BIHR4 nuclear receptor coordinates the timing of pupal ecdysis through ecdysteroid biosynthesis in Bombyx mori

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BHR4 is an ecdysteroid-induced orphan nuclear receptor whose expression appears in response to molting surges of ecdysteroid and then disappears as the ecdysteroid titer declines before each ecdysis. When the transgenic Bombyx larvae carrying an hsp70-BHR4 insert were heat-shocked during the pupal molt to misexpress BHR4, the pupal ecdysis was blocked due to a maintained ecdysteroid titer. The lack of decrease in the ecdysteroid titer was due to a stimulation of ecdysteroid biosynthesis caused by up-regulated ecdysteroid biosynthetic enzyme genes in the prothoracic glands rather than the disruption of the ecdysone cascade. Thus, the BHR4 expression during the pupal molt regulates the timing of pupal ecdysis through ecdysteroid synthesis.

Key words: BHR4, nuclear receptor, transcription factor, ecdysone, transgenic silkworm, Bombyx mori

INTRODUCTION

Molting and metamorphosis are important events in insect development that include the process of producing a new cuticle and culminate in ecdysis. Molting consists of a number of sequential events, including apolysis, new cuticle deposition, and ecdysis, followed by sclerotization and melanization of the newly formed cuticle. These events must occur in the proper order and during a certain time, which is primarily controlled by two hormones, ecdysteroid and juvenile hormone (JH). When both hormones are present in the hemolymph during the larval stages, they repeat larval molts whereas the presence of ecdysteroid alone causes pupal metamorphosis (Riddiford, 1994).

An active form of the ecdysteroid, 20-Hydroxyecdysone (20E), binds to the ecdysteroid receptor (EcR)/Ultraspiracle (USP) complex and activates a series of genes (Cherbas and Cherbas, 1996; Hiruma and Riddiford, 2009; Thummel and Chory, 2002). A small set of early-response genes are directly induced by the ecdysteroid, and are expressed immediately after ecdysteroid exposure. Many of these early genes encode transcription factors that repress their own expression and activate a large set of late-response genes. A subclass of early genes, early-late response genes are activated later than early genes (Huet et al., 1995). All of these classes of genes interact with each other; in particular, early-late genes transfer signals from early genes to late-response genes (Hiruma and Riddiford, 2001, 2007), and they play a central role in insect molting and metamorphosis.

The transcription factor HR4 was first identified as a novel monomeric orphan nuclear receptor in Bombyx mori and Tenebrio molitor (Charles et al., 1999; Mouillet et al., 1999); orthologs of this gene have also been found in other insects, such as Manduca sexta, Trichoplusia ni, Drosophila melanogaster, and Blattella germanica (Chen et al., 2002; Daniel et al., 2011; King-Jones et al., 2005; Weller et al., 2001). Studies on several insect species have revealed that HR4 contributes to the ecdysteroid signaling cascade as an early-late gene (Charles et al., 1999; Hiruma and Riddiford, 2001, 2007, 2009; King-Jones et al., 2005; Ou et al., 2011; Sullivan and Thummel, 2003; Weller et al., 2001). HR4 is expressed in response to the molting surge of ecdysteroid and then the expression ceases before ecdysis. In Manduca sexta, HR4 interactions with other genes are well characterized. Manduca HR4 (MHR4) is directly induced by 20E, but its expression is delayed due to the activation of another 20E-induced factor, Manduca HR3 (MHR3), which suppresses the MHR4 expression (Hiruma and Riddiford, 2007, 2009). In addition, MHR4 itself inhibits the dopa decarboxylase gene expression (Hiruma and Riddiford, 2007), which is the enzyme necessary for sclerotization and melanization of the cuticle (Hopkins and Kramer, 1992). Thus, MHR4 might play an important role in insect molting and sclerotization. Studies on the interactions between HR4 and other transcription factors in M. sexta have been performed using a cell line (Hiruma and Riddiford, 2007), but the actual role of HR4 in development of lepidopteran insect is poorly understood.

