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Metamorphosis of the Wing Disc and an Expressed Sequence Tag (EST) Database of Bombyx mori 【Review article】

家蠶生翅盤的變態與表現序列標幟資料庫【綜合論述】

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Abstract

In this review, I describe the wing discs of B. mori, their metamorphosis, ecdysone effect, expressed sequence tag (EST) database of Bombyx mori, microarray analysis and isolation of ecdysone-responsive genes through the EST database and microarray analysis. Wing discs show dynamic morphological changes during metamorphosis under fluctuating hemolymph ecdysteroid titers. In correlation with these morphological changes, gene expression patterns showed interesting changes. We isolated nine cuticle protein genes, two peptidase genes, and various tubulin isotypes through the EST catalog, and one cuticle protein gene and two protease genes through the microarray analysis.

摘要

在這篇評論，我描述家蠶的生翅盤，它的變態、蛻皮激素的影響、微陣列分析、表現序列標幟（EST）資料庫，及經由後兩者找出蛻皮激素反應基因。生翅盤在變態過程中，顯示變動的形態改變是隨著血淋巴中蛻皮激素濃度而變動著。與這些形態改變相關的基因表現形式，也呈現有趣的改變。我們經由EST資料庫找到9個表皮蛋白基因，2個胜?胸基因，和多種的同功型微管蛋白，以及利用微陣列分析找到1個表皮蛋白基因和2個蛋白胸基因。

Key words: EST, ecdysone, wing disc, Bombyx mori, metamorphosis
關鍵詞: 表現序列標幟、蛻皮激素、生翅盤、家蠶、變態

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Metamorphosis of the Wing Disc and an Expressed Sequence Tag (EST) Database of *Bombyx mori*

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ABSTRACT

In this review, I describe the wing discs of *B. mori*, their metamorphosis, ecdysone effect, expressed sequence tag (EST) database of *Bombyx mori*, microarray analysis and isolation of ecdysone-responsive genes through the EST database and microarray analysis. Wing discs show dynamic morphological changes during metamorphosis under fluctuating hemolymph ecdysteroid titers. In correlation with these morphological changes, gene expression patterns showed interesting changes. We isolated nine cuticle protein genes, two peptidase genes, and various tubulin isotypes through the EST catalog, and one cuticle protein gene and two protease genes through the microarray analysis.

Key words: EST, ecdysone, wing disc, *Bombyx mori*, metamorphosis

Introduction

Ecdysteroid plays an essential role during the metamorphosis of insects. The morphogenesis of adult organs such as the imaginal discs is regulated by ecdysteroids (Oberlander, 1985). The fluctuation in hemolymph ecdysteroid titers at the initiation of metamorphosis is well known in insects (Riddiford and Truman, 1993; Thummel, 1995). Molecular analyses showed that 20-hydroxyecdysone (20E) functions as a signal to activate or repress target genes, which regulate insect morphogenesis, at the level of transcription (Natzle, 1993; White *et al.*, 1997). The Ashburner model explains 20E's response in *Drosophila* salivary glands (Ashburner *et al.*, 1974; Ashburner, 1991), where the existence of primary response loci and secondary response loci was demonstrated. The ecdysone EcR/USP complex directly regulates the primary response genes: *BR-C*, *E75A*, *E75B*, *E75C*, *E74A*, and *E74B*. These early genes regulate secondary-response late genes (Thummel, 1995; White *et al.*, 1997; Jiang *et al.*, 2000). Ecdysone-dependent pathways exist in imaginal discs and are responsible for imaginal disc metamorphosis. Therefore, microarray analysis has been applied to *Drosophila* imaginal discs (Klebes *et al.*, 2002; Butler *et al.*, 2003).

The hemolymph ecdysteroid titer

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decreases to an undetectable level after the last molting and then begins to increase again after the start of spinning to a maximum level around the cessation of spinning: 2 days after the wandering stage (Kiguchi *et al*., 1985) in *B*. *mori*. During these stages, the morphological and related changes of gene expression patterns in wing discs are matters discussed in this review.

