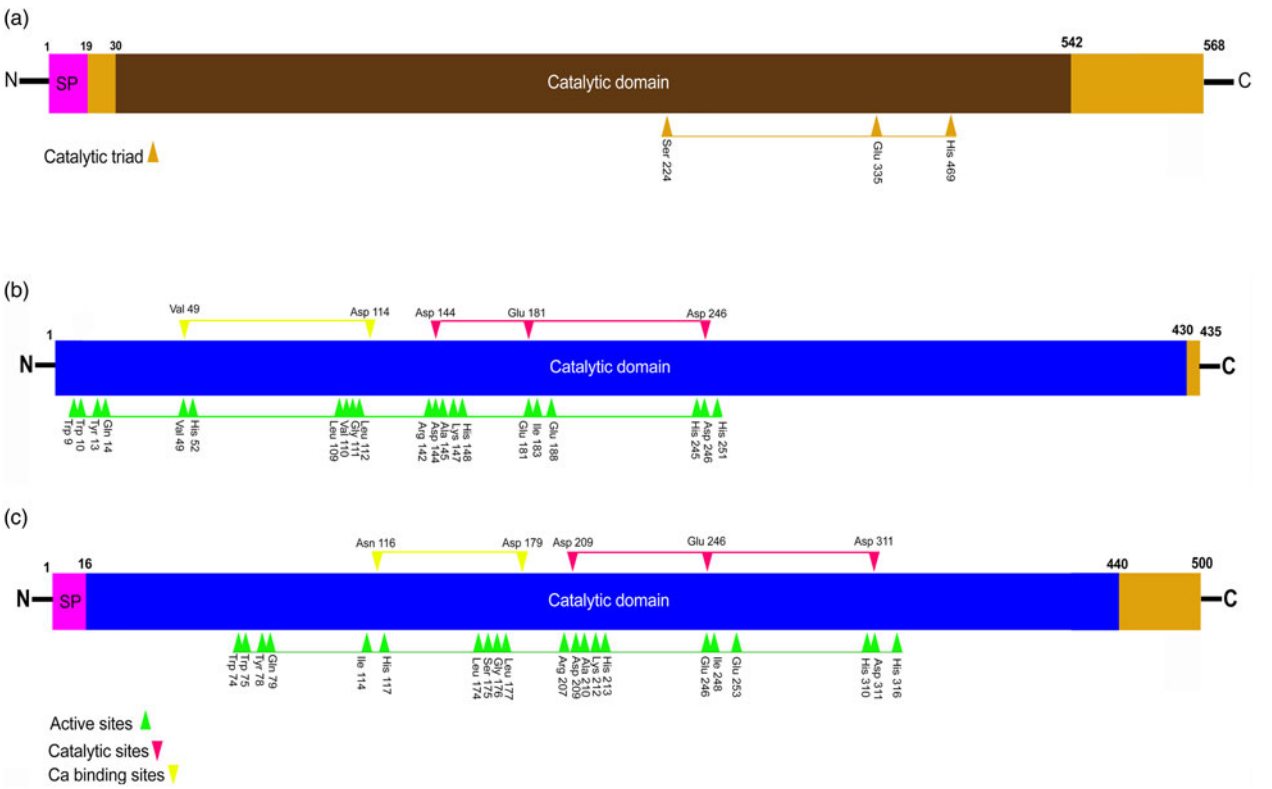

Fig. 1. Diagrams showing analysis of candidate enzymes amino acid sequences (a) *H. armigera* JH esterase protein (AEB77712), (b, c) *H. armigera* α-amylase proteins (ABU98613 & ABU98614) (Ha-AMY1 & 2), (SP; Signal peptide).

were removed. The midguts were then cleaned of adhering unwanted tissues and homogenized with a hand-held glass grinder on ice. The homogenates were centrifuged at 15,000 × *g* at 4°C for 15 min. The resulting supernatants were used as enzyme source for calculating α-amylase activity. Alpha-amylase activity was assayed according to Bernfeld (1955) using dinitrosalicylic acid (DNS) as the visualizing reagent and 1% soluble starch as the substrate. Briefly, 10 µl enzyme extract was incubated for 30 min at 35°C with 100 µl buffer and 15 ml soluble starch. The experiment was stopped with the addition of 75 µl DNS and heating in boiling water for10 min. The absorbance of the reaction mixture was read at 540 nm using a microplate reader. In the blanks, denatured enzyme extract (5 min heating in boiling water) was used instead of enzyme extract. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35°C. All assays were done in triplicate.

Visualization of α-amylase activity

In-gel α-amylase assay was performed using native-polyacrylamide gel electrophoresis (PAGE) for visualizing α-amylase activity. Enzyme extract was diluted in an electrophoresis sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue) and loaded in a gel of 5% stacking and 10% separating polyacrylamide gels. To

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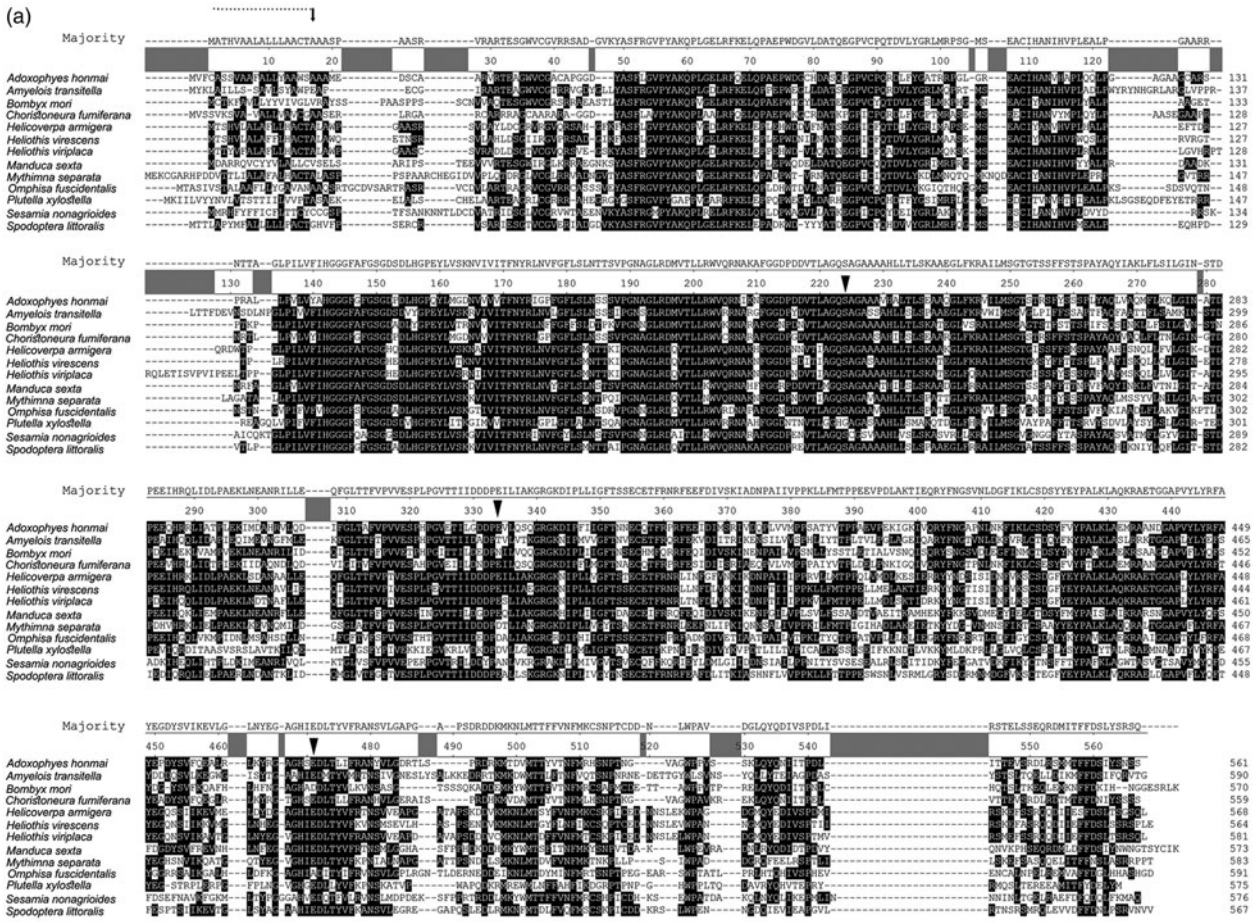

Fig. 2. Alignment and phylogenetic analysis of *Ha-JHE* and *Ha-AMY48 & 49* with its homologs from other insect species. (a, b) The identical amino acid residues that are conserved among all of the sequences are indicated by white letters in black background. The conserved α-amylase and JHE signal peptide (as indicated by a dotted line at the top of the upper panel) active site is indicated with arrowheads. The calcium-binding sites in α-amylase (AMY) seq.s are indicated with a black circle. The catalytic site is indicated with a gray diamond (a) alignment of JHE amino acid, (b) alignment of AMY amino acids). (c, d) The phylogenetic analysis is performed using the Seqman Clustal W program (DNAstar package, DNASTAR Inc., Madison) with 1000 bootstrap replicates at a Gap Penalty of 10 and a Gap Length Penalty of 0.2. (c) Alignment of AMY amino acids. (d) Alignment of JHE amino acid.

observe α-amylase activity bands after native PAGE, there solving gel was incubated in a 1% (w/v) starch solution for 60 min with gentle shaking. Finally, it was stained with a solution of 14 mM KI and 10 mM I_2. The bands of α-amylase activity appeared as clear areas in the field of black background of the gel.