Drosophila HR4 (DHR4) has different interactions with other ecdysteroid-induced transcription factors from that of Manduca. For example, the DHR4 expression is not
only inhibited by DHR3, but DHR4 induces the \( \beta \)FTZ-F1 expression (King-Jones et al., 2005). When the DHR4 was silenced by RNAi in the final instar larvae, the larvae began to metamorphose prematurely and resulted in small-sized individuals (King-jones et al., 2005; Ou et al., 2011).

In this study, we generated transgenic Bombyx mori; the expression of Bombyx HR4 (BHR4) can be induced by heat-shock. Our findings showed that the prevention of the decline in the BHR4 expression during the pupal molt caused the failure of the pupal ecdysis, which was due to the prevention of the decline in the ecdysteroid titer. We caused the failure of the pupal ecdysis, which was due to heat-shock. Our findings showed that the prevention of expression of gene. The F1s were backcrossed with \( w-I \) to segregate BF1 individuals into DsRed-positive and DsRed-negative siblings. These siblings were used as transgenic and non-transgenic animals, respectively.

**Staging animals and heat-shock protocol**

* B. mori larvae were reared on fresh mulberry leaves at 25°C. Larvae that entered the wandering stage were individually wrapped in a paper towel and were checked every 1 h to examine their gut purge. Larvae that just emptied their gut contents were considered larvae at 0 h after gut purge.

To induce BHR4 expressions, 10-20 larvae were placed in a square plastic dish (140 mm × 100 mm) subsequently sealed with parafilm, and then were submerged into a 42°C water bath for 1 h. To maintain continuous BHR4 expressions, a heat-shock treatment was repeated every 8 h until the last treatment at 72 h after gut purge.

**Hormones**

An ecdysteroid agonist, RH-2485 (a gift from Dr. Tateishi), was dissolved in 10% dimethyl sulfoxide (DMSO) at a concentration of 0.1 mg/mL and was subcutaneously injected into wandering larvae.

Total hemolymph ecdysteroid concentrations and HPLC-purified ecdysteroids (see below) were determined by ELISA (Shiotsuki et al., 2004). Total ecdysteroids were extracted from hemolymphs by mixing them with three volumes of methanol followed by centrifugation at 9,500 g for 10 min at room temperature; supernatants were used.

Both ecdysone (E) and 20E were separated and collected from the total ecdysteroids extracted from the hemolymph using HPLC (LC-10AT, Shimadzu, Kyoto, Japan; Kamimura et al., 2012). The extract was applied to a C18 reverse-phase column (TSK gel ODS-80Ts, 4.6 × 150 mm, TOSOH, Tokyo, Japan) and E and 20E were eluted with a 20-30% linear gradient of acetonitrile at a flow rate of 0.6 mL/min. The fractions corresponding to E and 20E were pooled, dried, dissolved in methanol, dried again, and dissolved in water. The samples were directly used for ELISA.

**Detection and quantification of BHR4 mRNA**

Total RNA was extracted from the epidermis or the prothoracic glands using ISOGEN (TaKaRa Bio Inc., Otsu, Japan) or a High Pure RNA Tissue kit (Roche, Mannheim, Germany) respectively, according to the manufacturers’ instructions. Total RNA was converted into cDNA using a Prime Script RT reagent kit (TaKaRa) using oligo dT and random hexamer primers. RT-PCR was performed to confirm the heat-induced BHR4 expression in the cDNA using Ex Taq (TaKaRa Bio Inc., Otsu, Japan). The condition was as follows: one cycle of 99°C for

**MATERIALS AND METHODS**

**The Bombyx line expressing BHR4**

BHR4 cDNA was isolated from the pGRF1-SK plasmid (Charles et al., 1999) by digestion with EcoRI and HindIII. Either the Bombyx hsp70 promoters or polyadenylation signal sequences were isolated from the carrying plasmid (a gift of Dr. K. Kojima) using EcoRI and SalI, or EcoRI and HindIII, respectively. The isolated hsp70 promoter was then cloned into a pBluescriptII SK+ (Agilent Technologies, Santa Clara, CA, USA) plasmid that was digested with EcoRI and HindIII. Then, the BHR4 cDNA and the polyadenylation signal were cloned into the plasmid between the HindIII and SalI sites by triple ligation. The whole expression cassette (hsp70-BHR4) was isolated by BamHI digestion and inserted into the BglII site of pBac[3xP3-DsRed] (Inoue et al., 2005) to construct pBac[hsp70-BHR4, 3xP3-DsRed].