1. Wing discs

Insects have two different types of organs besides reproductive ones: one is larval and degenerates, and the other is imaginal and develops during metamorphosis. Numerous studies on imaginal discs (Doctor and Fristrom, 1985; Oberlander, 1985) led us to construct cDNA libraries of different stages of wing discs of the final larval instar of *B*. *mori*. Imaginal discs have been studied as targets of ecdysone for a long time: their dynamic morphological changes as a result of ecdysone function, ecdysone sensitivity for the screening and isolation of several ecdysone-inducible genes (Natzle *et al*., 1986)

Wing discs exist in silence until the last larval ecdysis in holometabolous insects. After the disappearance of the juvenile hormone (JH), the rate of proliferation of disc cells increases rapidly (Kurushima and Ohtaki, 1975; Kawasaki and Iwashita, 1987), and the low concentration of ecdysteroids promotes cell proliferation after the wandering stage in *Bombyx* wing discs (Kawasaki, 1998). Cuticle deposition, evagination, changes in the cell shape, and the appearance of newly synthesized peptides were observed in *Bombyx* wing discs 2 days after the wandering stage (Kawasaki and Iwashita, 1987; Kawasaki, 1998), when the peak ecdysteriod level was observed (Quan *et al*., 1998).

Metamorphosis of lepidopteran imaginal discs such as cuticle deposition, and tracheole migration and evagination were induced after ecdysteroid treatment *in vitro* (Fristrom *et al*., 1973, Mandaron, 1973). Ecdysteroid accelerates mitosis in *Tenebrio* accessory glands (Szopa *et al*., 1985) and *Manduca* epidermis (Kato and Riddiford, 1987). Several ecdysteroid-inducible polypeptides and genes were detected and cloned in imaginal discs of *Drosophila melanogaster* (Natzle *et al*., 1986; Woods *et al*., 1987; Apple and Fristrom, 1991). Ecdysone-inducible polypeptides were detected (Lynn *et al*., 1982) or ecdysone inducible cDNA clones were obtained (Chareyre *et al*., 1993; Besson *et al*., 1996) in lepidopteran imaginal discs. Some of these cDNAs corresponding mRNAs were expressed in the spinning stage specifically in *Bombyx* wing disc (Chareyre *et al*., 1993; Besson *et al*., 1996). It can be assumed that ecdysteroid works both for controlling the cell cycle and for inducing new genes for material production in preparation for pupation. Therefore, for a better study of the effect of the hormone on genetic modulation during insect metamorphosis, we selected the wing discs of *B*. *mori* as a target organ.

Recently, ecdysteroid-regulating genes have been isolated, and their expression has been observed in *Bombyx* wing discs (Kamimura *et al*., 1996; Quan *et al*., 1998; Matsuoka and Fujiwara, 2000; Zhao *et al*., 2001; Takeda *et al*., 2001; Noji *et al*., 2003). Gaining a overview of ecdysone-dependent and -independent genes during wing metamorphosis will lead to a better understanding of insect metamorphosis. For this, we constructed four cDNA libraries from different stages of the wing disc of *B*. *mori*, sequenced approximately 1000 randomly selected cDNAs from each library, and compared the expression profiles at four different stages.

2. SilkBase

Data described above have been compiled into an EST database of *B*. *mori* together with other sequence data from

different cDNA libraries of *B. mori*, leading to the construction of SilkBase (http://www.ab.a.u-tokyo.ac.jp/silkbase/; Mita *et al.*, 2003). To build a foundation for the complete genomic analysis of *B. mori*, we have constructed an EST database. So far, the *Bombyx* EST database contains 35,000 ESTs from 36 cDNA libraries, which are grouped into 11,000 nonredundant ESTs with an average length of 1.25 kb. The comparison with FlyBase suggests that the present EST database, SilkBase, covers › 55% of all genes of *Bombyx*. Genomic information provides powerful tools for understanding biological mechanisms and functions and is essential for biology, medical science, and agriculture.

The cDNA catalog is very effective in listing the genes responsible for the metamorphosis of wing discs. Multiple cloning of cuticle protein genes was performed by sequencing cDNAs randomly selected from a cDNA library of wing discs just before pupation, and nine different cuticular protein genes were identified. By a comparison of the cDNA libraries prepared from wing discs before and after larval-pupal metamorphosis, we isolated *BmAcer* and *BmNepl* whose expression patterns were stage-specific and correlated with 20E levels. Also, this method was applied to isolate as many tubulin isotypes as possible and to examine the organ distribution of tubulin isotypes of *B. mori*.