Statistical analysis

Data were subjected to analysis of variance (ANOVA), and the means were compared by Student's *t*-test. Statistical analyses were performed using the software Statgraphics Plus 5.1.

Results

Sequences properties and phylogenetic analysis

A blast search using NCBI database determined three mRNA sequence for *H. armigera* α-amylase gene, *Ha-AMY* (Accession number: EF600048, EF600049 and EU325552) and one mRNA candidate genes with complete open reading frames (ORFs) for JHE gene, *Ha-JHE* (Accession no.: HaHM588760). Through this study for every gene one abbreviation was selected as following (EF600048: *Ha-AMY48*); (EF600049: *Ha-AMY49*); (EU325552: *Ha-AMY52*); (HM588760: *Ha-JHE*). In the case of *Ha-AMY*, two sequences, *Ha-AMY52* and *Ha-AMY49*, had 98% identity in Blast P when highly similarity option was selected. Between two this sequences *Ha-AMY49* was chosen. *Ha-AMY48* has 1485 bp in length and 1262 bp in ORFs. This sequence has three conserved domain. *Ha-AMY49* is some bigger than *Ha-AMY 48*, as 1502 bp located in it ORF region. *Ha-JHE* has 3011 bp of nucleotides about twofold rather than *Ha-AMY48* sequence and a long conserved domain with 512 bp. Each dsRNA were named with specific name related to their gene; ds*Ha-AMY48* for HaEF600048, ds*Ha-AMY49* for HaEF600049 and ds*Ha-JHE* for HaHM588760 mRNA. Translated amino acids of candidate genes were detected, and then the schematic pictures of amino acid sequences were prepared (fig. 1).

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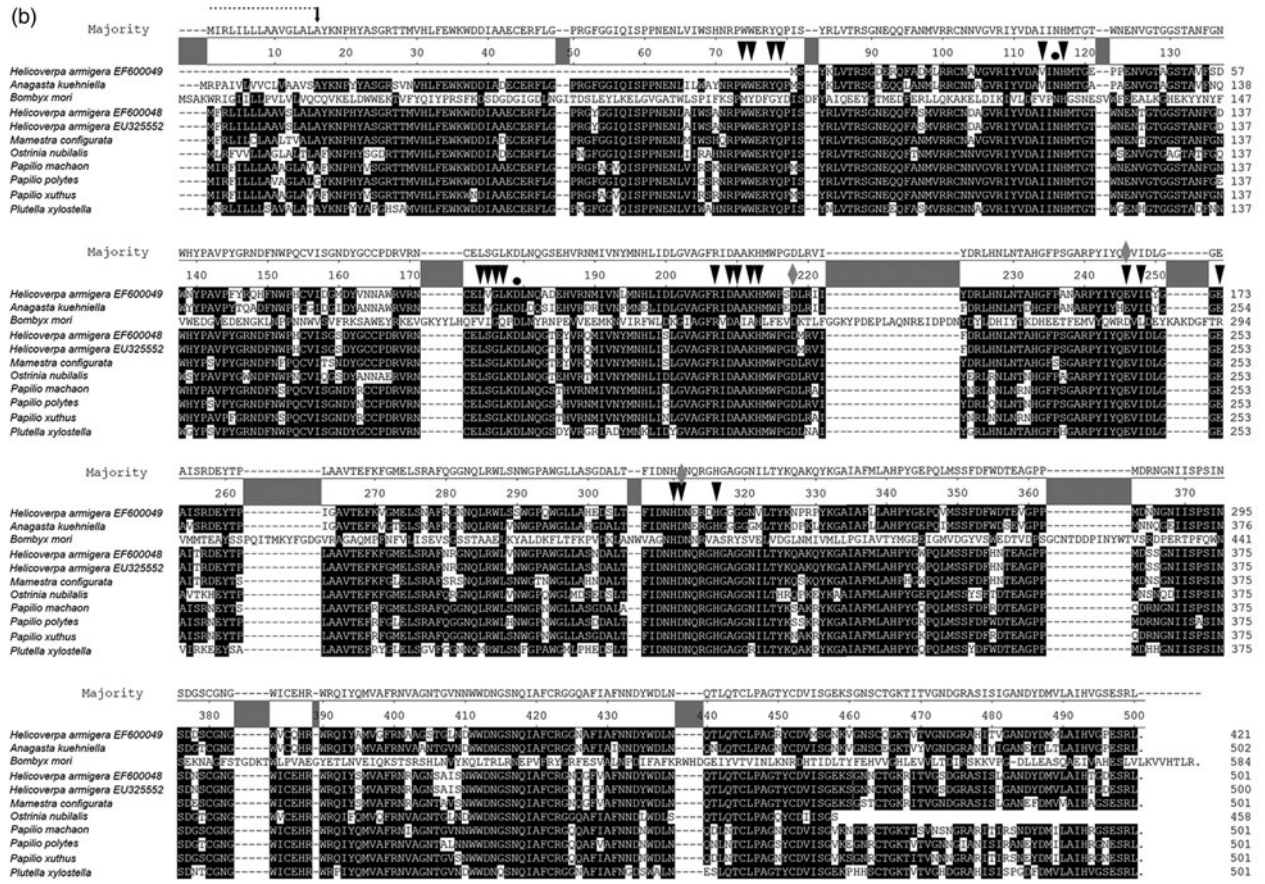

Fig. 2. Continued

Alignment and phylogenetic analysis tree for amino acid residues of candidate genes in *H. armigera* and another well-known animal proteins were drawn separately (fig. 2a, b). The GenBank numbers or NCBI reference sequence of alignment α-amylase proteins are following: (*H. armigera*: ABU98613, ABU98614, ACB54942) (*S. litura*: AGV76304); (*Spodoptera frugiperda*: AAO13754); (*Plutella xylostella*: XP_011548154); (*Papilio xuthus*: XP_013164404); (*Papilio polytes*: XP_013143960); (*Papilio machaon*: XP_014357550); (*Oatrinia nubilalis*: AAA03640); (*Mamestra configurata*: AEA76309); (*B. mori*: ACJ49024); (*Anagasta kuehniella*: ACL14798); (*Scirpophaga incertulas*: ABV24963); (*Apis mellifera*: BAA86909); (*D. melanogaster*: CAA59126); (*Diatraea saccharalis*: AAP92665); (*Homo sapiens* 1: AAA51724); (*H. sapiens* 2: BAA14130) and (*Mus musculus*: NP_001103975). The GeneBank number or NCBI reference sequence of JHE are provided: (*H. armigera*: AEB77712); (*Sesamia nonagrioides*: ABW24129); (*Spodoptera littoralis*: ACV60229); (*P. xylostella*: XP_011563398); (*P. machaon*: XP_014365132); (*Omphisa fuscidentalis*: ACB12192); (*Manduca sexta*: AAG42021); (*Mythimna separate*: AJR27471); (*Heliothis virescens*: AAB96654); (*Heliothis viriplaca*: AGB93712); (*Chilo suppressalis*: AIY69071); (*Choristoneura fumiferana*: AAD34172); (*B. mori*: AAR37335); (*Amyelois transitella*: XP_013197467); (*Agrotis ipsilon*: AGS49136); (*D. melanogaster*: AAK07833); (*A. mellifera*: AAU81605) and (*Adoxophyes honmai*: BAR88201.1). Neighbor-joining trees were constructed a Poisson-corrected distance with bootstrap test of 1000 replications. This analysis showed two α-amylases from *H. armigera* (ABU98614 and ACB54942) were closely related together but had not high relation with neighbor sequences. Our finding showed a neighbor relatively between *A. mellifera* and *H. armigera* amino acid seq. (ABU98613). All studied sequences divided into six groups that mammalian animals such as human and mice were in one group and near the two *H. armigera* seq.s. (fig. 2c). JHE amino acids divided into ten groups based on phylogenetic tree in (fig. 2d). JHE amino acid of our target pest is near to *Heliothis* genus amino acids.