To generate a transgenic silkworm line with the hsp70-BHR4, 3xP3-DsRed insert, the piggyBac vector pBac[hsp70-BHR4, 3xP3-DsRed] was co-injected with the helper plasmid, pHA3PIG, into pre-blastoderm stage embryos of the Bombyx mori strain, w-1; pnd as described previously (Tamura et al., 2000). The plasmids were dissolved in the injection buffer (5 mM KCl, 0.5 mM sodium phosphate, pH 7.0) at a concentration of 0.2 mg/mL. After injection, the embryos were allowed to develop at 25°C and were reared on an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan). Among the injected embryos, 104 fertile moths were recovered. The transgenic G1 embryos were screened under an MZ10F fluorescent stereomicroscope (Leica, Heerbrugg, Switzerland) equipped with filter sets to detect DsRed expressions in the stemmata of embryos. The established hsp70-BHR4, 3xP3-DsRed lines were maintained as homozygotes. To give rise to experimental animals, they were crossed with the w-I strain to make F1 progenies that are heterozygous for the transgenic and non-transgenic animals, respectively.
BHR4 stimulates ecdysteroid biosynthesis

1 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 10 min. The ef1α mRNA was used as a positive control. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to measure the amount of target mRNA using cDNA with a Light Cycler 480 (Roche Applied Science, Mannheim, Germany). The amplified PCR products were used as quantification standards at a concentration of 0.1 ng/μL to 0.1 fg/μL. The conditions were as follows: 95°C for 5 min, followed by 95°C for 5 sec and 60°C for 20 sec. The optimum cycle numbers were between 15 and 40. The rp49 mRNA was used as an internal control. The oligonucleotide primer sequences used in this study are listed in Table 1.

RESULTS

Roles of BHR4 during pupal molt

When transgenic Bombyx mori carrying the hsp70-BHR4 gene were heat-shocked (42°C, 1 h), the BHR4 expression was strongly induced in the epidermis and prothoracic glands 1 h after the first heat-shock (Fig. 1). Thus, heat-shock effectively induced the BHR4 expression. This was confirmed in every experiment described in this paper.

Some larvae failed to pupate when a high level of the BHR4 expression was maintained by heat-shock through the time in which the BHR4 expression normally declines (Table 2a). They were not only unable to shed the old larval cuticle, but also had pigmented Verson’s glands (Fig. 2). A similar phenotype was observed in a previous study on Manduca sexta final instar larvae injected with 20E (Nijhout, 1976). Therefore, these results indicate that misexpression of BHR4 phenocopies prevents ecdysteroid titer decline, leading to the failure of pupal ecdysis.

When an ecdysteroid agonist, RH-2485, was injected into larvae 30 h after they purged their gut contents, the larvae also failed pupal ecdysis (Table 2b); these individuals also had pigmented Verson’s glands, similar to larvae with BHR4 misexpression (Fig. 2). These results strongly suggest that the BHR4 misexpression has the same physiological impact as ectopic ecdysteroid signaling. Because BHR4 is an early-late gene in the ecdysteroid signaling cascade, misexpressed BHR4 could either transduce a signal downstream of the cascade to prevent the ecdysis or it could stimulate ecdysteroid synthesis.

