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Impairment of pupation by RNA interference-aided knockdown of *Broad-Complex* gene in *Leptinotarsa decemlineata* (Say)

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Abstract

Dietary delivery of bacterially expressed double-stranded RNA (dsRNA) has a great potential for management of *Leptinotarsa decemlineata*. An important first step is to discover possible RNA-interference (RNAi)-target genes effective against larvae, especially the old larvae. In the present paper, five putative *Broad-Complex* (*BrC*) cDNAs (*Z1-Z4*, and *Z6*) were identified in *L. decemlineata*. The expression of the five *LdBrC* isoforms was suppressed by juvenile hormone signaling, whereas the transcription was upregulated by 20-hydroxyecdysone signaling at the fourth (final) instar larval stage. Feeding of bacterially expressed ds*BrC* (derived from a common fragment of the five *LdBrC* variants) in the third- and fourth-instar larvae successfully knocked down the target mRNAs. For the fourth-instar *LdBrC* RNAi hypomorphs, they had a higher larval mortality compared with the controls. Moreover, most ds*BrC*-fed beetles did not pupate normally. After removal of the apolysed larval cuticle, a miniature adult was found. The adult head, compound eyes, prothorax, mesothorax, metathorax were found on the dorsal view. Distinct adult cuticle pigmentation was seen on the prothorax. The mouthparts, forelegs, midlegs, and hindlegs could be observed on the ventral view of the miniature adults. For the third-instar *LdBrC* RNAi specimens, around 20% moribund beetles remained as prepupae and finally died. Therefore, *LdBrC* is among the most attractive candidate genes for RNAi to control the fourth-instar larvae in *L. decemlineata*.

Keywords: *Leptinotarsa decemlineata*, *Broad Complex*, metamorphosis, pupation, RNA interference

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Introduction

In many potato producing areas around the world, defoliation by the Colorado potato beetle *Leptinotarsa decemlineata* (Say) can cause 40–100% yield losses in potato crops (Cingel *et al.*, 2016). Chemical insecticides are extensively used to control the beetles and protect potato production. However, this leads to rapid development of insecticide resistance (Clements *et al.*, 2017; Crossley *et al.*, 2017). In order to efficiently reduce the damage of *L. decemlineata*, novel management strategies must be explored (Xu *et al.*, 2017). RNA interference (RNAi) is a sequence-specific mRNA degradation process initiated by double-stranded RNA (dsRNA). It is being developed as a technology for pest management (Palli, 2014; Wang & Jin, 2017). In *L. decemlineata*, RNAi by the dietary introduction of bacterially expressed dsRNAs is able to effectively

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knockdown target genes (Meng *et al*., 2018*a*, *b*, Xu *et al*., 2018). Moreover, a dsRNA spraying method, the spray-induced gene silencing, has been proven to be effective to decrease the defoliation by *L. decemlineata* larvae on potato plant in the glass room (San Miguel & Scott, 2016). These demonstrate that RNAi can be developed against *L. decemlineata*.

However, RNAi efficiency is variable among life stages (Scott *et al*., 2013). In *Apis mellifera*, for example, when a vitellogenin-dsRNA is injected at the preblastoderm stage, 15% workers have strongly reduced levels of vitellogenin mRNA. In contrast, 96% individuals show the knockdown phenotype when dsRNA is introduced by intra-abdominal injection in newly emerged bees (Amdam *et al*., 2003). In *L. decemlineata*, dsRNA from a housekeeping gene *S-adenosyl-L-homocysteine hydrolase* (*LdSAHase*) causes lethality, inhibits growth and impairs pupation in an instar-dependent manner: the young larvae are more susceptible to dsRNA than the old ones (Guo *et al*., 2015). Similarly, knockdown of a chitin synthase gene *LdChSB* causes more serious defects and more mortality in the young larvae (Shi *et al*., 2016).

However, the first- to fourth (final)-instar *L. decemlineata* larvae frequently occur simultaneously in the potato field (Guo *et al*., 2013). Depending on climate, *L. decemlineata* beetles have several generations annually. Moreover, the females often lay eggs continuously for months. The generation overlapping makes young and old larvae, as well as adults, simultaneously feed foliage in the same potato field. Therefore, an RNAi-mediated management approach must be effective to both young and old larvae, and even to the adults. In the present paper, therefore, we intended to find attractive candidate genes for RNAi effective against third and final instar larvae.

Broad Complex (*BrC*) is an important metamorphic gene. It encodes a transcription factor which comprises a Bric-a-brac/Tramtrack/Broad complex (BTB) domain and an alternatively spliced C2H2 zinc finger domain (Z1–Z6) (DiBello *et al*., 1991; Bayer *et al*., 1996; Nishita & Takiya, 2004; Spokony & Restifo, 2007; Piulachs *et al*., 2010; Nagamine *et al*., 2014). In holometabolous insects, BrC controls pupal commitment and morphogenesis, and prevents adult differentiation (Kiss *et al*., 1976; Kiss *et al*., 1988; Zhou *et al*., 1998; Zhou & Riddiford, 2002; Zhou *et al*., 2004; Konopova & Jindra, 2008; Parthasarathy *et al*., 2008; Suzuki *et al*., 2008; Daimon *et al*., 2015). In *Manduca sexta*, *BrC* expression is among the first molecular events underlying pupal commitment of both epidermis and wing discs (Zhou & Riddiford, 2001). In *Drosophila melanogaster*, loss-of-function mutation of *BrC* gene (*nonpupariating* (*npr1*) mutant) can cause failure of pupariation (Kiss *et al*., 1988; DiBello *et al*., 1991; Bayer *et al*., 1997). In *Bombyx mori*, *BmBrC* RNAi in imaginal discs and primordia inhibits metamorphosis (Uhlirova *et al*., 2003). Likewise, the majority of the *TcBrC* RNAi larvae in *Tribolium castaneum* arrest development at the end of the prepupal stage or just after the pupal molt (Konopova & Jindra, 2008; Ureña *et al*., 2016). Comparable anti-development effects are also observed in the *BrC* RNAi *Oncopeltus fasciatus* nymphs (Erezyilmaz *et al*., 2006) and *Chrysopa perla* larvae (Konopova & Jindra, 2008). It appears that *BrC* is a potential gene for RNAi against the fourth-instar *L. decemlineata* larvae.

