Expressional and functional analysis of CYP15A1, a juvenile hormone epoxidase, in the red flour beetle *Tribolium castaneum*.

Chieka Minakuchi[1]*, Fumika Ishii[1], Yumiko Washidu[1], Akio Ichikawa[2], Toshiharu Tanaka[1], Ken Miura[1], and Tetsuro Shinoda[2]

[1] Graduate School of Bio-Agricultural Sciences, Nagoya University, Furocho, Chikusa, Nagoya 464-8601, Japan

[2] National Institute of Agrobiological Sciences, 1-2 Ohwashi, Tsukuba 305-8634, Japan

* Corresponding author: Chieka Minakuchi, Dr. Agr.
Graduate school of Bio-Agricultural Sciences, Nagoya University, Nagoya 464-8601, Japan; Tel.: +81-52-789-4035; Fax: +81-52-789-4032; e-mail: c_mina@agr.nagoya-u.ac.jp

Abstract

Juvenile hormone (JH) is synthesized and secreted by the corpora allata. In the final two steps of JH biosynthesis, farnesoic acid (FA) is converted to JH through methylation by JH acid *O*-methyltransferase (JHAMT) and epoxidation by the cytochrome P450 enzyme CYP15. In the present study, we identified a homolog of CYP15 from the red flour beetle *Tribolium castaneum* (*TcCYP15A1*), and analyzed its expression as well as its role in JH biosynthesis. Quantitative RT-PCR analysis showed that the level of *TcCYP15A1* mRNA was high in the embryonic stage as well as in the middle of the final larval instar. In the embryonic stage, the transcript level of *TcCYP15A1* started to increase 30 h after egg laying (AEL), peaked 54-60 h AEL, and was followed by an increase of *TcJHAMT* mRNA, suggesting that JH biosynthesis started at this time point. *TcCYP15A1* mRNA was present, but not exclusively so in the larval corpora allata. The recombinant TcCYP15A1 protein epoxidized both FA and methyl farnesoate (MF) in highly stereo-specific manners. These results confirmed that *TcCYP15A1* is involved in JH biosynthesis. The RNAi-mediated knockdown of *TcCYP15A1* in the pre-final larval instar did not result in precocious metamorphosis to pupa, indicating that MF may exhibit JH-like activity in order to maintain the larval status. The double knockdown of *TcJHAMT* and *TcCYP15A1* resulted in pupae and adults with shorter wings, suggesting that the precursors of JH, JH acid and MF, may be essential for wing expansion.

Keywords

Juvenile hormone; *Tribolium castaneum*; CYP15; epoxidation

1. Introduction

Juvenile hormone (JH) is synthesized via the mevalonate pathway and subsequent reaction steps that are specific to JH biosynthesis (Belles et al., 2005). In the final two steps, farnesoic acid (FA) is converted to JH via methylation and epoxidation. The genes that encode enzymes catalyzing these steps have already been identified. *JH acid O-methyltransferase* (*JHAMT*) was first identified and characterized in the silkworm *Bombyx mori* (Shinoda and Itoyama, 2003), and then in a wide variety of species such as the fruitfly *Drosophila melanogaster* (Niwa et al., 2008), red flour beetle *Tribolium castaneum* (Minakuchi et al., 2008), Eri silkworm *Samia cynthia ricini* (Sheng et al., 2008), mosquito *Aedes aegypti* (Mayoral et al., 2009), desert locust *Schistocerca gregaria* (Marchal et al., 2011), and honeybee *Apis mellifella* (Li et al., 2013). *CYP15A1* was first identified as a gene encoding a cytochrome P450 enzyme that epoxidizes methyl farnesoate (MF) to JH in the cockroach *Diploptera punctata* (Helvig et al., 2004). *CYP15C1*, which epoxidizes FA to JH acid, was subsequently identified and characterized in *B. mori* (Daimon et al., 2012). In *S. gregaria*. *CYP15A1* was identified and shown to be involved in JH biosynthesis (Marchal et al., 2011). Although *CYP15* genes are highly conserved among insects, except in higher Diptera such as *D. melanogaster* whose genome does not encode a *CYP15* gene (Daimon and Shinoda, 2013), they have not yet been characterized in other species including *T. castaneum*.

The transcriptional regulation of *JHAMT* and *CYP15* is important for the regulation of JH biosynthesis, and the relevance of these two genes varies with the physiological role of JH and insect species. For example, the level of *CYP15A1* transcription correlated well with JH synthesis in the corpora allata (CA) of adult females of *D. punctata* (Helvig et al., 2004), suggesting that the transcriptional regulation of