3. Microarray analysis

Microarray analysis was applied to quantify the expression of each gene in each stage in order to confirm whether the expression of the genes identified from the EST was induced by 20E in a stage-specific manner. Microarray analysis was applied to 5760 EST clones of SilkBase to compare 20E +/- cultured wing discs (Kawasaki *et al.*, 2004). After three microarray analyses, 110 identical clones from the EST database of *B. mori* had signals which were two times stronger against mRNAs from hormone-treated wing discs than from hormone-free wing discs. Among the 110 clones, 63 were from wing disc ESTs. Of these 63 clones, 46 were from the wing disc EST database at the W2 and W3 stages. Nine clones have already been reported to be ecdysone-inducible (*Urbain*, *BmAcer*, *BmNepl* and *BmWCP10*), and three (*Chitinase* and *BmChi-h*) have shown stage-specific expression in the *Bombyx* epidermis when the ecdysteroid titer was high (Daimon *et al.*, 2003). Results indicate that there are abundant ecdysone-inducible genes in wing discs when the hemolymph ecdysteroid titer is high. The evidence that microarray analysis identified a number of reported genes as ecdysone-inducible ones such as *Urbain*, *E75*, *BmAcer*, *BmWCP10*, *BmChi-h* and *BmNepl*, together with results of the RT-PCR showed the effectiveness of the microarray analysis using wing discs for the screening of ecdysone-inducible genes as reported by Li and White (2003). Krebs *et al.*, (2002) isolated developmentally specific genes from the mosquito using a microarray analysis.

On the other hand, cDNA microarrays constructed from 5086 ESTs (Ote *et al.*, 2004) were used to monitore gene expression profiles in wing discs of *B. mori* at 13 time points (day-4 fifth instar larvae to day-0 pupae) during pupal ecdysis. Of the 5086 ESTs on the microarrays, 2998 ESTs had significant signals in more than half of the experiments. Of the 2998 ESTs, genes represented by 683 ESTs showed significant perturbations during pupal ecdysis. Genes previously known to be induced during metamorphosis were identified, including *E75*, *Urbain*, *Chitinases*, and cuticle proteins. The expressions of genes represented by 59 ESTs induced at the beginning of the wandering stage contained genes predicted to be involved in protein degradation, amino acid metabolism, and amino acid transport.

The expressions of genes represented by 147 ESTs induced after the ecdysteroid peak had a role in cuticle synthesis, pigmentation, ion transport, protein transport, and transcription regulation. The expressions of genes represented by 85 ESTs, which are repressed after the ecdysteroid peak, were predicted to be involved in nucleotide and nucleic acid metabolism and the cell cycle. This indicates the involvement of several biological processes in wing disc development during metamorphosis. In *B. mori*, the ongoing EST project has identified over 10,000 non-redundant ESTs (Mita *et al.*, 2003).

4. Metamorphosis of the wing disc

To understand the metamorphosis on a molecular level, changes in the expression profiles in the imaginal disc during metamorphosis were investigated (Kawasaki *et al.*, 2004). For this purpose, we constructed cDNA libraries from four different stages of wing discs of *B. mori*, sequenced about 1000 cDNAs randomly collected from each library, and constructed an EST database. The morphological changes and expression profiles from EST were compared during those four stages. Wing discs showed dynamic morphogenesis in 4~5 days during the preparatory stage of metamorphosis, which was under the control of an ecdysteroid. Different expression profiles were observed in each of the four different stages by comparison of each EST clone. These profiles reflected the morphological changes of the *Bombyx* wing disc during metamorphosis. The results of expression profiles from the four stages suggested that the V4 stage was cell-proliferating; W0 was proliferating and the beginning of differentiation; W2 was morphologically changing; and W3 was cuticle-secreting. The wing disc of *B. mori* is an exceptionally suitable system for understanding the relationship between morphological changes and the distribution of mRNA.

5. Isolation of genes through the SilkBase catalog and microarray analysis

5.1. Through the EST catalog

5.1.1. *BMWCPs*

The insect cuticle is constructed from many cuticle proteins with various combinations of different temporal and spatial patterns (Willis, 1996). To elucidate this further, mass isolation of cuticle protein genes and their characterization were performed through an EST database of the W3 stage (Takeda *et al.*, 2001). Thirty-one clones of a cuticle protein gene were identified from among the 1050 EST clones; about 5% were cuticle protein genes in the W3-stage wing disc cDNA library. The sequence diversity of the deduced amino acid sequences of isolated *Bombyx* cuticle protein genes was examined along with the expression profiles. The deduced amino acid sequences of the nine cuticle protein genes contained a putative signal peptide at the N-terminal region and a very conserved hydrophilic region known as the R and R motif. The developmental expression of cuticle genes was classified into two types: pupation (five clones were expressed only around pupation) and pupation and mid-pupal (four clones were expressed around this stage). All of the isolated genes were expressed in the head, thoracic, and abdominal regions of the epidermis at different levels around pupation, but no expression was observed in the epidermis in the fourth molting stage.