Expression of candidate genes

We study on the expression of all candidate genes in development stages and some tissues of *H. armigera*. All target genes expressed in all larval stages from 1st to 5th and in pupa and adult, too. In the study of gene expression at some tissues, we found that all genes were expressed in midgut. Also *Ha-AMY49*, *Ha-AMY52* and *Ha-JHE* were expressed in epidermis and fat body (fig. 3).

Silencing of related mRNAs of H. armigera

Gene silence effect induced by dsRNA ingestion

For this experiment larvae were treated with poisoned AD to dsRNA. Gene expression analysis in most cases showed

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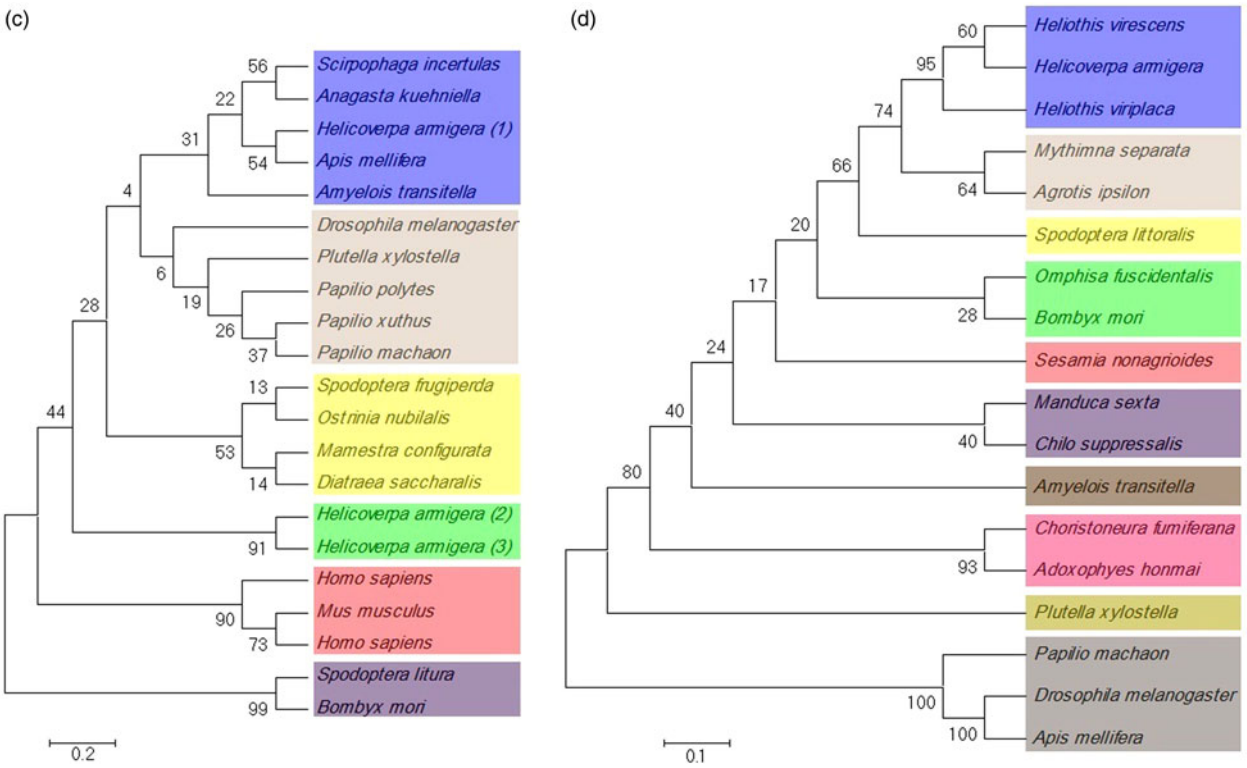

Fig. 2. Continued

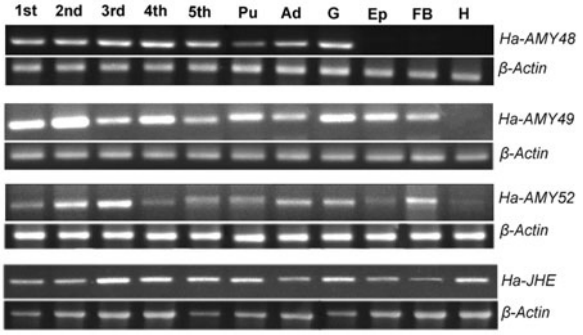

Fig. 3. Expression profiles of *Ha-AMY48*, *Ha-AMY49*, *Ha-AMY52* and *Ha-JHE* at different developmental stages from 1st-instar larvae to adult and in different tissues: midgut (G), epidermis (Ep), fat body (FB) and hemolymph (H).

significant RNAi effects of target sequences in fed larvae compared with adjacent larvae in the control group. After 24 h of third cube feeding, was observed decreasing in *HaAMY48*, *HaAMY49* and *Ha-JHE* expression 56, 59.7 and 14.4%, respectively. This slaking continued and were more after 48, 72 and 96 h after feeding last smeary cube to dsRNA. So that data after 96 h showed more than 96% RNAi effect on *HaAMY49* and a high suppression on *HaAMY48* (80%). Down regulation of *Ha-JHE* expression after 24, 48, 72 and 96 h had significant differences with control group. These results showed that dsRNA feeding induced interference effects on the expression of selective candidate with variable efficacy and time have an important role (fig. 4a–c).

Gene silence effect induced by dsRNA injection

In injection bioassay, three injections were done and the next experiment after third injection in three times, 24, 48 and 72 h, same ingestion bioassay, was done. All of gene expression results from Real-Time PCR analysis for these three candidates genes, showed significant suppression. *HaAMY48* mRNA expression was suppressed after 24, 48 and 72 h at a rate of 61.5, 71.5 and 74.3%, respectively. Same these results was happened for *HaAMY49* that was observed 70.5, 87.7 and 91.5% at 24, 48 and 72 h, respectively, after third injection of dsRNA compared with control groups. An impressive down regulation was observed about *Ha-JHE* expression, so that after 72 h, 75% of this gene was interfered by RNAi effects (fig. 5a–c). Figure 6 shows some phenotypic effects in larvae, pupa and adult that treated in the larval stage with *Ha-AMY* dsRNAs as injection.

Effects of ingestion and injection of candidate's genes dsRNA on survival, mass, growth performance and development of H. armigera

The effects of feeding bioassay on some biology aspects of *H. armigera* were studied. Among of these three genes, larvae that were reared on AD containing ds*Ha-JHE*, showed the lowest survival (60%), while this percent for larvae were fed with dsRNAs related to α-amylase, were about 80% for both *HaAMY48* and 49. The larvae growth on AD cubes containing an equal mix of these two dsRNAs with near 30% mortality, did not show significant effects. Same results were observed in injecting bioassay. About 50% of larvae after three injection