71 *CYP15A1* is critical for the regulation of JH biosynthesis in reproduction in this species.
72 In contrast, *JHAMT* mRNA has been shown to fluctuate synchronously with JH
73 biosynthesis during larval to pupal development in *B. mori* (Kinjoh et al., 2007; Shinoda
74 and Itoyama, 2003), while *CYP15C1* mRNA is constitutively present (Daimon et al.,
75 2012). This finding suggests that the transcriptional regulation of *JHAMT*, but not
76 *CYP15C1*, is critical for the regulation of JH biosynthesis during metamorphosis in *B.*
77 *mori*. In *T. castaneum*, expressional analysis and RNAi experiments on *JHAMT*
78 revealed that the transcriptional regulation of *JHAMT* was critical for metamorphosis
79 (Minakuchi et al., 2008) and reproduction in both the male and female adults of this
80 species (Parthasarathy et al., 2010; Parthasarathy et al., 2009). However, the
81 physiological function of *CYP15* has not yet been elucidated in *T. castaneum*.
82 The final steps in the JH biosynthetic pathway from FA to JH diverged among
83 certain insect taxa. The esterification of FA is assumed to precede epoxidation in most
84 insect species, including Orthoptera, Dictyoptera, Coleoptera, and Diptera (Belles et al.,
85 2005). In contrast, the presence of an enzyme that specifically epoxidizes FA to JH
86 acid in the tobacco hornworm, *Manduca sexta*, implies that the epoxidation of FA
87 precedes esterification in Lepidoptera (Reibstein et al., 1976; Schooley and Baker,
88 1985). Recent studies revealed that recombinant CYP15A1 of *D. punctata*
89 (Dictyoptera) catalyzed the epoxidation of MF to JH, but not FA to JH acid (Helvig et
90 al., 2004), while recombinant CYP15C1 of *B. mori* (Lepidoptera) preferably epoxidized
91 FA over MF (Daimon et al., 2012). Thus, the substrate specificity of CYP15 is
92 responsible for differences in the final JH biosynthetic steps between a primitive
93 hemimetabola and Lepidoptera. The substrate specificity of CYP15 in *T. castaneum*,
94 which represents a primitive holometabola, is of interest from an evolutionary point of
95 view.

In the present study, we cloned a homolog of *CYP15* from *T. castaneum*, and analyzed its spatial and developmental expression. We also characterized the enzymatic properties of recombinant CYP15A1 and elucidated its function *in vivo* using RNAi experiments. The physiological roles of *CYP15A1* in JH biosynthesis and development of *T. castaneum* are discussed.

2. Materials and Methods

2.1. Insect rearing

The wild-type strain of *T. castaneum* was provided by the National Food Research Institute (Ibaraki, Japan). *T. castaneum* was reared in whole wheat flour in the dark at 30 ºC (Minakuchi et al., 2008). *T. castaneum* larvae do not develop synchronously: in our hands, most of them pupated either at the seventh or eighth larval instar. To investigate the developmental profile using quantitative RT-PCR, the instar that pupated was distinguished by measuring the head capsule widths of larvae using a microscope [Leica Microsystems MZ16FA／DFC500 system (Leica Microsystems, Heerbrugg, Switzerland)] as described previously (Minakuchi et al., 2008); sixth instar larvae with head capsules 570-600 μm were considered to be penultimate instar larvae, and seventh instar larvae with head capsules 690-740 μm were considered to be final instar larvae. Staged larvae to be used for other purposes were reared in a container with 0.6 g of whole wheat flour per 100 larvae until the fifth instar, and were given 1.6 g of whole wheat flour in the sixth instar; most larvae pupated in the eighth larval instar under this rearing protocol.

2.2. cDNA cloning of the *CYP15* gene

121 The whole ORF of *T. castaneum CYP15* was amplified as follows. Total RNA
122 was extracted from the whole body of *T. castaneum* with TRIzol reagent (Life
123 Technologies). First strand cDNA was synthesized with an oligo dT primer and
124 PrimeScript II reverse transcriptase (Takara Bio, Shiga, Japan). RT-PCR was
125 performed with ExTaq HS DNA polymerase (Takara Bio) and primers designed based
126 on the predicted *TcCYP15* sequence (XM_965210) on the DDBJ/EMBL-Bank/GenBank
127 International Nucleotide Sequence Database. PCR products were purified, cloned into
128 a pGEM-T Easy vector (Promega, Madison, WI), and sequenced. This construct was
129 utilized in order to prepare template DNA for dsRNA synthesis and as a standard for
130 quantitative RT-PCR. Primer sequences are listed in Table S1.
131
132 2.3. Quantitative RT-PCR analysis
133 Total RNA was isolated from eggs with TRIzol reagent (Life Technologies), and
134 first strand cDNA was synthesized with an oligo dT primer and PrimeScript II reverse
135 transcriptase (Takara Bio). To examine other developmental expression profiles, a set
136 of cDNAs of larvae, pupae, and adults from a previous study (Minakuchi et al., 2008;
137 Minakuchi et al., 2009) was used: several insects were combined for RNA isolation of
138 the first, second, and third larval instars, whereas RNA was isolated from individuals for
139 the sixth and seventh larval instars, pupal and adult stages. To begin exploring
140 tissue-specificity of these genes in the sixth instar, four larvae were cut in half between
141 thoracic segments T2 and T3, and the anterior and posterior parts were collected
142 separately for RNA isolation.
143 Transcripts were quantified using a LightCycler 2.0 (Roche Diagnostics, Basle,
144 Switzerland) or Thermal Cycler Dice Real Time System (model TP800, Takara Bio).
145 Quantitative RT-PCR was carried out in a 12-µl reaction volume containing SYBR