In the work presented here, we identified five putative *BrC* cDNAs (*LdBrC-Z1*, *LdBrC-Z2*, *LdBrC-Z3*, *LdBrC-Z4*, and *LdBrC-Z6*) in *L. decemlineata*. We found that knockdown of all these *LdBrC* isoforms caused larval mortalities in resultant larvae. Our results imply that *LdBrC* gene is a potential target for a dsRNA-based control method to the final instar larvae in *L. decemlineata*.

Materials and methods

Insects and chemicals

The *L. decemlineata* beetles were kept in an insectary according to a previously described method (Shi *et al*., 2013), with potato foliage at the vegetative growth or young tuber stages in order to assure sufficient nutrition.

Ecdysteroid 20-hydroxyecdysone (20E) (Sigma-Aldrich, USA), a juvenile hormone (JH) analogue methoprene [isopropyl (2E, 4E, 7S)-11-methoxy-3, 7, 11-trimethyldodeca-2, 4-dienoate] (Shanghai Kewelchem Company, Shanghai, China), were purified by reversed-phase high-performance liquid chromatography or high-performance liquid chromatography before experiments.

Molecular cloning

The putative *LdBrC* isoforms were obtained from the genome (Schoville *et al*., 2018) and transcriptome data (Shi *et al*., 2013) of *L. decemlineata*. The correctness of the sequences was substantiated by polymerase chain reaction (PCR) using primers in table S1. The full-length cDNAs were obtained by 5′- and/or 3′-RACE, using SMARTer RACE kit (Takara Bio.), with specific primers listed in table S1. After obtaining full-length cDNAs, primer pairs (table S1) were designed to verify the complete open reading frames. All of the sequenced cDNAs were submitted to GenBank (accession numbers: *LdBrC Z1*, KP340512; *LdBrC Z2*, KP340513; *LdBrC Z3*, KP340514; *LdBrC Z4*, KX881916; *LdBrC Z6*, KX9881917).

Preparation of dsRNAs

The same method as previously described (Zhou *et al*., 2013) was used to express ds*JHAMT*, ds*EcR*, ds*BrC*-1, ds*BrC*-2 and ds*egfp*, derived respectively from a 261 bp fragment of *LdJHAMT*, a 344 bp fragment of *LdEcR*, a 249 bp and 312 bp fragments targeting two different regions in the common sequence of the five *LdBrC* isoforms, and a 414 bp fragment of enhanced green fluorescent protein gene from *Aequorea victoria*. The five dsRNAs were individually transcribed with specific primers in table S1, using *Escherichia coli* HT115 (DE3) competent cells lacking RNase III. Individual colonies were inoculated, and grown until cultures reached an OD600 value of 1.0. The colonies were then induced to express dsRNA by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.1 mM. The expressed dsRNA was extracted and confirmed by electrophoresis on 1% agarose gel. Bacteria cells were centrifuged at 5000*g* for 10 min, and resuspended in an equal original culture volume of 0.05 M phosphate buffered saline (PBS, pH 7.4). The bacterial solutions (at a dsRNA concentration of about 0.5 μg ml^{-1}) were used for the experiment.

Dietary introduction of dsRNA

The same method as described previously (Fu *et al*., 2015) was used to dietarily introduce dsRNA into third- and fourth-instar larvae. Ten newly-ecdysed third- or fourth-instar larvae were confined in a Petri dish (9 cm diameter and 1.5 cm height) to feed foliage immersed with a bacterial suspension

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containing ds*JHAMT*, ds*EcR*, ds*BrC*-1 or ds*BrC*-2. To test the transcription response of *LdBrC* to dietary methoprene (a juvenile hormone analog, JHA) or ds*JHAMT*, six repeats were set. Three replicates were used to collect hemolymph for JH measurement and three were used to extract total RNA, after 3 days' ingestion of treated foliage (replaced with freshly treated ones each day). To determine the response of *LdBrC* to dietary 20E or ds*EcR*, three replicates were performed, which were used to extract total RNA after 3 days' ingestion of treated foliage. For knockdown of *LdBrC*, six replicates were set. Three replicates were used to observe the pupation and adults by allowing the larvae to feed on treated leaves for 3 days; and then all the treatments were transferred to untreated foliage until reaching the wandering stage. The other three were collected for RNA extraction after continuously fed on treated foliage for 3 days.

The beetles were weighed 5 days after the initiation of the experiment. The larval mortality and pupation were recorded during a 2-week trial period. The samples on day 3 after the initiation of the experiments were collected. The effects of gene silencing, the levels of *LdJHAMT*, *LdEcR* and five *LdBrC* isoforms, and JH titer were determined.

For the above experiments, three biological replicates were carried out.

Real-time quantitative PCR (qRT-PCR)

For analysis of the tissue expression patterns, RNA templates were derived from the brain-corpora cardiaca-corpora allata complex, prothoracic gland, ventral ganglion, foregut, midgut, hindgut, Malpighian tubules, hemocytes, epidermis and fat body of the day 4 fourth-instar larvae. For analysis of the effects of treatments, total RNA was extracted from treated larvae. Each sample contained 5–10 individuals and repeated three times. The RNA was extracted using SV Total RNA Isolation System Kit (Promega). Purified RNA was subjected to DNase I to remove any residual DNA according to the manufacturer's instructions. Quantitative mRNA measurements were performed by qRT-PCR in technical triplicate, using four internal control genes (*LdRP4*, *LdRP18*, *LdARF1* and *LdARF4*, the primers listed in table S1) according to our published results (Shi *et al*., 2013). An RT negative control (without reverse transcriptase) and a non-template negative control were included for each primer set to confirm the absence of genomic DNA and to check for primer-dimer or contamination in the reactions, respectively.

