DIFFERENTIAL GENE EXPRESSION IN *Melipona scutellaris* (HYMENOPTERA, MELIPONINI): EFFECT OF JUVENILE HORMONE III

EXPRESSÃO GÊNICA DIFERENCIAL EM Melipona scutellaris (HYMENOPTERA, MELIPONINI): EFEITOS DO HORMÔNIO JUVENIL III

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ABSTRACT: The objective of the present study was to determine by differential display reverse transcriptase–polymerase chain reaction (DDRT-PCR) the effects of juvenile hormone (JH) III applied during the late larval 3 (L3) phase on gene expression in *Melipona scutellaris*. A temporal window of expression of feminizing genes exists during the late L3 and pre-defecating larval phases when these genes can be turned on or off by the action of JH, which is able to mediate the differentiation of female larvae into queens. Combination of the HT11A-AP4 primers revealed differential expression in L3 individuals treated with JH III for 1 h, with weak expression of the transcript, while intense expression was observed for controls and individuals treated for 4 h. Combination of the HT11G-AP4 and HT11G-AP5 primers showed suppression of the gene products for each primer combination in 1-h treated larvae compared to untreated control individuals of the same age and individuals treated for 4 h. Differential gene expression was also observed during development. These results demonstrate that the JH III may suppress or alter gene expression profiles during phase L3 of *M. scutellaris*.

UNITERMS: Gene Expression; DDRT-PCR; Juvenile Hormone; *Melipona*; Caste.

INTRODUCTION

In 1974, Kerr proposed a model to explain the segregation of 75 workers for 25 queens in each 100 births in *Melipona*. Two main genes, X^a and X^b, with two alleles each, are responsible for the production of feminizing substances in such a way that double heterozygous larvae, when well fed, develop into queens while poorly fed larvae or larvae homozygous for these genes become workers due to the low production of juvenile hormone (JH).

Topical application of JH to *Melipona* larvae during the phase of cocoon spinning (pre-defecating larvae), and even to larvae in the late L3 stage, confirms the influence of this hormone on the production of queens from worker larvae by triggering genetic mechanisms that promote the differentiation of these larvae into complete females, i.e., queens (BONETTI, 1982). The external anatomy of the ovaries of *M. quadrifasciata* queens induced by treatment with JH is identical to that of natural queens (BONETTI, 1984). In addition, the pattern of distribution of tergal glands is identical for queens induced by JH treatment and for natural queens (BONETTI; CRUZ-LANDIM; KERR, 1994).

The effects of JH I, II and III have been studied by Bonetti *et al*. (1995) who determined the minimum dose sufficient to produce queens from *Melipona* worker larvae. JH I was found to be the most efficient in producing *Melipona* queens, followed by JH III. According to Silva (1999), JH III at the dose of 0.5 µg/µl is able to induce the production of up to 100% *M. scutellaris* queens when applied topically to pre-defecating female larvae.

JH produced in the corpora allata and secreted in the hemolymph promotes, in some way, the interaction between the corpora allata and the genome, probably through nuclear receptors of the steroid superfamily (DAVEY, 2000).

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Received: 16/11/05 Accepted: 16/01/06

The genetic material of an individual is the same in all cells but is expressed differently in each cell type. The differential display reverse transcriptase–polymerase chain reaction (DDRT-PCR) technique developed by Liang and Pardee (1992) permits the detection of differentially expressed genes in cells, tissues and organisms under different conditions by means of the mRNA profile. Since the publication of this method, numerous optimizations of the technique have been proposed (MOU et al., 1994 e GUIMARÃES et al., 1995 e DOSS, 1996 e BONNET; PRÉVOT; BOURGOUIN, 1998 e SUNG, et al. 2004).

The aim of the present study was to determine the gene expression profile in *M. scutellaris* after topical application of JH III to L3 larvae using the DDRT-PCR technique.

MATERIAL AND METHODS

M. scutellaris beehives were maintained at the Uberlândia Meliponary, Uberlândia-MG, Brazil (S 180 55'/ W 480 17').

Late L3 larvae were topically and separately treated with 1 µl JH III (Sigma) dissolved in acetone P.A. (Merck) at the dose of 0.5 µg/µl. The larvae were maintained in an incubator at 31°C and a relative humidity of 80% obtained with a saturated KCl solution in a desiccator (American Society for Testing Materials, ASTM, 1951). Control larvae without treatment and treated only with acetone P.A. were identically manipulated. Treated and control larvae were collected after 1 and 4 h after treatment and stored frozen in an ultrafreezer at -80°C until the time of RNA extraction.

Total RNA was extracted from treated and control larvae by the TRIzol method (Gibco) according to manufacturer recommendations. The samples were submitted to DNAse I digestion for removal of chromosomal DNA.