146 Premix ExTaq (Takara Bio), 0.2 μM of each primer, and 1 μl of template cDNAs or
147 standard plasmids. PCR conditions were 95 ºC for 10 s, followed by 45 cycles at 95
148 ºC for 5 s and 60 ºC for 20 s for LightCycler, and 95 ºC for 30 s, followed by 45 cycles
149 at 95 ºC for 5 s and 60 ºC for 30 s for Thermal Cycler Dice. After PCR, the absence of
150 unwanted byproducts was confirmed by a melting curve analysis. Serial dilutions of a
151 plasmid containing the ORF of each gene were used as standards. The transcript levels
152 of the target genes were normalized to the amount of *rpL32* (*Rp49*) mRNA in the same
153 sample. Primers used to quantify *TcJHAMT*, *methoprene tolerant* (*TcMet*), *Krüppel*
154 *homolog 1* (*TcKr-h1*), and *TcrpL32* were described in previous studies (Minakuchi et al.,
155 2008; Minakuchi et al., 2009). Primer sequences and MIQE checklist are shown in
156 Tables S2 and S3.
157
158 2.4. Whole mount *in situ* hybridization
159 *In situ* hybridization of the larval stage was carried out as reported previously
160 (Minakuchi et al., 2008). The full coding regions of *TcJHAMT* and *TcCYP15A1* were
161 subcloned into the pGEM-T Easy vector, and a linearized plasmid was used as the
162 template for RNA synthesis. Digoxigenin (DIG)-labeled sense and antisense RNA
163 probes were prepared using a DIG RNA Labeling Kit and SP6 or T7 RNA polymerase
164 (Roche Applied Science, Mannheim, Germany), according to the manufacturer's
165 instructions. The heads of sixth instar larvae were dissected in PBS, and most of the
166 head capsules were carefully removed with forceps. Tissues were fixed in 4%
167 paraformaldehyde at 4 °C for 40 min, and treated with 5 μg/ml Proteinase K for 75 s.
168 Re-fixation, hybridization, and detection with pre-adsorbed, alkaline
169 phosphatase-conjugated anti-DIG FAB fragments and nitroblue
170 tetrazolium/5-bromo-4-chloroindol-2-yl phosphate (Roche Applied Science) were

171 performed as described previously (Lehmann and Tautz, 1994; Minakuchi et al., 2008;
172 Niwa et al., 2004). After hybridization and detection, the remaining head capsule, fat
173 body, and muscles were carefully removed with forceps so that the brain and CA could
174 be clearly seen.
175 The *in situ* hybridization of embryos was performed as described previously
176 (Lehmann and Tautz, 1994; Tautz and Pfeifle, 1989). Embryos 53-61 h after egg
177 laying (AEL) were dechorionated, fixed, and devitellinized on a double-sided sticky
178 tape with a forceps (Cerny et al., 2005). Hybridization and detection were carried out
179 as described previously (Lehmann and Tautz, 1994).
180
181 2.5. RNAi experiments
182 Template DNA fragments for the synthesis of dsRNA were prepared by PCR as
183 follows. A DNA fragment containing a portion of the *TcCYP15* ORF (751 bp) with a
184 T7 promoter sequence at the N-terminal end was amplified with TcCYP15_5T7 and
185 TcCYP15_3 primers to synthesize sense RNA, and a fragment containing the *TcCYP15*
186 ORF with a T7 promoter sequence at the C-terminal end was amplified with
187 TcCYP15_5 and TcCYP15_3T7 primers to synthesize antisense RNA. RNA was
188 synthesized using the T7 RiboMAX Express RNAi System (Promega Corporation).
189 The *in vitro* transcription reaction was carried out at 37 °C for 1 h with T7 RNA
190 polymerase, and template DNA was digested by DNase I. Both strands of *TcCYP15*
191 RNA were mixed and incubated at 70 °C for 10 min, and were gradually cooled to room
192 temperature for annealing. dsRNA in the mixture was then purified by ethanol
193 precipitation and dissolved in nuclease-free water. Alternatively, dsRNA was
194 synthesized as follows. A DNA fragment containing the 751-bp *TcCYP15* sequence
195 with T7 promoter sequences on both ends was amplified with TcCYP15_5T7 and

196 TcCYP15_3T7 primers, and used for dsRNA synthesis. dsRNA was synthesized using
197 the MEGAscript RNAi Kit (Ambion). dsRNA for TcJHAMT was synthesized in a
198 previously reported manner (Minakuchi et al., 2008). As a negative control, dsRNA
199 for *maltose binding protein E* (*malE*) or *enhanced green fluorescent protein* (*EGFP*)
200 was prepared as described previously (Minakuchi et al., 2008; Yokoi et al., 2012).
201 Larvae were anesthetized with ether for 3-4 min, aligned on double-sided sticky
202 tape, and approximately 5 μg/μl dsRNA solution (approximately 30-40 nL for the fourth
203 instar, 40–50 nL for the fifth instar, and 100-150 nL for the eighth instar) was injected
204 into the abdomens using a glass capillary tube pulled by a Narishige needle puller.
205
206 2.6. Chemicals
207 (2*E*, 6*E*)-farnesoic acid (FA) and (2*E*, 6*E*)-methyl farnesoate (MF) were purchased
208 from Echelon Research Laboratories (Salt Lake City), and racemic JH III from Sigma.
209 (10*R*)- and (10*S*)-JH III were prepared from the racemic JH III by enantioselective
210 HPLC (Ichikawa et al., 2007). (10*R*)- and (10*S*)-JH III acid were prepared from (10*R*)-
211 and (10*S*)-JH III, respectively, as described previously (Ichikawa et al., 2010).
212
213 2.7. Expression and enzyme assay of the TcCYP15A1 protein in Sf9 cells
214 The full ORF with a stop codon (TAA) of *TcCYP15A1* cDNA was subcloned into
215 the *Sac* I/*Not* I site of pIZT/V5-His vector (Life Technologies). The generated
216 pIZT/TcCYP15A1 plasmid was transfected into Sf9 cells using FuGENE HD
217 (Promega). The pIZT/BmCYP15C1 plasmid, which expressed the CYP15C1 of *B.*
218 *mori* (Daimon et al., 2012), and the empty pIZT/V5-His (negative control) plasmid were
219 transfected in parallel as controls. Sf9 cells were plated in a 6-well culture plate
220 (IWAKI) with 2 mL/well of SF900-II SFM medium (Life Technologies) at a cell

