

### Melanin Synthesis Pathway Interruption: CRISPR/Cas9mediated Knockout of *dopa decarboxylase* (*DDC*) in *Harmonia axyridis* (Coleoptera: Coccinellidae)

Meng-meng Wu,<sup>1,2,\*</sup> Xu Chen,<sup>3,\*</sup> Qing-xuan Xu,<sup>2</sup> Lian-sheng Zang,<sup>1,3,0</sup> Su Wang,<sup>2</sup> Ming Li,<sup>4,5</sup> and Da Xiao<sup>2,5,0</sup>

<sup>1</sup>Jilin Engineering Research Center of Resource Insects Industrialization, Institute of Biological Control, Jilin Agricultural University, Changchun 130118, China, <sup>2</sup>Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China, <sup>3</sup>Key Laboratory of Green Pesticide and Agricultural Bioengineering of Ministry of Education, Guizhou University, Guiyang 550025, China, <sup>4</sup>Division of Biological Sciences, Section of Cell and Developmental Biology, University of California, San Diego, La Jolla, CA 92093, USA, and <sup>5</sup>Corresponding author, e-mail: mingli@ucsd.edu (M.L.), xiaoda@ipepbaafs.cn (D.X.)

\*These authors contributed equally.

Subject Editor: Joanna Chiu

Received 2 May 2022; Editorial decision 14 July 2022.

#### Abstract

CRISPR/Cas9 technology is a very powerful genome editing tool and has been used in many insect species for functional genomics studies through targeted gene mutagenesis. Here, we successfully established CRISPR/Cas9 research platform in Asian multi-colored ladybird beetle, *Harmonia axyridis*, an important natural enemy in biological control. In this study, one pivotal gene *dopa decarboxylase (DDC)* in melanin synthesis was targeted by CRISPR/Cas9 to generate mutants in *H. axyridis* by CRISPR/Cas9 technology. Our results showed that injection of single guide RNA of the *DDC* and Cas9 protein into preblastoderm eggs induced one insertion and four deletion (indels) mutant *H. axyridis*. Mutations of *HaDDC* gene generated 25% mutant rate with melanin missing phenotype in larva, pupa,I and adult stage. The predation ability of the fourth instar larvae has no significant difference between wild (control) and mutant *H. axyridis* (G0), while these mutant fourth instar larvae had longer developmental period than that of the wild type. Consequently, the total predation of the fourth instar larvae was significantly increased in *H. axyridis* mutants comparing with the wild type. These results indicated that the success of CRISPR/Cas9 gene editing in *H. axyridis*. The gene editing platform in *H. axyridis* would facilitate the gene function research and promote special strain of predatory ladybird beetle generation.

Key words: CRISPR/Cas9, Harmonia axyridis, melanin, dopa decarboxylase (DDC)

CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) is an effective gene editing technology that originates from an old bacteria adaptive immune system (Jansen et al. 2002, Cong et al. 2013, Sander and Joung 2014, Taning et al. 2017). This system relies on a single guide RNA (sgRNA) chimera of mature CRISPR RNA (crRNA) and *trans*-activating crRNA (tracrRNA), combining with Cas9 to cleave complementary target DNA sequences in the genome. The guide RNA binds to target sequences, then Cas9 protein follows the guide RNA to the target DNA sequence and cuts it, thereby generate a double-strand break. Finally, the DNA repair machineries recognize and repair the DNA damage through nonhomologous end joining (NHEJ) or homology-directed repair (HDR) manner (Knott and Doudna 2018). CRISPR/Cas technology has been widely applied to functional genomics studies in many species, from microbes (Yin et al. 2019), plant

(Fauser et al. 2014, Jiang et al. 2014), insects to animals (Cong et al. 2013). Currently, CRISPR/Cas9 was also proposed as a therapeutic approach to treat human life-threatening diseases (Artegiani et al. 2020).

CRISPR/Cas9 is an effective approach to study gene function of many kinds of insect species (Sun et al. 2017), such as economic insect *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) (Daimon et al., 2014; Ma et al., 2017), *Apis mellifera* (Linnaeus) (Hymenoptera: Apidae) (Kohno et al., 2016); sanitary pest insect *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) (Kistler et al., 2015), *Anopheles gambiae* (Giles) (Diptera: Culicidae) (Hammond et al., 2016); agricultural pest insect *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Wang et al., 2020b), *Plutalla xylostella* (Linnaeus) (Lepidoptera: Plutellidae) (Huang et al., 2016; Wang et al., 2020a; Zou et al., 2020), *Spodoptera litura* (Fabricius)

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (https://creativecommons.org/licenses/ by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com (Lepidoptera: Noctuidae) (Bi et al., 2016; Zhu et al., 2016), and natural enemy insect *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) (Li et al., 2017). The successful gene editing was applied in healthy and economic important Diptera and Lepidoptera insect species (Sun et al., 2017; Li et al., 2021). In Coleoptera insect, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) was only species using CRISPR/Cas9 for gene editing research (Gilles et al. 2015, Adrianos et al. 2018).

In this study, CRISPR/Cas9 was first applied in another Coleoptera insect species, Asian multi-colored ladybird beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). *H. axyridis* has the remarkable predation ability to many kinds of insect pests (Bradley et al. 2003, Koch, 2003) as well as for high variation in elytra patterns (Niimi and Ando 2021). Currently, most ladybird beetle species were laboratory culturable for large scale field releasing (Wang et al. 2012). In order to improve field pest control efficacy of the released predatory ladybirds in the adverse environmental circumstances: such as low, or high temperature, drought etc. Special strains of ladybird beetles are needed in practical application. Thus, it is necessary to apply gene-editing technique in ladybird beetles to improve the tolerance to unfavorable circumstances.

