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Identification and Characterization of Novel Genes Expressed Preferentially in the Corpora Allata or Corpora Cardiaca During the Juvenile Hormone Synthetic Period in the Silkworm, *Bombyx mori*

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Juvenile hormone (JH) plays important roles in insect development and physiology. JH titer is tightly regulated to coordinately adjust systemic physiology and development. Although control of JH titer is explained by the expression of JH biosynthetic enzymes in the corpora allata (CA), molecular mechanisms that regulate the expression of these genes remain elusive. In the present study, to identify novel regulators of JH biosynthetic genes, we conducted a gene expression screen using the CA and corpora cardiaca (CC) of the silkworm, *Bombyx mori*, in the JH synthesis period. We identified seven candidate genes and characterized their properties through extensive expression analyses. Of these candidates, we found that a novel gene, which encodes type II phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2] 4-phosphatase, shows highly correlated expression with JH titer. In addition, expression of this gene was strongly upregulated by starvation, when JH biosynthetic enzyme genes are concurrently upregulated. These results, for the first time, imply possible involvement of phosphoinositol signal in regulation of JH biosynthesis, providing novel insights into molecular mechanisms of nutrition-dependent regulation of JH biosynthesis.

Key words: *Bombyx mori*, JH, corpora allata, phosphoinositol, development

INTRODUCTION

Postembryonic development in insects is regulated by coordinated actions of ecdysone and juvenile hormone (JH) (Nijhout, 1994). Active metabolite of ecdysone, 20-hydroxyexdysone (20E), triggers molting events, and JH exerts “status quo” effects on larval development. A rise of 20E titer promotes larva-to-larva development under a high JH condition, whereas it leads to larva-to-pupa metamorphosis under a low JH condition (Nijhout, 1994; Riddiford, 1996). In addition to its effects on larval development, JH exerts pleiotropic effects on insect physiology, such as reproduction, diapause, and polyphenism (Denlinger, 2005; Hartfelder, 2005; Raikhel, 2005). These functions of JH titer are tightly regulated by concerted actions of JH biosynthetic and degradative enzymes (Nijhout, 1994; Tan et al., 2005; Kinjoh et al., 2007).

JH is synthesized and secreted from the corpora allata (CA), a neurosecretory organ that is connected to the brain through the corpora cardiaca (CC). JH biosynthesis consists of a series of enzymatic reactions that are conventionally divided into two major phases: the early steps where farnesyl pyrophosphate (FPP) is synthesized through the mevalonate pathway and the late steps where FPP is converted to JH. The mevalonate pathway is highly conserved among animals; FPP is synthesized from acetyl-CoA or acetoacetyl-CoA through the actions of eight enzymes, as follows: acetoacetyl-CoA thiolase (AACT), HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGR), mevalonate kinase (MevK), phosphomevalonate kinase (MevPK), diphosphomevalonate decarboxylase (MevPPD), isopentenyl diphosphate isomerase (IPPI), and farnesyl diphosphate synthase (FPPS). The late steps are arthropod specific, where JH is synthesized from FPP through the actions of five enzymes as follows; FPP pyrophosphatase, farnesol dehydrogenase, farnesal dehydrogenase, farnesoic acid epoxidase, and juvenile hormone acid methyltransferase (JHAMT) (Shinoda and Itoyama, 2003; Kinjoh et al., 2007; Ueda et al., 2009; Daimon et al., 2012). Because JH is immediately secreted from the CA to hemolymph upon biosynthesis and is not stored, JH titer correlates with the levels of expression of JH biosynthetic enzyme genes (Shinoda and Itoyama, 2003; Kinjoh et al., 2007). Thus, JH biosynthetic activity of CA is controlled by the regulation of expression levels of these enzyme genes.

JH biosynthesis is significantly affected by nutritional signals. In the larvae of the tobacco hornworm, *Manduca sexta*, JH titer is increased by starvation and is subsequently decreased by re-feeding (Cymborowski, 1982). Furthermore, starvation-induced increase in JH titer inhibits imaginal disc formation, allowing larvae to conduct an extra molt until they reach the critical weight (Truman et al., 2006). These phenomena indicate that nutritional signals and inter-

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nal physiological states converge onto JH biosynthetic activity of CA to coordinate systemic physiology. However, the molecular mechanisms underlying the effects of nutritional signals on JH biosynthetic activity remain elusive.

JH biosynthesis is also modulated by various factors including neuropeptides, such as allatotropin (AT) and short neuropeptide F (sNPF) (Goodman, 2005; Stay and Tobe, 2007; Audsley et al., 2008; Yamanaka et al., 2008; Kaneko and Hiruma, 2014), neurotransmitters, (Granger et al., 1996; Gilbert et al., 2000; Granger et al., 2000), decapentaplegic (Huang et al., 2011), TGF- β signaling (Ishimaru et al., 2016), and the molting hormone 20E (Whisenton et al., 1985; Kaneko et al., 2011). A recent transcriptomic study revealed that AT receptor (ATR), which had long been believed to be expressed in the CA, is preferentially expressed in the CC, but not in the CA. In addition, sNPF released from ATR-positive CC cells functions as an allatoregulatory peptide, (Yamanaka et al., 2008). These studies indicate that both the CA and the CC are involved in JH biosynthesis.

In the present study, we identified genes preferentially expressed in the CA or CC during JH synthetic period in the larvae of silkworm, *B. mori*. One of these genes, which encodes type II phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2] 4-phosphatase, showed an expression pattern that is highly correlated with JH titer and strongly enhanced expression under starvation. These results imply that phosphoinositol signaling pathway may regulate JH biosynthetic activity of CA in the downstream of nutritional signals.