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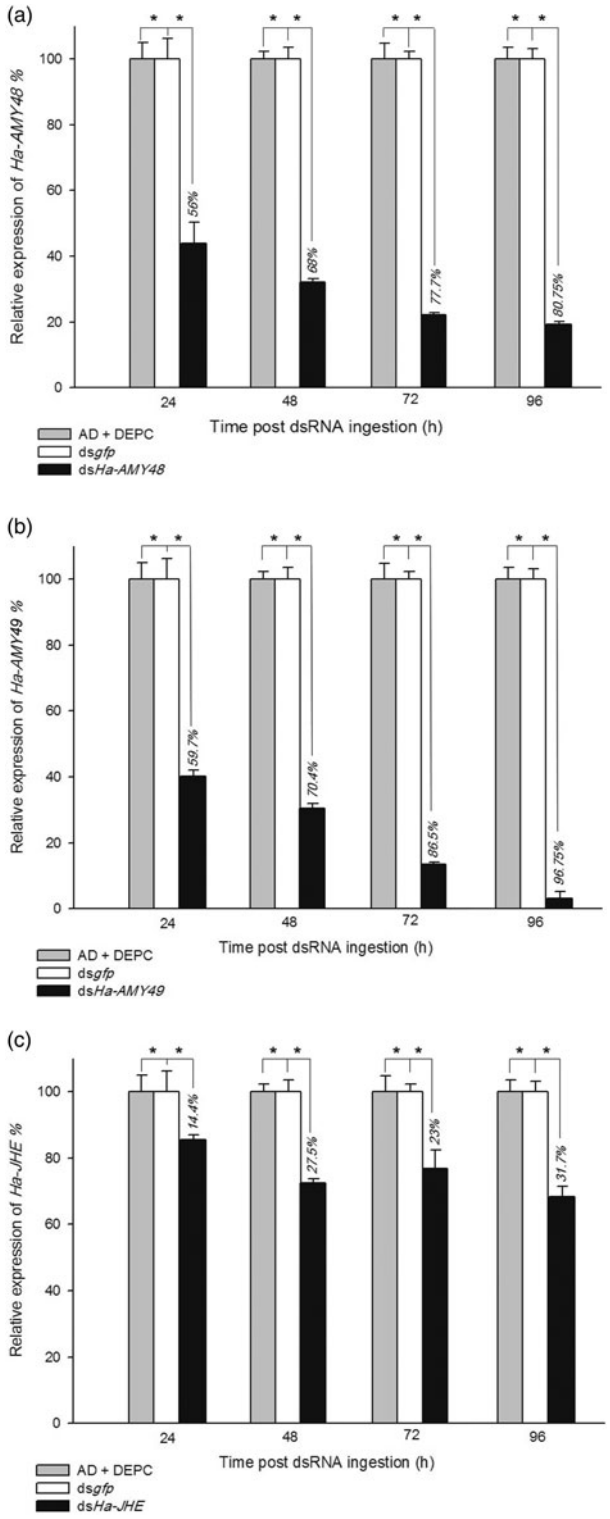

Fig. 4. Knockdown of *Ha-AMY48* (a), *Ha-AMY49* (b) and *Ha-JHE* (c) expression by ingestion of dsRNA. The larvae samples of each group were collected post after 24, 48, 72 and 96 h feeding of last treated AD cube. Non-dsRNA (DEPC) and ds *gfp* were used as control. Relativity was calculated basis of DEPC groups. All the experiments were triplicated. Asterisk indicates significant difference of relative expression ($P < 0.05$, Student's *t*-test).

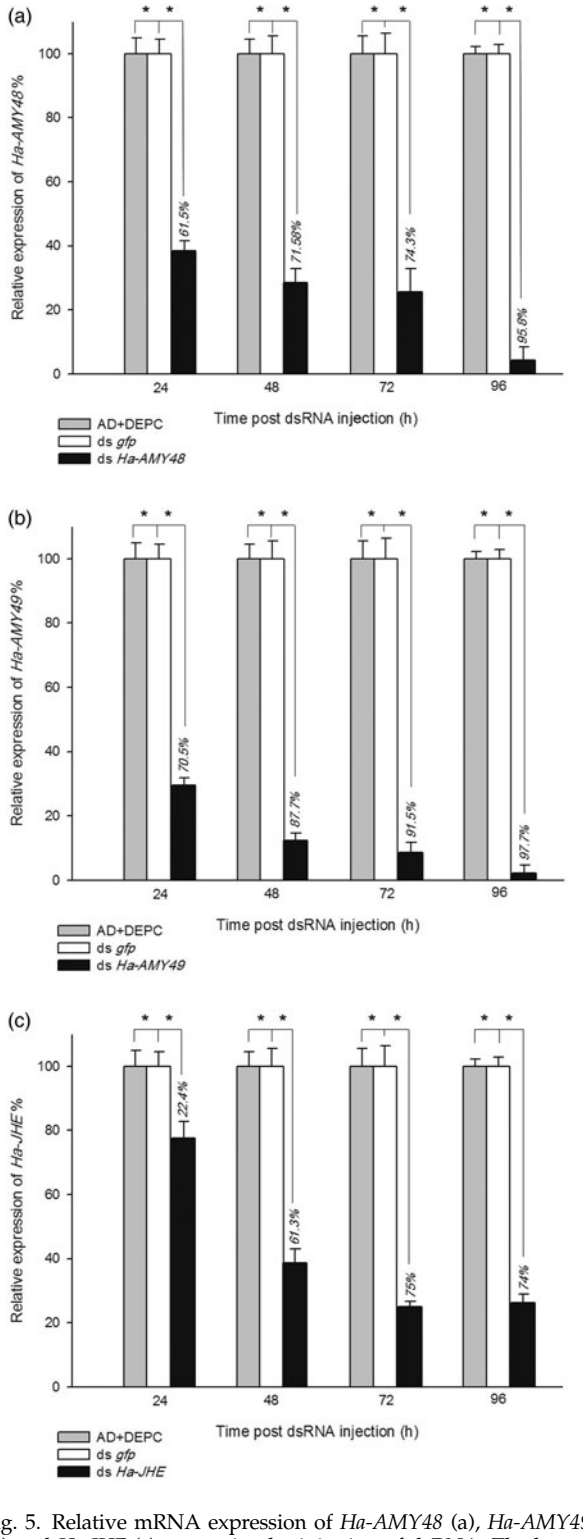

Fig. 5. Relative mRNA expression of *Ha-AMY48* (a), *Ha-AMY49* (b) and *Ha-JHE* (c) expression by injection of dsRNA. The larvae samples of each group were collected post after 24, 48, 72 and 96 h last dsRNA injection. Non-dsRNA (DEPC) and ds *gfp* were used as control. Relativity was calculated basis of DEPC groups. All the experiments were triplicated. The percentages on bars show knockdown % of gene. Asterisk indicates significant difference of relative expression ($P < 0.05$, Student's *t*-test).

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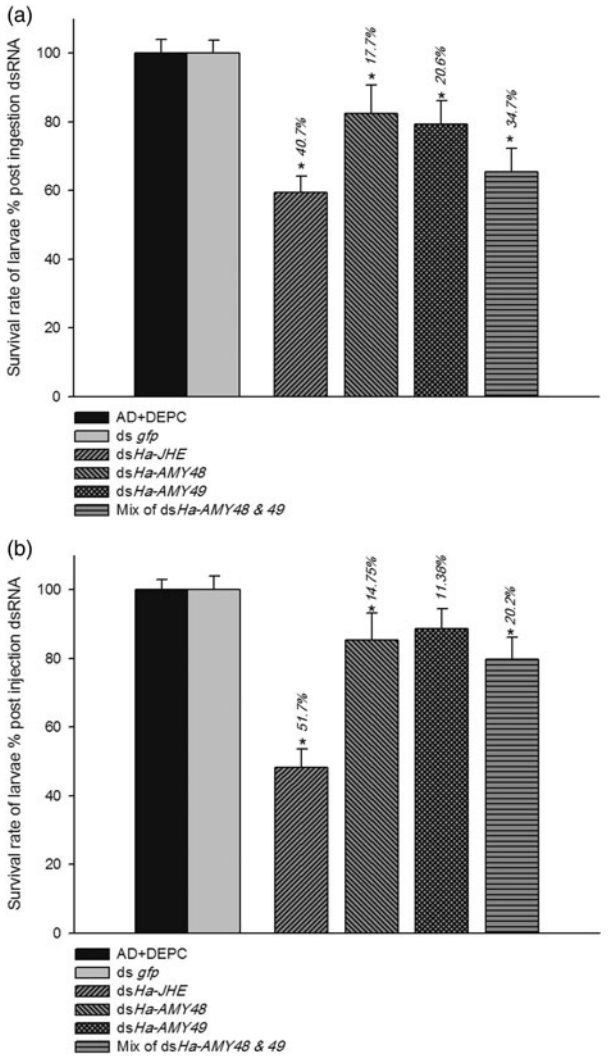

Fig. 6. Survival rate % of cotton bollworm larvae: after rearing on AD containing dsRNA (a) and after dsRNA injection (b). In feeding experiment dsRNA for each gene was applied thrice onto surface of AD cube after interval of 24 h and in injection case, dsRNA thrice injected to body cavity interval of 24 h and then larvae were reared on normal AD and their further growth and development and was observed until larvae were change to pupa. Non-dsRNA (DEPC) and ds *gfp* were used as control. Relativity was calculated basis of DEPC groups. The percentages on bars show mortality %. Asterisk indicates significant difference of relative expression ($P < 0.05$, Student's *t*-test).