Table 1 Primer sets used in this study

<table>
<thead>
<tr>
<th>gene</th>
<th>primer sequence</th>
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<tr>
<td>BHR4 (RT-PCR)</td>
<td>5'-GGAAAGTTGACTCCAGATGTAGCAG - 3'</td>
</tr>
<tr>
<td></td>
<td>5'-CGGATTCGCCACATTATAACAGCA - 3'</td>
</tr>
<tr>
<td>BHR4 (qRT-PCR)</td>
<td>5'-TCCCTCGTGCTAGTACGAACAGG - 3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCCTGCCCATAAGTGACGTCAA - 3'</td>
</tr>
<tr>
<td>neverland</td>
<td>5'-CGAAGCGTACATGTTTGAG - 3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCAAATTCTGAAGGTTCGT - 3'</td>
</tr>
<tr>
<td>spook</td>
<td>5'-CCCCACTCATACTCTCCAGCAAT - 3'</td>
</tr>
<tr>
<td></td>
<td>5'-CGGCTTCTTTGAGTGTCGTG - 3'</td>
</tr>
<tr>
<td>phantom</td>
<td>5'-GAGCCGAATATGCTCCTCAAGCA - 3'</td>
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<tr>
<td></td>
<td>5'-AAATCTGGTGTGGTGTGAGAG - 3'</td>
</tr>
<tr>
<td>disembodied</td>
<td>5'-TGAGGAGTACACCCGAGCCTTTCT - 3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGCGACCACTTACCTTCCATTTCCATTT - 3'</td>
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<tr>
<td>shadow</td>
<td>5'-GGTTTATATGCGCAAAAGACAT - 3'</td>
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<td></td>
<td>5'-GGTGAAATGATGCACCACTTT - 3'</td>
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<td>ef1α</td>
<td>5'-ACACGTCGACTCCGGCAAGT - 3'</td>
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<td>rp49</td>
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<td></td>
<td>5'-TGCTGGGCTTCTTCCACGA - 3'</td>
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Table 2 The failure of the pupal ecdysis by the expression of BHR4 and the injection of RH-2485

a. BHR4 expression

<table>
<thead>
<tr>
<th>gene</th>
<th>No.</th>
<th>% normally ecdysed</th>
<th>% non-ecdysed pupae</th>
<th>% died</th>
</tr>
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<tbody>
<tr>
<td>BHR4</td>
<td>48</td>
<td>64.6</td>
<td>35.4</td>
<td>0</td>
</tr>
<tr>
<td>HS</td>
<td>21</td>
<td>90.5</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>No HS</td>
<td>20</td>
<td>95.0</td>
<td>5.0</td>
<td>0</td>
</tr>
</tbody>
</table>

b. RH-2485 injection

<table>
<thead>
<tr>
<th>gene</th>
<th>No.</th>
<th>% normally ecdysed</th>
<th>% non-ecdysed pupae</th>
<th>% died</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH-2485</td>
<td>10</td>
<td>0</td>
<td>100.0</td>
<td>0</td>
</tr>
<tr>
<td>Control (10% DMSO)</td>
<td>10</td>
<td>90.0</td>
<td>10.0</td>
<td>0</td>
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</table>

1) Larvae were heat-shocked 18 h after purging the gut contents and the treatment was repeated every 8 h thereafter.
2) One microgram RH-2485 was injected to the larvae 30 h after the onset of gut purge, and 10 μl of 10% DMSO solution was injected as above as a control.
3) Some larvae in the controls died caused by the damage of either heat-shock (a) or injection (b).
controls were not significantly different; however, the ecdysone-expressing and heat-shocked and non-heat-shocked BHR4 60 h after ligation. Ecdysteroid concentrations in both ecdysteroid concentrations were determined 42, 48, and every 8 h to ensure a continuous expression. After ligation, both portions were heat-shocked and activated for 12 h after the gut purge, and then inactivated, we ligated the larvae on both sides of the fourth abdominal segment 18 h after gut purge, and then measured the ecdysteroid concentration in both the anterior and posterior portions with or without the BHR4 expression. After ligation, both portions were heat-shocked every 8 h to ensure a continuous BHR4 expression, and ecdysteroid concentrations were determined 42, 48, and 60 h after ligation. Ecdysteroid concentrations in both BHR4-expressing and heat-shocked and non-heat-shocked controls were not significantly different; however, the ecdysteroid concentration in the anterior portions were high because of the presence of the prothoracic glands (Fig. 5). Thus, the high level of E and 20E maintained by the BHR4 misexpression observed in the hemolymph (Fig. 4) was not due to the prevention of ecdysteroid inactivation.