MATERIALS AND METHODS

Insects

Eggs of a racial hybrid of *B. mori*, Kinshu x Showa, were purchased from a local dealer (Ueda Sanshu, Nagano, Japan). Larvae were reared on an artificial diet (Silkmate 2M, Nihon Nosan Kogyo, Yokohama, Japan) at 25°C under a 12 h light/12 h dark photoperiod. The day of ecdysis was designated as day zero (IV0 or V0).

Fluorescent differential display

The CAs and CCs (101–106 tissue samples, each) were collected from day four of fourth instar (IV4) or day five of fifth instar (V5) larvae. Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA), treated with DNase I, and reverses transcribed (RT) with SuperScript III (Invitrogen). Fluorescent differential display was performed as described previously using a Fluorescent Differential Display Kit and LA *Taq* polymerase (Takara-Bio, Shiga, Japan) with partial modification (Ito and Sakaki, 1999; Taguchi et al., 2011). To verify the reproducibility of experiments, screening was conducted in duplicate. Bands of interest were excised, reamplified, and subcloned into a pGEM-T vector (Promega, Madison, WI). Three clones for each band were isolated and the sequences of all clones were determined.

Semi-quantitative RT- PCR

Total RNA was isolated from CAs and CCs (30 tissue samples each) of IV4 or V5 larvae, treated with DNase I, and reverse transcribed with ReverTra Ace (Toyobo, Osaka, Japan). Semi-quantitative RT-PCR was performed with GoTaq Green Master Mix (Promega) using gene-specific primers (Table 1), following the manufacturer's protocol. PCR conditions were 33–45 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. Cycles of amplification was adjusted to the level of each gene expression, which was estimated by preliminary experiments. *Ribosomal protein L3* (*rpl3*) was used as an internal control. PCR without RT reaction was included and confirmed no genomic contamination. To secure the reproducibility of semi-quantitative RT-PCR, all experiments were conducted in triplicate.

Real-time RT-PCR

The precise levels of gene expression were determined by real-time RT-PCR. Total RNA was isolated from CA-CC complexes (20–40 each) or various tissues (3–7 each) for each sample. The total RNA was treated with DNase I, and reverse transcribed with PrimScript RT Master mix (Takara-Bio). Real-time RT-PCR was performed with and SYBR Premix Ex Taq™ II , using gene-specific primers (Table 2). Levels of JH biosynthetic enzyme gene expression were determined using the gene-specific primers described previously (Shinoda and Itoyama, 2003; Kinjoh et al., 2007; Daimon et al., 2012). PCR condition was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The fluorescent signals from samples (SYBR Green) were obtained using ABI7900HT (Applied Biosystems, Foster City, CA) with default settings. The level of gene

Table 1. Sequence of primers used for semi-quantitative RT-PCR.

Primers	Sequences (5′ to 3′)
BGIBMGA007861/Forward	AGTCGATGGAGTAGCGTTCTTTG
BGIBMGA007861/Reverse	GGCATCGTCCTTCAGTTCTTG
Nene/Forward	TCTGCGATCCTGTCCAGCCA
Nene/Reverse	ACAGCTCACCGCCTACCCGA
BGIBMGA005735/Forward	ATTGGCGCCGCTAGGCTTGG
BGIBMGA005735/Reverse	CCGTCGGCGAGAACCAGCTC
BGIBMGA002123/Forward	GGGCCAGCGACGGTCTGAAC
BGIBMGA002123/Reverse	AAAGGGACACGCGCAGCCTC
BGIBMGA008114/Forward	TACGGCGACGGAAAGGTGCG
BGIBMGA008114/Reverse	GGGAGAGGTTTCTCGCACGGG
BGIBMGA012888/Forward	AGCTCGCTATTCGCGCCCAC
BGIBMGA012888/Reverse	CGACGAGCCAGGTGCCGAAG
BGIBMGA007603/Forward	GAAGGCCGAACACCCTGGCG
BGIBMGA007603/Reverse	GCTCGTTGGTCCACTGCGGG
RpL3/Forward	AGCACCCCGTCATGGGTCTA
RpL3/Reverse	TGCTCCAAGCTCATCCTGC

Table 2. Sequence of primers used for real-time RT-PCR.

Primers	Sequences (5′ to 3′)
BGIBMGA007861/Forward	AGTCGATGGAGTAGCGTTCTTTG
BGIBMGA007861/Reverse	GGCATCGTCCTTCAGTTCTTG
Nene/Forward	TCTGCGATCCTGTCCAGCCA
Nene/Reverse	ACAGCTCACCGCCTACCCGA
BGIBMGA005735/Forward	AAGCCGAACCCTCACCAA
BGIBMGA005735/Reverse	TGCCGACTTCATCCACCA
BGIBMGA002123/Forward	GGGCCAGCGACGGTCTGAAC
BGIBMGA002123/Reverse	AAAGGGACACGCGCAGCCTC
BGIBMGA008114/Forward	ACTCGCAACCAGCATCTCC
BGIBMGA008114/Reverse	TCGGGAATAGGTGTGGATAAAAC
BGIBMGA012888/Forward	GCCAAGGTCTTCGTCCAATC
BGIBMGA012888/Reverse	TTGCTGTTTCGTAGATGCTGTTC
BGIBMGA007603/Forward	GAAGGCCGAACACCCTGGCG
BGIBMGA007603/Reverse	GCTCGTTGGTCCACTGCGGG
RpL3/Forward	AAGAGATCGTGGAGGCTGTCA
RpL3/Reverse	CGTAGTCCATGAGGGGTCTCA