In basic research application of CRISPR/Cas9, to select the appropriate target gene is a key issue. An ideal target gene selection should show the visible phenotype change but without any abnormal impact on insect development. Some studies have demonstrated that editing of genes involve in pigment synthesis showed the countable phenotype changes using CRISPR/Cas9 technique in Nilaparvata lugens (Stal) (Homoptera: Delphacidae) (Xue et al., 2018), N. vitripennis (Li et al., 2017), A. aegypti (Li et al., 2017), Vanessa cardui (Linnaeus) (Lepidoptera: Nymphalidae) (Zhang and Reed, 2016; Zhang et al., 2017), Aedes albopictus (Skuse) (Diptera: Culicidae) (Liu et al., 2019), Drosophila suzukii (Matsumura) (Diptera: Drosophilidae) (Yan et al., 2020), and T. castaneum (Adrianos et al., 2018). The Asian multi-colored ladybird beetle has various elytra patterns (Ando et al. 2018, Ando and Niimi 2019, Niimi and Ando 2021) with four common color elytra patterns: succinea, axyridis, spectabilis, conspicua (Gautier et al., 2018; Xiao et al., 2020). Therefore, the genes in pigmentation pathways could be considered as an ideal target gene in geneediting system establish in H. axyridis. In addition, studies have demonstrated that *dopa decarboxylase* (DDC) gene in dopamine melanin synthesis that plays a pivotal role in H. axyridis pigmentation. The suppression of DDC expression in the third or fourth instar larvae of H. axyridis resulted in melanin disappearance in elytra (Chen et al. 2019, Xiao et al. 2020). In this study, DDC was used as target gene in H. axyridis to develop an efficient gene editing protocol by CRISPR/Cas9 technology. This study provided strong evidences that CRISPR/Cas9 technology can be used as a promising approach for gene function research and special strain cultivate in predatory ladybird beetles.

#### **Materials and Methods**

#### Insect Culture

The *ladybird beetles* (*H. axyridis*) were collected from wheat experimental field (GPS location:  $39^{\circ}95_0$  N,  $116^{\circ}28_0$  E) at Beijing Academy of Agriculture and Forestry Sciences (BAAFS, Beijing, China) and fed with the cowpea aphid, *Aphis craccivora* Koch (Hemiptera: Aphididae) on broad bean leaves (Vicia faba L. 'LinCan-5') in the laboratory growth chamber at  $25 \pm 2^{\circ}$ C; 65% RH, and a photoperiod of 16:8 (L:D) h (L-100, Suntech, Beijing, China). The ladybird adults were reared in customized insect-proof cages (50 cm × 50 cm × 60 cm) covered by 100-micron nylon-mesh screen cages with 40 pairs each cage and fed on cowpea aphids daily.

#### Guide RNA design and synthesis

sgRNA target sequences were located in exon 6 of the HaDDC genes through the CHOPCHOP V3.00 website. The DNA template for in vitro sgRNA synthesis was obtained by fusion PCR with two oligonucleotides using the High-Fidelity DNA Polymerase PCR Mix (NEB, Ipswich, MA). One oligonucleotide was used as the forward primer that encoded the T7 polymerase binding site and the sgRNA target sequence, and the other common oligonucleotide was served as the reverse primer encoding the remaining sgRNA sequences (Table 1). The total volume of PCR is 50 µL that including 0.5 µL of Phusion DNA Polymerase, 1 µL of dNTPs (10  $\mu$ M), 2.5  $\mu$ L of forward primer (10  $\mu$ M), 2.5  $\mu$ L of reverse primer (10 µM), and 33.5 µL of nuclease-free H<sub>2</sub>O. The PCR program was performed with 3-step amplification protocol that consist of 98°C for 30 s; 35 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 15 s, and a final extension at 72°C for 10 min. The MinElute PCR Purification Kit was carried out to purify PCR products (Qiagen, Hilden, Germany). The concentration of the generated DNA template was measured using NanoDrop ONE spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Then, sgRNAs were in vitro transcribed by T7 MEGAscript Kit (Ambion, Foster City, CA). The synthesized sgRNAs were purified by the MEGAclear Transcription Clean-Up Kit (Ambion, Foster City, CA) and injected to H. axyridis embryos or stored in aliquots at -80°C until use. In this study, the recombinant Cas9 protein from Streptococcus pyogenes was obtained commercially (Thermo Fisher Scientific, Waltham, MA).

	Table '	<ol> <li>Sequences and</li> </ol>	d relevant parameters o	of the PCR primers used	for sgRNA syn	thesis and genotype analys	sis
--	---------	-----------------------------------	-------------------------	-------------------------	---------------	----------------------------	-----

Primer name	Sequence (5'-3')	Tm (°C)	Product size (bp)
PCR for sgRNA synthesis			
sgRNA-F (target GFP)	GAAATTAATACGACTCACTATAGGCATCG	56.0	142
	ACTTCAAGGAGGAGTTTTAGAGCTAGAAATAGC		
sgRNA-F (target DDC)	GAAATTAATACGACTCACTATAGAGTACA	55.0	115
	GGTATCTGATGAATTTTAGAGCTAGAAATAGC		
sgRNA-ComR	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACG	55.0	
	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC		
PCR for genotype analysis			
HaDDC-F	CGCCACCCTGGGAACAAC	59.5	500
HaDDC-R	GCCCTTTAGCCTGAAGCACA	57.5	
PCR for genotype analysis HaDDC-F HaDDC-R	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC CGCCACCCTGGGAACAAC GCCCTTTAGCCTGAAGCACA	59.5 57.5	500

#### H. axyridis Egg Collection and Microinjection

Forty pairs of *H. axyridis* adults in every cage were fed daily with cowpea aphids, on leaves of broad bean seedlings. Fresh preblastoderm stage eggs (15 min after egg laying) were collected on the double-sided sticky tape that attached to a microscopic slide (24 × 50 mm). ddH<sub>2</sub>O, mixture of sgRNA target GFP (300 ng/µL) and Cas9 (200 ng/µL) and mixtures of sgRNA target DDC (100, 300, 600 ng/µL), and Cas9  $(200 \text{ ng/}\mu\text{L})$  were injected into the posterior pole of each egg within 30 min-45 min after oviposition using a FemtoJet 4i and an InjectMan 4 microinjection system (Eppendorf, Hamburg, Germany). In order to obtain the higher survival rate after microinjection in H. axyridis eggs, we have developed the two-needle microinjection system in H. axyridis. Briefly, eggs were poked with the empty needle firstly to release egg pressure, then the solution (ddH<sub>2</sub>O or mixture of sgRNA and Cas9 protein) was injected at around the location of the first injection. After microinjection, the eggs were placed in a petri dish at 25°C, 65% RH, and a photoperiod of 16:8 (L:D) h. At 24 h after injection, flour was sprinkled on the double-sided adhesive microscope slides to help the newly hatched larvae (G0) move away the slides. The larva (G0) was fed with A. craccivora in a small round plastic box (diameter 9.0 cm × high 5.0 cm) individually. Approximate 500 eggs were collected and injected at a time.