For the reverse transcription reaction, 200 ng total RNA, 8 pmol oligo-dT primers with an anchor base (Table 1), 10 U RNAsin, 1X buffer, 10 mM DDT, and 200 mM dNTPs were used. After incubation at 42°C for 3 min, 200 U SUPERSCRIPT II reverse transcriptase (Gibco) was added, and the material was again incubated at 42°C for 50 min, followed by 15 min at 75°C. Negative controls without the addition of reverse transcriptase were prepared to verify the occurrence of nonspecific amplifications resulting from contaminating DNA.

Table 1. Sequences of the primers used for the reverse transcription reaction and PCR amplification.

***Primer*s**	**Seqüência 5' ⟶ 3'**
Poli A*	AAGCTTTTTTTTTTA
Poli G *	AAGCTTTTTTTTTTG
AP4 +	AAGCTTCTCAACG
AP5 +	AAGCTTAGTAGGC

*Primers for reverse transcription
+Arbitrary primers used for the amplification reaction

The cDNAs obtained were amplified by PCR in an MJ RESEARCH PTC 100 thermocycler using 1 µl of the reverse transcription reaction, 50 µM of each dNTP, 1 U Taq DNA polymerase (Gibco), 1X buffer, 2.5 mM MgCl, 8 pmol of the primers used in the reverse transcription reaction (HT11A or HT11G), and 8 pmol of the arbitrary primers, AP4 or AP5 (Table 1) in a final volume of 20 µl. The amplified products were submitted to 6% polyacrylamide/8 M urea gel electrophoresis at 120 V for 18 h. The bands were visualized by silver nitrate staining by the method of Blum; Beir and Gross (1987) modified by BASAM, Caetano-Anoles and Gresshoff (1991).

RESULTS

Combination of the HT11A and AP4 primers revealed a differential gene expression, with individuals treated with JH III for 1 h weakly expressing the transcript, while expression was more intense in controls and individuals treated for 4 h (Figure 1).

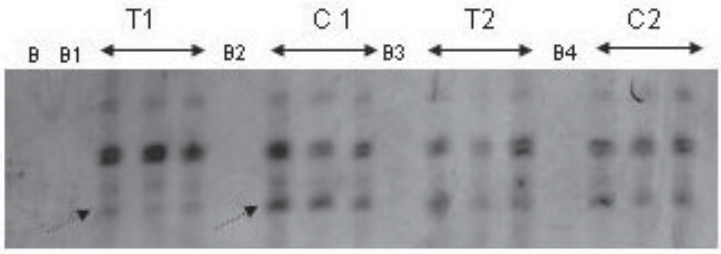

Figure 1. DDRT-PCR products (HT11A-AP4 primer combination) on 6% polyacrylamide/8 M urea gel stained with silver nitrate. B: blank (without RNA). B1, B2, B3 and B4: negative controls (without RT). T1: Larva 3 after 1 h of treatment with JH III; C1: control/1 h; T2: larva 3 after 4 h of treatment with JH III; C2: control/4 h. The arrows (.....) indicate differential expression.

The primer combinations HT11G-AP5 revealed suppression of a gene product in the larvae treated with JH III for 1 h compared to untreated individuals. After 4 h of treatment with HJ, the gene product turn on with the same expression observed for control of 1 h (Figure 2)

The HT11G-AP4 primer combination showed differential gene expression during larval development, with strong expression of bands 2 and 3in larvae treated Control/1 h compared to treated and Control4 h. In addition, this primer combination showed that another gene product (band 1) was suppressed only in individual after 1 h of treatment with JH (Figure 3).

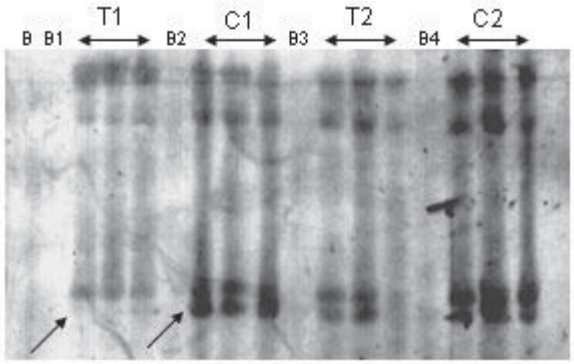

Figure 2. DDRT-PCR products (HT11G-AP5 primer combination) on 6% polyacrylamide/8 M urea gel stained with silver nitrate. B: blank (without RNA). B1, B2, B3 and B4: negative controls (without RT). T1: Larva 3 after 1 h of treatment with JH III; C1: control/1 h; T2: larva 3 after 4 h of treatment with JH III; C2: control/4 h. The arrows (⟶) indicate the position of the transcript in an individual treated for 1 h with JH III compared to control.