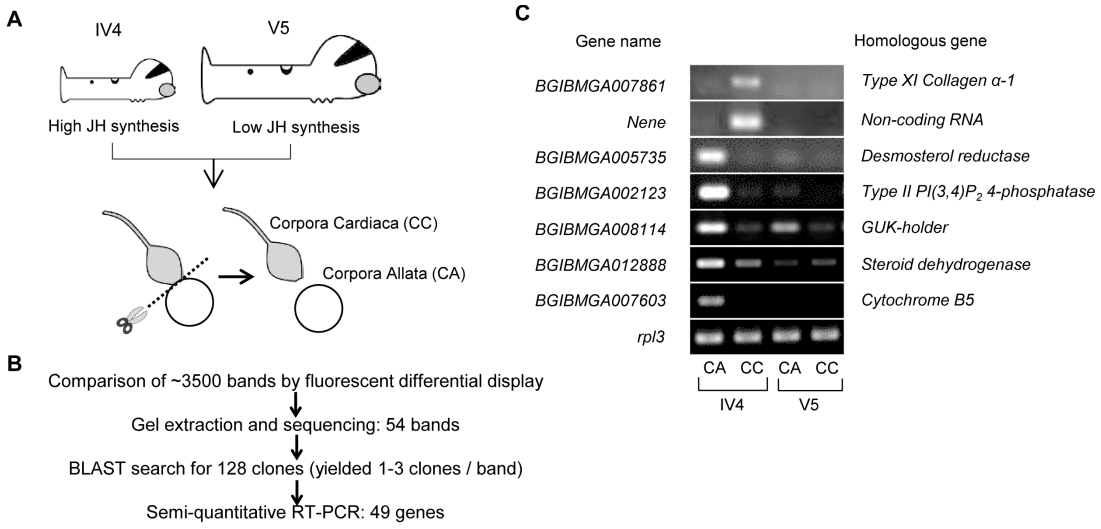

Fig. 1. Screening of genes expressed preferentially during the JH synthesizing stage in the CA and CC. **(A)** Schematic illustration of the screening procedures. The CA–CC complex was dissected from IV4 (high JH titer) or V5 (low JH titer) larvae. The CA and CC were separated and used for fluorescent differential display. **(B)** Flowchart of screening. **(C)** Results of semi-quantitative RT-PCR. Seven genes were confirmed to be expressed in a JH synthesizing stage-selective manner in the CA and CC. Gene names and their homologues were listed in the left and right side of band pictures, respectively. Experiments were conducted in triplicate (biological replicates). The bands of *rpl3* showed similar intensity among samples, indicating that similar amounts of RNA were used. PCR without RT reaction confirmed no genome contamination (not shown).

expression was determined using the serially diluted (10-fold dilutions for a dynamic range of 10^6) quantification standards, which was prepared from purified PCR products. The determined value of each sample was normalized to that of *rpl3*. The normalized values were again normalized to that of control sample, and shown as the relative expression. Statistical analyses were conducted by the *F*-test and *Student*'s *t*-test, using Microsoft Excel (Microsoft). All data are presented as mean ± standard error.

Rapid amplification of cDNA ends (RACE)

To identify the full-length cDNA sequence of candidate genes, 5′- and 3′-RACE were performed repeatedly using the CA-CC complex total RNA and the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA). The cDNA sequences were analyzed using Geneious Pro 7 (Biomatters, Auckland, New Zealand).

Whole-mount in situ hybridization

The digoxigenin (DIG)-labeled RNA probes were synthesized using a DIG RNA labeling mix (Roche). Template DNA fragments for RNA probes were obtained using gene-specific primers [BGIBMGA007861; 5′-CCAGGCAGACAAGGACGCCG-3′ and 5′-CGATTCGCCCGTGGCACCTT-3′, BGIBMGA005735; 5′-ATTGGCGCCGCTAGGCTTGG-3′ and 5′-TTAGGCAGCGAAGCCCGCAC-3′, BGIBMGA002123; 5′-GGGCCAGCGACGGTCTGAAC-3′ and 5′-AAAGGGACACGCGCAGCCTC-3′, BGIBMGA012888; 5′-AAGCTGTTAGCCGCCCGTGG-3′ and 5′-CGACGAGCCAGGTGCCGAAG-3′]. The PCR products were subcloned into a pGEM-T vector (Promega) and insert sequences were confirmed.

Whole-mount in situ hybridization was conducted as described previously (Ueda et al., 2009). CA-CC-brain complexes were dissected from IV4 or V5 larvae, fixed in 4% formaldehyde in phosphate-buffered saline with 0.1% Tween 20 (PBTw) overnight at 4°C, gradually