#### gDNA Isolation and Mutagenesis Detection

In order to detect the insertion or deletion mutations of the HaDDC gene induced by the CRISPR/Cas9 genome editing system, genomic DNA (gDNA) samples were extracted from the third instar larval exuviae of H. axyridis. Trace amounts of gDNA were isolated individually using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA fragments of HaDDC (500 bp) were amplified by genespecific primer pairs flanking the sgRNA target sites (Table 1). 300 ng of gDNA template were used in 50 µL Premix Taq (Takara, Dalian, China) reaction system with three-step PCR program: 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 10 min. The PCR products were purified using QIAEX II Agarose Gel Extraction Kit (Qiagen, Hilden, Germany) and subcloned into the pMD18-T cloning vector (Takara, Dalian, China), then, transformed into DH5a E. coli (Cwbio, Jiangsu, China). Plasmids were isolated and sequenced for indel mutation detection (Tsingke biotechnology company, Beijing, China).

#### **Evaluation of Biological Parameter**

To estimate the impact of *DDC* gene mutation on biological parameters (developmental time and predation ability) of *H. axyridis*, the neonates (G0) after sgRNA and Cas9 injection were reared in small plastic box individually and each neonate was fed with 50–120 *A. craccivora* daily. The survivor and development of

each larva of the control (wild type) and mutant (sgRNA and Cas9 protein injected) were recorded daily. Each treatment consisted of three replicates with 20 ladybird beetles in each replicate.

To examine the predation capacity, the fourth instar larvae of mutant and wild type *H. axyridis* were carried out to record predation number individually. Meanwhile, in order to avoid feeding bias of *H. axyridis* to *A. craccivora* occur, the other aphid – *Megoura japonica* were used in predation capacity assay. The fourth instar larvae of each treatment group (wild or mutant type) were starved for 24 h, then, 120 of the second instar nymph of *M. japonica* were provided to each larva in a petri dish, individually. The number of *M. japonica* total consumptions was recorded after 24 h feeding for each larva. Each treatment consisted of three replicates with 20 ladybird beetles in each replicate.

#### Image Processing

The phenotypes of all ladybird beetles were recorded by Zeiss Microscope SteREO Discovery V20 (Carl Zeiss, Oberkochen, Germany) with the following parameters set: identical magnification  $(10\times)$ , exposure time (10 s), and intensity of light (26%).

#### Statistical Analysis

The unpaired Student's *t*-test was used to analyze the differences in developmental time and predation assay between wild and mutant type of *H. axyridis* by using SPSS software (v. 22, IBM Corp. Armonk, NY). Means and standard error of development time and predation ability were calculated by the GraphPad Prism v.8 (GraphPad Software, California).

#### Results

# Rationally Chosen of *DDC* as Target to Develop a CRIPSR/Cas9 Based Gene Editing Technique in *H. axyridis*

In order to easily detect successful CRISPR/Cas9 mutagenesis and establish an efficient genome editing platform for *H. axyridis*, we chose to target a conserved *dopa decarboxylase* (*DDC*) gene, encoding a pivotal enzyme involved in dopamine-melanin synthesis. The length of *HaDDC* genomic DNA sequence is 18, 364 bp, which contains 6 exons and 5 introns (Fig. 1). The full-length of open read frame (ORF) of *HaDDC* is 1431 bp that encodes a protein of 476 amino acid residues. As well as studies have indicated that suppressed the expression level of *HaDDC* through RNAi technique resulting in distinct melanin missing phenotypes in *H. axyridis* (Chen et al., 2019; Xiao et al., 2020), thereby, *HaDDC* would be an optimal target for the first time CRIPSR/Cas9 gene editing technique application in *H. axyridis*.



Fig. 1. Schematic diagram for the organization of *DDC* gene in *H. axyridis*. Schematic of sgRNA/Cas9 target sites in exon 6 of the *HaDDC* gene. The sgRNA target sequences are highlighted in black; the PAM sequences are marked in red and underlined.

#### Mutagenesis Efficiency is sgRNA Dose-dependent

To develop new microinjection technique to reduce the mechanical damage in *H. axyridis* eggs is the critical step. Two-needle microinjection system was successfully used in silkworm (Kanda and Tamura 1991) for gene editing research to overcome the thick and hard egg shell (Tamura et al. 2007). Based on the idea of two-needle microinjection, we have developed a suitable approach in microinjection in *H. axyridis* eggs that poke with the empty needle firstly to release egg pressure, then inject the ddH<sub>2</sub>O (or sgRNA and Cas9 protein mixture) around the first needle injected location. This modified microinjection method significantly increased the egg survival rate from 25% to 50% approximately in *H. axyridis*, demonstrated that the potential success of CRISPR/Cas9 gene editing in *H. axyridis* at first step.

To disrupt the function of *DDC* gene, we designed the sgRNA by using the CHOPCHOP V3.00 (Nami et al. 2018) to target the exon 6 of *HaDDC* (Fig. 1). To test the relationship between sgRNA amount and mutagenesis ratio, three concentrations of sgRNA (100, 300, and 600 ng/ $\mu$ L) with Cas9 protein (200 ng/ $\mu$ L) were microinjected in preblastoderm stage in *H. axyridis*. The survival rate of injected eggs and the mutation rate mediated by CRISPR/ Cas9 were sgRNA dose-dependent (Table 2), with the increased concentration of sgRNA lead to the increased proportion of mutant individuals, but the survival rate of injected eggs concomitantly decreased (Table 2). Therefore, the combination of 300 ng/  $\mu$ L sgRNA and 200 ng/ $\mu$ L Cas9 protein was used as the optimal concentration in the following experiments. After micro-injection, 30% (580 out of 1960) eggs could successfully hatch, and the mutation rate was 25% (145 out of 580).