5.1.2 *BmGRPs*

BmGRP genes have different expression profiles from *BMWCP* genes. To address these expression differences, the developmental profiles and responses to hormonal stimulation of *BmGRP* genes were examined, by comparing them to *BMWCP* genes (Zhong *et al.*, in press). Three types of *GRP* (glycine-rich protein) cDNAs were

identified in the EST database of *B. mori*. These came from 21 ESTs in the W3-stage wing disc EST library. We named them *BmGRP1*, *BmGRP2* and *BmGRP3*. *BmGRP1* and *BmGRP2* had 57% identity in the deduced amino acid sequences. Expressions of all *BmGRPs* were observed in the epidermis in the fourth molting stage, and in the wing at pupation and mid-pupal stage. It is suggested that *BmGRPs* contribute to larval, pupal and adult cuticle formation together with other cuticle proteins. Transcripts of *BmGRP2* increased after 7 days of the pupal stage. *BmGRP2* is suggested to be associated with construction of the adult trachea in the wing. Hormonal responses of *BmGRPs* were compared with those of another group of cuticle protein genes, *BMWCPs*. *BmGRPs* were induced by a pulse of 20E. Induction of *BmGRP3* was observed in W1 wing discs in the presence of JHA which was added with 20E, whereas that of *BMWCP2* was inhibited in the presence of JHA. Induction of *BmGRPs* was observed in the wing discs of V3 and W1 stages, while that of *BMWCP2* was not observed in V3 wing discs. These differences between *BMWCPs* and *BmGRPs* in response to hormones at different developmental stages are discussed.

5.1.3. *BmAcer*

A clone encoding a putative angiotensin-converting enzyme-related gene was isolated from the wing disc cDNA library of the silkworm, *B. mori* (Quan *et al.*, 2001). The predicted open reading frame encoded 648 amino acids with about 50% identities with the *Drosophila melanogaster* angiotensin-converting enzymes, *Ance* and *Acer*. Northern analysis identified a 2.2-kb mRNA which was abundant in wing discs 2 days after the beginning of the wandering stage. An accumulation of the transcript was observed approximately 2 h after 20E exposure *in vitro* and was slightly blocked by a protein synthetic inhibitor. These data suggest that the transcription of the *BmAcer* gene is directly 20E-inducible.

5.1.4. *BmNEP-L*

In the process of comparing two cDNA libraries (W0 and W2), a clone from the wing discs of *B. mori* encoding a putative neutral endopeptidase 24.11-like gene was isolated (Zhao *et al.*, 2001). The predicted open reading frame encoded 772 amino acid residues, having about 53% identity with *Drosophila* GH07643, 36% with rat NEP, and 34% with rat ECE. This is the first *NEP* gene isolated in invertebrates. A 3.6-kb transcript was found to accumulate in the wing disc according to the increase in ecdysteroid titers during metamorphosis. Accumulation of the transcript was induced in wing discs with 20E about 20 h after incubation, which was inhibited by cycloheximide. This gene is ecdysone-inducible, appears to encode a functional protein, and may function during wing metamorphosis.

5.1.5. Tubulins

Four different types of β-tubulin and three types of α-tubulin were identified (Kawasaki *et al.*, 2003). Among isotypes of β-tubulin, *BMTUB3* has longer sequences than the other isotypes, from residues 56 to 61 and including c-terminal sequences, which are similar to *Drosophila* β3-tubulin. *BMTUB2* was ubiquitously observed in most cDNA libraries, while BMTUB1 was observed in libraries of the BmN cell, compound eye, fat body, hemocyte and wing disc. *BMTUB4* was observed in a testis cDNA library. Among isotypes of α-tubulin, *BMTUA1* was detected in most cDNA libraries of *B. mori*, while *BMTUA2* was detected in brain, compound eye and wing disc S0 libraries. *BMTUA3* was only detected in a testis library.