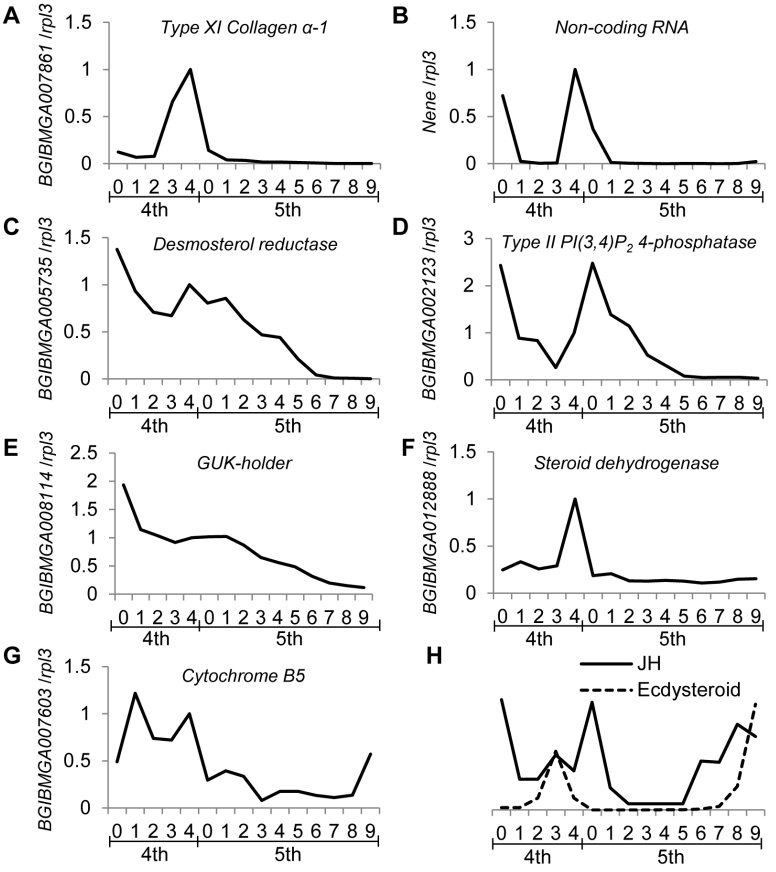

Fig. 2. Temporal expression profiles of candidate genes in the CA-CC complexes of fourth and fifth-instar larvae. **(A–G)** The levels of candidate gene expression were quantified by real-time RT-PCR. Relative values to IV4 larvae are shown. One sample (prepared from 20–40 CA–CC complexes) per stage was analyzed. **(H)** Temporal titer changes in the JH and ecdysteroid in the silkworm. The JH and ecdysteroid titer are depicted according to previous papers (Sakurai and Niimi, 1997; Satake et al., 1998; Koyama et al., 2004).

dehydrated with serial concentrations of ethanol/PBTw, and stored in 100% ethanol at –30°C until use. On the day of hybridization, the samples were gradually rehydrated with serial concentrations of ethanol/PBTw, permeabilized with proteinase K (5 μg/ml) in PBTw for 75 sec at room temperature, washed with PBTw, refixed in 4% formaldehyde in PBTw for 30 min, washed with PBTw, incubated in prehybridization solution (50% formamide, 5 × saline sodium citrate [SSC] [pH 7.0], 2% Blocking reagent [Roche], 0.1% TritonX100, 0.1% CHAPS, 1 μg/ml yeast tRNA, 5 mM EDTA, and 5 μg/ml heparin) for 1 h at 50°C, and hybridized with RNA probes (1 μg/ml) overnight at 50°C in prehybridization solution. After washes in SSCFS (50% formamide, 5 × SSC [pH 4.5], and 1% SDS) for 20 min at 50°C, the samples were washed three times in 2 × SSCC (2 × SSC [pH 7.0], 0.1% CHAPS) for 20 min at 50°C, three times in 0.2 × SSCC (0.2 × SSC [pH7.0], 0.1% CHAPS) for 20 min at 50°C, and three times in KTBT (50 mM Tris-HCl [pH7.5], 150 mM NaCl, 10 mM KCl, 0.1% Triton X-100) for 10 min at room temperature, then treated with blocking solution (1.5% Blocking reagent [Roche] in KTBT) for 1 h, and then incubated in alkaline phosphatase-conjugated anti-DIG antibody (1/2500, Roche) in blocking solution overnight at 4°C. After washes in KTBT for 1 h five times at room temperature and washes in NTMT (100 mM NaCl, 100 mM Tris-HCl [pH 9.5], 50 mM $MgCl_2$, 0.1% Triton X-100) for 5 min three times, signals were developed with NBT/BCIP solution (1/50 dilution in NTMT, Roche) for 1 h or overnight at room temperature with continuous agitation. The color-developed samples were washed in PBTw, fixed in 4% formaldehyde/PBTw, washed in PBTw, washed in serial concentrations of ethanol/PBTw, and mounted in 50% glycerol/PBTw. Images were obtained using the BZ-9000 microscope (Keyence, Osaka, Japan).

Hormone treatments

Fenoxycarb (Wako, Osaka, Japan), dissolved in acetone (1 mg/ml), was applied topically to IV1 larvae (5 μl each). 20E (Sigma, St. Louis, MO), dissolved in distilled water (1 mg/ml), was injected into IV1 larvae (3 μl). For control experiments, the same amount of solvent was topically applied or injected.

RESULTS

Identification of genes preferentially expressed in the CA or CC during JH biosynthetic stage

To identify novel genes that regulate JH biosynthesis, we conducted a fluorescent differential display screening using the CA and CC of silkworm larvae (Fig. 1A). We compared gene expression patterns between day four of fourth instar (IV4) and day five of fifth instar (V5) larvae, when JH biosynthesis is active and inactive, respectively (Sakurai and Niimi, 1997; Kinjoh et al., 2007). In addition, we separately compared gene expression patterns between CA and CC on these stages. We compared approximately 3500 bands and identified 49 candidate genes. 26, eight, eight, and seven candidates were identified from IV4 CA, IV4 CC, V5 CA, and V5 CC, respectively (Fig. 1B). *AACT* and *JHAMT*, known as JH biosynthetic enzyme genes and expressed specifically in the CA of JH biosynthetic stage, were included in these candidates, confirming that our screening was conducted appropriately (Shinoda and Itoyama, 2003; Kinjoh et al., 2007). After further verification by semi-quantitative RT-PCR, database search using the silkworm genome information, and determination of transcript sequences by RACE, we selected seven novel genes for further analyses; five and two genes expressed strongly in IV4 CA and IV4 CC, respectively (Fig. 1C). Since no homologous gene or clear open reading frame was identified for one of the IV4 CC-expressed genes, we named this gene *nene* (noncoding RNA expressed in the nervous sys-

