Roles of Krüppel Homolog 1 and Broad-Complex in the Development of Dendroctonus armandi (Coleoptera: Scolytinae)

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In insects, metamorphosis is controlled by juvenile hormone (JH) and 20-hydroxyecdysone (20E). Krüppel homolog 1 (Kr-h1), a key JH-early inducible gene, is responsible for the suppression of metamorphosis and the regulation of the Broad-Complex (Br-C) gene, which is induced by 20E and functions as a “pupal specifier”. In this study, we identified and characterized the expression patterns and tissue distribution of DaKr-h1 and DaBr-C at various developmental stages of Dendroctonus armandi. The expression of the two genes was induced by JH analog (JHA) methoprene and 20E, and their functions were investigated by RNA interference. DaKr-h1 and DaBr-C were predominantly expressed in the heads of larvae and were significantly downregulated during the molting stage. In contrast, the DaKr-h1 transcript level was highest in the adult anterior midgut. DaBr-C was mainly expressed in female adults, with the highest transcript levels in the ovaries. In the larval and pupal stages, both JHA and 20E significantly induced DaKr-h1, but only 20E significantly induced DaBr-C, indicating the importance of hormones in metamorphosis. DaKr-h1 knockdown in larvae upregulated DaBr-C expression, resulting in precocious metamorphosis from larvae to pupae and the formation of miniature pupae. DaKr-h1 knockdown in pupae suppressed DaBr-C expression, increased emergence, caused abnormal morphology, and caused the formation of small-winged adults. These results suggest that DaKr-h1 is required for the metamorphosis of D. armandi. Our findings provide insight into the roles of DaKr-h1 and DaBr-C in JH-induced transcriptional repression and highlight DaKr-h1 as a potential target for metamorphosis suppression in D. armandi.

Keywords: Broad-complex, Dendroctonus armandi, 20-hydroxyecdysone, juvenile hormone, Krüppel homolog 1, RNA interference

INTRODUCTION

Insect metamorphosis, including larval–larval molting and larval–pupal–adult metamorphosis, is synergistically regulated by several insect hormones, most notably juvenile hormone (JH) and ecdysone (Riddiford, 1994; Riddiford et al., 2000). Juvenile hormone is secreted by the corpora allata in all insects from silverfish to Drosophila although their anatomical location differs slightly (Riddiford, 2012). It is considered a “status quo” hormone that maintains larval properties and
inhibits metamorphosis during molting (Riddiford, 1996). 20-Hydroxycycloxydiene (20E), the active metabolite of ecdysone, induces larval–larval molting at high levels of JH, whereas it initiates larval–pupal and pupal–adult metamorphosis when the JH concentration drops sharply (Riddiford, 1994; Riddiford et al., 2010; Smykal et al., 2014; Daimon et al., 2015; Liu et al., 2018). Thus, the main function of JH is to prevent the premature metamorphosis (Riddiford, 1994). Krippel homolog 1 (Kr-h1), a C2H2 zinc finger transcription factor, plays an important role in the larval development of Drosophila melanogaster and Tribolium castaneum. Treatment with the JH analog pyrroxyphenyl during pupal development in D. melanogaster and T. castaneum resulted in Kr-h1 upregulation and the formation of a “second pupa” rather than an adult. In the JH signaling pathway, Kr-h1 is an important early responder gene (Pecasse et al., 2000; Minakuchi et al., 2008; Minakuchi et al., 2009; Zhu et al., 2010; Lozano and Belles, 2011; Zhang et al., 2011). Kr-h1 homologs have been identified in several species, including Aps mellifera (Grozinger and Robinson, 2006), Aedes aegypti (Zhu et al., 2010), Frankliniella occidentalis, and Haplothrips brevitubus (Minakuchi et al., 2011).

The JH receptor Methoprene-tolerant regulates Kr-h1, which then regulates Broad-Complex (Br-C) expression (Abdou et al., 2011). The pupal specifier Br-C (Minakuchi, 2008; Minakuchi et al., 2009; Kayukawa et al., 2016) and the adult specifier Ecdysone-induced Protein 93F (E93) (Ureña et al., 2014; Ureña et al., 2016; Kayukawa et al., 2017) both rely on Kr-h1 as a transcription repressor. Br-C, which is consisted of the Bric-a-brac-Trimtrack-Broad (BTB) complex and a zinc finger structure, is induced by 20E and functions as a “pupal specifier” during the larval–pupal transition (Kiss et al., 1976; Kiss et al., 1988; DiBello et al., 1991; Zhou and Riddiford, 2002). The structure and function of numerous Br-C genes have been characterized and analyzed from several insect species, including Aedes aegypti, Blattella germanica, Bombyx mori, Drosophila melanogaster, Frankliniella occidentalis, Haplothrips brevitubus, Lymantria dispar and Manduca sexta (Bayer et al., 1997; Zhou et al., 1998; Chen et al., 2004; Minakuchi et al., 2011; Yang et al., 2014; Ding et al., 2020). In D. melanogaster, depletion of DmKr-h1 with reduced DmBr-C levels in the anterior compartment and RNA interference (RNAi) affected larval pupation (Ureña et al., 2016). RNAi analysis in the Blattella germanica and Pyrocorhis apterus revealed that Br-C is specifically required for regulation of wing development, in particular size, shape and vein formation (Konopova et al., 2011; Huang et al., 2013). Kr-h1 was also found to be induced by 20E. It was first postulated in the 1970s that 20E plays a molecular role in target cells during the larval–pupal transition. Gene expression analysis following 20E stimulation supports a model in which the ternary complex EcR/USP/20E activates transcription of E75 and Hr3, which together control the delayed expression of βFTZ-F1 (King-Jones and Thummel, 2005). Dynamic expression of βFTZ-F1 is dependent on Hr3 stimulation, and Hr3 plays a key role in regulating the developmental switch by repressing 20E transcription of early response genes E75, E74 and Br-C and activates the downstream late response factor FTZ-F1 (White et al., 1997; Lam et al., 1999; Kageyama et al., 2003; Parvy et al., 2014).

