Molecular Characterization of the cDNA Encoding Ecdysone Receptor Isoform B1 and Its Expression in the Oriental Fruit Fly, Bactrocera dorsalis (Diptera: Tephritidae)

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Abstract

The 20-hydroxyecdysone (20E) plays a critical role in a series of biological processes, via the ecdysone receptor/USP heterodimeric complex in arthropod. In order to clarify the regulatory mechanism of 20E, we characterized a full-length cDNA encoding a putative ecdysone receptor isoform B1 and named it as *BdEcR-B1* in the oriental fruit fly, *Bactrocera dorsalis*. The *BdEcR-B1* gene was 3,111 bp long, with an open reading frame of 2,304 bp, which encoded 767 amino acids with a predicated molecular weight of 83.3 kDa and an isoelectric point of 6.74. Alignment analysis revealed that the deduced protein sequence had 80% identity to EcR-B1 isoforms of various dipteran species, indicating that this gene was highly conserved during the evolution of the Diptera. Phylogenetic analysis suggested that *BdEcR-B1* was orthologous to the EcR-B1 proteins identified in other insect species. Quantitative real-time PCR showed that *BdEcR-B1* was expressed at all tested developmental stages, and the expression of *BdEcR-B1* reached a significantly higher level just prior to the larval-pupa molt stage and in 4-d old pupa than those in other stages. Moreover, the *BdEcR-B1* gene much more strongly expressed in gut and Malpighian tubule than those in the trachea and fat body, which suggests that this gene may be involved in tissue-specific function during larval development.

Key Words: *Bactrocera dorsalis*, ecdysone receptor, cloning, characterization, RT-qP-CR

Resumen

El 20-hydroxyecdysone (20E) juega un papel crítico en una serie de procesos biológicos, a través del complejo receptor de ecdisona heterodimérica / USP en los artrópodos. Para aclarar el mecanismo de regulación del 20E, se caracterizó una codificación de cDNA de longitud completa de un receptor putativo de ecdisona isoforma B1 y se le nombró como *BdEcR-B1* en la mosca oriental de la fruta, *Bactrocera dorsalis*. El gen *BdEcR-B1* fue de 3111 pb de largo, con un marco de lectura abierto de 2304 pb, que codifica 767 aminoácidos con un peso molecular predicho de 83.3 kDa y un punto isoeléctrico de 6.74. El análisis de alineación reveló que la secuencia de la proteína deducida tenía 80% de identidad a isoformas EcR-B1 de insectos del orden Diptera, que indica que este gen es bastante conservado durante la evolución de Diptera. El análisis filogenético sugiere que *BdEcR-B1* fue ortólogos a las proteínas EcR-B1, identificadas en otras especies de insectos. El análisis de PCR cuantitativo en tiempo real mostró que *BdEcR-B1* se expresó en todas los etadios de desarrollo probados, y la expresión de *BdEcR-B1* llegó a un nivel significativamente mayor en el estadio un poco antes de la muda de la larva a la pupa, y en las pupas de cuarto días de edad que en los de otros estadios. Por otra parte, el *BdEcR-B1* mostró una mayor expresión en el intestino y túbulo de Malpighi que en las tráqueas y la grasa corporal, lo que sugiere que este gen puede estar implicado con una función específica de los tejidos en el desarrollo de las larvas.

Palabras clave: *Bactrocera dorsalis*, receptor de la ecdisona, clonación, PCR cuantitativa, tiempo real

As the most active form of ecdysteroids in insects, 20-hydroxyecdysone (20E) plays a critical role in a series of biological processes during the whole life cycle, such as metamorphosis, development and reproduction (Henrich et al. 1998). The effect of 20E is transduced through a heterodimeric complex of 2 nuclear receptors, the ecdysone receptor (EcR) and ultraspiracle protein (USP) (Yao et al. 1992; Yao et al. 1993). The receptors directly initialize the expression of ecdysone-response gene cascades, which ultimately affect cell proliferation, differentiation and apoptosis

on an organism-wide scale (Riddiford et al. 2000; Schwedes et al. 2011).

In *Drosophila melanogaster*, three EcR isoforms were identified: EcR-A, EcR-B1, EcR-B2, according to different promoters and splicing patterns (Talbot et al. 1993). All these isoforms share an identical C-terminal region, which include a highly conserved DNA binding domain (DBD) and a structurally conserved ligand binding domain (LBD). However, they have isoform-specific regions in the N-terminal, which is indicative of alternative splicing (Hu et al. 2003; Bain et al. 2007). It has been shown that different isoforms have different tissue-specific and development-specific expression patterns (Bender et al. 1997) . Unlike EcR-A, which is highly expressed in adult stage, the EcR-B isoform, which participates in tissue remodeling in the larval stage, is highly expressed in larval-specific tissue, and mutations or RNAi against EcR-B will cause obvious defects in larval metamorphosis, such as arrested development and pupation failure (Bender et al. 1997; Schubiger et al. 1998; Tan & Palli 2008).