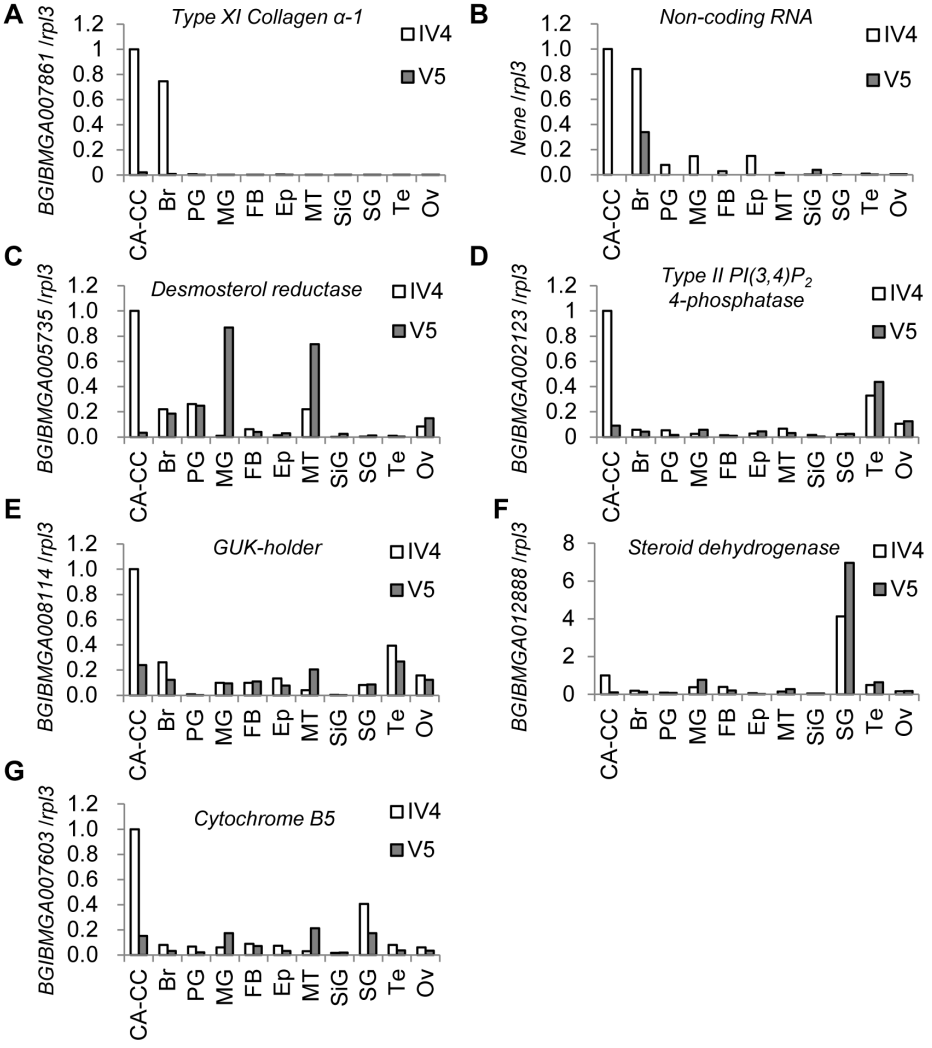

Fig. 3. Tissue expression profiles of candidate genes in the IV4 and V5 larvae. **(A–G)** The levels of identified gene expression were precisely quantified by real-time RT-PCR. Relative values to CA-CC complex of IV4 larvae are shown. Br, brain; PG, prothoracic gland; MG, midgut; FB, fat body; Ep, epidermis; MT, Malpighian tubule; SiG, silk gland; SG, salivary gland; Te, testis; Ov, ovary. One sample (prepared from 20–40 CA–CC complexes or three to seven tissues) per stage/tissue was analyzed.

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Temporal expression profiles of candidate genes

Expression levels of known JH biosynthetic enzyme genes correlate with JH titer and show dynamic changes throughout development. In order to investigate whether the newly identified candidate genes show similar patterns in expression levels, we investigated temporal expression profiles of the candidate genes from IV0 to V9 with real-time RT-PCR using the CA-CC complexes (Fig. 2). *BGIBMGA007861* (*Type XI Collagen α-1*), *nene*, *BGIBMGA005735* (*Desmosterol reductase*), *BGIBMGA002123* (*Type II PI(3,4)P_2 4-phosphatase*), and *BGIBMGA012888* (*Steroid dehydrogenase*) showed correlated expression profile with JH titer until the first half of fifth instar. Especially, the expression of *BGIBMGA002123* highly correlated with JH titer, peaking in V0 instar. In contrast, expression levels of these genes did not correlate with the rise of JH titer in the latter half of fifth instar. Since increase in JH titer in late fifth instar is derived from the gonad and not the CA (Sakurai and Niimi, 1997; Kinjoh et al., 2007), the low expression of candidate genes can be reflecting the low JH production in the CA. These results suggest that these genes are not upregulated in response to increase in JH titer, but functions upstream or parallel of the JH biosynthesis pathway in the CA-CC complex. *BGIBMGA008114* (*GUK-holder*) and *BGIBMGA007603* (*Cytochrome B5*) did not show correlated expression profile with JH titer, suggesting that these genes are not related to JH biosynthesis.

Tissue expression profiles of candidate genes

Next, we performed quantitative RT-PCR to reveal tissue expression profiles of the candidate genes (Fig. 3). *BGIBMGA007861* (*Type XI Collagen α-1*) and *nene* were preferentially expressed in the nervous tissues of IV4 larvae. *BGIBMGA002123* (*Type II PI(3,4)P_2 4-phosphatase*), *BGIBMGA008114* (*GUK-holder*) and *BGIBMGA007603* (*Cytochrome B5*) were preferentially expressed in the CA-CC complex of IV4 larvae. In contrast, *BGIBMGA005735* (*Desmosterol reductase*) and *BGIBMGA012888* (*Steroid dehydrogenase*) were highly expressed in tissues other than the CA-CC complex.