density of approximately 3.5 x 10^5/mL 24 h before transfection. The cells were transfected by adding a mixture of plasmid DNA (3 μg) and 9 μL of FuGENE HD in 150 μL of the medium. Sixty hours after transfection, the cells were detached from the bottom of the plate by pipetting, and 0.4 mL of the cell suspension was transferred to a siliconized glass test tube (10 mm ID x 120 mm). FA or MF (1 mg/mL in MeOH, 4 μL) was added to the test tubes (10 μg/mL at final concentration, triplicates) and mixed briefly. After being incubated at 27°C for 3 h, an equal volume (0.4 ml) of CH_3CN was added to the test tube and vigorously mixed to stop the enzymatic reaction. The test tubes were centrifuged at 4,800 rpm for 10 min to remove cell debris, and the supernatants were used to analyze the products, as described below.

2.8. Identification and quantification of JH III and JH III acid by HPLC, GC/MS, and LC/MS

The identification and quantification of JH III and JH III acid produced in the medium was performed on a reversed-phase HPLC system essentially as described previously with slight modifications (Shinoda and Itoyama, 2004; Daimon et al., 2012). The above-mentioned supernatants (10 μL) were directly subjected to the Shimadzu LC10 HPLC system equipped with an ODS UG80 column (150 mm × 3.0 mm ID, Shiseido). The HPLC conditions were: solvent, CH_3CN:20 mM CH_3COONH_4 (pH 5.5), 60:40 for JH III, and 40:60 for JH III acid; flow rate, 0.5 ml/min; detector, UV 219 nm. Under these conditions, standard JH III and JH III acid gave a single peak with a retention time of approximately 11.4 min and 9.8 min, respectively. The amounts of JH III and JH III acid in the supernatant were calculated based on the peak areas obtained from known amounts of the respective standards. To further confirm the chemical nature of the products, the peaks corresponding to the standard JH III and JH

III acid were collected by reversed-phase HPLC and analyzed by GC/MS and LC/MS. A GCMS-QP2010 Plus system (Shimadzu) equipped with an OCI/PTV-2010 on-column injector and DB-35ms capillary column (30 m x 0.25 mm ID, Agilent J&B) was used to analyze JH III. Helium was used as a carrier gas at a flow rate of 1.3 ml/min. The GC oven was set to an initial temperature of 40°C for 1 min and was ramped at 10°C/min to 250°C. The on-column injector was set to an initial temperature of 40°C for 0.5 min and was ramped at 170°C/min to 250°C. One microliter of the sample dissolved in hexane was injected and the full scan spectrum was obtained in the electron impact (EI) ionization mode. A HP 1100 series LC/MSD system (Agilent) was used to analyze JH III acid. The analytical conditions were as follows: column, ODS UG80, 150 mm × 3.0 mm ID (Shiseido); solvent, CH_3CN:20 mM CH_3COONH_4 (pH 5.5), 40:60; flow rate, 0.5 ml/min. Ten microliters of the sample dissolved in CH_3CN was injected and the ESI-MS spectrum was obtained in the negative mode.

2.9. Enantioselective-HPLC analysis of JH III and JH III acid

JH III and JH III acid produced in the medium by recombinant TcCYP15A1 were purified by reversed-phase HPLC as described above. The stereo-specificity of the epoxide group in JH III and JH III acid was analyzed by reversed-phase chiral-HPLC. The HPLC conditions were as follows: column, Chiralpak AD-3R, 150 × 2.1 mm ID (DAICEL); solvent, CH_3CN:20 mM CH_3COONH_4 (pH 5.5), 60:40 for JH III, and 50:50 for JH III acid; flow rate, 0.2 ml/min; detector, UV 219 nm.

3. Results

3.1. cDNA sequence and developmental expression profiles of *T. castaneum CYP15*

271 cDNA containing the full ORFs of *TcCYP15* was amplified by RT-PCR using the
272 primers designed from the genomic sequence and then sequenced. The sequence was
273 deposited in the DDBJ/EMBL-Bank/GenBank International Nucleotide Sequence
274 Database (accession number, AB987827). The deduced amino acid sequence was
275 99% identical to the predicted sequence (XP_970303), which had been defined as the
276 probable cytochrome P450 305a1. Blastx searches revealed that it was orthologous to
277 CYP15 in other insects rather than CYP305a1 of *D. melanogaster* (AAF49108). The
278 deduced amino acid sequence showed the highest identity (57%) to CYP15A1 of *D.*
279 *punctata* (AAS13464). It was also highly similar (43%) to CYP15C1 of *B. mori*
280 (NP_001140197), but slightly less similar to the CYP305a1 (40%). Based on the
281 nomenclature of P450 genes, we designated it as *T. castaneum* CYP15A1.
282 The amount of the *TcCYP15* transcript was high in the embryonic stage, declined to
283 a low level in the 1st larval instar, and remained low during the larval period examined
284 here (Figs. 1A and 1B). It started to increase on Day 2 of the final larval instar, peaked
285 on Day 3, and maintained at a low level in the prepupal and pupal stages (Fig. 1B).
286 *TcCYP15* mRNA increased before ecdysis to the adult stage in both males and females
287 (data not shown), then decreased to a low level in adults.
288 The expression profile of *TcCYP15A1* mRNA in the embryonic stage was
289 examined in detail (Fig. 2). The transcript of *TcCYP15A1* was undetectable until 30 h
290 AEL. It started to increase 30-36 h AEL, peaked 54-60 h AEL, and decreased to a low
291 level by 66-72 h AEL. We also examined the expression profiles of *TcJHAMT*, *TcMet*,
292 and *TcKr-h1* (Fig. 2). TcMet was previously identified as the principal component of
293 the JH receptor complex (Jindra et al., 2013), and *TcKr-h1* is an early JH-inducible
294 transcription factor (Minakuchi et al., 2009). The expression profile of *TcJHAMT* was
295 similar to that of *TcCYP15A1*, but the increase in *TcCYP15A1* mRNA preceded that of