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), is one of the most important pests of the horticultural industry in Asian and Pacific countries, because its larvae are frugivorous on more than 250 plant species including citrus and mango. Fruit infestation by fruit fly larvae causes direct fruit damage, fruit drop and export barriers in international trade through quarantine restrictions, all of which result in serious economic loses (Chen 2006; Stephens 2007). Having high mobility and fecundity as well as being very polyphagous, the oriental fruit fly is a dreaded invasive species and a major quarantine target (Clarke et al. 2005). Because of the economic importance of *B. dorsalis* and its development of resistance to insecticides, it is urgent to open new potential avenues for control. Here, we report the cloning and characterization of this insect's EcR-B1 cDNA and its expression patterns in different developmental stages and in the tissues of larvae using quantitative real-time PCR with indications that it provides a potential molecular target for controlling this pest.

MATERIALS AND METHODS

Insects

Specimens of *B. dorsalis* were originally obtained in Fujian Province, People's Republic of China. The adult colony was reared at 27 ± 1 °C, 70 ± 5% RH, and 14:10 h L:D. Adults were fed an artificial diet, which on the basis of weight consisted of water, yeast, honey, and Vitamin C in the ratio: 500: 15:15:1. Eggs were placed for larval eclosion onto an artificial larval diet, which on the basis of weight consisted of corn flour, agar, wheat-germ flour, yeast, sugar, nipagin, sorbic acid, Vitamin C, and linolenic acid in the ratio: 600: 60: 250: 200: 200: 14: 7: 10: 1. This diet was poured into Petri dishes, allowed to cool, and then eggs from the adult colony were placed for hatching onto the surface of the cooled diet, and the larvae developed on it. Each petri dish was placed into a pot filled with sand until the larvae had pupated. After pupation, pupae were sieved out, placed into plastic cups and covered with sand. The plastic cups were placed in a cage to collect adults.

One-, 3-, 5-, and 7-d old larvae, 1-, 4-, 7-, and 10-d old pupae, and newly emerged female adults were chosen and stored at -80 °C until use. The fat body, gut, Malpighian tubule and trachea from the 7-d old larvae were dissected in 0.75% NaCl and immediately used for RNA isolation.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from larvae using RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany). The total RNA was dissolved in 40 μL DEPC-treated water. The purity and quality of the RNA were tested by the ratio of OD_{260}/OD_{280} and electrophoresis. High quality RNA was stored at -80 °C. Two μg of RNA was used to synthesis cDNA by means of the PrimeScript® 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) with oligo $(dT)_{18}$ primer and by means of the SMARTer™ RACE cDNA Amplification Kit (Clontech, California, USA), respectively. All reaction conditions were as stipulated by the manufacturer's instrucions, and the synthesized cDNA was kept at -20 °C for future use.

Obtaining Full-Length *BdEcR-B1* and Validation of Opening Reading Frame (ORF)

Based on the transcriptome data of *B. dorsalis*, 2 pairs of gene-specific primers were designed to obtain the fragments of *BdEcR-B1* (Shen et al. 2011). Gene-specific primers EcR-F/R1 and EcR-F/R2 (Table 1) were designed to amplify 2 cDNA fragments (nucleotide 150 F- 851 F and 1094 F-1621 F), and to generate 702 bp and 528 bp fragments, respectively.

The missing part was amplified using gene-specific primers EcR-3R1, EcR-3R2 (Table 1) and RACE (Rapid Amplification of cDNA Ends) technology were applied to obtain 3´-RACE of *BdEcR-B1*. In the first round reaction, 3´-RACE was performed by using 1 μL of 3´-RACE -ready-cDNA with Universal Primer Mix (UPM, Clontech) and EcR-3R1. Then nested PCR was conducted with the Nested Universal Primer (NUP, Clontech) and EcR-3R2.

PCR amplifications were carried out in a total volume of 25 μL, containing 15 μL ddH$_2$O, 2.5 μL 10 × PCR Buffer (Mg^{2+} free), 2.5 μL Mg^{2+} (2.5

TABLE 1. SEQUENCE OF ALL PRIMERS USED IN THE STUDY OF ECDYSONE RECEPTOR ISOFORM B1 IN *BACTROCERA DORSALIS*.

Primer	Sequence	Function
EcR-F1	5'-CACAACCCAATCAAACTTCA-3'	Fragment cDNA cloning
EcR-R1	5'-GCCGAATAGCCGTTGAGA-3'	Fragment cDNA cloning
EcR-F2	5'-AATGTCTGGCAGTGGGTATG-3'	Fragment cDNA cloning
EcR-R2	5'-TACGCAACATCATCACTTCC-3'	Fragment cDNA cloning
EcR-3R1	5'-AGTGATGATGTTGCGTATGG-3'	3'-RACE of *BdEcR-B1*
EcR-3R2	5'-GCACGCTTGGCAATCAGA-3'	3'-RACE of *BdEcR-B1*
EcR-FL-F	5'-CAACCCAATCAAACTTCAA-3'	Full-length validation
EcR-FL-R	5'-CCCATACCCCACCGCTTA -3'	Full-length validation
EcR-Q-F	5'-GAAAATGAAAGCCCGAATGA-3'	Q-PCR
EcR-Q-R	5'-GCCATACGCAACATCATCAC-3'	Q-PCR
α-tubulin-F	5'-CGCATTCATGGTTGATAACG-3'	Internal control
α-tubulin-R	5'-GGGCACCAAGTTAGTCTGGA-3'	Internal control

mM), 2 µL dNTP (2.5 mM), 1 µL each primer (10 mM), 1 µL cDNA, and 0.25 µL rTaq™ polymerase (TaKaRa, Dalian, China). PCRs were conducted by the following thermal cycles: 3 min initial denaturation at 95 °C, followed by 34 cycles of 30 s at 95 °C, 30 s annealing at 58 °C, 1.5 min extension at 72 °C, with a final extension at 72 °C for 10 min.