RNA interference (RNAi) technology has now become a widely used tool to analyze the gene functions of Chinese white pine beetle (Dendroctonus armandi Tsai and Li)). Chen’s team used RNAi to investigate and analyze the olfactory receptor coreceptor (DarmOrco), chemoreceptor (DarmCRSP2), aquaporins (DaAqgs), antifreeze protein genes (DaAFP), Capa peptide receptors (DaCapaRs), nepropeptide F (DaNPF), and 3-hydroxy-3-methylglutaryl coenzyme A reductase genes (HMGR) in D. armandi (Zhang et al., 2016; Li et al., 2018; Fu et al., 2019, 2020, 2021; Liu et al., 2021; Sun et al., 2021). Sun et al. (2022) discovered that allatostatin C (PISCFC/AST) and juvenile hormone acid O-methyltransferase (JHAMT) were major regulators of juvenile hormone synthesis in D. armandi after obtaining dsRNA technology via the L4440 vector construction.

RNAi technique was used to characterize the activities of two genes in D. armandi, DaKr-h1 and DaBr-C. Their expression induced by JHA and 20E were analyzed in different tissues at different developmental stages by a series of RNAi experiments. While JH may prevent premature larval–adult metamorphosis by direct Kr-h1-dependent Br-C gene repression, JH-induced transcriptional repression of the target genes leads to the emergence of supernumerary pupae during the pupal–adult transition. Furthermore, ingesting bacterially generated dsRNA could be an effective RNAi-based method for controlling insect pests.

**MATERIALS AND METHODS**

**Insects**

We collected Pinus armandii Franch infested with D. armandi on the southern slopes of central Qinling Mountains (33°18′–33°28′ N, 108°21′–108°39′ E) in Shaanxi, China, and placed the specimens in a greenhouse. The adult insects were collected after they emerged and stored on moist paper at 4°C. The sex of adults was based on external genitalia and male-specific auditory cues (Dai et al., 2014; Zhao et al., 2017). Larvae and pupae were collected from under the bark of infected P. armandii.

**Ribonucleic Acid Isolation and cDNA Synthesis**

Total RNA was isolated from three beetles by the UNIQ-10 Column Trizol Total RNA Isolation Kit (Sangon Biotech, Shanghai, China) in accordance with the manufacturer’s protocol. Its integrity was checked on 1% agarose gels and quantified using NANO DROP 2000 spectrophotometry (Thermo Scientific, Pittsburgh, Pennsylvania, United States of America). The purity was calculated by mean of relation A260/A280 ratio (μg/mL = A260 × dilution factor × 40). The synthesized cDNA obtained from the sample was used as the template using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China).

**Amplification of Genes, Cloning and Sequence Analyses**

cDNA synthesized from the sample was used as a template for PCR reaction. In Primer Premier 5.0, specific primers
(Supplementary Table S1) were designed based on Kr-h1 and Br-C sequences of Dendroctonus ponderosae from NCBI (http://www.ncbi.nlm.nih.gov/). PCR amplifications were performed in a C1000 thermocycler (Bio-Rad, Hercules, CA, United States), cDNA amplification was performed in a 20 μl reaction volume: 1 μl cDNA, 0.25 μM each primer, 10 μl Eco‘Taq PCR SuperMix (TransGen Biotech, Beijing, China), with ddH2O added to 20 μl. The reaction conditions were as follows: 94°C for 5 min, 30 cycles of 94°C for 30 s, TM of each pair of primers for 30 s and 72°C for 30 s with a final extension for 10 min at 72°C for 30 s. The PCR products were visualized on 1% agarose gels stained with 1× DuRed and compared with a 2 K plus DNA marker (TransGen Biotech, Beijing, China).

Single-stranded 5’and 3’ RACE-ready cDNA was synthesized from RNA using a SMARTer RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain, CA, United States) according to the manufacturer’s protocol. Partial sequences were used in the primer design, and the PCR was performed as described in the SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain, CA, United States). The amplicons were purified, cloned and sequenced. Sequences were manually edited with EditSeq from DNASTAR (https://www.dnastar.com/) to obtain inserts, which were then BLASTed against the NCBI database. The complete sequences were compared using a BlastP search with those deposited in GenBank (Altschul et al., 1990).

Sequence Analyses of the Genes
The molecular mass (kDa) and isoelectric point (IP) of the two sequences were determined by the ProtParam program (Gasteiger et al., 2005). Kr-h1 and Br-C of D. armandi were checked for likely subcellular localization using Target P1.1 software (http://www.cbs.dtu.dk/services/TargetP/) with the default parameters (Emanuelsson et al., 2000).

In order to identify Kr-h1 and Br-C in D. armandi, a phylogenetic inference analysis of 11 full-length sequences was performed by the neighbor-joining method with MEGA7.0 (Le and Gascuel, 2008; Kumar et al., 2016). To estimate the support for each node, bootstrap values were calculated after 1,000 pseudoreplicates.