According to a previously described method (Bustin *et al*., 2009), the generation of specific PCR products was confirmed by gel electrophoresis. The primer pair for each gene was tested with a tenfold logarithmic dilution of a cDNA mixture to generate a linear standard curve (crossing point plotted vs. log of template concentration), which was used to calculate the primer pair efficiency. All primer pairs amplified a single PCR product with the expected sizes, showed a slope less than −3.0, and exhibited efficiency values ranging from 2.7 to 2.8. Data were analyzed by the $2^{-\Delta\Delta CT}$ method, using the geometric mean of the four internal control genes for normalization.

Quantitative determination of JH

We quantified JH titers in the beetles according to a previously reported method (Zhou *et al*., 2013). Hemolymph in three replicates (a total of 30 larvae) was respectively collected and transferred to a glass vial containing 400 µl of methanol, with 30 ng fenoxycarb (Syngenta Co. Ltd, China) as an internal standard, and then 100 µl of 2% NaCl was added. They were extracted three times with 300 µl of hexane. After adding hexane, the sample was vortexed vigorously incubated for 5 min at room temperature, and centrifuged (965 *g*) for 5 min. The hexane (upper) phase was collected in a new glass vial. The combined hexane extract (900 µl) was dried completely under vacuum and dissolved in 30 µl of acetonitrile. A liquid chromatography mass spectrometry was used to quantify JH titers (ng per ml hemolymph) (Cornette *et al*., 2008).

Data analysis

The data were given as means ± SE, and were analyzed by one-way ANOVA followed by the Tukey–Kramer test, using SPSS for Windows (SPSS, Chicago, IL, USA). Since no significant difference between dsRNAs targeting two different regions of *LdBrC* (ds*BrC*-1 and ds*BrC*-2) was found, the data of each dsRNA were combined.

Results

Identification of LdBrC

Five full-length *LdBrC* cDNAs were cloned from *L. decemlineata*. The *LdBrC* gene contained 11 exons. The first six exons encoded BTB core region, whereas a specific exon encoded zinc finger in each *LdBrC* isoform (fig. S1). The homologs of Z1–Z6 were extensively searched in public databases using tblastx with the deduced amino acid sequences of *LdBrC* isoforms as the query. The Z1–Z5 sequences were respectively obtained from various orders of insects including holometabolan and hemimetabolan. Z6 was only found in a single species, *Blattella germanica*. The phylogenetic tree of the Z1-Z6 sequences in various insects was constructed based on amino acid sequences of zinc fingers. Based on the phylogenetic analysis, the Z1–Z6 groups were respectively monophyletic. Accordingly, the five *LdBrC* isoforms in *L. decemlineata* were named as *LdBrC-Z1*, *LdBrC-Z2*, *LdBrC-Z3*, *LdBrC-Z4*, and *LdBrC-Z6*, respectively (fig. 1).

Tissue expression profiles of LdBrC

To identify tissues at which *LdBrC* is expressed and potentially required, cDNA samples were prepared from the brain-corpora cardiaca-corpora allata complex, prothoracic gland, ventral ganglion, foregut, midgut, hindgut, Malpighian tubules, hemocytes, epidermis, and fat body of the day 4 fourth-instar larvae analyzed. The five isoforms could be detected at all examined tissues. The highest level of *LdBrC-Z1* was found in fat body; the most abundant mRNA levels of *LdBrC-Z2*, *LdBrC-Z3*, *LdBrC-Z4*, and *LdBrC-Z6* were noted in the brain-corpora cardiaca-corpora allata complex. The lowest levels were observed in midgut (for *LdBrC-Z1*, *LdBrC-Z3* and *LdBrC-Z6*), ventral ganglion (for *LdBrC-Z2*) or hemocytes (for *LdBrC-Z4*) (fig. 2).

JH downregulates the expression of LdBrC *isoforms*

In *L. decemlineata*, *JHAMT* expression levels are highly correlated to circulating JH titers. Therefore, it seems that JHAMT is responsible for JH biosynthesis (Fu *et al*., 2016). We tested *in vivo* effects of methoprene (a JH analog) and ds*JHAMT* ingestion (fig. S2) on the transcription of *LdBrC* isoforms in the

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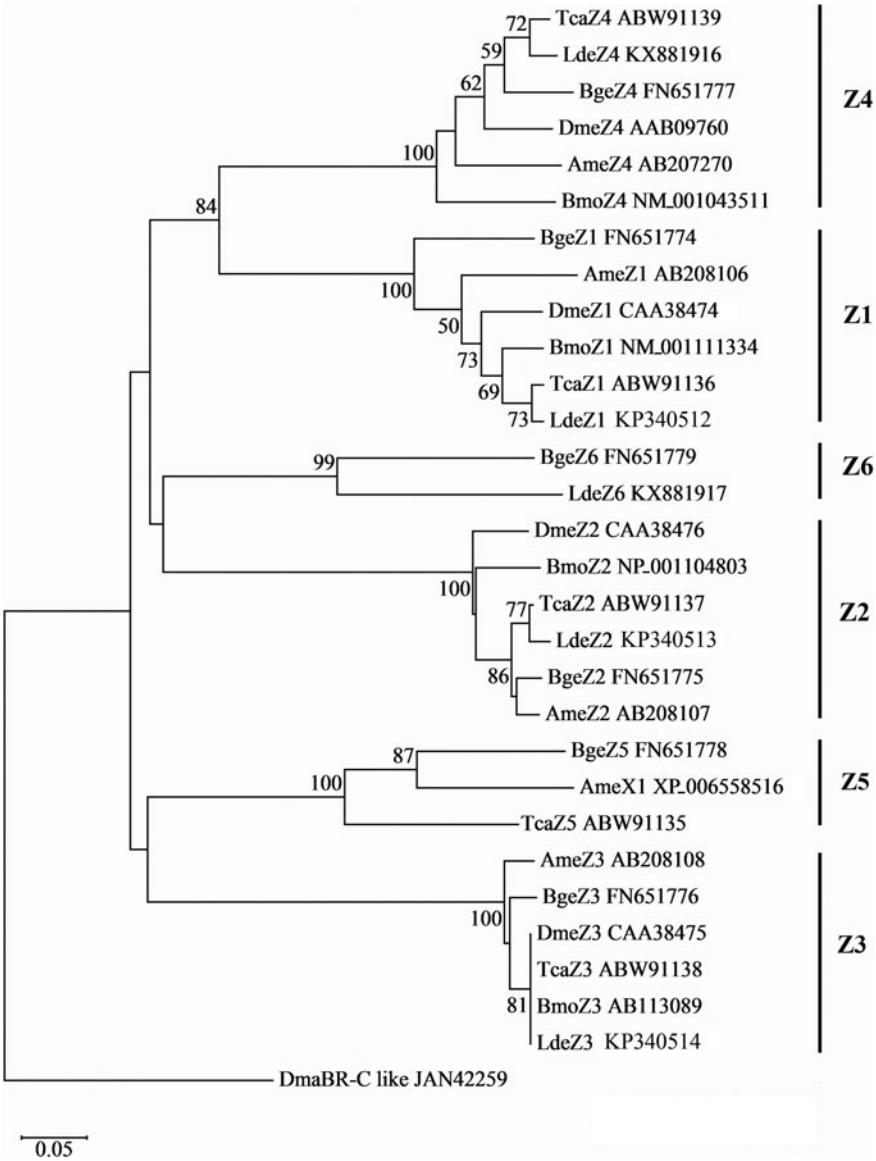