The PCR products were analyzed on 1.0% agarose gel and stained with GoodView™ (SBS Genetech, Beijing, China). The target band of cDNAs were excised and recovered with the Gel Extraction Mini Kit (Watson Biotechnologies, Shanghai, China).

The purified cDNA fragments were cloned into pGEM®-T Easy vector (Promega, Madison, Wisconsin, USA). The ligation reactions were transformed into DH5α competent cells (Transgen, Beijing, China). Successful clones were picked out by standard ampicillin selection and PCR with gene-specific primers used before, and further sequenced in both directions with an ABI Model 3100 automated sequencer (Invitrogen Life Technologies, Shanghai, China).

DNAMAN 5.2.2 (Lynnon, BioSoft, Quebec, Canada) was applied to assemble the putative full-length of *BdEcR-B1*. To verify the full-length of cDNA, the primers EcR-FL-F and EcR-FL-R (Table 1) were designed to amplify the ORF of *BdEcR-B1*, which contained partial 5´ and 3´-UTR. The PCR products were purified, cloned, and sequenced as described above.

Sequence Analysis

The full-length of the amino acid of *BdEcR-B1* was compared with other EcR sequences by means of the "BLAST-P" tool at NCBI website (http://www.ncbi.nlm.nih.gov/). The sequence was edited with DNAMAN 5.2.2. SUMOylation motifs were predicted by SUMOsp 2.0.4 (http://sumosp.biocuckoo.org/). The Neighbor-Joining method was carried out to construct a phylogenetic tree with 1000 replications as the bootstrap value by MEGA 5.04.

RT-qPCR Analysis

Developmental and tissue expression patterns of *BdEcR-B1* mRNA were assessed by RT-qPCR. Total RNA was isolated from different tissues (fat body, gut, Malpighian tubule and trachea of the third instar larvae), 1- and 4-d larvae, using RNeasy® Plus Micro Kit with gDNA Eliminator spin column (Qiagen, Hilden, Germany). Total RNA for 7-d larvae, pupae and adults was extracted using TRzol (Biomed, Beijing, China), and then treated with RQ1 DNase (TaKaRa, Dalian, China). First-strand cDNAs were synthesized by PrimeScript® RT reagent Kit (TaKaRa, Dalian, China). RT-qPCR was conducted using SYBR Green detection system (iQ™ SYBR® Green Supermix, BIO-RAD, Hercules, California, USA) with Mx3000P thermal cycler (Stratagene, La Jolla, California, USA) and gene-specific primers EcR-Q-F and EcR-Q-R. The PCR amplifications were performed in a total volume of 20 µL, including 10 µL SYBR Green Mix, 7 µL ddH$_2$O, 1 µL of each primer (10 mM) and 1 µL of template cDNA, following the common cycles: pre-incubation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s. After reaction, a melting curve analysis from 60 °C to 95 °C was applied to all reactions to ensure consistency and specificity of the amplified product. The α-tubulin gene was used as reference gene (GenBank accession number: GU269902) (Table 1), and the data was calculated using $\triangle\triangle$Ct method (Pfaffl 2001). All reactions were replicated 3 times.

Furthermore, the data of developmental expression and tissue-specific expression patterns were subjected an analysis of variance (ANOVA) (SPSS 12.0 for Windows) with the use of the LSD test to separate significantly different means.

RESULTS

Sequence Analysis of *BdEcR-B1* cDNA

The *BdEcR-B1* cDNA (GenBank accession number: JQ034623) was obtained by PCR. The full-length cDNA of *BdEcR-B1* contains 3,111 nucleotides, including 192 bp of 5´-UTR and 615 bp of 3´-UTR. The 2,304 bp open reading frame (ORF) encoded a protein of 767 amino acids with a predicted molecular weight of 83.3 kDa and an isoelectric point of 6.74. The putative polyadenylation signal "AATAAA" motif was detected in the 3´-UTR.

The polypeptide exhibited 5 domains (A/B, C, D, E, F), which were characteristic of a member of the nuclear receptor (Fig. 1). In the A/B domain, *BdEcR-B1* contained a highly conserved (K/R) RRW (K3-W6) motif, which belonged to the EcR-B1 isoform. In addition, several B1 isoform-specific regions were found in the N-terminus, such as the S-rich (E18-S28), which consisted of acidic residues, the SP residues (S38-P39), and the modified DL-rich motif (V45-D55), which was composed of repeats of several bulky hydrophobic residues and acidic residues. Two zinc finger motifs (E240-V315) were found in the C domain. Furthermore, an "EGCKG" (P-box, E260-G264) and a "KFGRS" (D-box, K289-S283) were identified in C domain. Two heptapetide sequences, the potential nuclear location signal (NLS), "MKRREKK" (M320-K326) and "QKEKEKQ" (Q328-Q324) were found in the D hinge domain. SUMOylation sites were predicted by SUMOsp 2.0.4. The result indicated that the protein has 4 potential SUMOylation sites (ΨKxE, where Ψ indicates one or more large hydrophobic amino acids) at positions 326, 709, 729 and 750.