296 *TcJHAMT* by approximately 12 h. In contrast, *TcMet* mRNA was constitutively
297 present with few fluctuations. The *TcKr-h1* transcript was detected at a low level at
298 the beginning (0-12 h AEL), and then decreased and was maintained at a marginal level
299 until 30-36 h AEL. It began to increase 36-42 h AEL, reached a plateau by 54-60 h
300 AEL, and remained high until 90 h AEL.
301
302 3.2. Spatial expression profiles of *TcCYP15A1*
303 The tissue specificity of *TcCYP15A1* mRNA was examined by quantitative
304 RT-PCR and *in situ* hybridization. Quantitative RT-PCR showed that the *TcCYP15A1*
305 transcript was more abundant in the anterior part of the sixth larval instar than in the
306 posterior part (Fig. 3A). The spatial expression of *TcCYP15A1* mRNA was further
307 examined in sixth instar larvae by *in situ* hybridization. With the antisense RNA probe,
308 mRNA localization was observed in a pair of small organs on the ventral side of the
309 brain (Fig. 3C), while no obvious signal was detected for the sense RNA probe (data not
310 shown). We assumed that this organ was the CA. Similar staining patterns were
311 observed for the antisense probe for *TcJHAMT* (Fig. 3D), which appeared to be more
312 intense than the signals for the *TcCYP15A1* probe.
313 As described above, the transcript levels of *TcJHAMT* and *TcCYP15A1* peaked
314 54-60 h AEL in the embryos (Figs. 2A and 2B). We performed *in situ* hybridization at
315 this stage. With the antisense probe for *TcCYP15A1*, mRNA localization was
316 observed in the putative primordial CA in the anterior part (Fig. 4B), while no obvious
317 signal was found for the sense probe (Fig. 4A). Similar staining patterns in the
318 putative primordial CA were observed with the antisense probe for *TcJHAMT* (Fig. 4D)
319 while no obvious signal was found for the sense probe (Fig. 4C).
320

321 3.3. Enzymatic properties of the TcCYP15A1 protein

322 The TcCYP15A1 protein was transiently expressed in Sf9 cells using an expression
323 plasmid vector (pIZT/V5-His), and its enzymatic properties were examined in the
324 presence of two potential substrates, MF and FA. To clarify substrate specificity, the
325 enzymatic properties of BmCYP15C1 were also examined in parallel. A
326 reversed-phase HPLC analysis detected a major peak, which co-migrated with standard
327 JH III (Fig. 5A), in the media containing MF, which was incubated with Sf9 cells
328 transfected with the expression plasmid for TcCYP15A1 (Fig. 5C). This peak was
329 purified and unequivocally determined to be JH III by GC/MS-EI (data not shown). In
330 contrast, JH III was not detected in the media incubated with cells transfected with the
331 original plasmid (control, Fig. 5B), or with the plasmid for BmCYP15C1 (Fig. 5D).
332 Similarly, a major peak, which co-migrated with standard JH III acid (Fig. 5E), was
333 detected in the media containing FA when incubated with cells expressing TcCYP15A1
334 (Fig. 5G). This peak was purified and determined to be JH III acid by LC/MS (data
335 not shown). The generation of JH III acid from FA was also detected by cells
336 expressing BmCYP15C1 (Fig. 5H), but not by control cells (Fig. 5F). Sf9 cells
337 expressing TcCYP15A1 produced similar amounts of JH III and JH III acid from MF
338 and FA, respectively, in a 3-h incubation, while BmCYP15C1 only produced JH III acid
339 from FA under the same conditions (Fig. 6), as reported previously (Daimon et al.,
340 2012). Since the generation of neither JH III nor JH III acid was detected in control
341 cells, the innate P450 activity of Sf9 cells was negligible in this study (Fig. 6). Thus,
342 the recombinant TcCYP15A1 protein can catalyze the epoxidation of not only FA but
343 also of MF, a distinctive feature of the coleopteran enzyme relative to that of *Bombyx*.

344 We then examined the stereo-specificity of the TcCYP15A1 protein by analyzing
345 the products by chiral liquid chromatography. Purified JH III produced from MF by