Fig. 1. Phylogenetic analysis of representative insect Broad-Complexes. An unrooted phylogenetic tree is generated by MEGA 6 using the neighbor-joining method based on amino acid sequences of zinc fingers. Bootstrap analyses of 1000 replications are carried out and bootstrap values >50% are shown on the tree. The proteins originate from *Blattella germanica* (Bge), *Tribolium castaneum* (Tca), *Leptinotarsa decemlineata* (Say) (Lde), *Drosophila melanogaster* (Dme), *Apis mellifera* (Ame), *Bombyx mori* (Bmo), and *Daphnia magna* (Dam).

newly-ecdysed fourth-instar larvae. We found that methoprene ingestion significantly reduced the expression of these transcripts ($P < 0.05$, ANOVA followed by the Tukey–Kramer test), whereas ds*JHAMT* feeding significantly increased the transcription of *LdBrC* isoforms ($P < 0.05$, ANOVA followed by the Tukey–Kramer test) in the treated beetles, compared with those in the control larvae (fig. 3).

20-Hydroxyecdysone activates LdBrC

LdEcR has been identified as the functional 20E receptor in *L. decemlineata* (Ogura *et al*., 2005). Here we determined *in vivo* effects of 20E and ds*EcR* ingestion (fig. S3) on the transcription of *LdBrC* isoforms in the newly-ecdysed fourth-instar larvae. As expected, 20E ingestion significantly upregulated the expression of these transcripts ($P < 0.05$, ANOVA followed by the Tukey–Kramer test), whereas ds*EcR* feeding significantly diminished the transcription of *LdBrC* isoforms in the treated larvae ($P < 0.05$, ANOVA followed by the Tukey–Kramer test), compared with those in the controls (fig. 4).

*Ingestion of ds*BrC *by the final larval instars completely inhibits pupation*

We dietarily introduced each of the two dsRNAs of *LdBrC* directed against the variable common region of all five variants into the newly-molted fourth-instar larvae. Combined

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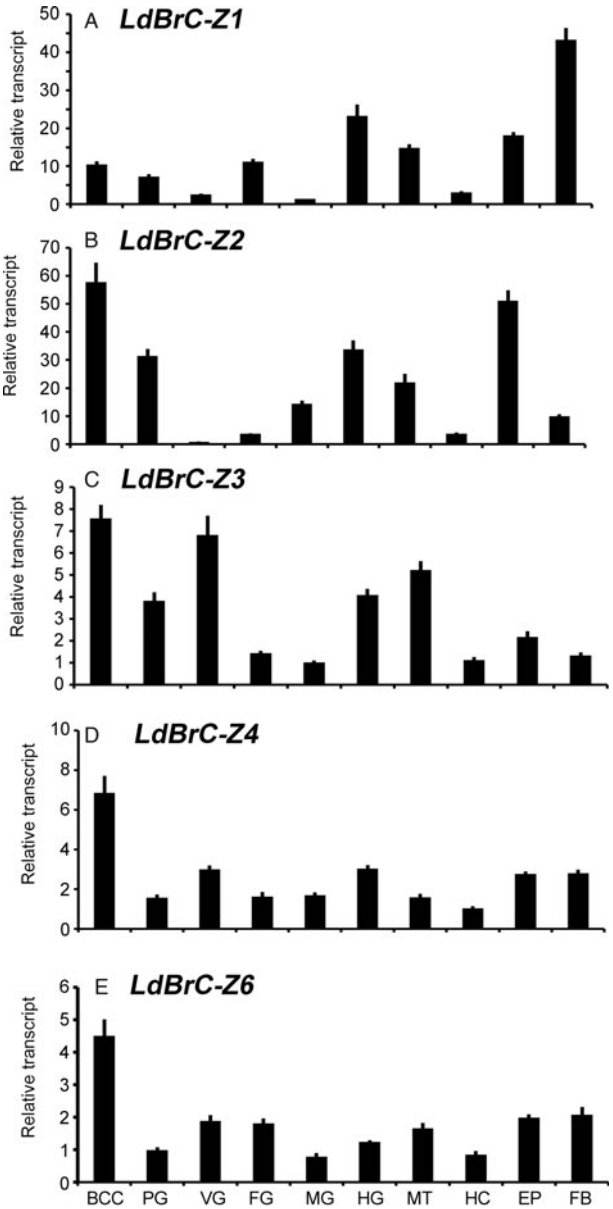