Phylogenetic Analysis

A phylogenetic analysis was conducted to clarify the relationships of *BdEcR-B1* with other homologs from GenBank. Neighbor-joining algorithm analysis yielded that the *BdEcR-B1* was most closely related to *Ceratitis capitata* EcR (CAA11907) (Fig. 2). Dipteran EcRs were clustered separately from the EcRs of insects in other Orders. In addition, BLAST-P analyses in the NCBI database showed that the deduced amino acid sequence of *BdEcR-B1* shared 86% identity with *C. capitata* EcR (CAA11907), 83% identity with *Drosophila sechellia* (XP002043398), and 83% identity with *Drosophila yukuba* (XP001961340).

Expression Patterns of *BdEcR-B1*

To clarify the expression pattern of *BdEcR-B1* gene in various developmental stages and tissues, RT-qPCR was performed. The results showed that transcripts of *BdEcR-B1* were detected from the first day of larval to the newly emerged female adult (Fig. 3). Significantly higher expression of *BdEcR-B1* gene appeared on the last day of larval stage and on the fourth day of the pupa stage (LSD test, $P < 0.05$). During the larval stage, the expression level of *BdEcR-B1* appeared to increase progressively with each successive instar. However, there was no significant difference in the expression levels of *BdEcR-B1* among the 1-d to 5-d old larvae, 1-d, 7-d, 10-d old pupae and the adult (Fig. 3).

The relative expression patterns of *BdEcR-B1* in different tissues were shown in Fig. 4. *BdEcR-B1* transcripts were observed in all tissues analyzed in this research. The expression levels of *BdEcR-B1* in the gut and the Malpighian tubule greatly exceeded those in the trachea and fat body. Further, the expression of these transcripts in the trachea was significantly higher than in the fat body (LSD test, $P < 0.05$).

DISCUSSION

Physiological processes and development are coordinated by means of endocrine regulation mechanisms in response to internal and environment signals. Plasticity in endocrine regulation enables organisms to survive, thrive and reproduce while encountering environment changes (Gáliková et al. 2011). In insects, the ecdysone titer tightly coordinates physiological changes throughout the whole life cycle by transducing signals to target genes via the edysone receptor proteins (EcRs) and the Ultraspiracle protein (USP) (Yao et al. 1992; Thomas et al. 1993; Cruz, 2006). The EcRs in the 3 isoforms (EcR-A, EcR-B1, and EcR-B2), are produced under the control of the EcR gene by using 2 different promoters and alternative splicing (Talbot et al. 1993). In *D. melanogaster*, isoform EcR-A acts as a weak activator and strong repressor, while isoform EcR-B acts either as a weak repressor or a strong activator (Hu et al. 2003). In this study, we cloned and characterized *BdEcR-B1* cDNA from the oriental fruit fly. Sequence analysis showed that the *BdEcR-B1* contains an open reading frame of 767 amino acid sequences with a predicted molecular weight of 83.3 kDa and a *pI* of 6.74. Like *Aedes aegypti*, *C. capitata* and *Lucilia cuprina*, the *BdEcR-B1* exhibits typical domain structure (A/B, C, D, E, F domain) of the nuclear receptor family and belongs to the hormone receptor family with a double methionine in the initial position of the protein sequence (Cho et al. 1995; Hannan & Hill. 1997; Verras et al. 1999).

EcR isoforms have been identified in more than 51 species of insects and other arthropods, such as *L. cuprina*, *Tribolium castaneum*, *Choristoneura fumiferana*, and *Spodoptera litura*. EcR