To confirm the mutant phenotype was induced by the genomic mutagenesis of the *DDC*, genomic DNA of *H. axyridis* was extracted from exuviae of several mutant larvae (G0), and used as PCR template to individually amplify the genomic DNA fragment. The sequencing results were confirmed by the indels (insertion or deletion) at the target site of mutant individuals (Fig. 2). Five main mutation models, namely nucleotide deletions (-3, 4, 9, 13 bp) and insertion (+1bp) occurred in ladybird beetle mutants (G0), and most of mutant genotypes, deletion of 3 bp and 13 bp were the predominant genotypes, approximately 31% and 17%, respectively.

These successful gene edited ladybirds showed uniform phenotype that the melanin was disappeared in the whole body as compared with the wild type (Fig. 3). In this study, the images of larvae were taken in the second day of each instar. To our knowledge, the first and second instar larvae of *H. axyridis* make preparation for molting during the second day of each instar and the old cuticle may detach from body and become dark through autoxidation as reported in the silkworm (Futahashi and Fujiwara 2006, Chen et al. 2015). Thus, it seems that no difference of cuticle melanin between wild and mutant type in the first and second instar larvae. In addition, approximately 30% (44 out of 145) mutant ladybird beetles could emerge as adults, but the old exuvium was attached to their bodies after eclosion. The eclosion rate of mutant type (G0) was significantly decreased as compared with wild type of 85% in *H. axyridis*. After manually remove of old exuviae from those deformed individuals, they moved slowly, lost mating and egg laying capacity. Around 70% mutant individuals (G0) even died at larval or pupal stage.

After micro-injection of 300 ng/ $\mu$ L sgRNA and 200 ng/ $\mu$ L Cas9 protein, approximate 70% eggs could not hatch into larval stage. In order to analyze the reasons of un-hatch eggs caused by mutagenesis in *HaDDC*, genomic DNA from un-hatched eggs were used as a template to amplify the sgRNA target region individually. Our results showed that 25% (7 out of 30) un-hatched eggs have deletion mutations (-4, -9 bp) that were caused by gene editing in *HaDDC*.

### DDC Gene Editing Effects on the Development and Predation Ability of *H. axyridis*

To investigate whether HaDDC gene editing could influence the development of H. axyridis fitness, the developmental time of each larva and pupa was recorded. Firstly, the development duration of each instar larvae and pupal stage of ddH<sub>2</sub>O injected individuals were recorded and calculated. Our results showed no significant difference of development duration of each instar larvae and pupal stage between ddH<sub>2</sub>O injected and wild type ladybirds. Thus, the effect of microinjection injure on development was excluded. Our results showed that DDC mutant ladybird beetles (G0) have longer larval developmental time than that of wild type. The average development time of the fourth instar larva was 7.4 ± 1.07 days in mutant H. axyridis which was significantly longer than that of wild type H. axyridis, with  $4.5 \pm 0.503$  days (T = -18.519, P < 0.001) (Fig. 4). Moreover, the development time of some fourth instar mutant larvae was up to 11 d or more, and some of them did not successfully pupate and died in larval stage.

Predation ability was another important biological trait of *H. axyridis*. The predation numbers of *H. axyridis* to its prey *M. japonica* were recorded and the fitness cost of *DDC* gene editing on predation ability in *H. axyridis* was evaluated. The results showed that there was no significant difference in the fourth instar larvae predation ability between wild and mutant type (G0) *H. axyridis* (wild type and mutants *H. axyridis* consumed 105 ± 12.93 and 96 ± 16.20 *M. japonica* per day, respectively. T = -3.383, P = 0.107). However, the total predation number of the fourth instar larva was significantly higher in mutant individuals than that of wild type individuals due to the prolonged larval developmental period (T = -105.112, P < 0.01) (Fig. 5).

Table 2. Effect of sgRNA and Cas9 protein concentration on hatching and mutant ratio in H. axyridis

	Concentration of sgRNA (ng/uL)	Concentration of Cas9 protein (ng/µL)	Number of	Number of hatching eggs	Hatching	Number of mutant larvae	Mutan
	ogra (11 (11g, p.2.)	ouss protoni (ng/µ2)	injected 0885	nutening 0885	runo		ratio
Water (ddH2O)	0	0	500	245	49%	0	0
sgRNA target	300	200	500	210	42%	0	0
GFP	100	200	2,000	700	35%	126	18%
sgRNA target	300	200	1,960	580	30%	145	25%
DDC	600	200	2,000	300	15%	90	30%



**Fig. 2.** Phenotype of the DDC mutation with abnormal pigmentation induced by sgRNA and Cas9 injection in H. axyridis. (A) Anterior view of the wild type (WT) and mutant type (MT) at second day of first, second, third, and fourth instar larvae. Bar = 1,000 μm. (B) Anterior view of the wild type (WT) and mutant type (MT) on pre-pupae and the second day of pupae. Bar = 1,000 μm. (C) Anterior view of the wild type (WT) and mutant type (MT) at second day of adult (f. *conspicua* and f. *succinea*). Bar = 1,000 μm. The mutant ladybird beetles were abnormal that melanin missing in body wall and body length was decreased compared with control.



Fig. 3. Identification of the mutant genotypes of DDC gene in *H. axyridis*. Five types of mutation were identified in *H. axyridis* using the flat end cloning, including 3, 4, 9, and 13-base deletion and 1-base insertion.