Fig. 2. Tissue expression profiles of *LdBrC* variants in *L. decemlineata*. The cDNA templates were derived from the brain-corpora cardiaca-corpora allata complex (BCC), prothoracic gland (PG), ventral ganglion (VG), foregut (FG), midgut (MG), hindgut (HG), Malpighian tubules (MT), hemocytes (HC), epidermis (EP) and fat body (FB) of the day 4 fourth-instar larvae. The lowest expression levels in MG for *LdBrC-Z1*, *LdBrC-Z3*, and *LdBrC-Z6*, in VG for *LdBrC-Z2* and in HC for *LdBrC-Z4* are set as 1. For each sample, three independent pools of 5–10 individuals are measured in technical triplicate using qRT-PCR. The bars represent $2^{-\Delta Ct}$ method (±SE) normalized to the geometrical mean of housekeeping gene expression.

data revealed that continuous ingestion of a ds*BrC* for 3 days significantly downregulated its target gene (fig. 5a).

The ds*BrC*-fed fully-grown larvae obtained lighter fresh weights than the controls (fig. 5b). While the control larvae pupated 7 days after initiation of bioassay (fig. 5e), around 30% of *LdBrC* RNAi hypomorphs normally pupated (fig. 5c). The remaining prepupae were completely wrapped in the larval cuticle. After 9 and 11 days after initiation of the experiment, the RNAi beetles were somewhat withered, dried, and darkened (fig. 5f).

A prepupa is a pharate pupa in which larval cuticle has been apolysed and the newly deposited cuticle has attained pupal characters. However, after removal of the apolysed larval cuticle, an arrested *LdBrC* RNAi hypomorph is a miniature adult (fig. 5g). Distinct adult cuticle pigmentation was seen on the prothorax (fig. 5h, i). The adult head, compound eyes, prothorax, mesothorax, and metathorax were found on the dorsal view. The discs of forewing and hindwing were attached on the mesothorax and metathorax, respectively (fig. 5j). The mouthparts, forelegs, midlegs, and hindlegs could be observed on the ventral view of the miniature adults (fig. 5k).

Approximately 60% of RNAi pupae emerged as adults (fig. 5d). All these *LdBrC* RNAi adults eventually died within 1 week after molting.

*Ingestion of ds*BrC *by the third larval instars causes lethality*

Continuous ingestion of a ds*BrC* for 2 days significantly reduced its target gene (fig. 6a). Normal control larvae ecdysed 2 days after initiation of bioassay (fig. 6e), around 20% of the *LdBrC* RNAi moribund beetles remained as prepupae at 13 days after initiation of the experiment (fig. 6f). They were wrapped in the old larval cuticle and finally died. The remaining RNAi larvae grew (fig. 6b) and pupated (fig. 6d) normally.

Discussions

BrC controls pupal commitment and pupal morphogenesis, and inhibits adult differentiation (Kiss *et al.*, 1976; Kiss *et al.*, 1988; Zhou *et al.*, 1998; Zhou & Riddiford, 2002; Zhou *et al.*, 2004; Konopova & Jindra, 2008; Parthasarathy *et al.*, 2008; Suzuki *et al.*, 2008; Daimon *et al.*, 2015). Therefore, the feasibility of using an RNAi-based pest-control strategy targeting *LdBrC* gene in the third- and fourth-instar larvae in *L. decemlineata* was carefully examined in this study.

LdBrC *should be an important regulator to* L. decemlineata *metamorphosis*

In the present paper, we provided three lines of evidence to suggest that *Ld*BrC was an essential regulator of the larval-pupal metamorphosis in *L. decemlineata*. Firstly, we cloned five full-length *LdBrC* cDNAs (*LdBrC-Z1*, *LdBrC-Z2*, *LdBrC-Z3*, *LdBrC-Z4*, and *LdBrC-Z6*) in *L. decemlineata*. These *BrC* isoforms (Z1–Z6) are conserved in both hemimetabolan and holometabolan insects (DiBello *et al.*, 1991; Bayer *et al.*, 1996; Nishita & Takiya, 2004; Spokony & Restifo, 2007; Piulachs *et al.*, 2010; Nagamine *et al.*, 2014). In *Psacothea hilari*, although seven *PhBrC* isoforms (referred to as Z1, Z2, Z3, Z2/Z3, Z4, Z5/Z6, and Z6, respectively) are cloned, the Z5/Z6 isoform is aberrant in that it contained a premature stop codon (Nagamine *et al.*, 2014).

The second line of evidence was that all isoform-specific mRNAs were easily detected in all tested tissues. The wide distribution of *LdBrC* variants among various tissues indicates its requirement for pupal differentiation in *L. decemlineata*. Similar tissue expression patterns of *BrC* isoforms have also been reported in other insect species (Emery *et al.*, 1994).

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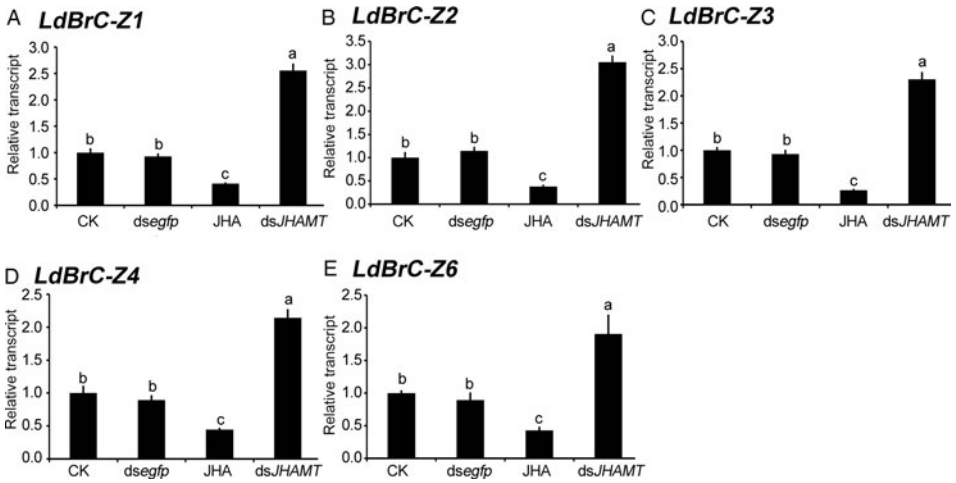