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   1  aaaattctcaaaaaagcaaaatttacacacaataaaaattttcaaaaaaaagtgtttaaaaaagtagttctacaagtctgctgtgcagcg
  91  caaactacgctaacaagtgaacaacgtgtctaccaaatacacaccccacacacacatacacaacccaatcaaacttcaaaaacttgact
 181  ggccgaacaacaATGatgaagcgacgttggtcgaataacggcggtttcgcctttcgcatgctggaagaatcctccaccgaagtgagctca
   1                MM  K  R  R  W  S  N  N  G  G  F  A  F  R  M  L  E__E__S__S__T__E__V__S__S
 271  tcctccaacgggctcgtgctcagcacggcttttgtcgccctcaccgctcgactcacccgttatgtgaacaagatctgtggtatgatgcc
  27   _S__S_ N  G  L  V  L  S  T  A  L  S  P__S__P__L__D__S__P  V  Y  G  E  Q  D  L  W  Y  D A
 361  gcattcaatggacacggtggccatagcgtgatcaccgcggcacatggttgcgctgcgctgctgccgcccacaaaccacaataataccgttg
  57      A  F  N  G  H  G  G  H  S  V  I  T  A  A  H  G  C  A  A  L  L  P  P  Q  T  T  I  I  P  L
 451  ccacctttgggtggtggcataaacaatattaatgtcaatggcgctggcaataacacaaatggcgcgaattttggtcatggcgctggtggt
  87      P  P  L  G  G  G  I  N  N  I  N  V  N  G  A  G  N  N  T  N  G  A  N  F  G  H  G  A  G  G
 541  ggcggcggcggtgtaatgggccatggcaatcagctgagcaacgggcatctcggcaatcaacaaaatgcacagaataataacaataataat          A/B
 117     G  G  G  G  V  M  G  H  G  N  Q  L  S  N  G  H  L  G  N  Q  Q  N  A  Q  N  N  N  N  N  N
 631  aatttgaatatgtataacaacaacaacacaaatatgttgcatgcaacattaaatcagggtttaattaatggtttgatgggtagtgtgttg
 147     N  L  N  M  Y  N  N  N  N  T  N  M  L  H  A  T  L  N  Q  G  L  I  N  G  L  M  G  S  V  L
 721  ggtgcaaatgcaggcatgcaacataatggactcaacatgcaacagcatacgccgcgtagcgattcggcgaattcaatatcgtcaggccgc
 177     G  A  N  A  G  M  Q  H  N  G  L  N  M  Q  Q  H  T  P  R  S  D  S  A  N  S  I  S  S  G  R
 811  gatgatctctcaccgtcgagcagtctcaacggctattcggcaaatgaaagttgtgatgtcaaaaagatcaaaaagggtccagcgccgcgt
 207     D  D  L  S  P  S  S  S  L  N  G  Y  S  A  N  E  S  C  D  V  K  K  I  K  K  G  P  A  P  R
 901  ctgcaggaggagctgtgtctggtgtgcggcgatcgggcatccggctatcattacaatgcgctcacctgcgaaggttgtaagggtttcttt
 237     L  Q  E  E  L  *  C  L  V  *  C  G  D  R  A  S  G  Y  H  Y  N  A  L  T  *  C  E  G  *  C  K  G  F  F          C
 991  cggcgcagcgttacgaagaacgcagtatattgttgtaaattcgggcgctcctgtgaaatggacatgtatatgcgacgaaaatgtcaagag
 267     R  R  S  V  T  K  N  A  V  Y  C  *  C  K  F  G  R  S  *  C  E  M  D  M  Y  M  R  R  K  *  C  Q  E
1081  tgtcgcatgaagaaatgtctggcagtgggtatgcgaccggaatcgtgtggtgccggagaatcaatgcgctatgaagcgacgggagaagaag
 297     *  C  R  M  K  K  C  L  A  V  G  M  R  P  E  C  V  V  P  E  N  Q  C  A  M  K  R  R  E  K  K
1171  gcgcaaaaggagaaagagaaacaaactactggcaattcgccgatcatatgcaaaagcgaatccattaagaatgaaatactcgaactgatg
 327   A  Q  K  E  K  E  K  Q  T  T  G  N  S  P  I  I  C  K  S  E  S  I  K  N  E  I  L  E  L  M
1261  aactgtgaaccgccgtcgcatccaacatgtccgttgctacctgatgatattgtggccaagtgcaggggcgagcaacattccgccgctcacg          D
 357     N  C  E  P  P  S  H  P  T  C  P  L  L  P  D  D  I  V  A  K  C  R  A  S  N  I  P  P  L  T
1351  cgtaaccagttggcggtcatatacaaactgatctggtatcaggatggctatgaacagccatcggacgaagatctgaagcgttataccgagc
 387     R  N  Q  L  A  V  I  Y  K  L  I  W  Y  Q  D  G  Y  E  Q  P  S  D  E  D  L  K  R  I  T  S
1441  accccccgatgaaaatgaaagcccgaatgatgtcagcttttcggcatataacccgaaattaccatttttgacagtacaactgattgtggagtt
 417     T  P  D  E  N  E  S  P  N  D  V  S  F  R  H  I  T  E  I  T  I  L  T  V  Q  L  I  V  E  F
1531  gcaaaaggtttaccggcatttacgaaaattccacaagaggatcaaattacgttgctgaaggcctgctcatcggaagtgatgatgtttgcgt
 447     A  K  G  L  P  A  F  T  K  I  P  Q  E  D  Q  I  T  L  L  K  A  C  S  S  E  V  M  M  L  R
1621  atggcccgacgttacgatcacaattcggattccatattcttcgccaacaatcgttcatatacgcgtgatgcgtacaaaatggccggtgtg