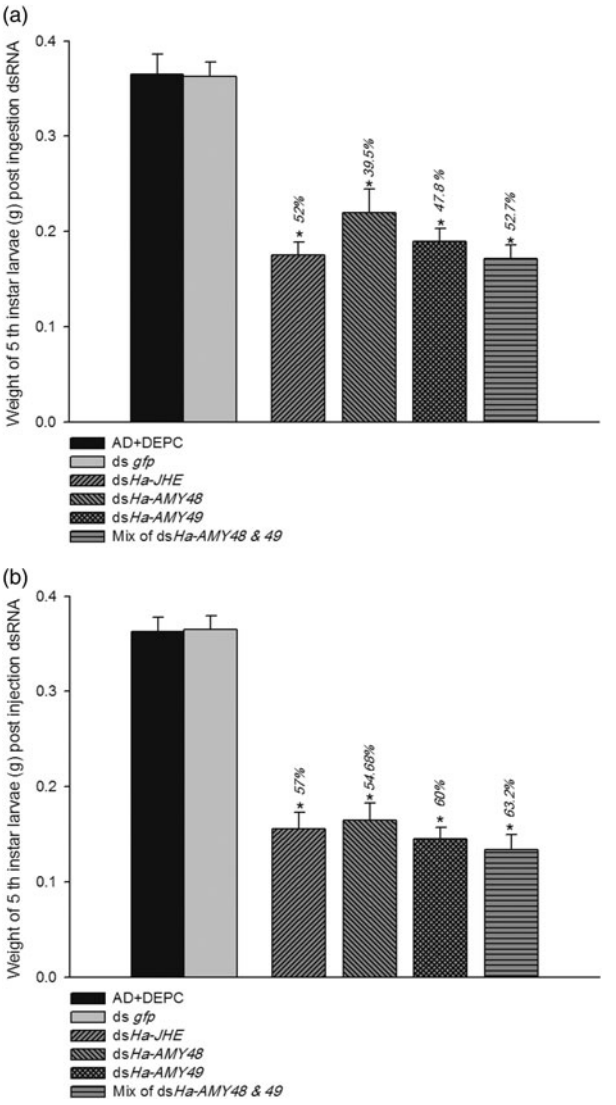

Fig. 7. Silencing effects of *Ha-AMY48*, *Ha-AMY49* and *Ha-JHE* on larval mass. After rearing on AD containing dsRNA (a) and after dsRNA injection (b). In feeding experiment dsRNA for each gene was applied thrice onto surface of AD cube after interval of 24 h and in injection case, dsRNA thrice injected to body cavity interval of 24 h and then larvae were reared on normal AD. The larvae of 5th day of 5th instar were weighted. Non-dsRNA (DEPC) and ds *gfp* were used as control. The percentages on bars show weight loss % that were calculated basis of DEPC groups. Asterisk indicates significant difference of relative expression ($P < 0.05$, Student's *t*-test).

of *HaJHE* dsRNA, were survived and were able change to pupa. Injection of ds*HaAMY48, 49* and mixing of them (ds*Ha-AM48 + 49*), had not high effects on mortality of larvae (about 10%) (fig. 7a, b).

There were found impressive results for effects of ingestion and injection dsRNA. The findings suggest these candidate genes have high effects on reduction of larval weight. Fifth instars of *H. armigera* that had 72 h feeding on AD cubes containing ds*Ha-JHE* during their developmental period, lost about 53% of weight. Three groups of larvae that were fed with *HaAMY48, 49* dsRNA and an equal mix of these two dsRNAs were weight loss (40, 48 and 53%, respectively) (fig. 8a, b). Effects of RNAi on some larvae, pupa and adult were showed in (fig. 8c). Some malformations on adult moths were observed in both ingestion and injection of dsRNAs in compare with control groups. The highest malformations of moths happened for ds*Ha-JHE* injection, about 49% (table 2).

Silencing effects of α-amylase candidate genes on midgut enzyme activity

Midgut crude extract of the larvae that fed with cubes containing ds*Ha-AMY48, 49* and equal mix of them showed

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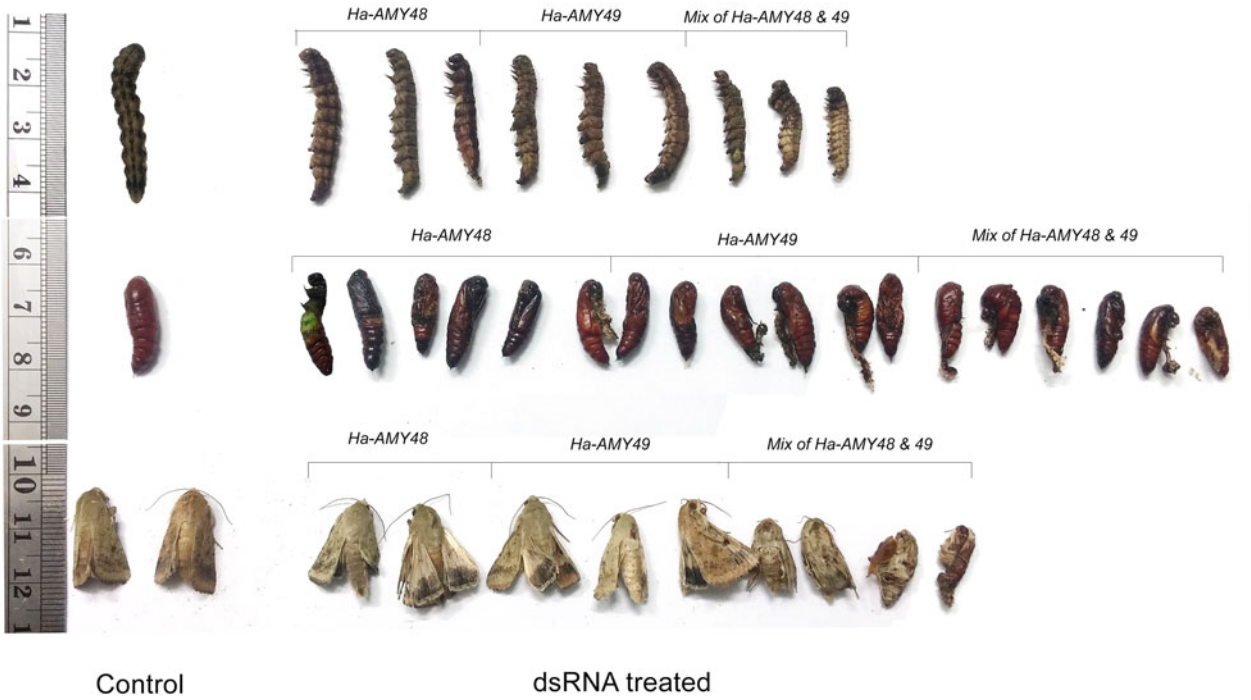

Fig. 8. RNAi of candidate genes triggered some malformation and abnormality on larvae, pupa and moth of *H. armigera* in injecting bioassay. These insects are the representative of all treated insects.

Table 2. Malformation % on moth after feeding and injecting bioassay of candidate genes.