## Development of an CRISPR/Cas9 Embryo Microinjection Protocol in *H. axyridis*

The whole protocol of CRISPR/Cas9 gene editing in *H. axyridis* has 6 procedures need approximate 25 d (Fig. 6). The protocol includes 1) pairing male and female adults to mate ( $\sim$ 1 d), 2) providing fresh leaves of broad bean for females eggs laying and collecting

(~15 min), 3) aligning fresh eggs on double-sided sticky tape (~30 min), 4) micro-injecting eggs with CRISPR/Cas9 components (injecting twice), 5) incubating injected eggs at  $25 \pm 1^{\circ}$ C, with 60% relative humidity to prevent dehydration of the injected embryos, and 6) transferring hatched larvae to fresh leaves of broad bean and fed with cowpea aphids daily.

ported in Archaea and Bacteria about 20 years ago (Jansen et al. 2002), it has rapidly applied as a powerful tool to study gene function, regulation, and interaction in vitro and in vivo among various organisms (Reid and O'Brochta et al., 2016, Taning et al., 2017). The research of insect gene function and molecular mechanism has made a rapid progress with the aid of CRISPR/Cas9 technique (Sun et al. 2017, Taning et al. 2017). *H. axyridis* is an important natural enemy insect that displays remarkable control ability to many kinds of pest insects (Koch et al. 2003, Evans 2009), and functional research in *H. axyridis* is limited to the RNAi technology with transient gene suppress effect (Chen et al. 2019, Xiao et al. 2020). In this study, we developed a complete protocol for permanent gene knockout by CRISPR/Cas9 in *H. axyridis*.

Many artificial factors may also affect the efficiency of gene editing, such as the reduction of egg collection, mechanical damage caused by microinjection, and the injection time etc. The introduction of sgRNA and Cas9 at an earlier stage of embryonic development can increase the probability of genetic mutations occurring. Previous studies had demonstrated that the best injection time was around 30-45 min after eggs laying in D. melanogaster (Kiehart et al. 2007) and A. gambiae or A. aegypti, respectively (Lobo et al. 2006). In our study, we injected at 45 min after laying eggs in H. axyridis. However, embryo survival rate is an important factor in gene-editing research in insect. The egg of H. axyridis is relatively small (1.26 mm in length and 0.60 mm in width) and the pressure of egg is relatively high. There were two challenges for egg injection: 1) the egg shell breaks needles easily, 2) the high pressure inside the egg causes the needle clog due to cytoplasm sucking back to the needle. Therefore, it is very difficult to obtain the higher survival rate after microinjection in H. axyridis eggs. Based on the idea of two-needle microinjection, we have developed a suitable approach in microinjection in H. axyridis eggs. This modified microinjection method significantly increased the egg survival rate from 25% to 50% in H. axyridis (ddH<sub>2</sub>O injection). Previous studies demonstrated that embryos survival rate after injection is estimated at 3% in N. lugens (Xue et al., 2018), 15-20% in D. melanogaster (Bassett et al., 2013), and approximate 10% in A. aegypti (Kistler et al., 2015). Our study demonstrated that appropriate modify injection procedure could



Fig. 6. Procedures for DDC gene editing in H. axyridis. The whole protocol of gene editing has 6 procedures.



**Fig. 4.** Effects of HaDDC knockout on developmental time of each instar larvae and pupa in *H. axyridis*. The results are presented as the mean and standard errors of three replicates, with 20 ladybird beetles in each replicate. Asterisk above the standard error bars indicate significantly differences based on Student's *t*-test (P < 0.05).



**Fig. 5.** Effects of HaDDC knockout on predation ability of the fourth instar larvae in *H. axyridis*. The results are presented as mean and standard errors of three replicates, with 20 ladybird beetles in each replicate. Asterisk above the standard error bars indicate significantly differences based on Student's *t*-test (P < 0.05) between wild and mutant type.

increase embryos survival rate and improve successful ration of gene editing in insect.

Not all genes could be successfully knockout or mutated by CRISPR/Cas9 in insect, e.g., the function of nicotinic acetylcholine receptor subunit (*nAChR*) study in *D. melanogaster* showed *Da5* gene was not able to generate mutant type after multiple attempts (Perry et al. 2021). Other studies suggested the target at 5'-end CDS sequence would have satisfactory editing efficiency (Li et al. 2017). However, our studies demonstrated that target at 3'-end CDS also obtained the satisfactory editing efficiency with visible phenotype changes in *H. axyridis* (Fig. 3). It indicated that editing efficiency due to the PAM site, but not the location of CDS.

Except the target site affects CRISPR/Cas9 editing efficiency, the injected egg survival rate and cleavage rate are sgRNA dosedependent. Injected eggs with the higher concentrations of sgRNA and Cas9 combinations had the higher mutagenesis rates, but the lower survival rates. A similar dose-dependent manner was also reported in *D. melanogaster* (Bassett et al., 2013), *N. vitripennis* (Li et al., 2017), *N. lugens* (Xue et al., 2018), and *S. litura* (Bi et al., 2016). Our study indicated that an optimal concentration of 300 ng/µL for sgRNA accompany with 200 ng/µL for Cas9 could achieve an acceptable mutation rate while minimize the mortality of *H. axyridis* after injection (Table 2).

To examine gene editing efficacy, DNA extraction from sgRNA and Cas9 injected *H. axyridis* is a very crucial step for mutant successful detection. In our study, the exuviae DNA of third instar larvae to fourth instar larvae were successfully extracted and used as PCR template to amplify the targeted genomic region. The results were consistent with those reported in both *H. armigera* (Wang et al., 2016) and *P. xylostella* (Guo et al., 2019) using exuviae DNA as template. Thereof, the DNA samples' extraction from old exuviae is an ideal approach without any damage or interruption on insect development.

The target gene involving in pigmentation pathway may generate obvious phenotype changes after the CRISPR/Cas9 gene editing. Some studies have demonstrated that DDC plays the pivotal role in melanin synthesis in H. axyridis by RNAi (Chen et al. 2019, Xiao et al. 2020). In the present study, DDC was successfully editing using CRISPR/Cas9 also resulting in melanin disappeared in the whole body of H. axyridis (Fig. 4). Our results were consistent with other studies about pigment synthesis pathway in other insect species, like DDC gene editing in V. cardu (Zhang and Reed, 2016), yellow gene editing in A. aegypti (Li et al., 2017), and A. albopictus (Liu et al., 2019). Moreover, the ABC transporter genes that encode eye pigment receptors were also successfully editing and showed visible phenotype changes in D. melanogaster (Bassett et al., 2013; Kondo and Ueda, 2013), D. suzukii (Yan et al., 2020), H. armifera (Khan et al., 2017), N. lugens (Xue et al., 2018), and N. vitripennis (Li et al., 2017). For CRISPR/Cas9 protocol establishment, the target genes selection should be considered having the visible phenotype changes without development disruption.