These results are based on immunoreactive ecdysteroids. To confirm the stimulative action of BHR4 on ecdysteroid synthesis, we measured the hemolymph concentration of E and 20E separately in larvae 42 h after the gut purge fractionated by HPLC. BHR4-expressing larvae contained 40-500 ng/mL each of E and 20E, whereas only trace levels of both E and 20E were found in the control larvae (Fig. 4), indicating that BHR4 stimulated ecdysteroid biosynthesis at the time of the declining phase of the ecdysteroid titer and that the relatively high amount of immunoreactive ecdysteroid detected by ELISA assays in both larvae might be due to 20E metabolites.

To rule out the possible effect of BHR4 on ecdysteroid inactivation, we ligated the larvae on both sides of the fourth abdominal segment 18 h after gut purge, and then measured the ecdysteroid concentration in both the anterior and posterior portions with or without the BHR4 expression. After ligation, both portions were heat-shocked every 8 h to ensure a continuous BHR4 expression, and ecdysteroid concentrations were determined 42, 48, and 60 h after ligation. Ecdysteroid concentrations in both BHR4-expressing and heat-shocked and non-heat-shocked controls were not significantly different; however, the ecdysteroid concentration in the anterior portions were high because of the presence of the prothoracic glands (Fig. 5). Thus, the high level of E and 20E maintained by the BHR4 misexpression observed in the hemolymph (Fig. 4) was not due to the prevention of ecdysteroid inactivation.

BHR4 misexpression leads to altered mRNA expression of ecdysteroid biosynthetic enzymes

Since BHR4 stimulated ecdysteroid biosynthesis in vivo, we determined the mRNA expression of enzymes involved in ecdysteroid biosynthesis in the prothoracic glands in order to investigate whether or not BHR4 induction affected enzyme expression. We analyzed the expressions of neverland (nvd), spook (spo), phantom (phm), disembodied (dib), and shadow (sad) (Gilbert and Warren, 2005; Ono et al., 2006; Warren et al., 2004; Yoshiyama et al., 2006) using qRT-PCR.

In the control larvae that were either heat-shocked or not, the fluctuation patterns of these enzyme genes were similar except for phm (Fig. 6). The heat-shocked control larvae showed an exceptionally high level of expression of phm at 30 h after gut purge from unknown reason. When the ecdysteroid titer was high, the gene expression of nvd, spo, and dib were also high, and all declined simultaneously with the ecdysteroid titer (32-42 h after gut purge; Figs. 3 and 6). Conversely, in larvae with a high ecdysteroid titer 36-48 h after the gut purge induced by heat-shocked BHR4 expression, the expression of all enzymes was high following a slight time lag, except for sad, which was nonresponsive (Figs. 3 and 6). These results strongly suggest that a BHR4 misexpression leads to the prolonged expression of many ecdysteroid biosyn-

**Fig. 1.** Induction of the recombinant BHR4 by heat-shock treatment in vivo. Transgenic (hsp-BHR4) and non-transgenic (-) day two fifth instar larvae were heat-shocked, and the prothoracic glands and epidermis were dissected 1 h later for RNA extraction. The BHR4 expression in these tissues was analyzed by qRT-PCR.

<table>
<thead>
<tr>
<th>hsp-BHR4</th>
<th>-</th>
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<tr>
<td>♂</td>
<td>♂</td>
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<td>a</td>
<td>b</td>
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<td>♀</td>
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<td>a</td>
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*Fig. 2.* Phenotypes of BHR4-misexpressed and RH-2485-injected larvae. (a) A larva that failed pupal ecdysis due to BHR4 misexpression. Heat-shock treatment began 18 h after gut purge to induce the BHR4 expression, and the treatment was repeated every 8 h to maintain a continuous BHR4 expression. The image was acquired 105 h after gut purge. Verson’s glands in each segment (arrows) were pigmented. (b) A larva that was injected with RH-2485. RH-2485 was injected 30 h after gut purge, and the image was acquired 120 h after gut purge. The larva failed pupal ecdysis and Verson’s glands were also pigmented (arrows).
BHR4 stimulates ecdysteroid biosynthesis