346 TcCYP15C1 showed a major peak co-migrating with standard (10*R*)-JH III, but not
347 with (10*S*)-JH III (*R*:*S*=100:0) (Fig. 7A-D). Similarly, purified JH III acid produced
348 from FA by TcCYP15A1 showed a major peak co-migrating with standard (10*R*)-JH III
349 acid, but not with (10*S*)-JH III acid (*R*:*S*=100:0) (Fig. 7E-H). These results indicated
350 that TcCYP15A1 epoxidizes both MF and FA in a highly stereo-specific manner.
351
352 3.4. Effects of RNAi-mediated knockdown on larva-pupa metamorphosis
353 The RNAi-mediated knockdown of *TcCYP15A1* was performed by injecting
354 dsRNA into the 4th and 5th larval instars. Most larvae that received *TcCYP15A1*
355 dsRNA on Day1_4th pupated in the 7th instar, and eclosed normally without undergoing
356 precocious pupation (Table 1). Similarly, all the larvae that received *TcCYP15A1*
357 dsRNA on Day0_5th pupated in the 7th or 8th instar, and eclosed normally. The
358 injection of *TcCYP15A1* dsRNA into the 3rd larval instar could not cause precocious
359 metamorphosis either (data not shown), although the transcript level of *TcCYP15A1* was
360 significantly reduced 7 days after treatment (Fig. S2): the transcript level of *TcCYP15A1*
361 in the treated animals was reduced to less than 10% of the control animals injected with
362 *EGFP* dsRNA (Fig. S2). In contrast, *TcJHAMT* RNAi in the 5th instar caused
363 precocious pupation in the 6th instar (Table 1), which was consistent with our previous
364 findings (Minakuchi et al., 2008).
365 Since the transcript level of *TcCYP15A1* was relatively high in the prepupal stage
366 (Fig. 1), we attempted the knockdown of *TcCYP15A1* in the final larval instar. All
367 animals that received either *TcCYP15A1* or *TcJHAMT* dsRNA on Day0_8th pupated and
368 eclosed normally (Table 2, Figs. 8B and 8C). In contrast, most animals (30/39) that
369 received a mixture of *TcCYP15A1* dsRNA and *TcJHAMT* dsRNA became pupae with
370 shorter wings and finally arrested either at the pupal stage or adult eclosion (Fig. 8D and

371 8E), or arrested at pupal ecdysis (Fig. 8F). A few animals (9/39) that received
372 *TcCYP15A1+TcJHAMT* dsRNA became adults, but 4 out of 9 adults died within a
373 month, while 98% of the control animals survived for more than a month.
374
375
376 **4. Discussion**
377 *CYP15* has been identified as a gene that encodes a cytochrome P450 enzyme
378 involved in epoxidation in JH biosynthesis in the cockroach *D. punctata* (Helvig et al.,
379 2004) and silkworm *B. mori* (Daimon et al., 2012). In this study, we performed
380 expressional and functional analyses of a *CYP15* gene from *T. castaneum*, and proved
381 its involvement in JH biosynthesis.
382 We examined temporal expression patterns in *T. castaneum* larvae. Quantitative
383 RT-PCR revealed that *TcCYP15* mRNA abundance was relatively high in the embryonic
384 stage and in the middle of the final larval instar, but was constitutively present at a low
385 level in all developmental stages examined (Fig. 1). This was in contrast to the
386 expression profile of *JHAMT* in *T. castaneum*: *JHAMT* mRNA was present in the larval
387 stage, decreased in the final larval instar, and was undetectable in the pupal stage, which
388 correlated well with the expression of the JH-inducible transcription factor *Kr-h1*, and is
389 likely to reflect JH biosynthetic activity in the CA (Minakuchi et al., 2008; Minakuchi et
390 al., 2009). In *B. mori*, *CYP15C1* mRNA was constitutively expressed in the corpus
391 cardiacum (CC)-CA complex from the first larval instar to the adult, while *JHAMT*
392 mRNA disappeared in the final larval instar when JH was not produced (Daimon et al.,
393 2012). Thus, JHAMT, rather than CYP15, may be the rate-limiting enzyme in JH
394 biosynthesis in *T. castaneum* as well as in *B. mori*.
395 *In situ* hybridization revealed that *TcCYP15A1* mRNA was highly expressed in the

CA of the penultimate (6th) instar larvae, but quantitative RT-PCR suggested that it was also present in other tissues (Figs. 3A and 3C). In contrast, *TcJHAMT* was exclusively expressed in the CA at this stage (Fig. 3D) (Minakuchi et al., 2008). The tissue-specific expression of *CYP15* mRNA has been reported in other insect species: *CYP15C1* mRNA is specifically expressed in the CA of *B. mori* (Daimon et al., 2012), while in *D. punctata*, *CYP15A1* transcripts were detected only in the CA of day 5-mated female adults (Helvig et al., 2004). Regarding the presence of *CYP15A1* mRNA in non-CA tissues, it should be pointed out that some insects are known to synthesize JH in tissues other than the CA. For example, the male accessory gland of *Hyalophora cecropia* (Weirich and Culver, 1979) and imaginal discs of *M. sexta* (Sparagana et al., 1985) converted JH acid to JH. In the mosquito *A. aegypti*, JH was shown to be synthesized *de novo* in the male accessory gland (Borovsky et al., 1994). In the longhorned beetle *Apriona germari*, JH was reported to be synthesized in the adult testes, male accessory glands, and ovaries (Tian et al., 2010). *TcJHAMT* was found to be expressed in non-CA tissues in the final instar larvae (Minakuchi et al., 2008). Thus, TcCYP15A1 may contribute to the *de novo* synthesis of JH in non-CA tissues in conjunction with TcJHAMT.

The temporal and spatial expression patterns of *TcCYP15A1* mRNA in the embryonic stage were also examined. *In situ* hybridization revealed that *TcCYP15A1* and *TcJHAMT* mRNA were colocalized in the putative primordial CA (Fig. 4). The overall temporal expression profiles of *TcCYP15A1* and *TcJHAMT* were similar, suggesting that both genes contributed to the synthesis of JH in the primordial CA. Interestingly, *TcCYP15A1* mRNA started to increase 36-42 h AEL, which was approximately 12 h earlier than the increase in *TcJHAMT* mRNA (Figs. 2A and 2B). The transcript level of the JH response gene *TcKr-h1* also increased 36-42 h AEL (Fig.