Unexpected, we found 25% un-hatched eggs were successfully gene editing. A similar phenomenon also found in *abdominal-A* homeotic gene editing in *P. xylostella* study (Huang et al. 2016). Previous study reported that dopamine is the product of *DDC* in melanin synthesis and dopamine works as neurotransmitter and plays a crucial role in insect development (Hodgetts and O'Keefe 2006). Therefore, the successful *HaDDC* editing resulted in absence of dopamine and further impact the embryogenesis process. Beyond, our results also suggested that the mutant ratio should carefully consider the mutants of embryos after microinjection, especially, the un-hatched eggs.

When *DDC* gene was successfully edited in *H. axyridis* by CRISPR/Cas9, the mutant ladybirds (G0) showed fitness cost on developmental time. The development time of mutant *H. axyridis* larval stages was significantly prolonged comparing with the controls, especially in the fourth instar larvae (Fig. 5). The fourth instar larvae are the developmental stage to prepare for transformation from larvae to pupa because of the titer changes in juvenile hormone, ecdysone, and others. Previous studies had demonstrated that *DDC* plays a crucial role in molting because it is closely related with the regulation of ecdysone in the molting process (Hiruma and Riddiford 2009, Wang et al. 2013, Sterkel et al 2019). Here, *DDC* mutant in *H. axyridis* might result in the disruption of ecdysone production and further affect molting.

*H. axyridis* was famous for its predation ability on various sucking mouthpart pest insects, e.g., aphid. Whether *DCC* mutants can affect predation ability on *H. axyridis*, the fourth instar larvae of *H. axyridis* in wild and mutant type (G0) were selected to evaluate predation ability. Our results demonstrated that the mutants of the fourth instar ladybird larvae (G0) have equal predation ability with wild type (Fig. 6). Therefore, we speculate absence of *DDC* has no significant effect on predation ability in mutant *H. axyridis* (G0).

In summary, we successfully established CRISPR/Cas9 gene editing platform in Asian-multi colored ladybird *H. axyridis*. The *HaDDC* mutants generated by CRISPR/Cas9 gene editing significantly affect metamorphosis from larvae to pupae stage in *H. axyridis* and the detailed regulation mechanism should be investigated in future research.

#### Acknowledgments

We thank Doctor Dan Sun (Department of Plant Protection, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences) for helping us to design needle in micro-injection in *H. axyridis*. Technical innovation program of Beijing Academy of Agriculture, and Forestry Sciences (KJCX20200110), National Natural Science Foundation of China (32072479), and the Beijing technology program (Z201100008020014).

#### **Author Contributions**

Conceived and designed the experiments: M.-M.W., X.C., L.-S.Z., S.W., D.X.; Performed the experiments: M.-M.W., X.C.; Analyzed the data: M.-M.W., Q.-X.X., D.X.; Contributed reagents/materials / analysis tools: Q.-X.X., S.W., D.X.; Wrote the paper: M.-M.W., X.C., S.W., M. L., D.X.; Contributed with revisions: M.-M.W., X.C., S.W., D.X.

#### **References Cited**

- Adrianos, S., M. Lorenzen, and B. Oppert. 2018. Metabolic pathway interruption: CRISPR/Cas9-mediated knockout of tryptophan 2, 3-dioxygenase in *Tribolium castaneum. J. Insect Physiol.* 107: 104–109.
- Ando, T., T. Matsuda, K. Goto, K. Hara, A. Ito, J. Hirata, J. Yatomi, R. Kajitani, M. Okuno, K. Yamaguchi, et al. 2018. Repeated inversions within a pannier intron drive diversification of intraspecific colour patterns of ladybird beetle. Nat. Commun. 9: 3843.
- Ando, T., and T. Niimi. 2019. Development and evolution of color patterns in ladybird beetles: a case study in *Harmonia axyridis*. Dev. Growth Differ. 61: 73–84.
- Artegiani, B., D. Hendriks, J. Beumer, R. Kok, X. Zheng, I. Joore, S. C. S. Lopes, J. Zon, S. Tans, and H. Clevers. 2020. Fast and efficient generation

of knock-in human organoids using homology-independent CRISPR/Cas9 precision genome editing. *Nat. Cell Biol.* 22: 321–331.