A number of transcription factors appear and disappear during insect molting and metamorphosis, which are regulated by ecdysteroid and JH. Ecdysteroid causes molting and metamorphosis through the EcR/USP complex, and binding to the EcR induces activation of the cascade of these transcription factors (Dubrovsky, 2005; Hiruma and Riddiford, 2009, 2010; King-Jones and Thummel, 2005; Riddiford et al., 2003). During a larval molt in lepidopteran insects such as Manduca, when the ecdysteroid titer begins to increase, the E75A expression also increases in the epidermis and subsequently declines as the ecdysteroid titer becomes high (Zhou et al., 1998). E75A suppression and high ecdysteroid titer trigger the MHR3 expression, such that the timing of the MHR3 expression is regulated by both the ecdysteroid and the E75A (Hiruma and Riddiford, 2007). MHR3 suppresses ecdysteroid-induced MHR4 whose mRNA appears in response to ecdysteroid during the declining phase of MHR3 mRNA when E75B expression begins to appear; MHR3, E75B, and MHR4 mRNA expression occur sequentially during the larval molt. MHR3 and E75B form a heterodimer (Hiruma and Riddiford, 2007), and this dimerization prevents the inhibitory action of MHR3 on the MHR4 expression; therefore, the timing of the MHR4 expression is determined by the cessation of the MHR3 expression and E75B activation (Hiruma and Riddiford, 2007). These results suggest that MHR4 plays an important role in the genetic cascades responsible for molting. We showed a different role of HR4 in Bombyx (BHR4), which functions as a regulator of ecdysteroid synthesis to determine the timing of pupal ecdisis.

**DISCUSSION**

**BHR4 action on ecdysteroid synthesis**

The MHR4 expression is directly induced by 20E *in vitro*, but its expression declines in the continuous presence of 20E if protein synthesis is not prevented (Hiruma and Riddiford, 2001). Therefore, ecdysteroid action on MHR4...
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fits well with the Ashburner model, in which early and early-late gene products engage in negative feedback to suppress gene expression with their own products (Ashburner et al., 1974). The ecdysteroid acts on the \textit{BHR4} gene in a similar fashion to \textit{MHR4} (Charles et al., 1999; Muramatsu and Hiruma, unpublished). During the last larval and pupal molt in \textit{Bombyx mori}, the \textit{BHR4} mRNA expression pattern mirrors that of the ecdysteroid titer in the hemolymph (Charles et al., 1999; Fig. 3), supporting not only the Ashburner model, but suggesting that the inactivation of the \textit{BHR4} expression corresponds with decline in ecdysteroid titer as found in \textit{M. sexta} (Hiruma and Riddiford, 2001).

Of interest is the finding that hemolymph ecdysteroid titer increased rather than decreased, and the larvae failed to pupate when the \textit{BHR4} was missexpressed at and after the peak of both the ecdysteroid titer and the \textit{BHR4} expression during the pupal molt. As shown in Figure 5, this increase in ecdysteroid titer was not due to ecdysteroid stabilization; instead, it was due to the activation of the ecdysteroid synthesis. We tried to measure the ecdysteroid synthesis by the prothoracic glands \textit{in vitro} with the expression of \textit{BHR4}, but the results showed inconsistent activation of the ecdysteroid synthesis by \textit{BHR4} (Yokoyama, unpublished). Yet, many of the ecdysteroid biosynthetic enzymes in the prothoracic glands, such as \textit{nvd}, \textit{spo}, and \textit{dib}, were up-regulated by the \textit{BHR4} expression and corresponded with the increase in ecdysteroid titer (Figs. 3 and 6), evidence that the ecdysteroid synthesis was activated. \textit{shadow}, which converts 3-dehydroecdysone (3DE) into \textit{E}, did not respond to the \textit{BHR4} misexpression. The expression of \textit{shadow} was maintained at a low level that was detected even during high ecdysteroid titers in normally developing larvae. Therefore, these results and those of the \textit{in vivo} heat-shock experiments (Figs. 3 and 4) show that ecdysteroid biosynthesis is stimulated by \textit{BHR4} at the declining time of ecdysteroid synthesis.