Genes	Sequence of primers (5′ to 3′)	PCR type	Amplification size (bp)
HaAM49	F: GTGGCAACATCCTCACATACA R: GTGGAAGTCGAAACTGCTCATA	qPCR	110
HaAM48	F: CGCATCATCTATGATCGTCTCC R: GCTGATAGCTTCACCTCCATAG	qPCR	111
HaJHE	F: TTCAGCATGTCTCCAGCTTAC R: GGTCGATGAGCTTCCTATGTATTT	qPCR	106
Ha18s	F: CACCACTAGAGGACACAGA R: CACCACTAGAGGACACAGAG	qPCR	95
T7HaAM49	F: TAATACGACTCACTATAGGGAGACGACAGGCACTTGGAACGAGA R: TAATACGACTCACTATAGGGAGACTAGACGGTCGAAGATGACGC	RT-PCR	546
T7HaAM48	F: TAATACGACTCACTATAGGGAGAATGGTCCTACAACCGTCCCT R: TAATACGACTCACTATAGGGAGAAACCCGTGAGCTGTGTTCAA	RT-PCR	550
T7HaJHE	F: TAATACGACTCACTATAGGGAGACCCAGAGTTCACAGACCGAC R: TAATACGACTCACTATAGGGAGATTCCTCGGGGTCCGTATCTT	RT-PCR	545

significant reduction of α-amylase-specific activity on specific substrate, starch, to their respective control, after 24, 48, 72 and 96 h of the latest feeding and ingestion bioassay. Specific activity of α-amylase decreased about 35% after 72 h of feeding dsRNA. But after 96 h insects recovered the level of α-amylase enzyme about 14% in maximum condition. Enzyme inhibition quantity arrived more than 87% in injection bioassay after 96 h. In the feeding bioassay significant difference was not observed between effects of ds*HaAMY48* to ds*HaAMY49* and ds*HaAMY48 + 49* on enzyme activity if compared together. In injection assay, these results were impressive. The high reduction of enzyme activity was observed after 96 h, for ds*HaAMY49*, ds*HaAMY48* and equal mix of them together in the treated group (59, 79 and 87.5%). In both, group of bioassay was recorded significant reduction to their respective controls in all tested times (fig. 9a, b)

Effects of injection bioassay on visualization of midgut α-amylase activity

The zymogram analysis showed that after DEPC injection, three bands of α-amylase activity in the midgut of cotton bowl worm larvae were active. However after 24 h, the third band was weak and after 48 and 72 h in addition to the third band, the second band was also fade (fig. 10).

Discussion

In the present paper, α-amylase and JHE genes were chosen as candidate for RNAi. One of the important reasons for this selection is critical roles of these proteins in insects. In recent years, varying RNAi studies were done on insects, especially on lepidopteran pests such as *H. armigera*. Considering

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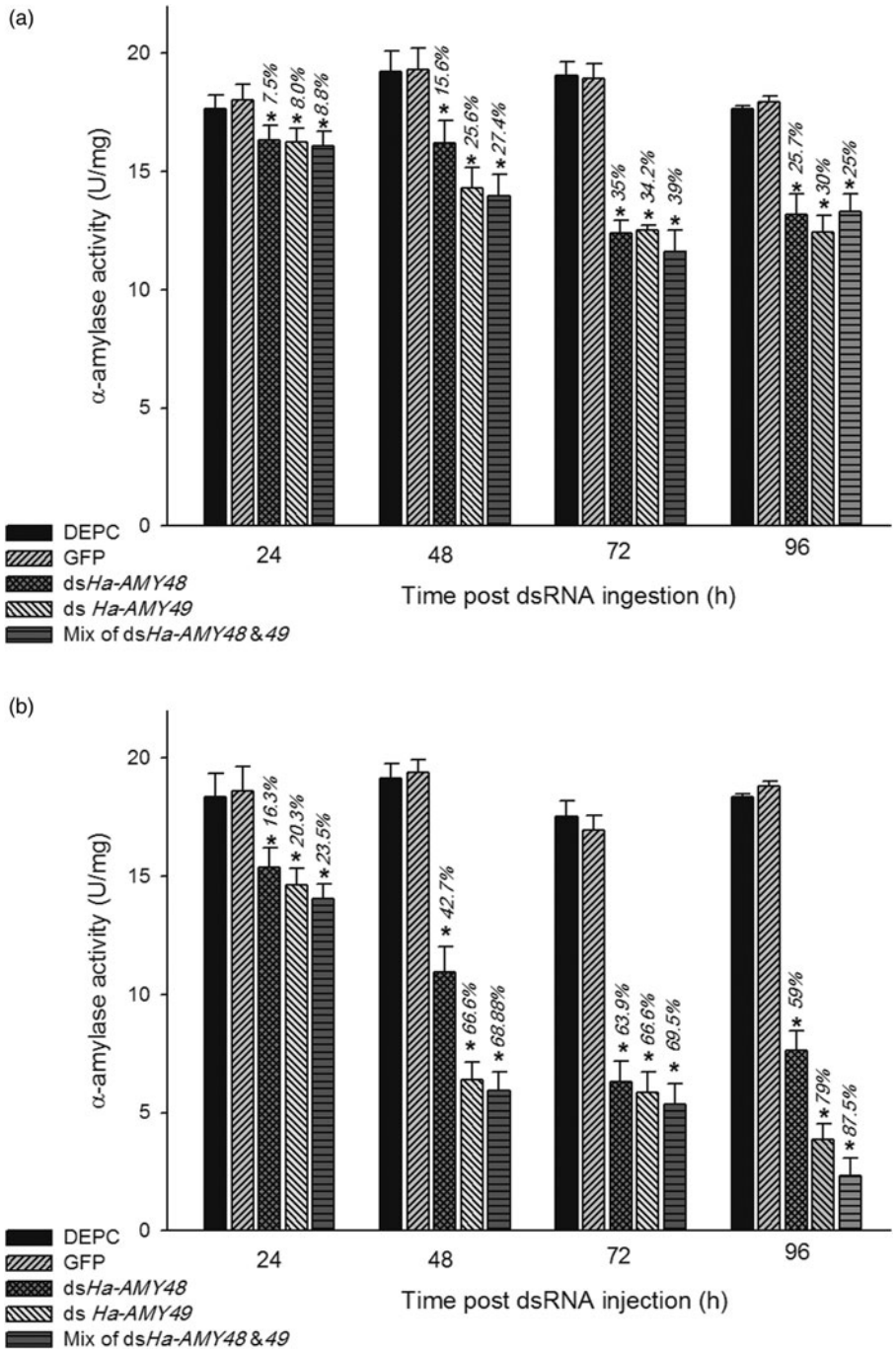

Fig. 9. RNAi effects on α-amylase-specific activity post 24, 48, 72 and 96 h of last treatment; midgut enzyme activity was calculated after dsRNA treated as feeding (a) and injecting (b) on specific substrate, starch. The percentages on bars show inhibition % of enzyme activity that were calculated basis of DEPC groups. Asterisk indicates significant difference of relative expression ($P < 0.05$, Student's *t*-test).

the importance of this pest, various genes that are responsible for varied functions such as digestion, detoxification, metamorphosis, lipid transport, etc. were selected to silencing (Chikate *et al*., 2016).

In this study, two dsRNAs were synthesized for interfering of midgut α-amylase expression and one more related dsRNA was designed for whole-body JHE in cotton bollworm larvae, too, that to achieve these goals, were used feeding and injecting bioassay. After feeding bioassay, the results of qPCR showed that significant down regulation of transcripts was happened after 24 (except *Ha-JHE*), 48, 72 and 96 h. Suppression of mRNA expression was also observed in injecting bioassay results. We found that the injection of dsRNA to hemocoel is more effective to outbreak RNAi than when it is

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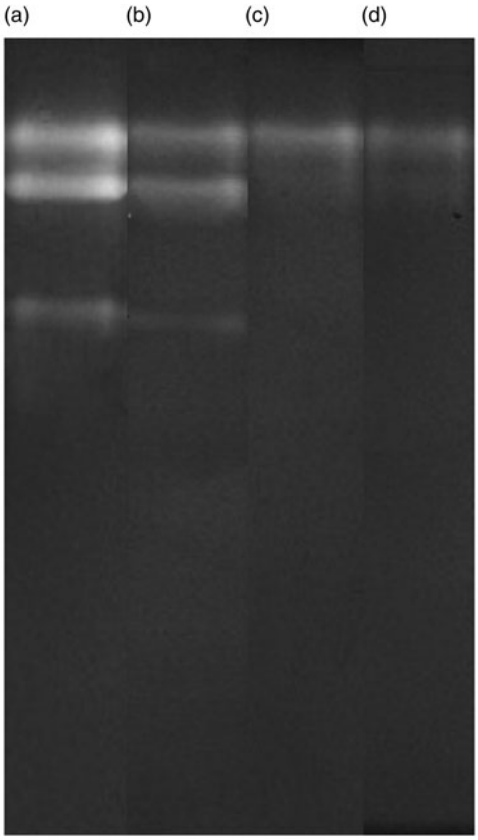

Fig. 10. Visualization of midgut α-amylase activity undergoes RNAi effects as injection bioassay. After 3rd injection in the treated larvae silencing effect of midgut α-amylase activity was studied by native PAGE profile after 24, 48 and 72 h in compare with the control group. Dsgfp (lane a), α-amylase activity in larval midgut after 24 h (lane b), after 48 h (lane c) and post 72 h (lane d).

fed to larvae. Down regulation of *Ha-JHE* after dsRNA injection was about three times, than feeding bioassay (75 and 23%, respectively). The delivery methods of dsRNA are effective for RNAi.