Fig. 3. Ingestion of a juvenile hormone analog (JHA) methoprene and knockdown of *LdJHAMT* on the expression of *LdBrC* isoforms in *L. decemlineata*. The newly-ecdysed fourth-instar larvae are allowed to ingest potato foliage treated with water (blank control, CK), ds*egfp* (negative control), 100 ng ml^{-1} JH analog (JHA) methoprene, or ds*JHAMT* for three days. For each sample, three independent pools of 5–10 individuals are measured in technical triplicate using qRT-PCR. The bars ($2^{-\Delta\Delta Ct}$ method values ± SE) marked by different letters indicate significant difference at *P* value <0.05.

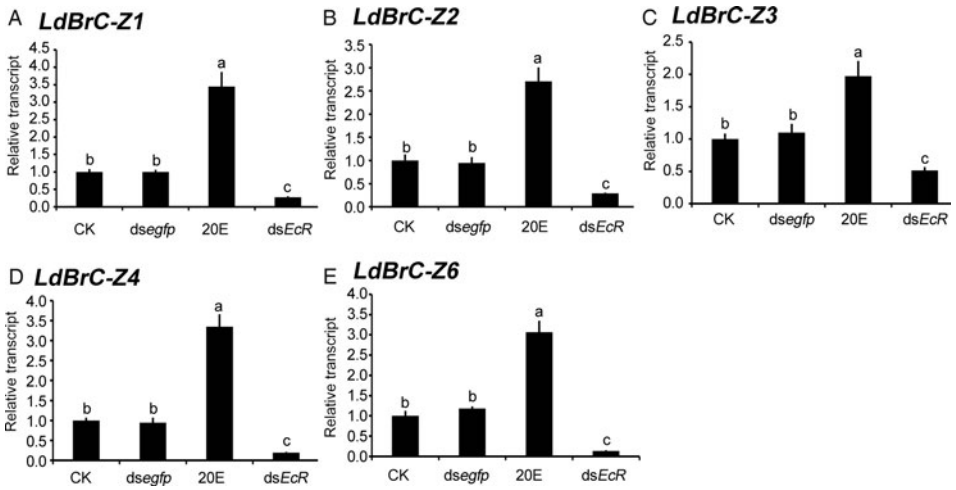

Fig. 4. Influence of 20-hydroxyecdysone (20E) signaling on the expression of *LdBrC* isoforms in *L. decemlineata*. The newly-ecdysed fourth-instar larvae are allowed to ingest potato foliage treated with water (blank control, CK), ds*egfp* (negative control), 100 ng ml^{-1} 20E, or ds*EcR* for three days. For each sample, three independent pools of 5–10 individuals are measured in technical triplicate using qRT-PCR. The bars ($2^{-\Delta\Delta Ct}$ method values ± SE) marked by different letters indicate significant difference at *P* value <0.05.

Thirdly, we found that the expression of the five *LdBrC* isoforms was suppressed by JH signaling, whereas the transcription was upregulated by 20E signaling. Tight regulation of the expression levels indicates important physiological functions of the five *LdBrC* isoforms in the larval-pupal metamorphosis in *L. decemlineata*. Consistent with our results, removal of the corpora allata (the primary organs for JH synthesis) in *M. sexta* and *B. mori* induces *BrC* expression and subsequent precocious pupation, and application of exogenous JH to allatectomized larvae inhibits *BrC* transcription and pupation in these larvae (Zhou *et al.*, 1998; Reza *et al.*, 2004). Moreover, treatment with methoprene at the onset of the adult molt causes re-expression of *BrC* in some insect species (Zhou & Riddiford, 2002; Konopova & Jindra, 2008; Minakuchi *et al.*, 2011). JH-mediated repression of *BrC* transcription and activity has also been reported in cultured insect epidermis (Zhou & Riddiford, 2001; Muramatsu *et al.*, 2008).

For 20E signaling, it is known that *BrC* is one of the early 20E response genes in holometabolous insect species (Karim *et al.*, 1993; Bayer *et al.*, 1996). In *B. mori*, the expression pattern of *BmBrC* in the carcass and silk gland roughly coincides with the fluctuation of 20E titers in the hemolymph (Nishita & Takiya, 2004). Moreover, an induction of *BmBrC* by 20E is detected in the *Bombyx* culture cell line BM-N (Nishita, 2014).

It can accordingly be hypothesized that JH represses *LdBrC* in young larvae. When JH declines at the final larval instar,