Analysis of the DaKr-h1 and DaBr-C Genes

Expression Patterns of Different Life Stages and Tissues
During development, D. armandi larvae were separated into three sub-stages: small larvae (SL: penultimate (or pre-final) instar larva weighing less than 2.5 mg); large larvae (LL: final instar, feeding larva weighing 5.0–7.0 mg); mature larva (ML: post-feeding final instar larva). Pupae were separated into five sub-stages: P0: pupae, P1: Day 1 of the pupal stage, P2: Day 2 of the pupal stage, P3: Day 3 of the pupal stage and P4: Day 4 of the pupal stage. D. armandi adults were separated into four sub-stages: teneral adults (TA: body color still light), dark brown adults (Dba: Adults darkened to dark brown, but were still under the bark and had not migrated), emergent adults (EA), and feeding adults (invading a new host). The difference between the teneral adults and dark brown adults is the difference in body color. The difference between dark brown and emergent is that the former is dark brown and still under the bark, while the emergent adults are black and have already emerged from the bark. The difference between emergent adults and feeding adults is that emergent adults is when the insect has just emerged from the bark and emerged, while new feeding adults is when the insect has emerged and invaded a new host and fed. There were three biological replicates per developmental stage, each containing three insects (Dai et al., 2014).

In terms of tissue distribution, for tissue-specific analysis of DaKr-h1 and DaBr-C genes, 60 males and 60 females that had emerged as adults (head, anterior midgut, hindgut, Malpighian tubule, fat body, reproductive organ (testes of males and ovaries of females and antennae), 30 larvae and 30 pupae (head, gut, fat body, epidermis) were dissected, frozen immediately in liquid nitrogen and stored at -80°C. Each tissue was replicated three times, and a pool of total RNA extracted from different tissues was used per replicate. RNA isolation and cDNA synthesis followed the protocols described above.

Effects of JH Analog Injection on Transcript Levels of DaKr-h1 and DaBr-C
Solutions of the stock juvenile hormone analog JHA methoprene (Sigma, Saint Louis, United States), were separately diluted to 5, 25 and 100 μg/μl concentrations using acetone (Huang et al., 2016). Next, 0.1 μl of each JHA dilution was injected into D. armandi larvae (mature larvae) and pupae (newly pupated pupae) through the ventral abdomen using Hamilton Microliter syringes (700 series, RN) with 32G sharp-point needles (Hamilton, Switzerland) to a final JHA content of 0.5, 2.5 or 10 μg. Meanwhile, an equal amount of acetone was injected as the solvent control. To analyze the expression of the JH-induced genes, the total RNA was extracted after 0, 24, 48 and 72 h of JHA or acetone treatment and subjected to cDNA synthesis and qRT-PCR.

Effects of 20E Injection on Transcript Levels of DaKr-h1 and DaBr-C
Solutions of the stock Ecdysterone (20E, 20-Hydroxyecdysone; Sangon Biotech, Shanghai, China) were separately diluted to 5, 25 and 100 μg/μl concentrations using ethanol. Next, 0.1 μl of each 20E dilution was injected into D. armandi larvae (mature larvae) and pupae (newly pupated pupae) through the ventral abdomen using Hamilton Microliter syringes (700 series, RN) with 32G sharp-point needles (Hamilton, Switzerland) to a final 20E content of 0.5, 2.5 or 10 μg. Meanwhile, an equal amount of ethanol was injected as the solvent control. To analyze the expression of the 20E-induced genes, the total RNA was extracted after 0, 24, 48 and 72 h of 20E or ethanol treatment and subjected to cDNA synthesis and qRT-PCR. Three biological replicates were measured, each containing three beetles.

dsRNA Synthesis

Target Genes
The Krüppel homolog 1 (Kr-h1) and Broad-Complex (Br-C) genes of D. armandi were identified in 2.3 above. The sequences of
**TABLE 1 |** Amino acid identity of putative DaKr-h1 and DaBr-C with related sequences in other insect species.

| Genes     | Species                          | Gene   | Accession No       | Identity in the Full length<sup>a</sup> |
|-----------|----------------------------------|--------|--------------------|----------------------------------------|
| DaKr-h1   | Dendroctonus ponderosae          | Kr-h1  | XP_017,756,355.1   | 96.13                                   |
|           | Sitophilus oryzae                | Kr-h1  | XP_030,765,511.1   | 79.00                                   |
|           | Anoplophora glabripennis        | Kr-h1  | XP_018,575,408.1   | 67.85                                   |
| DaBr-C    | Dendroctonus ponderosae          | Br-C   | XP_019,758,737.1   | 98.35                                   |
|           | Sitophilus oryzae                | Br-C   | XP_030,752,066.1   | 88.41                                   |
|           | Anoplophora glabripennis        | Br-C   | XP_018,566,190.1   | 75.00                                   |

*aAs predicted by BLAST (www.ncbi.nlm.nih.gov) (Altschul et al., 1990).

**TABLE 2 |** Physicochemical properties and cellular localization of DaKr-h1 and DaBr-C of D. armandi.

| Gene Name | ORF Size [Aa/Bp]<sup>a</sup> | Mw [kDa]<sup>b</sup> | I.P<sup>c</sup> | Signal Peptide Prediction<sup>d</sup> |
|-----------|-------------------------------|----------------------|--------------|-------------------------------------|
| DaKr-h1   | 491/1,460                     | 53.90                | 8.71         | SP 0.9988 mTP 0.001 other 0.0001   |
| DaBr-C    | 434/1,305                     | 47.75                | 5.80         | SP 1.0000 mTP 0.000 other 0.0000   |

*aAs predicted by the ProtParam program (Easteiger et al., 2005).
*bAs predicted by Target p 1.1 program (Emmanuelsson et al., 2003).
%c: isoelectric point; Mw: molecular weight; ORF: open reading frame; SP: secretory pathway signal peptide; mTP: mitochondrial targeting peptide.