In a previous study in *S. exigua*, it was observed that ingestion of dsRNA did not induce any silencing effects on amino peptidase-N gene in midgut of larvae but micro injection could down regulate this gene very well (Rajagopal *et al.*, 2002). Jing & Zhao-jun, 2014 studied on efficiency of different method to delivery of dsRNA in cotton bollworm and in they concluded that ultraspiracle protein gene (*USP*) could be suppressed by single injection of *in vitro* synthesized dsRNA but no phenotypic effect was observed by single ingestion. Considering of RNAi studies in *H. armigera* illustrated that dsRNA applied dose, type of delivery method of dsRNA and exposure time of dsRNA with site of expression are effective on triggering silencing effects on target genes. A review on RNAi in lepidopteran order showed positive correlation between amount of applied dsRNA and silencing efficiency (Terenius *et al.*, 2011). In a same study, when was applied 15 μg g^{-1} of a 667 bp dsRNA of transferrin gene with AD, after 12, 24, 36 and 48 h was observed 0, 95, 4 and 0% mRNA decrease but changing amount of dsRNA to 35 μg g^{-1} caused 99, 99, 99 and 45% reduction of the mRNA level, respectively (Zhang *et al.*, 2015). Briefly 8.5, 4.4, 62.2 and 59.5% down regulation of chitinase gene with applying 0.425, 21.25, 10.625 and 53.125 μg g^{-1}, respectively, was observed by feeding of transformed bacteria that expression related dsRNAs (Yang & Han, 2014). Feeding of transplastomic leaves that expressed a 19 bp dsRNA for chitin synthase, cytochrome P450 and v-ATPase, showed phenotypic effects on *H. armigera* such as mortality and growth inhibition (Jin *et al.*, 2015). Lim *et al.*, 2016 published comprehensive review paper that in this paper they mentioned some study about progresses and challenges related to applying of diet delivery of dsRNA in *H. armigera* through dsRNA solution, transgenic plant carrying a hairpin construct and bacterial suspension. Some phenotypic effects of candidate genes silencing was observed such as abnormality at larval, pupal and adult stages. These effects were occurred as impressive weight losing in larvae, too. *Ha-JHE* selected gene trigger more malformation, mortality and abnormality to cotton bollworm metamorphosis stages than each *Ha-AMY*s genes candidate. JHE is crucial importance in the regulation of development, metamorphosis and reproduction (Gilbert *et al.*, 2000). In a same study, RNAi of JH acid methyl transferase at *H. armigera* made some phenotypic effects such as growth inhibition and decreased pupation rate (Asokan *et al.*, 2013). Suppression of amylase candidate genes, had phenotypic effects as well as *Ha-JHE* even though when larvae were fed or were injected with mixing of dsRNA (*Ha-AMY49:48(1:1)*) most abnormality was observed. Zhu *et al.* (2005) mentioned that α-amylases presence in insect's crucial processes such as digestion and help them to survive in varied situations and increase their fitness rate, and this enzyme is active in non-feeding phases such as pupa. Undoubted, considering to this important roles, each interfering in α-amylases pathway causes negative effects on insect's fertility and fecundity. We found significant reduction of α-amylases activity on the larvae that fed on AD cubes containing ds*HaAMY48*, 49 and ds*Ha-AMY49:48(1:1)* and treated larvae with these dsRNA as injection. Output of this experiment confirmed RNAi effect on α-amylases in this paper. Interestingly, silencing effects on decreasing of catalysis effects of this enzyme on a specific substrate in the treated larvae with injection bioassay was appeared more than the dietary introduction in compare with control groups. Jing & Zhao-jun (2014) mentioned that dsRNA of USPs was degraded faster in midgut juice of *H. armigera* than hemolymph. Microinjection assay had 75% reduction on target gene in *Rhodnius prolixus* (Hemiptera), but dsRNA feeding only showed 42% reduction (Araujo *et al.*, 2006). Zymogram analysis showed three isozyme activity bands for α-amylases in control population of this species, while it was observed only two weak bands outcome of this enzyme activity of midgut crude extract from the larvae that were injected by *Ha-AMY49:48(1:1)* dsRNA, actually expression of one isozyme was suppressed 100%.

Conclusions

Our studies showed that both diet and injected delivered dsRNA effectively silenced candidate gene. But injection bioassay was more effective than ingestion method. Findings of this paper showed exposure time of dsRNA have a positive correlation with expression of interfering effects such as lose weight, adult malformations and specific enzyme activity. Certainly employed dose of dsRNA has a fundamental role

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on RNAi that it is better to considered. We understood that two candidate genes for α-amylase are different compare together because applying of their dsRNA showed an accumulative effect. Due to the mortal and destructive phenotypic effects of looking for silencing of *Ha-AMY* and *Ha-JHE* genes, we found that these genes can be candidates for new strategies to control of *H. armigera*.

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References

Araujo, R.N., Santos, A., Pinto, F.S., Gontijo, N.F., Lehane, M.J. & Pereira, M.H. (2006) RNA interference of the salivary gland nitrophorin 2 in the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) by dsRNA ingestion or injection. *Insect Biochemistry and Molecular Biology* **36**, 683–693.

Aravin, A.A., Naumova, N.M., Tulin, A.V., Vagin, V.V., Rozovsky, Y.M. & Gvozdev, V.A. (2001) Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *Drosophila melanogaster* (Diptera: Drosophilidae) germline. *Current Biology* **11**, 1017–1027.

Asokan, R., Sharath, C.G., Manamohan, M. & Krishna, N.K. (2013) Effect of diet delivered various concentrations of double-stranded RNA in silencing a midgut and a non-midgut gene of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Bulletin of Entomological Research* **103**, 555–563.

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T. & Pleau, M. (2007) Control of coleopteran insect pests through RNA interference. *Nature Biotechnology* **25**, 1322–1326.

Bernfeld, P. (1955) Amylase α and β. *Methods in Enzymology* **1**, 149–151.

Challa, M.M., Sanivada, S.K. & Koduru, U.D. (2013) Total soluble protein profiles of *Beauveria bassiana* and their relationship with virulence against *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Biocontrol Science and Technology* **23**, 1169–1185.

Chen, X., Tian, H., Zou, L., Tang, B., Hu, J. & Zhang, W. (2008) Disruption of *Spodoptera exigua* larval development by silencing chitin synthase gene A with RNA interference. *Bulletin of Entomological Research* **98**, 613–619.

Chikate, Y.R., Dawkar, V.V., Barbole, R.S., Tilak, P.V., Gupta, V. S. & Giri, A.P. (2016) RNAi of selected candidate genes interrupts growth and development of *Helicoverpa armigera* (Lepidoptera: Noctuidae. *Pesticide Biochemistry and Physiology* **133**, 44–51.

Denlinger, D.L. (1985) Hormonal control of diapause. pp. 353–412 *in* Kerkut, G.A. & Gilbert, L.I. (*Eds*) *Comprehensive Insect Physiology, Biochemistry and Pharmacology*. Oxford, Pergamon Press.

Devi, V.S., Sharma, H.C. & Rao, P.A. (2011) Interaction between host plant resistance and biological activity of *Bacillus thuringiensis* (Bacillales: Bacillaceae) in managing the pod borer *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. *Crop Protection* **30**, 962–969.

Dietz, F.J. & van der Straaten, J. (1992) Rethinking environmental economics: missing links between economic theory and environmental policy. *Journal of Economic Issues* **26**, 27–51.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. & Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans* (Rhabtida: Rhabditidae). *Nature* **391**, 806–811.

Franco, O.L., Riggen, D.J., Melo, F.R., Bloch, C., Silva, C. & Grossi, D.S. (2000) Activity of wheat α-amylase inhibitors towards bruchid α-amylases and structural explanation of observed specificities. *European Journal of Biochemistry* **267**, 1466–1473.

Geley, S. & Muller, C. (2004) RNAi: ancient mechanism with a promising future. *Experimental Gerontology* **39**, 985–998.

Ghanim, M., Kontsedalov, S. & Czosnek, H. (2007) Tissue-specific gene silencing by RNA interference in the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Insect Biochemistry and Molecular Biology* **37**, 732–738.