To further confirm the tissue-preferential expression, we conducted whole mount in situ hybridization using the CA-CC complex of IV4 and V5 larvae (Fig. 4). Consistent with the results of RT-PCR, signals of *BGIBMGA007861* (*Type XI Collagen α-1*) were preferentially detected in the CC of IV4 larvae (Fig. 4A). In addition, *BGIBMGA005735* (*Desmosterol reductase*), *BGIBMGA002123* (*Type II PI(3,4)P_2 4-phosphatase*), and *BGIBMGA012888* (*Steroid dehydrogenase*) were preferentially expressed in the CA of IV4 larvae (Fig. 4B–D). In contrast, signals were not detected for *nene*, *BGIBMGA008114* (*GUK-holder*), and *BGIBMGA007603* (*Cytochrome B5*), possibly due to low abundance of transcripts. These results collectively indicate that expression of *BGIBMGA007861* (*Type XI Collagen α-1*) and *BGIBMGA002123* (*Type II PI(3,4)P_2 4-phosphatase*) spatiotemporally correlate with JH biosynthesis, implying a possible involvement of these genes in JH production and/or regulation.

Response of candidate genes to JH or 20E treatment

We next investigated the possibility that correlated expression of candidate genes with JH titer is not due to their induction by JH. Topical application of a JH analog, fenoxycarb, to IV1 larvae had no effect on expression levels of candidate genes in the CA–CC complexes 24 h after treatment (Fig. 5A), indicating that expression of these genes are not regulated by JH. In addition, we investigated

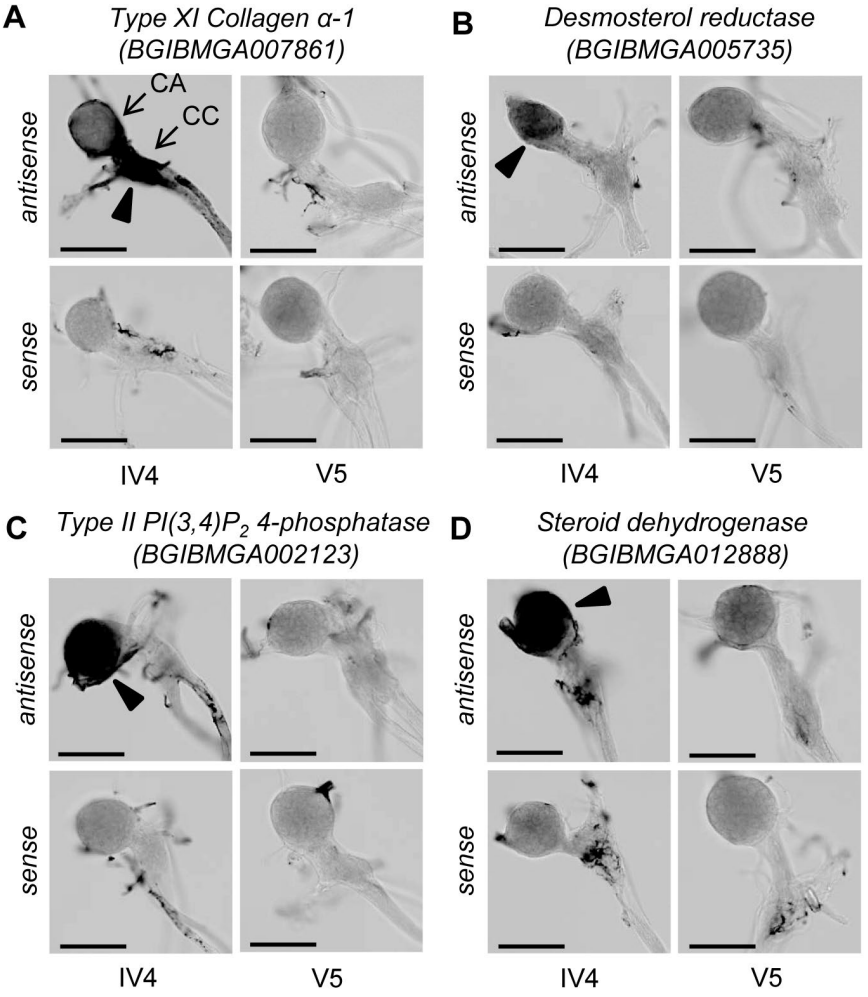

Fig. 4. Whole-mount in situ hybridization of candidate genes in the CA-CC complexes of IV4 and V5 larvae. **(A–D)** Upper and lower panels indicate results using antisense and sense probes, respectively. Left and right panels are the CA–CC complexes of IV4 and V5 larvae, respectively. Arrowheads indicate stained tissues. Scale bars: 100 μm.

the effects of 20E treatment, to examine if the developmental changes in gene expression levels are due to the dynamic change in ecdysteroid titer from IV3 to IV4. Expression levels of *BGIBMGA007861* (*Type XI Collagen α-1*) and *BGIBMGA012888* (*Steroid dehydrogenase*) in the CA–CC complexes were upregulated 24 h after 20E injection, but other candidate genes were not (Fig. 5B). These results collectively indicate that only *BGIBMGA002123* (*Type II PI(3,4)P_2 4-phosphatase*) remains as the most suitable candidate for upstream regulators of JH biosynthesis. We did not detect expression of *nene* in these analyses because of its lack of expression in the CA–CC complexes of IV1 larvae.