 477     M  A  R  R  Y  D  H  N  S  D  S  I  F  F  A  N  N  R  S  Y  T  R  D  A  Y  K  M  A  G  V
1711  gccgataataattgaggatctattgcattttttgtcggcagatgtactcgatgaaggtcgacaacgtcgaatacgctctcctcactgccatt          E
 507     A  D  N  I  E  D  L  L  H  F  C  R  Q  M  Y  S  M  K  V  D  N  V  E  Y  A  L  L  T  A  I
1801  gtgatctttctcgatcggccgggactcgaaaaggccgaactagtcgaagcgatacaaaaattactacatcaatacgtcgcgtatatatt
 537     V  I  F  S  D  R  P  G  L  E  K  A  E  L  V  E  A  I  Q  N  Y  Y  I  N  T  L  R  V  Y  I
1891  ataaatcgacattgtggcgataccaagagtctggtcttcttcgccaaattactctccatattaacggaactgcgcacgcttggcaatcag
 567     I  N  R  H  C  G  D  T  K  S  L  V  F  F  A  K  L  L  S  I  L  T  E  L  R  T  L  G  N  Q
1981  aatgccgaaatgtgttttctcattgaaattgaagaatcgtaaactgccgaaattcctcgaagaaatctgggacgtacatgcggtgccacca
 597     N  A  E  M  C  F  S  L  K  L  K  N  R  K  L  P  K  F  L  E  E  I  W  D  V  H  A  V  P  P
2071  tccgtgcaatcacacatacaggcaatgcaagccgaacaggaaagtctggaggccaatgcggccgcagtcgccaccacatccacatcagcc
 627     S  V  Q  S  H  I  Q  A  M  Q  A  E  Q  E  S  L  E  A  N  A  A  A  V  A  T  T  S  T  S  A
2161  gccgcaagttcgtccgcctccggttcacccactgacatgattggcgttggctttgcgaataattcacccaattccacatcggtctcaacg          F
 657     A  A  S  S  A  S  G  S  P  T  D  M  I  G  V  G  F  A  N  N  S  P  N  S  T  S  V  S  T
2251  agcgctatcggcatgaacggtggtctcgtcagcgacaacggcccggtacgtctagcttatacggcatgccattcaaacaggagtacata
 687     S  A  I  G  M  N  G  G  L  S  S  A  T  T  A  G  T  S  S  L  Y  G  M  P  F  K  Q  E  Y  I
2341  atggtcccggcagtggtatcggcattggcggcaatctgacgctcaagtcagagcatttaattggcatgcagctgtcgaatgatcatcac
 717     M  G  P  G  S  G  I  G  I  G  G  N  L  T  L  K  S  E  H  L  I  G  M  Q  L  S  N  D  H  H
2431  atgatgggcatacaactgaagcaagagcatatgatgggtggcgtggatcacaataacggcggcaTAGcatcacgcgcacgtgcaacaacag
 747     M  M  G  I  Q  L  K  Q  E  H  M  M  G  G  V  D  H  N  T  A  A  *
2521  tcgctgctgctcggtggcagtggcgaatgtagtggtggtggtggtgttgatggtgttgttgatgatgttgttggcggcggtcaagggctcggaatgagcgcagctagtg
2611  atgcatatggatgacggcatggatgttgccatggatggtggtggtgatgtcgttgttggcggcggtcaagggctcgaatgagcgcagctagtg
2701  tggcaacgacgcgctaattgcttgtggtacgctgtctgcgtagcgcccattttttattttggtctaatatattaagctttcgttcgttgtt
2791  aagcggtggggtatgggcgctgcctaagcacagttgttacgcaaggcaatgcgttgcagttgggattttttgtatggcagctagacatgca
2881  atcaagacgatttaaagtgaacttggtcggcatggcaatgtgtgcgcgtaccgttgtatgtatgtatatgtgaggcgtgtactttaaattt
2971  tgttgatttacggtacaagctacaataaaaactgcagttatttagtgattaagtgagcgcgcgcgcgcaagcgttgcggcatgcaccaag
3061  cggtggtagcaaaacacaatcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 1. The complete cDNA sequence and deduced amino acid sequence of *B. dorsalis* ecdysone receptor (EcR) B1 gene (GenBank accession number: JQ034623). The 5´-UTR and 3´-UTR are lowercase. The start codon is indicated with bold and the stop codon is indicated with bold and asterisk. In the A/B domain, the (K/R) RRW motif is light shaded. The EcR-B1 isoform-specific regions: the S-rich motif, the SP-residues and the DL-rich motif are indicated with dotted line, wavy line and dark shaded, respectively. The zinc finger motifs are underlined and four cysteine residues are marked with asterisk. Both of the hepapeptide sequences are boxed. Four SUMOylation sites are double lined. The putative polyadenylation signal "AATAAA" in 3´-UTR is underlined.
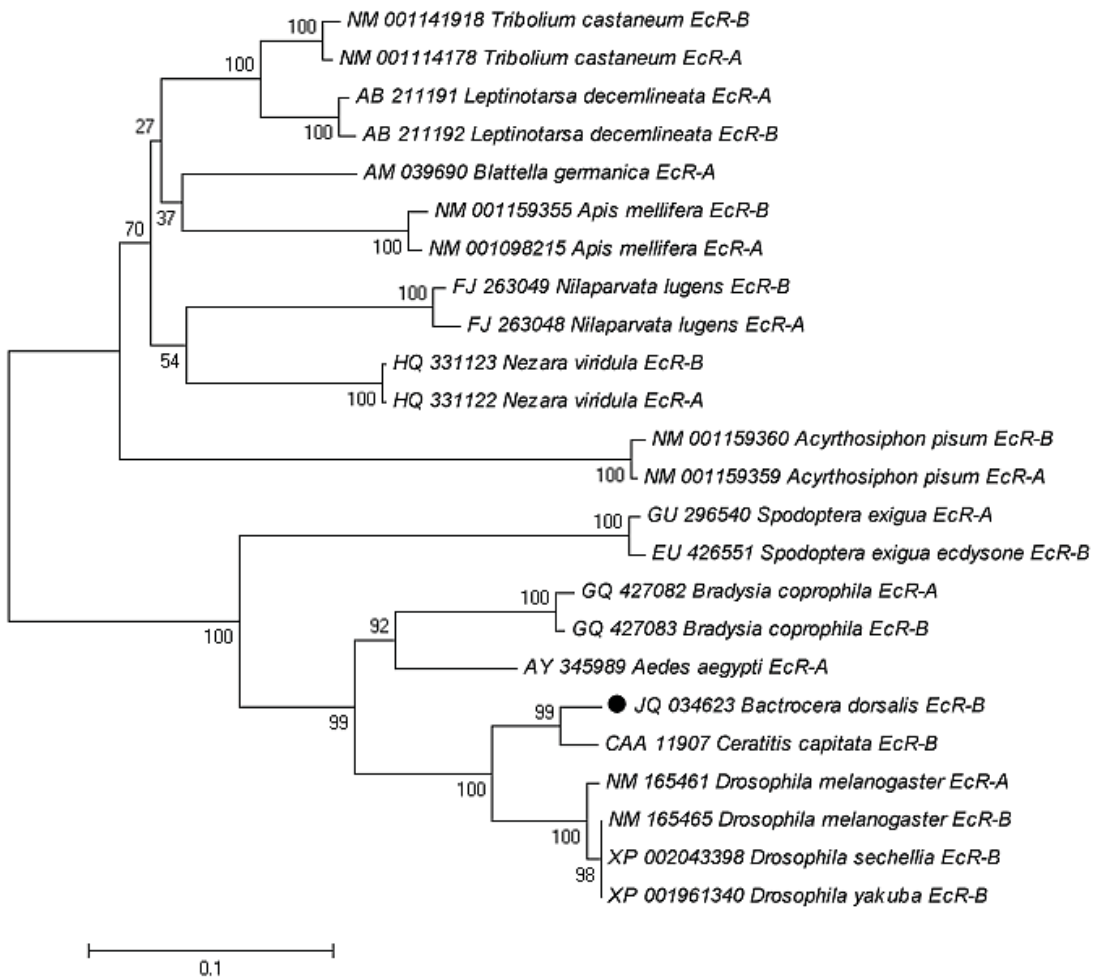
Fig. 2. Phylogenetic tree based on the deduced amino acid sequence comparison of homologous EcR isoforms genes from various orders of insect. The topology was tested by bootstrap analysis with 1000 replicates. The numbers above the branch indicated bootstrap percentages.