Ghosh, A., Chatterjee, M. & Roy, A. (2010) Bio-efficacy of spinosad against tomato fruit borer *Helicoverpa armigera* (Lepidoptera: Noctuidae) and its natural enemies. *Journal of Horticulture and Forestry* **2**, 108–111.

Gilbert, L.I., Granger, N.A. & Roe, R.M. (2000) The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochemistry and Insect Molecular Biology* **30**, 617–644.

Gordon, K. H. & Waterhouse, P. M. (2007) RNAi for insect-proof plants. *Nature Biotechnology* **25**, 1231–1232.

Hannon, G. J. (2002) RNA interference. *Nature* **418**, 244–251.

Huvenne, H. & Smagghe, G. (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *Journal of Insect Physiology* **56**, 227–235.

Heckel, D.G. (2012) Insecticide resistance after Silent spring. *Science* **337**, 1612–1614.

Jin, S., Singh, N.D., Li, L., Zhang, X. & Daniell, H. (2015) Engineered chloroplast dsRNA silences cytochrome p450 monooxygenase, V-ATPase and chitin synthase genes in the insect gut and disrupts *Helicoverpa armigera* (Lepidoptera: Noctuidae) larval development and pupation. *Plant Biotechnology Journal* **13**, 435–446.

Jing, Y. & Zhao-jun, H. (2014) Efficiency of different methods for dsRNA delivery in cotton bollworm (*Helicoverpa armigera*). *Journal of Integrative Agriculture* **13**, 115–123.

Kotkar, H.M., Bhide, A.J., Gupta, V.S. & Giri, A. (2012) Amylase gene expression patterns in *Helicoverpa armigera* (Lepidoptera: Noctuidae) upon feeding on a range of host plants. *Gene* **501**, 1–7.

Kotwica-Rolinska, J., Gvakharia, B.O., Kedzierska, U., Jadwiga, M.G. & Piotr, B. (2013) Effects of period RNAi on V-ATPase expression and rhythmic pH changes in the vas deferens of *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Insect Biochemistry and Molecular Biology* **43**, 522–32.

Kumar, M., Gupta, G.P. & Rajam, M.V. (2009) Silencing of acetyl cholinesterase gene of *Helicoverpa armigera* (Lepidoptera: Noctuidae) by siRNA affects larval growth and its life cycle. *Journal of Insect Physiology* **55**, 273–278.

Lim, Z.X., Robinson, K.E., Jain, R.G., Chandra, S.G., Asokan, R., Asgari, S. & Mitter, N. (2016) Diet-delivered RNAi in *Helicoverpa armigera* – progresses and challenges. *Journal of Insect Physiology* **85**, 86–93.

Livak, K.L. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25**, 402–408.

Mizoguchi, A. (2001) Effects of juvenile hormone on the secretion of prothoracico-tropic hormone in the last- and penultimate-instar larvae of the silkworm *Bombyx mori* (Lepidoptera: Bombycidae). *Journal of Insect Physiology* **47**, 767–775.

Price, D.R. & Gatehouse, J.A. (2008) RNAi-mediated crop protection against insects. *Trends in Biotechnology* **26**, 393–400.

Rajagopal, R., Sivakumar, S., Agrawal, N., Malhotra, P. & Bhatnagar, R.K. (2002) Silencing of midgut aminopeptidase N of *Spodoptera litura* (Lepidoptera: Noctuidae) by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. *Journal of Biological Chemistry* **277**, 46849–46851.

Riddiford, L.M. (1996) Molecular aspects of juvenile hormone action in insect metamorphosis. pp. 223–251 *in* Gilbert, L.I., Tata, J.R. & Atkinson, B.G. (*Eds*) *Metamorphosis: Post-embryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*. San Diego, Academic Press.

Riddiford, L.M., Himura, K., Zhou, X. & Nelson, C.A. (2003) Insights into the molecular basis of the hormonal control of molting and the metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology* **33**, 1327–1338.

Roe, R.M. & Benkatesh, K. (1990) Metabolism of juvenile hormones: degradation and titer regulation. pp. 126–179 *in* Gupta, A.P. (*Ed.*) *Morphogenetic Hormones of Arthropods*. New Brunswick, Rutgers University Press.

Scott, J.G. & Wen, Z.M. (2001) Cytochromes P450 of insects: the tip of the iceberg. *Pest Management Science* **57**, 958–967.

Siomi, H. & Siomi, M.C. (2009) On the road to reading the RNA interference code. *Nature* **457**, 396–404.

Terenius, O., Papanicolaou, A., Garbutt, J.S., Eleftherianos, I., Huvenne, H., Kanginakudru, S., Albrechtsen, M., An, C., Aymeric, J.L., Barthel, A., Bebas, P., Bitra, K., Bravo, A., Chevalier, F., Collinge, D.P., Crava, C.M., de Maagd, R.A., Duvic, B., Erlandson, M., Faye, I., Felföldi, G., Fujiwara, H., Futahashi, R., Gandhe, A.S., Gatehouse, H.S., Gatehouse, L.N., Giebultowicz, J.M., Gómez, I., Grimmelikhuijzen, C. J.P., Groot, A.T., Hauser, F., Heckel, D.G., Hegedus, D.D., Hrycaj, S., Huang, L., Hull, J.J., Iatrou, K., Iga, M., Kanost, M.R., Kotwica, J., Li, C., Li, J., Liu, J., Lundmark, M., Matsumoto, S., Meyering-Vos, M., Millichap, P.J., Monteiro, A., Mrinal, N., Niimi, T., Nowara, D., Ohnishi, A., Oostra, V., Ozaki, K., Papakonstantinou, M., Popadic, A., Rajam, M.V., Saenko, S., Simpson, R.M., Soberón, M., Strand, M.R., Tomita, S., Toprak, U., Wang, P., Wee, C.W., Whyard, S., Zhang, W., Nagaraju, J., French-Constant, R.H., Herrero, S., Gordon, K., Swevers, L. & Smagghe, G. (2011) RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. *Journal of Insect Physiology* **57**, 231–245.

Wang, Z., Dong, Y., Desneux, N. & Niu, C. (2013) RNAi silencing of the HaHMG-CoA reductase gene inhibits oviposition in the *Helicoverpa armigera* cotton bollworm. *PLoS ONE* **8**, 1–9.

Yamaguchi, J., Mizoguchi, T. & Fujiwara, H. (2011) siRNAs Induce Efficient RNAi Response in *Bombyx mori* Embryos. *PLoS ONE* **6**, 1–7.

Yang, J. & Han, Z. (2014) Optimisation of RNA interference-mediated gene silencing in *Helicoverpa armigera*. *Austral Entomology* **53**, 83–88.

Yoo, B.C., Kragler, F., Varkonyi-Gasic, E., Haywood, V., Archer-Evans, S., Young, M.L., Lough, T.J. & Lucas, W.J. (2004) Asystemic small RNA signaling system in plants. *The Plant Cell* **16**, 1979–2000.

Yu, N., Christiaens, C., Liu, J., Niu, J., Cappelle, K., Caccia, S., Huvenne, H. & Smagghe, G. (2013) Delivery of dsRNA for RNAi in insects: an overview and future directions. *Insect Science* **20**, 4–14.

Zeng, F. & Cohen, A.C. (2000) Partial characterization of α-amylase in the salivary glands of *Lygus hesperu*s and *L. lineolaris*. *Comparative Biochemistry and Physiology* **126**, 9–16.

Zhang, L., Shang, Q., Lu, Y., Zhao, Q. & Gao, X. (2015) A transferrin gene associated with development and 2-tridecanone tolerancein *Helicoverpa armigera*. *Insect Biochemistry and Molecular Biology* **24**, 155–166.

Zhu, Y.C., Liu, X., Maddurb, A.A., Oppert, B. & Chen, M.S. (2005) Cloning and characterization of chymotrypsin- and trypsin-like cDNAs from the gut of the Hessian fly (*Mayetiola destructor*). *Insect Biochemistry and Molecular Biology* **35**, 23–32.

Zhu, J.Q., Liu, S., Ma, Y., Zhang, J.Q., Qi, H.S., Wei, Z.J., Yao, Q., Zhang, W.Q. & Li, S. (2012) Improvement of pest resistance in transgenic tobacco plants expressing dsRNA of an insect-associated gene EcR. *PLoS ONE* **7**, 1–9.