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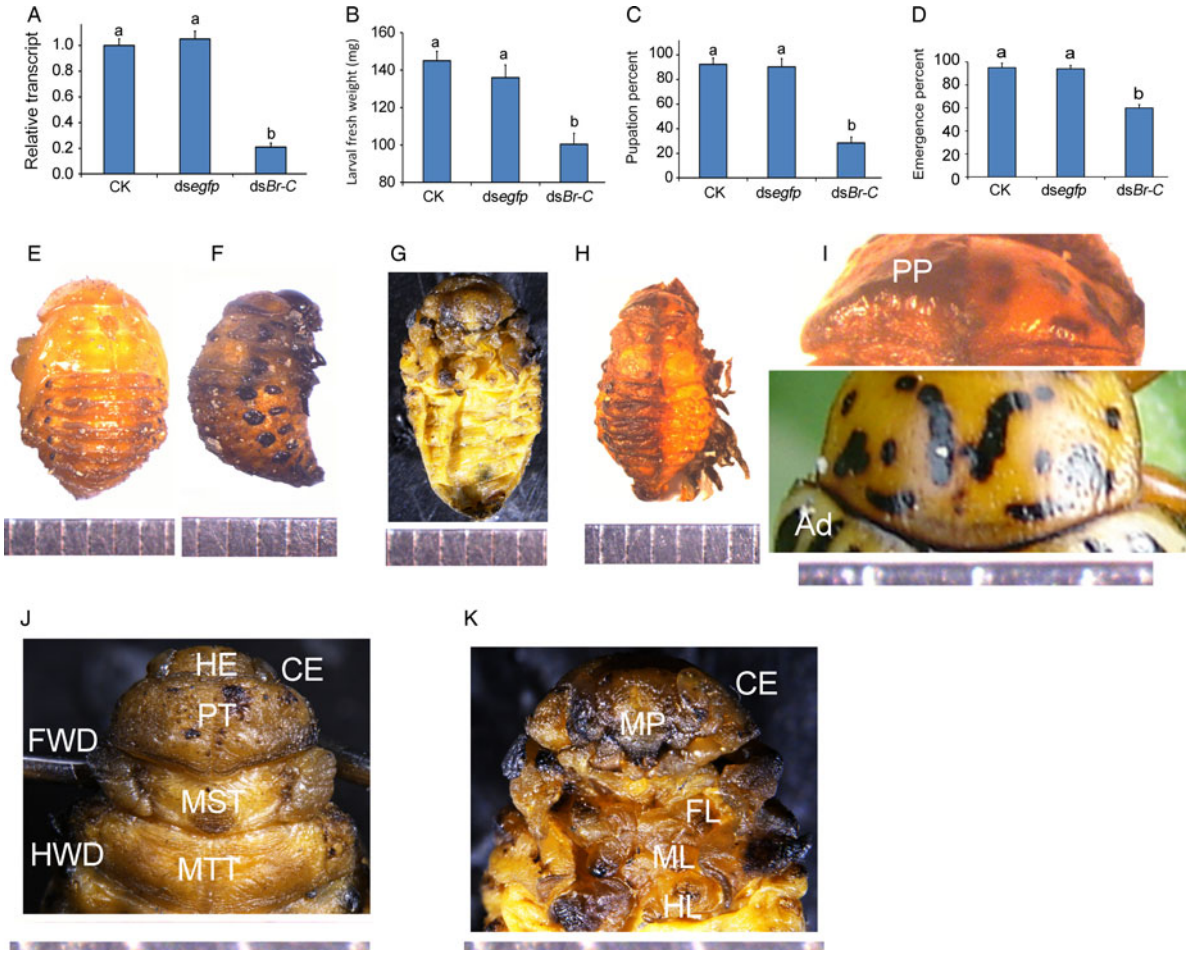

Fig. 5. The negative effects of the *LdBrC* RNAi on the fourth-instar *L. decemlineata* larvae. The newly-ecdysed fourth-instar larvae were allowed to ingest PBS (CK)-, ds*egfp*-, and ds*BrC*-dipped leaves for 3 days. The larvae were then transferred to untreated foliage if necessary. The relative transcript (a), larval fresh weights (b), pupation (c), and emergence (d) percentages were determined. The bars represent values (±SE). Different letters indicate significant difference at P value <0.05. While the CK larvae pupated 7 days after initiation of bioassay (e), around 70% of the *LdBrC* RNAi larvae fail to ecdyse. After 9 and 11 days after initiation, these beetles are somewhat withered, dried, and darkened (f). Upon removal of the whole apolysed larval cuticle, a miniature adult is seen (ventral view) (g). Prothorax of the prepupae (PP) shows distinct adult (Ad, the normal adult) cuticle pigmentation (dorsal view) (h, i). The head (HE), compound eyes (CE), prothorax (PT), mesothorax (MST), and metathorax (MTT) are formed. The discs of forewing (FWD) and hindwing (HWD) are attached on the mesothorax and metathorax, respectively (dorsal view) (j). The mouthparts (MT), forelegs (FL), midlegs (ML), and hindlegs (HL) are observed at the miniature adults (ventral view) (k). Scale bar is 1 mm. Approximately 60% of RNAi pupae emerged as adults (d).

LdBrC isoforms are expressed. The resultant proteins control pupal commitment and pupal morphogenesis in *L. decemlineata*, resembling other holometabolous insect species (Huang *et al*., 2013; Ureña *et al*., 2016).

Targeting LdBrC *with dsRNA is a potential control strategy*

In *Drosophila*, *Bombyx* and *Tribolium*, all BrC isoforms share some functions, or at least have partially overlapping functions during metamorphosis (Bayer *et al*., 1997; Parthasarathy *et al*., 2008; Suzuki *et al*., 2008). In *T. castaneum*, for example, all larvae treated for a single isoform ecdyse into pupae displaying a degree of aberrancies increasing in the order of isoforms: Z5 < Z1 < Z4 < Z3 < Z2. The most visible effect is the shortening of the wings and legs (Parthasarathy *et al*., 2008; Suzuki *et al*., 2008). Injection of ds*BrCZ1* and ds*BrCZ2*, ds*BrCZ1* and ds*BrCZ3*, ds*BrCZ2* and ds*BrCZ4*, and ds*BrCZ3* and ds*BrCZ4* results in individuals that resemble the complete *BrC* knockdown phenotypes, except that the degrees of pigmentation and segmentation are weaker and the urogomphi are more pupal-like in character (Parthasarathy *et al*., 2008; Suzuki *et al*., 2008).