5.2 Through microarray analysis

5.2.1. *BMWCP10*

Microarray analysis was used to isolate an ecdysone upregulated cuticle protein gene from wing discs of *B. mori* (Noji *et al.*, 2003). Transcripts of isolated cDNAs were identified by Northern blot analysis. Expression of the *BMWCP10* gene was observed during the W0~W3 stage, and the strongest signal was observed in the W2 stage. In contrast, expression of the *BMWCP2* gene was observed in the W3 and P0 stages. Expression of *BMWCP10* was observed after 20E treatment, while that of *BMWCP2* was observed after 20E removal *in vitro*. Induction of *BMWCP10* by 20E was observed in 30 min and was not inhibited by cycloheximide. Expression of *BMWCP2* was observed within wing discs cultured for more than 18 h in a hormone-free medium after 20E removal. 20E was required at least 4 h before removal for induction of *BMWCP2*. Induction of *BMWCP2* required protein synthesis. Thus, different ecdysone-responsive cuticle protein genes in wing discs of *B. mori* were isolated.

5.2.2. *BmADAMs*

By microarray analyses, two genes (*BmADAMTS-1* and *BmADAMTS-like*) encoding a protein, which were induced during pupal ecdysis in wing discs of *B. mori,* were identified (Ote *et al.*, 2005a); these genes were homologous to ADAMTS family members (a disintegrin and metaloprotease domain, with thrombospondin type-1 repeats). A complete metal-binding motif of the ADAM-type metaloprotease domain (HEXXHXXGXXHD) was contained in both amino acid sequences. However, thrombospondin type 1 (TSP-1) repeats were observed only in *BmADAMTS-1*. The B*mADAMTS-1* gene was expressed in hemocytes and the midgut of larvae on day 2 of the wandering stage (W2), and strongly induced during pupal ecdysis in the hemolymph. The *BmADAMTS-like* gene was expressed in epithelial tissues of the larvae on W2, and had expression peaks slightly later than the *BmADAMTS-1* gene. These results indicated that *BmADAMTS-1* and *BmADAMTS-like* might cleave the extracellular matrix (ECM) when degenerating and remodeling tissues during the molting periods.

5.2.3. *BmCPA*

Using microarray analyses, a gene encoding carboxypeptidase A (*BmCPA)* was identified, which was induced during pupal ecdysis in the wing discs of *B. mori* (Ote *et al.*, 2005b). The functional characterization of *BmCPA* showed that it had amino acid sequence similarities with the proteins in the carboxypeptidase A/B subfamily, from nematodes to humans. The *BmCPA* gene was expressed during molting periods in epithelial tissues. *BmCPA* was detected in the molting fluid, which fills the space between the old and new cuticle during molting. Western blot analysis showed that *BmCPA* was secreted as a zymogen and processed in the molting fluid. Recombinant *BmCPA* expressed in the insect cells had carboxypeptidase A activity. It was proposed that *BmCPA* degrades the proteins from the old cuticle during molting periods and contributes to recycling of the amino acids.

Conclusions

Wing discs show dynamic morphogenesis in 4-5 days during the preparatory stage of metamorphosis, which is under the control of an ecdysteroid. Different expression profiles were observed in each of the four different stages by comparison of each EST clone. These profiles reflect the morphological changes in the *Bombyx* wing disc during metamorphosis. The results of expression profiles from the four stages suggest that the V4 stage is cell-proliferating; W0 is proliferating and the beginning of differentiation; W2 is

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We isolated nine cuticle protein genes, two peptidase genes and tubulin isotypes through the EST catalog, and one cuticle protein gene and two protease genes through microarray analysis.

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家蠶生翅盤的變態與表現序列標幟資料庫

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摘　要

在這篇評論，我描述家蠶的生翅盤，它的變態、蛻皮激素的影響、微陣列分析、表現序列標幟（EST）資料庫，及經由後兩者找出蛻皮激素反應基因。生翅盤在變態過程中，顯示變動的形態改變是隨著血淋巴中蛻皮激素濃度而變動著。與這些形態改變相關的基因表現形式，也呈現有趣的改變。我們經由 EST 資料庫找到 9 個表皮蛋白基因，2 個胜肽脢基因，和多種的同功型微管蛋白，以及利用微陣列分析找到 1 個表皮蛋白基因和 2 個蛋白脢基因。

關鍵詞：表現序列標幟、蛻皮激素、生翅盤、家蠶、變態。