421 2D), suggesting that JH acid, which was supposedly converted from FA by TcCYP15A1
422 in the absence of TcJHAMT at this stage, may exhibit JH activity and induce the
423 expression of *TcKr-h1*.
424 Enzyme assays demonstrated that TcCYP15A1 epoxidized both FA and MF (Figs. 5
425 and 6). CYP15C1 of *B. mori* specifically epoxidized FA (Figs. 5D and 5H) (Daimon
426 et al., 2012), while CYP15A1 of *D. punctata* specifically epoxidized MF (Helvig et al.,
427 2004). Thus, the substrate specificity of the CYP15 enzyme varies among insect
428 orders. In *B. mori*, the enzyme properties of JHAMT and CYP15C1 indicated that FA
429 was epoxidized into JH acid, which was then methylated into JH in the final steps of JH
430 biosynthesis (Daimon et al., 2012; Shinoda and Itoyama, 2003). In *D. punctata*,
431 methylation appeared to precede epoxidation since CYP15A1 epoxidized MF, but not
432 FA (Helvig et al., 2004). TcJHAMT was previously shown to be able to methylate
433 both FA and JH acid with similar efficiencies (Minakuchi et al., 2008); therefore, it is
434 possible that the final two steps of JH biosynthesis proceeded in two ways in *T.*
435 *castaneum*: FA was initially epoxidized to JH acid, followed by methylation to JH, or
436 FA was initially methylated to MF, followed by epoxidation to JH (Fig. 9). We also
437 showed that the epoxidation of TcCYP15A1 produced (10*R*)-JH III and (10*R*)-JH III
438 acid in a highly stereo-specific manner (Fig. 7). The stereo-specific production of the
439 (10*R*)-isomer has also been reported in *D. punctata* (Helvig et al., 2004) and *B. mori*
440 (Daimon et al., 2012). To date, the absolute configuration of the chiral epoxide of
441 natural JH III has been reported to be 10*R* in several species such as *M. sexta* (Judy et
442 al., 1973) and *Tenebrio molitor* (Judy et al., 1975). We previously demonstrated that
443 the recombinant TcJHAMT protein methylated (10*R*)-JH III acid more favorably than
444 (10*S*)-JH III acid (Minakuchi et al., 2008). Although the stereo-chemistry of JH in *T.*
445 *castaneum* has not yet been elucidated, the enzymatic properties of TcCYP15A1 as well

446 as those of TcJHAMT suggest that the *T. castaneum* JH has the same stereo-chemical
447 properties as JHs from other insect species.
448 We previously reported that the RNAi-mediated knockdown of *TcJHAMT* in the
449 pre-final larval stage caused precocious larva-pupa metamorphosis due to a precocious
450 decrease in the JH titer (Minakuchi et al., 2008). The knockdown of *TcCYP15A1* did
451 not cause precocious pupation in this study. Since the RNAi-mediated knockdown of
452 *TcCYP15A1* was efficient (Fig. S2), it is not likely that the low efficiency of
453 RNAi-mediated knockdown resulted in a failure to induce precocious metamorphosis.
454 However, we cannot exclude the possibility that small quantities of TcCYP15A1
455 remaining after *TcCYP15A1* knockdown can still produce JH III, and that other enzymes
456 are able to convert MF to JH III in peripheral tissues outside the CA. Alternatively, FA
457 could still be methylated to MF after *TcCYP15A1* knockdown, and MF may then display
458 status-quo activity similar to JH (Fig. 9). Meanwhile, in *TcJHAMT* knockdown
459 animals, FA could be epoxidized into JH acid, which may have had some JH activity in
460 the embryonic stage as discussed above; however, JH acid may not be potent in terms of
461 maintaining the larval status. In *D. melanogaster*, the continuous exposure of larvae to
462 MF prevented normal pupariation, and MF was as potent as JH III (Harshman et al.,
463 2010; Jones et al., 2010). In contrast, a *B. mori* mutant strain lacking CYP15C1
464 function underwent precocious larva-pupa metamorphosis (Daimon et al., 2012),
465 although MF appeared to be produced in this mutant since JHAMT was able to
466 methylate FA and JH acid (Shinoda and Itoyama, 2003). The status-quo properties of
467 MF may differ among insect species.
468 The double knockdown of *TcJHAMT* and *TcCYP15A1* in the final larval instar
469 prevented wing expansion, whereas the single knockdown of either of them did not
470 have obvious effects (Fig. 8). This result suggested that the precursors of JH, i.e. MF

471 and JH acid, were necessary for wing expansion, and that their absence under the
472 double knockdown of *TcJHAMT* and *TcCYP15A1* prevented normal wing expansion.
473 JH appeared to be present in the prepupal stage of the Cecropia silkworm and tobacco
474 hornworm *M. sexta*, and allatectomy in the final larval instar resulted in precocious
475 differentiation of the adult structure, suggesting that JH plays a significant role in
476 preventing the precocious adult development of certain imaginal structures (Kiguchi
477 and Riddiford, 1978; Williams, 1961). In *T. castaneum*, the knockdown of *Met* in the
478 final larval instar resulted in precocious adult differentiation (Parthasarathy et al., 2008).
479 However, in the present study, we did not observe precocious differentiation of the adult
480 structure by the double knockdown of *TcJHAMT* and *TcCYP15A1*, possibly because
481 there was a trace amount of JH and its precursors under the double knockdown of
482 *TcJHAMT* and *TcCYP15A1*.
483 In summary, we characterized the *CYP15* gene from *T. castaneum*, and examined
484 its role in JH biosynthesis. TcCYP15A1 was able to epoxidize both FA and MF with
485 similar efficiencies and in a highly stereo-specific manner. Since the substrate
486 specificity of CYP15 against FA and MF appears to be diverse among insect species,
487 this may be a novel target for insect growth regulators with selective toxicity among
488 insect species.
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491
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622 **Figure legends**

623 Figure 1 Developmental expression profile of *T. castaneum CYP15A1*.

624 Transcript levels of *TcCYP15A1* were analyzed by quantitative RT-PCR, and signal

625 intensity was normalized to that of *TcrpL32*. (A) In the embryonic stage and the first,

626 second, and third larval instars, RNA was isolated from a mass of eggs or larvae. (B)

627 From the sixth larval instar until the adult stage, RNA was isolated from individuals

628 (three larvae in the sixth and seventh larval instars, and three males and three females

629 for pupae and adults at each time point), and the means and standard deviations of

630 expression are shown. The highest values during development (54-60 h after egg lay,

631 see Figure 2) were designated 100%.