Therefore, we dietarily introduced each of the two dsRNAs of *LdBrC* direct against the variable common region of all five variants into the newly-molted third- and fourth-instar larvae in the present paper, to test the lethal effects on the old larvae in *L. decemlineata*. Our results revealed that the fourth-instar ds*BrC*-fed fully-grown larvae obtained lighter fresh weights and approximately 30% *LdBrC* RNAi larvae did not get buried into the soil for pupation and finally died at the larval stage. Moreover, most remaining ds*BrC*-fed larvae did not normally pupate, or did not emerge as adults. Furthermore, around 20% third-instar *LdBrC* RNAi larvae remained as moribund beetles

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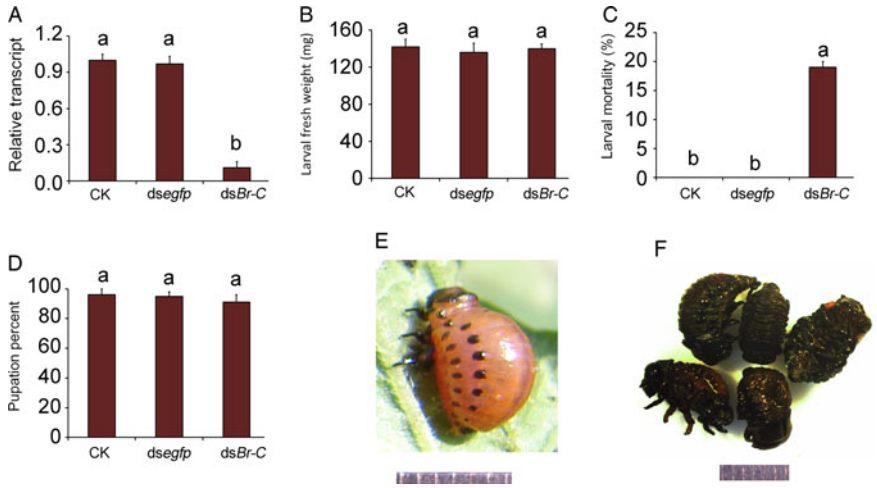

Fig. 6. The negative effects of the *LdBrC* RNAi on the third-instar *L. decemlineata* larvae. The newly-ecdysed third-instar larvae are allowed to ingest PBS (CK)-, ds*egfp*-, and ds*BrC*-dipped leaves for 3 days. The larvae are then transferred to untreated foliage if necessary. The relative transcript (a), larval fresh weights (b), larval mortalities (c), and pupation percent (d) are determined. The bars represent values (±SE). Different letters indicate significant difference at P value <0.05. While the CK larvae ecdysed to fourth larvae instars 2 days after initiation of bioassay (e), around 20% of the RNAi larvae failed to ecdyse, became moribund and finally died (f). Scale bar is 1 mm.

and finally died. Similar to our results, 98% of the *TcBrC* RNAi larvae in *T. castaneum* eventually die during the prepupal stage (Konopova & Jindra, 2008). Moreover, 95% of the ds*BrC* injected *Chrysopa* larvae arrest at the prepupal stage, and 41% of them fail to complete or even initiate spinning their cocoons (Konopova & Jindra, 2008).

In the present paper, we found that an arrested fourth-instar *LdBrC* RNAi hypomorph was a miniature adult in *L. decemlineata*, after removal of the apolysed larval cuticle. In agreement with our results, it is well known that BrC is involved in the metamorphic control of pupal commitment, pupal morphogenesis and the inhibition of adult differentiation (Kiss *et al*., 1976; Kiss *et al*., 1988; Zhou *et al*., 1998; Zhou & Riddiford, 2002; Zhou *et al*., 2004; Konopova & Jindra, 2008; Parthasarathy *et al*., 2008; Suzuki *et al*., 2008; Daimon *et al*., 2015). In the present paper, we found that the *LdBrC* RNAi larvae bypassed pupal stage, and became a miniature adult, although some structures, such as wing discs, were not fully developed in *L. decemlineata*. Similarly, *BrC* RNAi prepupae show a blend of larval, pupal, and partially even adult features in *Tribolium* and *Chrysopa* (Konopova & Jindra, 2008; Suzuki *et al*., 2008).

In short, exposure the fourth-instar larvae to ds*BrC*s killed most treated beetles. Therefore, our results demonstrate that targeting *LdBrC* with dsRNA can be used to control the final instar larvae in *L. decemlineata*.

However, there are still ample amount concerns and risks before application of ds*BrC* to control *L. decemlineata* larvae. Among them we will focus on three issues here. Firstly, expression in plants, chemical synthesis and production in bacteria and other microorganisms are the three major methods that are being developed to produce dsRNA. It is undoubted that the expression of dsRNA in transgenic plants might work well for commercial crops such as cotton. In contrast, dsRNA synthesized in bacteria may be a better choice for pests attacking food crops that is consumed directly by humans, such as potato (Palli, 2014). Our results in the present paper raise the possibility to control *L. decemlineata* larvae by bacterially-produced dsRNA.

Secondly, the first through fourth larval instars often occurred simultaneously in the potato field in Xinjiang Uygur Autonomous Region in China in *L. decemlineata* (Guo *et al*., 2013). The practical implementation of RNAi to protect potato necessarily requires the development of broad-spectrum larvicidal effects against both young and old larvae. A way is to construct a fused dsRNA including sequences from several genes respectively effective to young and old larvae. In fact, transgenic potato lines (*Solanum tuberosum* cv. Desiree) expressing fused viral coat protein coding sequences from *Potato virus X*, *Potato virus Y* and *Potato virus S* resulted in nearly 100% resistance against the infection of the three virus, when compared with untransformed controls (Hameed *et al*., 2017). Moreover, spraying barley (*Hordeum vulgare*) with a 791 nt long dsRNA (*CYP3*-dsRNA) targeting three fungal ergosterol biosynthesis genes (*CYP51A*, *CYP51B*, *CYP51C*) successfully knocked down the three genes in *Fusarium graminearum*, and inhibited fungal growth (Koch *et al*., 2016). We will conduct further experiments to test this issue.

The third concern is how to develop species-specific dsRNA pesticide given that BrC is conserved among insects. Since RNAi operates in a very sequence-specific manner, dsRNAs specific to the unique 5′- and/or 3′-untranslated region of BrCs could selectively kill only one species without adversely affecting other closely related species. Furthermore, when dsRNAs are harnessed as potential pesticides, it will also be important to assess the likelihood of cross-reactivity with other species (Whyard *et al*., 2009).

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485318001050.

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