**DaKr-h1 and DaBr-C genes** were digested with *Xba*I and *Sma*I. The *DaKr-h1* sequences were amplified with primers (Supplementary Table S1) using EcoTaq PCR SuperMix (TransGen Biotech, Beijing, China) and a C1000 thermo cycler (Bio-Rad, Hercules, CA, United States). The polymerase chain reaction (PCR) amplification reaction conditions were as mentioned earlier.

**Vector Construction and Expression**

**Construction of Transformed E. Coli Expressing dsRNA**

PCR products obtained in the previous steps were ligated into the plasmid vector, L4440 (Wuhan Miaoling Biotechnology Co., Ltd., Wuhan, China), between the *Xba*I and *Sma*I restriction sites. Successful cloning was verified through PCR and sequencing. Plasmids containing the correct insert were extracted and transformed into *E. coli* strain HT115 (DE3) strain (Shanghai Weidi Biotechnology Co., Ltd., Shanghai, China). Positive clones were isocultured at 37°C until the mid-exponential phase (OD600 = 0.4). To activate the T7 promoter for RNA transcription, IPTG (isopropyl-β-D-1-thiogalactopyranoside) was added to a final concentration of 0.8 mM and then incubated for an additional 4 h under the same conditions. Each bacterial cultures (100 ml) was transferred into a 50-ml Falcon tube and centrifuged at 4,000×g for 10 min at 4°C.

**Isolation of dsRNA Using Conventional Method**

Cells were harvested via centrifugation at 4,000 g and 4°C for 10 min. Bacteria were 10× concentrated and split into two vials. One vial (1 ml cell suspension) was used to extract RNA by UNIQ-10 Column Trizol Total RNA Isolation Kit (Sangon Biotech, Shanghai, China) according to the manufacturer’s protocol. The extracted RNA was compared with the dsRNA not induced by IPTG to determine whether IPTG had been successfully induced. Its integrity was checked on 1% agarose gels, and quantification was performed by spectrophotometry with a NANO DROP 2000 (Thermo Scientific, Pittsburgh, Pennsylvania, United States). The successfully induced vial were centrifuged at 4°C, 4,000 × g for 10 min. The supernatant was discarded, 500 μL of Trizol was added to bacterial pellet, and total RNA was extracted and subjected to DNase treatment. The reactions were allowed to proceed overnight at 42°C, followed by both the RNase and DNase digestion and purification steps to obtain the dsRNA. The dsRNA was spectrophotometrically quantified before injection.

**RNAi Experiment**

Synthesized dsRNA (0.2 μL) were injected into the ventral abdomen of the larvae on the first day of the last instar or pupae using a 10 μL Hamilton Microliter syringes (700 series, RN) with 32G sharp-point needles (Hamilton, Switzerland). dsRNA of L4440-Kr-h1 and L4440-Br-C (IPTG was not added) were used as negative controls. Untreated beetles were used as blank controls. Each beetle was injected only once. For each dose, three of the treated beetles were randomly selected at 24 and 72 h, frozen immediately in liquid nitrogen and stored at −80°C. The expression levels of *DaKr-h1* and *DaBr-C* were quantified first, and the expression of *DaBr-C* was quantified only in beetles, which *DaKr-h1* were successfully knocked down. Twenty-five larvae were observed and the survival rate was recorded, and 25 pupae were observed for defective wings after plumentation and repeated three times.

**Real-Time Polymerase Chain Reaction**

Specific qRT-PCR primers were designed by Primer Premier 5.0 on the basis of the obtained nucleotide sequences (Supplementary...
FIGURE 1 | Structures of DaKr-h1 and DaBr-C in D. armandi. (A) Alignment of the putative DaKr-h1 sequence in beetle species and their consensus sequences with D. armandi identified sequences. Black lines represent zinc finger structures C2H2 1–8, marked with numbers 1–8, respectively. (B) Alignment of the DaBr-C sequence. The green box represents the Bric-a-brac–Tramtrack–Broad complex.
Table S1). The melting curve analysis was performed to ensure that only a single product corresponding to the target sequence was amplified. All primer pairs were tested in advance to obtain close to 100%. The expression of the CYP4G55 (Dai et al., 2015) and β-actin (Dai et al., 2014) genes was used as an internal control. Real-time PCR was performed in triplicate according to the manufacturer’s instructions using TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) on a CFX96TM Real-Time qPCR Detection System (Bio-Rad, Hercules, California, United States). The qPCR was performed using the following program: 95°C for 10 min; 40 cycles at 95°C for 5 s, TM of each pair of primers (Supplementary Table S1) for 15 s and 72°C for 20 s.

Statistics
The 2^ΔΔCt method was used to determine the effect of interference. According to a role of thumb, transcript levels below 0.5 relative to the control were considered to indicate a significant effect of RNAi. One-way analysis of variance (ANOVA) (p < 0.05) and two-way analysis was used to determine significance of different treatments. For gene silencing analysis, an unpaired t-test was used to compare differences of two groups. The Kaplan-Meier method (log rank (Mantel–Cox)) was used to analyze the survival rates (p < 0.05) (Gillespie and Fisher, 1979). All statistical analyses were performed using SPSS Statistics 21.0 (IBM, California, IL, United States) and plotted using Prism 5.0 (GraphPad Software, CA, United States).