isoforms have common C/D, E and F domains, yet they have different N-terminal regions (Verras et al. 1999; Nakagawa & Henrich 2009; Hwang et al. 2010; Watanabe et al. 2010). In the C domain (DNA binding domain, DBD), there are two C_4 zinc finger modules, which show sequence-specific DNA binding activity. In the E domain (ligand binding domain, LBD), there are some motifs for specific ligand binding, dimerization and transactivation. Another transactivation site is located in the A/B domain of the amino acid sequence. Comparison of *BdEcR-B1* A/B domain to the corresponding sites of the B1 isoforms of other Dipteran species, revealed that *BdEcR-B1* displays a highly conserved isoform-specific B1 microdomain, including the (K/R)RRW motif, the S-rich motif, the DL-rich motif, the SP residues and a modified EESS(T/S)EV(V/T)SS motif. The S-rich and DL-rich conserved motifs in the EcR-B1 iso-

forms are structurally and functionally independent of each other, but they interact with essential co-regulatory proteins and play an important role in transcriptional regulation; and they have the similar functions in these species (Watanabe et al. 2010).

Amino acid sequence alignments were utilized to construct a phylogenic tree with *BdEcR-B1* and 8 other insect EcRs. The data indicate that *BdEcR-B1* conforms to the taxonomic analysis of various insects, and it shows high similarity to the other dipteran EcRs, which form a single cluster. In contrast, *BdEcR-B1* shows much less similarity to the EcRs of species belonging to the Coleoptera, Homoptera, Hemiptera, Lepidoptera, Hymenoptera and Blattaria.

As a potential molecular target of ecdysone, the regulation mechanism of EcR isoforms in arthropod development have been well studied
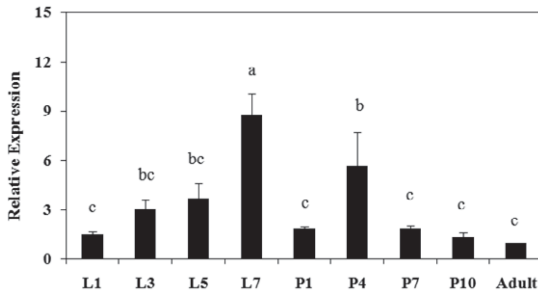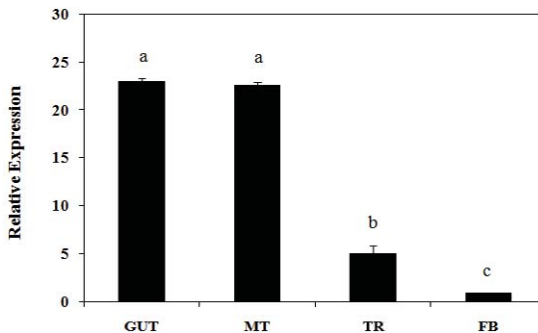
Fig. 3. Expression patterns of the *BdEcR-B1* in different developmental stages of *B. dorsalis* (bar graph represented Mean ± SE; different letters above each bar indicated the significant difference, *P* < 0.05, LSD in ANOVA). L and P represent larval and pupa, respectively.