632

633 Figure 2 Expression profile in the embryonic stage of *T. castaneum*.

634 Transcript levels of *TcCYP15A1* (A), *TcJHAMT* (B), *TcMet* (C), and *TcKr-h1* (D) were

635 analyzed by quantitative RT-PCR, and signal intensity was normalized to that of

636 *TcrpL32*. RNA was isolated from at least 20 eggs for each replicate, and the means

637 and standard deviations of expression are shown (N=3).

638

639 Figure 3 Spatial expression pattern in *T. castaneum*.

640 (A) Spatial expression pattern of *TcCYP15A1* mRNA. RNA was isolated from four

641 larvae in the sixth instar, which were cut in half between thoracic segments T2 and T3,

642 and the transcript levels of *TcCYP15A1* in the anterior and posterior parts were

643 examined by quantitative RT-PCR. A, anterior part; P, posterior part.

644 (B-D) *In situ* hybridization of *TcCYP15A1* (C) and *TcJHAMT* (D), and schematic

645 illustration of organs (B). In (C) and (D), the heads of sixth instar larvae were

646 dissected, fixed, hybridized with antisense RNA probes for each gene, and detected.

647 Ventral and lateral views of the same individual were shown. mRNA localization in
648 the putative corpora allata was indicated by yellow arrows, and the brain and
649 subesophageal ganglion were outlined with dashed lines. In (C), subesophageal
650 ganglion has been removed during dissection. A, anterior; BR, brain; D, dorsal; ES,
651 esophagus; P, posterior; SG, subesophageal ganglion; V, ventral.
652
653 Figure 4 *In situ* hybridization in embryos. Embryos 53-61 h AEL were fixed,
654 hybridized with sense and antisense RNA probes for each gene, and detected. (A)
655 Sense probe for *TcCYP15A1* mRNA, (B) antisense probe for *TcCYP15A1* mRNA, (C)
656 sense probe for *TcJHAMT* mRNA, and (D) antisense probe for *TcJHAMT* mRNA.
657 mRNA localization in the putative primordial corpora allata is indicated by arrows. A,
658 anterior; D, dorsal; P, posterior; V, ventral. Scale bar, 200 μm.
659
660 Figure 5 Production of JH III and JH III acid from MF and FA, respectively, by
661 TcCYP15A1 and BmCYP15C1.
662 Sf9 cells expressing TcCYP15A1 were incubated with MF (C) or FA (G), and the
663 metabolites were analyzed by reversed-phase HPLC. Similarly, Sf9 cells expressing
664 BmCYP15C1 were incubated with MF (D) or FA (H), and the metabolites were
665 analyzed by HPLC. The chromatograms of standard JH III (10 ng, A) and JH III acid
666 (50 ng, E), and the chromatograms of the metabolites of cells transfected with the
667 original pIZT vector (B, F) were shown. Arrows indicate the peak of JH III, and the
668 arrowheads indicate the peak of JH III acid.
669
670 Figure 6 Relative amount of JH III and JH III acid produced from MF and FA,
671 respectively, by TcCYP15A1 and BmCYP15C1.

672 Sf9 cells expressing either TcCYP15A1 or BmCYP15A1 and control cells (pIZT) were
673 incubated with medium (0.4 ml) containing either MF or FA (4,000 ng) for 3 hr. Black
674 and gray bars indicate the total amount of JH III and JH III acid produced in the culture
675 media, respectively (Mean ± SD, n=3).
676
677 Figure 7 Stereo-specificity of TcCYP15A1 against MF and FA.
678 Sf9 cells expressing TcCYP15A1 were incubated with MF (D) or FA (H), and the
679 metabolites were analyzed by chiral column HPLC. The chromatograms of standard
680 racemic JH III (A), (10*S*)-JH III (B), (10*R*)-JH III (C), racemic JH III acid (E), (10*S*)-JH
681 III acid (F), and (10*R*)-JH III acid (G) were shown.
682
683 Figure 8 Effects of RNAi-mediated knockdown.
684 Pupae that were injected with the dsRNA of *malE* (A), *TcCYP15* (B), *TcJHAMT* (C), or
685 the mixture of *TcCYP15* and *TcJHAMT* (D-F), on Day 0 of the eighth larval instar (Day
686 0_8th). Arrows indicate the edge of the elytron.
687
688 Figure 9 Diagram of the JH biosynthetic pathway in *T. castaneum*.
689 In normal individuals, the final two steps of JH biosynthesis proceed in two ways: FA is
690 initially epoxidized to JH III acid, followed by methylation to JH, or FA is initially
691 methylated to MF, followed by epoxidation to JH. Under *TcCYP15A1* knockdown, FA
692 was still methylated into MF. MF may have status-quo activity to prevent
693 metamorphosis.
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695
696