Starvation upregulates expression of BGIBMGA002123 (Type II PI(3,4)P_2 4-phosphatase) and JH biosynthetic genes

Nutritional conditions influence JH biosynthesis. To explore possible connections between nutritional conditions and the expression of candidate genes, we examined whether the levels of candidate genes are affected by starvation. Here, we used fourth instar larvae, as the CA of this stage possess JH synthetic activity and is suitable for assessment of whether starvation further promotes JH biosynthesis. Levels of candidate gene expression in the CA-CC complexes of larvae starved for two days (from IV1 to IV3) were compared with those of control (fed larvae). Expression of *BGIBMGA005735* (*Desmosterol reductase*) and *BGIBMGA002123* (*Type II PI(3,4)P_2 4-phosphatase*) was significantly increased in the starved larvae (Fig. 6A). Notably, expression of *BGIBMGA002123* (*Type II PI(3,4)P_2 4-phosphatase*) was highly upregulated by starvation. These results suggest that starvation strongly affects phosphoinositol signaling in the CA, since PI(3,4)P_2 4-phosphatase is the only pathway to metabolize PI(3,4)P_2 to form PI3P and play important roles in phosphoinositol metabolisms (Hawkins and Stephens, 2016). We also investigated levels of JH biosynthetic enzyme genes in these samples, to examine whether JH biosynthesis is influenced by starvation (Fig. 6B). Although the levels of HMG-CoA synthase (HMGS) and farnesyl acid epoxidase (CYP15C1) expression were significantly decreased by starvation, expression of other enzyme genes were upregulated or unchanged, suggesting that the JH biosynthesis is prone to upregulation by starvation. These results suggest the possibility that starvation affects phosphoinositol signaling in the CA, which results in upregulation of JH biosynthesis.

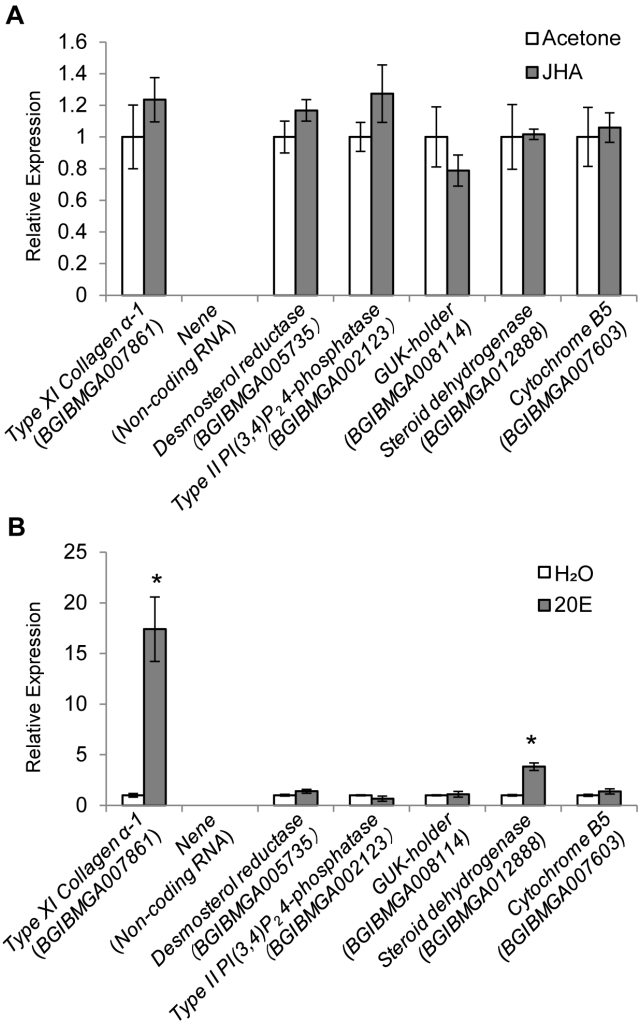

Fig. 5. Effects of hormone treatment on gene expression. Effects of fenoxycarb (JHA) **(A)** or 20-hydroxyecdysone (20E) **(B)** treatment on gene expression. The IV1 larvae were treated with JHA (5 μg) **(A)** or 20E (3 μg) **(B)**, and the levels of gene expression in the CA-CC complexes were measured 24 h after treatment. Relative values to control treatment (**(A)** acetone, **(B)** H_2O) are shown. Experiments were conducted in triplicate (biological replicates). *: $P < 0.05$, *Student*'s *t*-test.

DISCUSSION

In the present study, we conducted a screen to identify novel regulators of JH biosynthesis using silkworm larval CA and CC. Through a detailed expression profiling of candidate genes, we identified several novel genes that are preferentially expressed in the CA or CC of JH biosynthetic period. Interestingly, expression of *BGIBMGA002123*, which encodes type II PI(3,4)P_2 4-phosphatase, showed high correlation with JH titer and was highly upregulated by starvation, when JH biosynthetic enzyme genes are concurrently upregulated. Although functional analysis using knockout or overexpression animals is necessary, these results imply a novel signaling pathway, where phosphoinositol signaling regulates JH biosynthesis in the CA.

JH exerts pleiotropic effects on insect development and physiology (Nijhout, 1994). Therefore, JH titer is tightly controlled by both synthesis and degradation. Although functional analyses of genes for JH biosynthesis and degradation are intensively performed (Shinoda and Itoyama, 2003; Tan et al., 2005; Kinjoh et al., 2007; Niwa et al., 2008; Ueda et al., 2009; Daimon et al., 2012; Daimon et al., 2015), it is still obscure how the expression of JH biosynthetic enzyme genes in the CA is regulated to accommodate developmental and physiological demands. Developmental fluctuation of JH titer is attributable to dynamic changes in expression of JH biosynthetic enzyme genes (Kinjoh et al., 2007). In the present study, we identified several genes whose expression correlate with JH titer and characterized their response to hormones, 20E and JH, and starvation. Our results provide for the first time insights into possible