- Bassett, A. R., C. Tibbit, C. P. Ponting, and J. L. Liu. 2013. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep.* 4: 220–228.
- Bi, H. L., J. Xu, A. J. Tan, and Y. P. Huang. 2016. CRISPR/Cas9-mediated targeted gene mutagenesis in Spodoptera litura. Insect Sci. 23: 469–477.
- Bradley, J. C., T. H. Chad, G. Kevin, and R. I. Anthony. 2003. Biodiversity and biocontrol: emergent impacts of a multi-enemy assemblage on pest suppression and crop yield in an agroecosystem. *Ecol. Lett.* 6: 857–865.
- Chen, P., J. Wang, H. Li, Y. Li, P. Chen, T. Li, X. Chen, J. Xiao, and J. Zhang. 2015. Role of GTP-CHI links PAH and TH in melanin synthesis in silkworm, *Bombyx mori. Gene.* 567: 138–145.
- Chen, X., D. Xiao, X. Du, X. Guo, F. Zhang, N. Desneux, L. Zang, and S. Wang. 2019. The role of the dopamine melanin pathway in the ontogeny of elytral melanization in *Harmonia axyridis*. Front. Physiol. 10: 1066.
- Cong, L., F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. B. Wu, W. Y. Jiang, L. A. Marraffini, et al. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 339: 819–823.
- Daimon, T., T. Kiuchi, and Y. Takasu. 2014. Recent progress in genome engineering techniques in the silkworm, *Bombyx mori. Dev. Growth Differ*. 56: 14–25.
- Evans, E. W. 2009. Ladybeetles as predators of insects other than *Hemiptera*. *Biol. Control.* 51: 255–267.
- Fauser, F., S. Schiml, and H. Puchta. 2014. CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in Arabidopsis thaliana. *Plant J*. 79: 348–359.
- Futahashi, R., and H. Fujiwara. 2006. Expression of one isoform of GTP cyclohydrolase Icoincides with the larval black markings of the swallowtail butterfly, *Papilio xuthus. Insect Biochem. Mol. Biol.* 36: 63–70.
- Gautier, M., J. Yamaguchi, J. Foucaud, A. Loiseau, A. Ausset, B. Facon, B. Gschloessl, J. Lagnel, E. Loire, H. Parrinello, *et al.* 2018. The genomic basis of color pattern polymorphism in the *Harlequin Ladybird*. *Curr. Biol.* 28: 3296–3302.
- Gilles, A. F., J. B. Schinko, and M. Averof. 2015. Efficient CRISPR-mediated gene targeting and transgene replacement in the beetle *Tribolium castaneum*. *Development*. 142: 2832–2839.
- Guo, Z., D. Sun, S. Kang, J. Zhou, L. Gong, J. Qin, L. Guo, L. Zhu, Y. Bai, L. Luo, et al. 2019. CRISPR/Cas9-mediated knockout of both the PxABCC2 and PxABCC3 genes confers high-level resistance to Bacillus thuringiensis Cry1Ac toxin in the diamondback moth, Plutalla xylostella (L.). Insect Biochem. Mol. Biol. 107: 31–38.
- Hammond, A., R. Galizi, K. Kyrou, A. Simoni, C. Siniscalchi, D. Katsanos, M. Gribble, D. Baker, E. Marois, S. Russell, et al. 2016. CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. Nat. Biotechnol. 34: 78–83.
- Hiruma, K., and L. M. Riddiford. 2009. The molecular mechanism of cuticular melanization: the ecdysone cascade leading to dopa decarboxylase expression in *Manduca sexta*. *Insect Biochem. Mol. Biol.* 39: 245–253.
- Hodgetts, R. B., and S. L. O'Keefe. 2006. DOPA DECARBOXYLASE: a model gene-enzyme system for studying development, behavior, and systematics. *Annu. Rev. Entomol.* 51: 259–284.
- Huang, Y., Y. Chen, B. Zeng, Y. Wang, A. A. James, G. M. Gurr, G. Yang, X. Lin, Y. Huang, and M. You. 2016. CRISPR/Cas9 mediated knockout of the abdominal-A homeotic gene in the global pest, diamondback moth (*Plutella xylostella*). *Insect Biochem. Mol. Biol.* 75: 98–106.
- Jansen, R., J. D. Embden, W. Gaastra, and L. M. Schouls. 2002. Identification of genes that are associated with DNA repeats in prokaryotes. *Mol. Microbiol.* 43: 1565–1575.
- Jiang, W. Z., B. Yang, and D. P. Weeks. 2014. Efficient CRISPR/Cas9-mediated gene editing in Arabidopsis thaliana and inheritance of modified genes in the T2 and T3 generations. *PLoS One*. 9: e99225.
- Kanda, T., and T. Tamura. 1991. Microinjection method for DNA in early embryos of the silkworm, bombyx mori, using air-pressure. *Bull. Natl. Inst. Sericult. Entomol. Sci.* 2: 31–46.
- Khan, S. A., M. Reichelt, and D. G. Heckel. 2017. Functional analysis of the ABCs of eye color in *Helicoverpa armigera* with CRISPR/Cas9-induced mutation. *Sci. Rep.* 7: 40025.