RESULTS
Identification of DaKr-h1 and DaBr-C Genes
DaKr-h1 and DaBr-C were identified from D. armandi, and the full-length amino acid sequences shared the highest identity (96.13–98.35%) with D. ponderosae (Table 1, Supplementary Figure S1). From the obtained values, DaKr-h1 and DaBr-C were assigned as corresponding homologs of D. ponderosae Kr-h1 and Br-C.

Analysis of the deduced amino acid sequences of DaKr-h1 revealed the presence of eight adjacent Cys/His2 zinc finger DNA-binding domains numbered from Z1 to Z8. These zinc finger regions, often called CysX2-CysX2-HisX2, indicate the spacers between the zinc-binding residues (Duportets et al., 2012) (Figure 1A). DaBr-C, an insect-specific transcription factor, has a BTB structural domain, which is a protein–protein interaction motif, at the N-terminus (Zhou et al., 1998) (Figure 1B). The full-length open reading frames (ORFs) of DaKr-h1 and DaBr-C were 1460 bp and 1305 bp, encoding 491 and 434 amino acids. Respectively, the predicted molecular mass were 53.90 and 47.75 kDa, and the isoelectric point were 8.71 and 5.80; Target P 1.1 program the predicted subcellular location of DaKr-h1 and DaBr-C suggest cytoplasmic location (Table 2).

DaKr-h1 and DaBr-C Transcript Levels in Different Tissues of D. armandi at Different Life Stages
Transcript levels were measured by qRT-PCR. Relative to the larval stage, one-way ANOVA showed statistically significant differences in transcript levels among the developmental stages (DaKr-h1: F statistic (F) = 6.128, degree of freedom (df) = 14, significance level (p) < 0.0001; DaBr-C: F = 2.402, df = 14, p = 0.022). DaKr-h1 and DaBr-C expression tended to increase from the small larval stage, reached the highest value in the large larval stage, decreased in the mature larval stage, and remained stable from the pupal to adult stage. No significant difference was observed in the expression between males and females (Figure 2).
To understand the functional roles of DaKr-h1 and DaBr-C, we studied the tissue-specific expression in three developmental stages (i.e., larva, pupa, and adult). qRT-PCR analysis showed statistically significant differences between different tissues at all developmental stages. In larvae, the DaKr-h1 and DaBr-C transcript levels were highly expressed in the head and gut (DaKr-h1: $F = 4.386$, $df = 3$, $p = 0.042$; DaBr-C: $F = 4.386$, $df = 3$, $p = 0.042$). DaKr-h1 and DaBr-C were highly expressed in the head of pupae (DaKr-h1: $F = 15.022$, $df = 3$, $p = 0.001$; DaBr-C: $F = 15.022$, $df = 3$, $p = 0.001$) (Figures 3A,C). In adults, the DaKr-h1 transcript level was higher in the midgut and in the Malpighian tubules, than in the head, hindgut, fat body, testes, ovaries, and antennae (Figure 3B). Whereas, the DaBr-C transcript level was highest in the ovaries of females and testes of males ($F = 15.369$, $df = 6$, $p < 0.0001$) (Figure 3D).

Thus, the DaKr-h1 gene exhibited a broad tissue expression pattern, reflecting the possible pleiotropic action of Kr-h1 in D. armandi.

**Effects of JH Analog Injection on DaKr-h1 and DaBr-C Transcript Levels**

To reveal the molecular mechanism of the influence of JHA on DaKr-h1 expression, the relative expression profile of DaKr-h1 was analyzed by qRT-PCR at more time points after JHA treatment. qRT-PCR analysis showed that low level of methoprene (0.5 µg) suppress DaKr-h1 expression for the first 48 h after injection in the larva, then it returns to normal levels at 72 h. By contrast, 10 µg methoprene induces it by 48 h after which it declines back to normal, likely indicating that the methoprene has been metabolized or excreted by that time (Figure 4A). In the pupa Figure 4B shows clearly that all doses of the JH analog induce DaKr-h1 mRNA with the lower doses being more effective earlier (24 h). Similarly, for DaBr-C expression, methoprene treatment had no effect on broad expression in the larva but in the pupa the lower doses appeared to depress DaBr-C expression at 48 h (Figures 4C,D).
Effects of 20E Injection on DaKr-h1 and DaBr-C Transcript Levels

qRT-PCR analysis showed that 2.5 µg of 20E suppress DaKr-h1 expression for 72 h after injection in the larvae but other dose (0.5 and 10 µg) of 20E treatment had no effect on DaKr-h1 expression in the larva (Figure 5A). In the pupa, Figure 5B clearly shows that low levels of 20E (0.5, 2.5 µg) induced DaKr-h1 expression by 72 h after injection. Also, low levels of 20E (0.5 µg) induced DaBr-C expression only at 48 h after the larval injection. By contrast, 2.5 and 10 µg of 20E suppress DaBr-C expression for 48 h, after which it rose back to normal levels. In the pupa, low level of 20E (0.5 µg) induced DaBr-C expression during the first 24 h after injection, and then returned to normal levels within 72 h. However, other dose (2.5 and 10 µg) of 20E treatment had no effect on the broad expression of pupa (Figures 5C,D).

dsRNA Expression

Two expression vectors L4440–Kr-h1 and L4440–Br-C corresponding to DaKr-h1 and DaBr-C were constructed on the basis of the L4440 vector. The plasmid was digested with restriction endonucleases XbaI and SmaI, and gel electrophoresis showed that one line was about 390 bp from L4440–Kr-h1 and the other 489 bp from L4440–Br-C. After HT115-carrying plasmids L4440–Kr-h1 and L4440–Br-C were induced by IPTG, the total RNA (containing dsKr-h1 or dsBr-C) was extracted from engineered bacteria. Gel electrophoresis showed that the residual RNA were dsKr-h1 and dsBr-C bands (Figure 6).