by mutational analysis in *D. melanogaster*, *S. exigua*, *Tigrious japonicus*, *Nilaparvata lugens*, *Laodelphgax striatellus*, and *Sogatella furcifera*. This analysis shows that some mutations result in the arrest of ecdysone-responses during metamorphosis (e.g., larval molting) and tissue remodeling (Hwang et al. 2010; Lam & Thummel. 2000; Schubiger et al. 1998; Verras et al. 1999; Wu et al. 2012; Yao et al. 2010). In insects, increased 20E levels can induce the expression of genes, which is essential for the synthesis of the new cuticle and the production of ecdysis-triggering hormone receptors preparatory to molting. The situation in *B. dorsalis* is similar to that in *C. capitata,* in which significantly high levels of *CcEcR-B1* are found in late third instar larvae and in the middle of the pre-pupal stage, and these levels are higher than in earlier larval stages, or in the pupal stages or in the adult (Verras et al. 1999). In the water flea, *Daphnia magna* Strauss, the EcR-B expression is

Fig. 4. Expression patterns of the *BdEcR-B1* in different tissues of larval *B. dorsalis* (bar graph represented Mean ± SE; different letters above each bar indicate significant difference, *P* < 0.05, LSD in ANOVA). FB, GUT, MT, and TR represent fat body, gut, Malpighian tubule, and trachea, respectively.

up-regulated more than 20-fold relative to initial the molt (Kato et al. 2007). A similar expression pattern occurs in *D. melanogaster* in which EcR-B isoforms are highly expressed in the second and third instar larvae. The larvae with EcR-B mutants fail to molt with a newly duplicated cuticle. Interestingly, EcR over-expression during some sensitive stages also caused developmental arrest and lethality in *Drosophila* (Schubiger et al. 2003). Relatively high expressions of *BdEcR-B1* gene occur in *B. dorsalis* just prior to the last larval instar to pupal molt and again during the mid-pupal stage. This suggests that these 2 critical times in metamorphosis, which might be vulnerable to interventions in the regulation of the ecdysone-mediated signal transduction via an increased EcR expression strategy. All the results indicate that normal levels of *BdEcR-B1* in different developmental stages are crucial to ensure normal metamorphosis in *B. dorsalis*.

EcR proteins are extensively expressed in different tissues, but those 3 isoforms are each expressed in a tissue- or cell-specific manner. Unlike EcR-A isoform, which predominately expresses in proliferative tissues, the EcR-B isoforms are expressed in larval-specific tissues that are dependent on 20E-triggered larval-specific remodeling (Schubiger et al. 1998; Cruz et al. 2006). In holometabolous insects, midgut remodeling is important during metamorphosis, especially in mosquitoes; and EcR-B has been indicated to play an essential role in both histolysis of larval tissue and differentiation of imaginal diploid cells (Parthasarathy & Palli 2007). The EcR-B1 of *Chilo suppressalis* is expressed more highly in the midgut than either in the fat body or in the epidermis, which indicates that EcR-B1 may play an important role in remodeling (Minakuchi et al. 2002). At the onset of the larval-pupal molt in *Bombyx mori*, the EcR-B1 gene is significantly expressed in larval tissue, like fat body, epidermis, and salivary glands, and especially in wing discs in the last larval instar. This gene is also involved in programmed cell death in the anterior silk gland (Jindra & Riddford 1996; Goncu & Parlak 2009). Besides, EcR genes participate in the chitin biosynthesis pathway, which is the major polysaccharide layer in the trachea, muscle, cuticle and peritrophic matrix (Yao et al. 2010). In this study, we found that during the last larval stadium, the mRNA transcript of *BdEcR-B1* is regulated in a tissue-specific manner in that relatively high amounts of mRNA are found in the gut and Malpighian tubule, and this indicates that the *BdEcR-B1* is expressed in a tissue-specific manner, i.e., that the gut and the Malpighian tubule are the main expressing tissues, and that *BdEcR-B1* may be essential in tissue remodeling.

In conclusion, we cloned and characterized the cDNA of ecdysone receptor isform B1 in the orien-

tal fruit fly, and elucidated its temporal and tissue-specific expression pattern. Our present work suggests that *BdEcR-B1* plays an essential role in larval molting and tissue remodeling. However, our data do not cover the details of other isoforms, nor elucidate its relationship to the USP throughout the whole life cycle. Further investigations will be focused on the EcR/USP relationship and the ecdysone-triggered gene cascade.

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