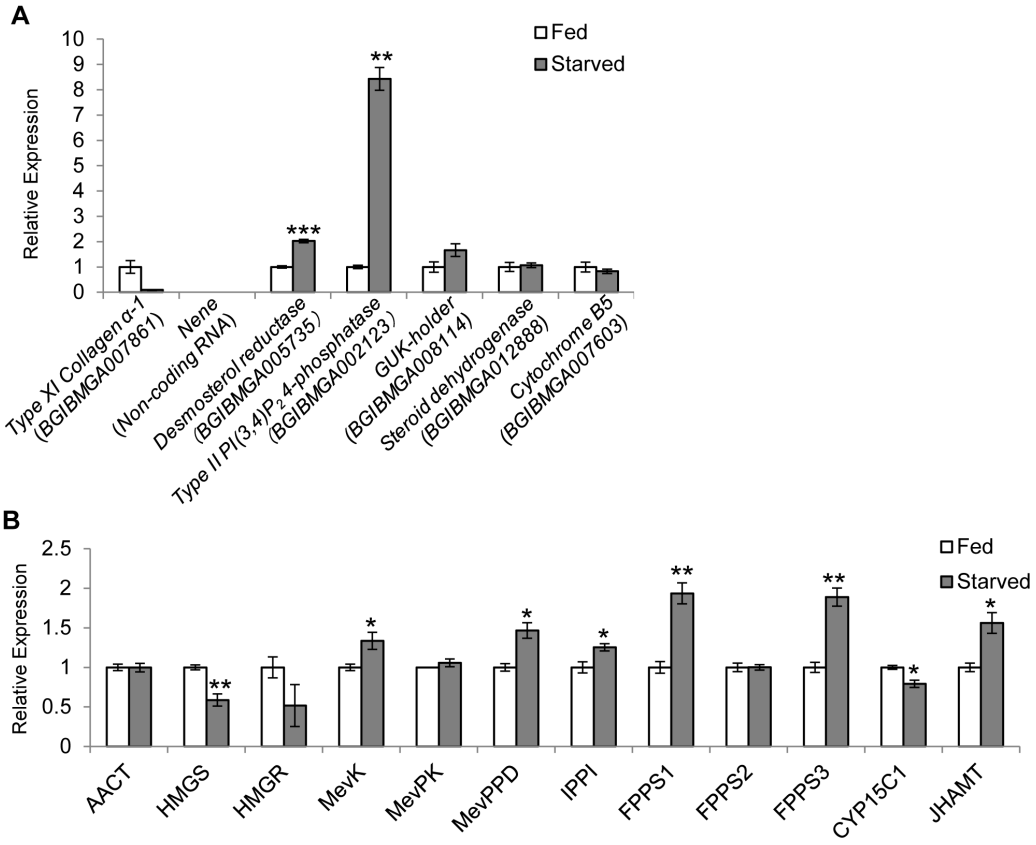

Fig. 6. Starvation regulates gene expression. Effects of starvation on expression levels of candidate genes **(A)** and JH biosynthetic enzyme genes **(B)**. **(A)** IV1larvae were starved for 48 hours, and levels of gene expressions in the CA–CC complexes were measured with real-time RT-PCR. **(B)** The levels of JH biosynthetic enzyme expression in the CA-CC complexes of starved larvae. AACT, acetoacetyl-CoA thiolase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MevK, mevalonate kinase; MevPK, phosphomevalonate kinase; MevPPD, diphosphomevalonate decarboxylase; IPPI, isopentenyl diphosphate isomerase; FPPS, farnesyl diphosphate synthase; CYP15C1, farnesyl acid epoxidase; JHAMT, JH acid *O*-methyltransferase. Experiments were conducted in triplicate (biological replicates). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, *Student*'s *t*-test.

upstream regulators of JH biosynthesis in the silkworm, *B. mori*.

In some insects, number of larval molting is plastically regulated (Nijhout, 1994). For example, in the last instar larvae of *M. sexta*, additional larval molting is induced when the larval body weight is below the appropriate level for pupation, which is known as the critical weight. Lack of nutrition is sensed by the larval body and induces JH titer increase, which leads to inhibition of pupal molting and imaginal disc formation (Cymborowski, 1982; Truman et al., 2006). This coordination of systemic physiology and developmental process provides larvae with another chance of pupation under a more appropriate condition. Although this nutrition-dependent regulation of development is a well-known phenomenon, molecular mechanisms controlling the upregulation of JH biosynthesis in the CA, is unknown. Therefore, our identification of type II PI(3,4)P_2 4-phosphatase as a possible candidate for nutrition-dependent JH regulator provides insights into molecular mechanisms where systemic physiology adjusts insect development.

How can phosphoinositol signaling regulate JH biosynthesis in the CA? Type II PI(3,4)P_2 4-phosphatase has PH (pleckstrin homology) domain and is the major pathway to dephosphorylate PI(3,4)P_2 to form PI3P in the plasma membrane. Increase in type II PI(3,4)P_2 4-phosphatase expression is thus expected to decrease the level of PI(3,4)P_2, and increase that of PI3P, respectively. One possible target of PI(3,4)P_2 is Akt/PI3K pathways (Hawkins and Stephens, 2016), which regulates FOXO (forkhead box O) and TOR (target of rapamycin) pathways. Since FOXO and TOR pathways regulate JH biosynthesis in the adult German cockroach *B. germanica* (Maestro et al., 2009; Suren-Castillo et al., 2012), we speculate that nutrition-dependent change in phosphoinositol signaling may regulate JH biosynthesis through FOXO and TOR pathway in the CA. In future studies, it will be essential to address this hypothesis using transgenic or knockout silkworms, in which *BGIBMGA002123* is overexpressed in the CA or mutated.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

ST performed all experiments. ST and TK designed and wrote the paper. MI advised on the study.

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