Effects of RNAi on DaKr-h1 and DaBr-C Expression

Determination of DaKr-h1 and DaBr-C Silencing by qRT-Polymerase Chain Reaction

Analysis of DaKr-h1 and DaBr-C expression after injection of dsKr-h1 confirmed that DaKr-h1 and DaBr-C were knocked down at all developmental stages (Figure 7). Compared with the negative control and as determined by qRT-PCR, DaKr-h1 and DaBr-C transcript levels at 72 h were significantly lower than those at 24 h (p < 0.05). These results indicate that DaKr-h1 and DaBr-C gene silencing can reduce target gene expression.
Knockdown Effect of Injecting dsKr-h1 and dsBr-C Separately

In this study, RNAi was used to determine DaEcR, DaE75, DaHr3, and DaFTZ-F1 gene expression after DaKr-h1 or DaBr-C knockdown in larval, pupal and adult stages. In the larva: DaKr-h1 knockdown suppressed DaEcR and DaFTZ-F1 expression and significantly upregulated DaBr-C expression but had no effect on DaE75 and DaHr3 expression (Figure 8A, Supplementary Table S2). DaBr-C knockdown significantly upregulated DaKr-h1 expression but had no effect on DaEcR, DaE75, DaHr3 and DaFTZ-F1 expression (Figure 8E, Supplementary Table S2). In the pupa: DaKr-h1 knockdown significantly upregulated DaFTZ-F1 expression but had no effect on DaEcR, DaE75, DaHr3 and DaBr-C expression (Figure 8B, Supplementary Table S2). DaBr-C knockdown significantly upregulated DaKr-h1 and suppressed DaFTZ-F1 expression (Figure 8F, Supplementary Table S2). In adult female: dsKr-h1 injection significantly upregulated DaBr-C and DaE75 expression, with no significant effect on DaEcR, DaHr3 and DaFTZ-F1 expression (Figure 8C, Supplementary Table S2). After dsBr-C injection, DaKr-h1 expression was significantly upregulated (Figure 8G, Supplementary Table S2). In adult male: dsKr-h1 injection significantly upregulated DaBr-C expression, whereas DaEcR, DaE75, DaHr3 and DaFTZ-F1 expression did not change significantly (Figure 8D, Supplementary Table S2). DaEcR expression was significantly upregulated after dsBr-C injection (Figure 8H, Supplementary Table S2).

Effects of dsKr-h1 and dsBr-C RNAi on the Development of D. armandi Larvae and Pupae

After larvae were treated with engineered bacteria, the survival rate of the vector (control), dsKr-h1, and dsBr-C groups on day 5 was 20.0, 16.7, and 43.3%, respectively (Figure 9A). The Kaplan-Meier method (log-rank Mantel–Cox test) was used to analyze the survival rate. No significant difference was observed in the survival rate in larvae of the dsKr-h1 and dsBr-C groups compared with the control group (DaKr-h1: \( \chi^2 = 0.089, df = 1, p = 0.766 \); DaBr-C: \( \chi^2 = 2.404, df = 1, p = 0.121 \)). After the pupae were treated with dsRNA, the survival rate of the vector (control), dsKr-h1, and dsBr-C groups on day 9 in pupae was 60, 58, and 42.5%, respectively (Figure 9B). No significant difference was observed in the survival rate in pupae of...
the dsKr-h1 and dsBr-C groups compared with the control group (DaKr-h1: $\chi^2 = 0.824$, df = 1, $p = 0.364$; DaBr-C: $\chi^2 = 0.048$, df = 1, $p = 0.826$). The survival rate was 20% lower in the dsKr-h1 group than in the vector group (control). In addition to analyzing the larval and pupal survival rates, the effects of dsKr-h1 and dsBr-C on the emergence and abnormal morphology rates of pupae were analyzed. The emergence rate of the control, dsKr-h1, and dsBr-C groups was 68.0, 72.0, and 62.7%, respectively. No significant difference was observed in the emergence rate of the dsKr-h1 and dsBr-C groups compared with the control group ($F = 2.313$, df = 1, $p = 0.180$; Figure 9C). The abnormal morphology rate of the dsKr-h1 and dsBr-C groups was 63.0 and 25.5% ($F = 54.605$, df = 1, $p < 0.0001$; Figure 9D). Taken together, these data suggest that DaKr-h1 and DaBr-C gene silencing affects the growth and development of D. armandi.

**Adult Development of dsRNA Phenotypes**

The phenotypes of D. armandi pupating larvae and adults produced by dsRNA-mediated silencing of transcripts are shown in Figure 10. DaKr-h1 silencing resulted in early pupation of D. armandi in larvae (It took only 2 days for the larvae to pupate early), and the pupae were significantly smaller than the control pupae (Figure 10A). dsRNA injection of DaKr-h1 into D. armandi pupae produced approximately 63% of deformed adults. Compared with the control group, the aberrant beetles had shorter carapace lengths and were neither tanned nor sclerotized (Figure 10B). Figure 10B shows the phenotypes of D. armandi adults produced by dsRNA-mediated transcript silencing. The injection of pupae with dsRNA for DaBr-C resulted in shape abnormalities in 25.5% of the treated beetles. Many parts of their appendices, including the wings and parts of the legs, were deformed or partly covered with old epicuticle.