- Kieharrt, D. P., J. M. Crawford, and R. A. Montague. 2007. Quantitative microinjection of *Drosophila* embryos. *Cold Spring Harb. Protoc.* 4 pdb: Prot4718.
- Kistler, K. E., L. B. Vosshall, and B. J. Matthews. 2015. Genome engineering with CRISPR-Cas9 in the mosquito Aedes aegypti. Cell Rep. 11: 51–60.
- Knott, G. J., and J. A. Doudna. 2018. CRISPR-Cas guides the future of genetic engineering. *Science*. 361: 866–869.
- Koch, R. L. 2003. The multicolored Asian lady beetle, *Harmonia axyridis*: a review of its biology, uses in biological control, and non-target impacts. J. Insect Sci. 3: 1–16.
- Kohno, H., S. Suenami, H. Takeuchi, T. Sasaki, and T. Kubo. 2016. Production of knockout mutant by CRISPR/Cas9 in the European honeybee, *Apis mellifera L. Zool. Sci.* 33: 505–512.
- Kondo, S., and R. Ueda. 2013. Highly improved gene targeting by germlinespecfic Cas9 expression in Drosophila. *Genetics*. 195: 715–721.
- Li, J. J., Y. Shi, J. N. Wu, H. Li, S. Guy, and T. X. Liu. 2021. CRISPR/Cas9 in lepidopteran insects: Progress, application and prospects. J. Insect Physiol. 135: 104325.
- Li, M., L. Y. C. Au, D. Douglah, A. Chong, B. J. White, P. M. Ferree, and O. S. Akbari. 2017. Generation of heritable germline mutations in the jewel wasp *Nasonia vitripennis* using CRISPR/Cas9. *Sci. Rep.* 7: 901.
- Li, M., M. Bui, T. Yang, C. S. Bowman, B. J. White, and O. S. Akbari. 2017. Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, *Aedes aegypti. Proc. Natl. Acad. Sci.* 114: E10540–E10549.
- Liu, T., W. Q. Yang, Y. G. Xie, P. W. Liu, L. H. Xie, F. Lin, C. Y. Li, J. B. Gu, K. Wu, G. Y. Yan, et al. 2019. Construction of an efficient genomic editing system with CRISPR/Cas9 in the vector mosquito Aedes albopictus. Insect Sci. 26: 1045–1054.
- Lobo, N. F., J. R. Clayton, M. J. Fraser, F. C. Kafatos, and F. H. Collins. 2006. High efficiency germ-line transformation of mosquitoes. *Nat. Protoc.* 1: 1312–1317.
- Ma, S., Y. Liu, Y. Liu, J. Chang, T. Zhang, X. Wang, R. Shi, W. Lu, X. Xia, P. Zhao, et al. 2017. An integrated CRISPR Bombyx mori genome editing system with improved efficiency and expanded target sites. Insect Biochem. Mol. Biol. 83: 13–20.
- Nami, F., M. Basiri, L. Satarian, C. Curtiss, H. Baharvand, and C. Verfaillie. 2018. Strategies for in vivo genome editing in nondividing cells. *Trends Biotechnol.* 36: 770–786.
- Niimi, T., and T. Ando. 2021. Evo-devo of wing colour patterns in beetles. Curr. Opin. Genet. 69: 97–102.
- Perry, T., W. Chen, R. Ghazali, Y. T. Yang, D. Christesen, F. Martelli, C. Lumb, H. N. B. Luong, J. Mitchell, J. K. Holien, et al. 2021. Role of nicotinic acetylcholine receptor subunits in the mode of action of neonicotinoid, sulfoximine and spinosyn insecticides in *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 131: 103547.
- Reid, W., and D. A. O'Brochta. 2016. Applications of genome editing in insects. Curr. Opin. Insect Sci. 13: 43–54.
- Sander, J. D., and J. K. Joung 2014. CRISPR-Cas systems for editing regulating and targeting genomes. Nat. Biotechnol. 32: 347–355.
- Sterkel, M., S. Ons, and P. L. Oliveira. 2019. DOPA decarboxylase is essential for cuticle tanning in *Rhodnius prolixus* (Hemiptera: Reduviidae), affecting ecdysis, survival and reproduction. *Insect Biochem. Mol. Biol.* 108: 24–31.
- Sun, D., Z. Guo, Y. Liu, and Y. Zhang. 2017. Progress and prospects of CRISPR/ Cas system in insects and other arthropods. *Front. Physiol.* 8: 608.
- Tamura, T., N. Kuwabara, K. Uchino, I. Kobayashi, and T. Kanda 2007. An improved DNA injection method for silkworm eggs drastically increases the efficiency of producing transgenic silkworms. *Insect Biotechnol. Sericol.* 76: 155–159.
- Taning, C. N. T., B. V. Eynde, N. Yu, S. Ma, and G. Smagghe. 2017. CRISPR/ Cas9 in insects: applications, best practices and biosafety concerns. J. Insect Physiol. 98: 245–257.
- Wang, H. T., W. D. Zhang, C. X. Zhong, J. F. Zheng, L. Miao, Q. L. Qin. 2012. Mass rearing the multicolored Asian lady beetle on beet armyworm larvae. *J. Appl. Entomol.* 49: 1726–1731.
- Wang, M., Z. Cai, Y. Lu, H. H. Xin, R. Chen, S. Liang, C. O. Singh, J. N. Kim, Y. Niu, Y. Miao. 2013. Expression and functions of dopa decarboxylase

in the silkworm, *Bombyx mori* was regulated by molting hormone. *Mol. Biol. Rep.* 40: 4115–4122.

- Wang, J., H. Zhang, H. Wang, S. Zhao, Y. Zuo, Y. Yang, Y. Wu. 2016. Functional validation of cadherin as a receptor of *Bt* toxin Cry1Ac in *Helicoverpa armigera* utilizing the CRISPR/Cas9 system. *Insect Biochem. Mol. Biol.* 76: 11–17.
- Wang, X., X. Cao, D. Jiang, Y. Yang, and Y. Wu. 2020a. CRISPR/Cas9 mediated ryanodine receptor I4790M knockin confers unequanl resistance to diamides in *Plutella xylostella*. *Insect Biochem. Mol. Biol.* 125: 103453
- Wang, J., H. Ma, S. Zhao, J. Huang, Y. Yang, B. E. Tabashnik, Y. Wu. 2020b. Functional redundancy of two ABC transporter proteins in mediating toxicity of Bacillus thuringiensis to cotton bollworm. *PLoS Pathog.* 16: e1008427.
- Xiao, D., X. Chen, R. B. Tian, M. M. Wu, F. Zhang, L. Zang, J. D. Harwood, S. Wang. 2020. Molecular and potential regulatory mechanisms of melanin synthesis in *Harmonia axyridis*. Int. J. Mol. Sci. 21: 2088.
- Xue, W. H., N. Xu, X. B. Yuan, H. H. Chen, J. L. Zhang, S. J. Fu, C. X. Zhang, H. J. Xu. 2018. CRISPR/Cas9-mediated knockout of two eye pigmentation genes in the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae). *Insect Biochem. Mol. Biol.* 93: 19–26.

- Yan, Y., J. Ziemek, and M. F. Schetelig. 2020. CRISPR/Cas9 mediated disruption of the white gene leads to pigmentation deficiency and copulation failure in *Drosophila suzukii*. J. Insect Physiol. 126: 104091.
- Yin, H., W. Xue, and D. G. Anderson. 2019. CRISPR-Cas: a tool for cancer research and therapeutics. Nat. Rev. Clin. Oncol. 16: 281–295.
- Zhang, L. L., A. Mazo-Vargas, and R. D. Reed. 2017. Single master regulatory gene coordinates the evolution and development of butterfly color and iridescence. *Proc. Natl. Acad. Sci.* 114: 10707–10712.
- Zhang, L. L., and R. D. Reed. 2016. Genome editing in butterflies reveals that spalt promotes and Distal-less represses eyespot colour patterns. *Nat. Commun.* 7: 11769.
- Zhu, G. H., J. Xu, Z. Cui, X. T. Dong, Z. F. Ye, D. J. Niu, Y. P. Huang, S. L. Dong. 2016. Functional characterization of SlitPBP3 in *Spodoptera litura* by CRISPR/Cas9 mediated genome editing. *Insect Biochem. Mol. Biol.* 75: 1–9.
- Zou, M. M., Q. Wang, L. N. Chu, L. Vasseur, Y. L. Zhai, Y. D. Qin, W. Y. He, G. Yang, Y. Y. Zhou, L. Peng, et al. 2020. CRISPR/Cas9-induced vitellogenin knockout lead to incomplete embryonic development in *Plutella xylostella*. *Insect Biochem*. Mol. Biol. 123: 103406.