The \textit{BHR4}-induced stimulation of ecdysteroid biosynthesis seems to be stage-dependent, as heat-shock induction of \textit{BHR4} only slightly affected the increasing phase of the ecdysteroid titer, which was slightly lower than that in the controls, but markedly stimulated the ecdysteroid synthesis during the declining phase of the titer (Fig. 3). These results suggest that the responsiveness of the prothoracic glands to \textit{BHR4} changes around the peak time of the ecdysteroid synthesis from being unresponsive to being stimulated. Yet the mechanism of this switch is unknown. A similar function of a nuclear receptor was observed in the ecdysteroid-induced E75A, which acts in a feed-forward pathway to amplify or maintain ecdysteroid titer during \textit{Drosophila melanogaster} larval development to ensure proper temporal progression through the life cycle (Bialecki et al., 2002).

\textbf{HR4 determines the timing of pupal ecdysis by changing ecdysteroid production}

The increase in the ecdysteroid titer during molt programs many events associated with the molting process, but its decline is required for these events to occur (Hiruma et al., 1995; Hiruma and Riddiford, 1985; Schwartz and Truman, 1983; Slama, 1980). During the pupal molt, the molting surge of the ecdysteroid stimulates the \textit{BHR4} expression, followed by its decline corresponding to the declining ecdysteroid titer. This \textit{BHR4} decline is essential to induce the ecdysteroid level decline in order to ensure normal pupation, as the decline in the ecdysteroid molting
BHR4 stimulates ecdysteroid biosynthesis

surge is required for normal molting and metamorphosis; this nuclear receptor is apparently involved in this inhibitory action of ecdysteroid.

HR4, including BHR4, binds to the FTZ-F1 response element (Charles et al., 1999; Mouillet et al., 1999). The βFTZ-F1 expression is induced by the decline in the ecdysteroid titer after its exposure (Hiruma and Riddiford, 2001; Sun et al., 1994), and appears after the peak of the HR4 expression. Therefore, BHR4 is likely a regulator of βFTZ-F1. This mid-prepupal factor is required for cuticle formation during the molting period (Kageyama et al., 1997; Yamada et al., 2000). In particular, it is responsible for the induction of the pupal cuticle gene, EDG-84A, in D. melanogaster (Kawasaki et al., 2002; Murata et al., 1996). Thus, BHR4 not only determines the timing of the ecdysteroid titer decline, but might indirectly regulate the regulation of pupal cuticle formation.

Mutations in the DHR4 nuclear receptor in D. melanogaster result in larvae that precociously leave food to form prepupae, producing smaller and lighter pupae (King-Jones et al., 2005), which is due to a premature increase in the ecdysteroid titer (Ou et al., 2011). These results are different from our results in B. mori, probably because of different HR4 interactions with other transcription factors in the ecdysteroid cascade. In D. melanogaster, the DHR4 expression is stimulated by the ecdysteroid at the onset of metamorphosis, acting as both a repressor of early ecdysteroid-induced regulatory genes, such as

Fig. 6. Effect of BHR4 misexpression on the mRNA expression of ecdysteroid biosynthetic enzymes in the prothoracic glands. The time course of the mRNA expression of neverland, spook, phantom, disembodied, and shadow were analyzed by qRT-PCR. ○, heat-shocked transgenic expressing BHR4; ●, heat-shocked non-transgenic control; ▲, transgenic without heat-shock control. Error bars represent S.D. (n = 3).
EcR, E74, and E75A, and as an inducer of the β-FTZ-F1 mid-prepupal competence factor (King-Jones et al., 2005).

HR4 plays a central role in insect development, as it not only acts as an important member of the ecdysteroid signaling cascade that interacts with other factors involved in the process of molting and metamorphosis, but also regulates ecdysteroid synthesis to determine the proper timing of pupal ecdysis. Other BHR4 actions during the pupal molt were not determined in this study. For unknown reasons, BHR4 RNAi constructs were unable to suppress BHR4 expression in transgenic B. mori lines (Yokoyama and Tomita, unpublished), which is not uncommon among other RNAi constructs in Bombyx (H. Sezutsu, personal communication). Further studies on the coordination of BHR4 with other factors will shed light on the regulation of ecdysteroid synthesis by this orphan receptor.

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BHR4 stimulates ecdysteroid biosynthesis