**DISCUSSION**

In this study, we performed expressional and functional analysis of Kr-h1 and Br-C identified from D. armandi. A phylogenetic tree constructed by aligning DaKr-h1 and DaBr-C amino acid sequences with amino acid sequences from other insects showed that DaKr-h1 and DaBr-C amino acid sequences cluster with known D. ponderosae Kr-h1 and Br-C proteins, indicating that the identified sequences are genuine Kr-h1 and Br-C orthologs of D. armandi.

To investigate DaKr-h1 and DaBr-C expression patterns in D. armandi, DaKr-h1 and DaBr-C expression was examined at different developmental stages (Figure 7). The expression levels were determined by qRT-PCR analysis, and the results showed that the expression levels of DaKr-h1 and DaBr-C were significantly different among the different stages. The expression levels were highest in the larvae and decreased in the pupae and adults. The differences were statistically significant (One-way ANOVA, $p < 0.05$, with Tukey’s test of multiple comparisons).

**FIGURE 6** | Confirmation of dsRNA produced in HT115 cells. The recombinant plasmids were transformed into HT115 competent cells. Individual transformants were cultured on 2 x yeast–tryptone media with addition of isopropyl β-D-1-thiogalactopyranoside. The cell cultures were processed for total RNA extraction. Lane M: 2 kb Plus DNA marker (TransGen Biotech, Beijing, China). Arrowhead indicates the position of the dsRNA band.

**FIGURE 7** | qRT-PCR analysis of DaKr-h1 and DaBr-C transcript patterns in D. armandi after being injected with dsRNA for 24 and 72 h. (A) DaKr-h1; (B) DaBr-C. Error bars indicate standard error of the mean of three biological replicates (One-way ANOVA, $p < 0.05$, with Tukey’s test of multiple comparisons).
**FIGURE 8** | *DaKr-h1* and *DaBr-C* knockdown affects JH signaling pathway-related genes. The larvae, pupae, and emerged adults were allowed to ingest with L4440 (negative control), dsKr-h1, and dsBr-C for 3 days. *DaKr-h1, DaBr-C, DaEcR, DaE75, DaHr3,* and *DaFTZ-F1* transcript levels were measured. *DaKr-h1* knockdown in larvae (A), pupae (B), adult females (C), and adult males (D). *DaBr-C* knockdown in larvae (E), pupae (F), adult females (G), and adult males (H). The columns represent means with vertical lines indicating the standard error. Asterisks denote significant differences (unpaired t-test; *p* < 0.05, **p** ≤ 0.01, ***p** ≤ 0.001).
different developmental stages. Temporal expression profiles showed that DaKr-h1 and DaBr-C expression were predominantly expressed in the final larval stage (final instar, feeding larva), decreased to low levels at mature larvae (post-feeding final instar larva), and became low in the pupal stage, with expression remaining stable from the pupal to adult stage. This was similar to Kr-h1 expression in D. melanogaster (Minakuchi et al., 2008), T. castaneum (Minakuchi et al., 2009), Bombyx mori (Kayukawa et al., 2014), and Helicoverpa armigera (Zhang et al., 2018). The temporal expression profile of DaBr-C was similar to M. sexta (Zhou et al., 1998; Zhou and Riddiford, 2001; Zhou and Riddiford, 2002) and T. castaneum (Konopova and Jindra, 2008; Suzuki et al., 2008). DaBr-C expression was prominent during the larval–pupal transition but decreased as pupae began to develop to the adult. These results suggest that DaKr-h1 and DaBr-C were essential for the metamorphosis of D. armandi, especially during the final larval stage.

In this study, we demonstrated that DaKr-h1 was highly expressed in the heads of larvae and pupae. This was consistent with kr-h1 gene expression in the brains of D. melanogaster larvae and A. mellifera worker honeybees (Grozinger et al., 2003; Shi et al., 2007). The DaKr-h1 transcript level in adults was higher in the midgut and in the Malpighian tubules than in the head, hindgut, fat body, testes, ovaries, and antennae. Thus, the DaKr-h1 gene exhibited a broad tissue expression pattern, reflecting a possible pleiotropic role for Kr-h1 in D. armandi. Interestingly, the DaKr-h1 gene was slightly expressed in the antennae, which are chemosensory organs bearing sensilla specialized for the detection of olfactory signals by the antennal lobes—the main olfactory center of the brain (Duportets et al., 2012).

DaBr-C expression was highest in the heads of larvae and pupae, and in the reproductive organs of male and female adults. Br-C is widely distributed in several tissues from the last larval stage of development to the pupal stage (Bayer et al., 1996; Zhou and Riddiford, 2001; Reza et al., 2004). Studies have shown that the transcription factor Br-C has several roles in insect oogenesis. One is the formation of the dorsal appendage of the egg chorion in Drosophila (Deng and Bownes, 1997; Ward and Berg, 2005). It also plays a role in the effect of nutrition on oogenesis in Drosophila melanogaster (Terashima and Bownes, 2005). The Broad Complex isomform 2 (BrC-Z2) transcriptional factor plays a critical role in vitellogenin transcription in the silkworm Bombyx mori (Yang et al., 2014). Tissue distribution studies in spotted shrimp revealed that Br-C was expressed in the ovaries and was higher in the ovaries than in the testes, suggesting that Br-C plays an important role in its reproductive development and is important in ovarian and testicular development (Buaklin et al., 2013).