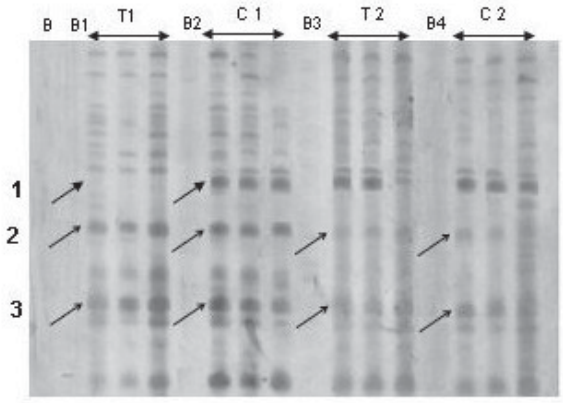

Figure 3. DDRT-PCR products (HT11G-AP4 primer combination) on 6% polyacrylamide/8 M urea gel stained with silver nitrate. B: blank (without RNA). B1, B2, B3 and B4: negative controls (without RT). T1: Larva 3 after 1 h of treatment with JH III; C1: control/1 h; T2: larva 3 after 4 h of treatment with JH III; C2: control/4 h. The arrows (⟶) indicate the position of the transcript in the control and the corresponding position in the treated individuals. The arrows (→) indicate the intensity polymorphisms during larval development.

DISCUSSION

JH is associated with the development, morphology and physiology of insects and its effects have been extensively investigated (BONETTI, 1982 , 1984; BONETTI; CRUZ-LANDIM; KERR, 1994 e BONETTI; KERR; MATUSITA, 1995 e HEPPERLE; HARTFELDER, 2001 e TOZETTO; PUCCIA; SIMÕES, 2000). In addition, the gene expression pattern in *Apis* species during caste differentiation under natural conditions has been studied by different authors (CORONA; ESTRADA; ZURITA, 1999 e EVANS; WHEELER, 1999 e EVANS; WHEELER, 2001 e TAKEUCHI et al., 2001).

In the present study, topical application of JH III to late L3 *M. scutellaris* larvae caused alterations in the mRNA profile (Figure 1, 2 and 3). JH was found to act only on 1-h treated larvae, suppressing gene expression, while no effect of this hormone was observed in larvae treated for 4 h. Bonetti (1990) demonstrated suppression of adult-specific esterases in 8-day-old *M. quadrifasciata* individuals treated with JH I during the larval stage. Furthermore, this author observed less intense expression of some regions with esterase activity in individuals treated with JH I. Jones, Schelling and Chhokar (1996) also found suppression of storage proteins during larval development of *Trichoplusia ni* after topical application of a JH analog. Comas, Piulachs and Bellés (1999), studying cardioallatectomized *Blattella germanica* treated with JH III, observed elevated vitellogenin mRNA levels after 2 h of treatment, demonstrating the gonadotropic effect of this hormone. Vermunt et al. (1999) observed an increase in the expression of the JH esterase gene in larvae of the beetle *Leptinotarsa decemlineata* and suppression of expression in adults after application of a JH analog. Similar alterations induced by JH or its analogs have also been reported by De Kort Koopmaschap and Vermut. (1997) for *Leptinotarsa decemlineata* and by Hepperle and Hartfelder (2001) for *Apis mellifera*.

Differential gene expression was also observed during 1 and 4 h of development (Figure 3, band 2 and 3) under natural conditions as showed in *Apis* by different authors (CORONA; ESTRADA; ZURITA, 1999 e EVANS; WHEELER, 1999 e EVANS; WHEELER, 2001; TAKEUCHI et al., 2001). These genes probably are turn on or turn off in response of manipulation due to stress conditions.

Identification and sequencing of the genes that are activated or turned off during development are necessary in order to determine the molecular mechanisms that govern development in the direction of differentiation.

ACKNOWLEDGEMENTS

We thank CAPES, FAPEMIG and CNPq for financial support. C. U. Vieira, received a scholarship from FAPEMIG.

RESUMO: O objetivo desse trabalho foi detectar, pela técnica de *Differential Display Reverse Transcriptase - Polymerase Chain Reaction* (DDRT-PCR), os efeitos do Hormônio Juvenil (HJ) III na expressão gênica, quando aplicado no estágio tardio de larva 3 (L3) de *Melipona scutellaris.* Nas fases que abrangem L3 e larva pré-defecante (LPD) há uma janela temporal de expressão dos genes feminizantes, durante a qual eles podem ser ligados ou desligados por ação do HJ, o qual é capaz de promover a diferenciação das larvas fêmeas, em rainhas. A combinação dos *primers* HT11A-AP4 revelou uma expressão diferencial no indivíduo tratado com HJ III, com fraca expressão do transcrito após l hora de tratamento do indivíduo, enquanto que no Controle e indivíduos com 4 horas de tratamento, a expressão foi mais intensa. As combinações dos *prímers* HT11G-AP4 e HT11G-AP5 revelaram, em cada uma dessas combinações, a supressão de um produto gênico na larva após l hora de tratamento com HJ III em relação ao expresso nos indivíduos de mesma idade não tratados e no indivíduo com quatro horas após o tratamento. Foi observado, também, expressão diferencial de transcritos durante o desenvolvimento ontogenético. Esses resultados demonstram que o HJ III suprime e/ou altera o perfil de expressão gênica durante a fase de L3 *de Melipona scutellaris.*

UNITERMOS: Expressão Gênica; DDRT-PCR; Hormônio Juvenil; *Melipona;* Castas.

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