JH stimulates Kr-h1 expression in various insects (Kayukawa et al., 2012). For example, JH treatment increased Kr-h1 expression in Blattella germanica, H. armigera, and Nilaparvata lugens late instar larvae (Lozano and Belles, 2011; Jin et al., 2014; Zhang et al., 2018). Interestingly, the results of the
This situation was similar to the precocious metamorphosis reported in insects such as *T. castaneum*, *Pyrrhocoris apterus*, and *B. germanica* (Minakuchi et al., 2008; Minakuchi et al., 2009; Konopova et al., 2011; Lozano and Belles, 2011). RNAi-mediated DaKr-h1 gene silencing at the pupal stage (newly pupated pupae) promoted insect metamorphosis in the present study. In *N. lugens*, dsKr-h1-treated individuals had smaller wings, and the depletion of NlKr-h1 resulted in the partial formation of early adult features (Jin et al., 2014). Furthermore, *B. mori* with transgenes overexpressing *Kr-h1* failed to pupate, suggesting that *Kr-h1* was involved in the suppression of metamorphosis in *B. mori* (Kayukawa et al., 2014).

These studies suggest that *Kr-h1* is a master repressor of insect morphogenesis. Although *Kr-h1* plays a role in suppressing insect metamorphosis in a JH-dependent manner, studies have proposed that it suppresses metamorphosis by modifying the expression of 20E-inducible genes. The *Kr-h1* protein molecule interacts with the *Br-C* gene at the *Kr-h1* binding site and suppresses *Br-C* expression in larvae (Minakuchi et al., 2009; Zhu et al., 2010; Kayukawa et al., 2016). In *D. armandi*, DaBr-C expression was upregulated by DaKr-h1 knockdown in the larval stage, whereas it was downregulated by DaKr-h1 knockdown in the pupal stage. In *Drosophila*, larvae survive until pupation after knockdown of all *Br-C* isoforms, suggesting that *Br-C* is not essential for early postembryonic development (Kiss et al., 1988). In *T. castaneum*, knockout of *Br-C* in larvae affects 20E-mediated midgut remodeling during larval-pupal metamorphosis (Parthasarathy et al., 2008). Similarly, knockdown of *Br-C* in late 4th instar *L. dispar* larvae resulted in developmental defects, *epidermis* remodelling failure, and molting disruption (Ding et al., 2020). In conclusion, *Br-C* is required for insects to complete metamorphic processes involving growth, differentiation, and tissue remodelling (Konopova and Jindra, 2008). Here, we found that *Br-C* is not essential for early postembryonic development of *D. armandi* in early larvae that survive until pupation after knockout of *Br-C*. However, knockdown of *Br-C* at the pupal stage resulted in developmental defects and wing deformities. These results suggest that DaBr-C plays a critical role in epidermal and wing remodeling during *D. armandi* development and molting.

After *Kr-h1* knockdown, *Br-C* expression was downregulated in the last nymphal instar of *B. germanica* (Huang et al., 2013). In the pupal stage of *T. castaneum*, exogenous JH analogs mediated *Kr-h1* upregulation and induced *Br-C* transcription (Minakuchi et al., 2009). In *M. sexta* and *B. mori*, the removal of the corpus allatum (i.e., the main organ of JH synthesis) induced *Br-C* expression and precocious metamorphosis (Zhou et al., 1998; Reza et al., 2004). Moreover, knocking down *Kr-h1* in the larval and adult stages of *D. armandi* reduced DaEcR expression, but knocking down *Kr-h1* in the pupal stage reduced DaEcR, DaE75, and DaHr3 expression. In *D. melanogaster*, *Kr-h1* mutations resulted in changes in the expression patterns of ecdysone-inducible genes, such as EcR, E74A, E75B, Hr3, which together control the delayed expression of *BFTZ-F1* (King-Jones and Thummel, 2005) during the metamorphosis stage (Pecas et al., 2000; Liu et al., 2018). These studies suggest that *Kr-h1* suppresses metamorphosis by modifying the expression of early ecdysone-inducible genes.

Based on the results of this study and previous research, it is reasonable to speculate on the hormonal regulation mechanisms of DaKr-h1 and DaBr-C. DaKr-h1 expression may be induced by
JH via the Methoprene-tolerant–steroid receptor coactivator complex (Kayukawa et al., 2012; Kayukawa and Shinoda, 2015), and DaKr-h1 molecules may subsequently repress DaBr-C expression. Because of JH persistence, DaKr-h1 expression can be maintained at high levels during this stage. A decrease in JH concentration at the onset of the last instar larval stage can lead to a temporary absence of DaKr-h1 (Kayukawa et al., 2014), contributing to the induction of DaBr-C by 20E during the larval–pupal transition (Reza et al., 2004; Muramatsu et al., 2008). Any apparent inconsistencies in the inter-regulatory roles of genes at different developmental stages may be due to cellular factors such as transcription factors, coactivators, repressors, promoters, epigenetic modifications, or different cellular environments, including endocrine, paracrine, and nutritional factors (Kayukawa and Shinoda, 2015).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

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Y-YS and HC conceived and designed the experiment; D-YF, BL and L-JW performed the investigation; Y-YS performed data analyses; Y-YS wrote the original draft; HC reviewed and edited the document.

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SUPPLEMENTARY MATERIAL

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