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Evolution of Developmental Control Mechanisms

Dramatic changes in patterning gene expression during metamorphosis are associated with the formation of a feather-like antenna by the silk moth, *Bombyx mori*

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ABSTRACT

Many moths use sex pheromones to find their mates in the dark. Their antennae are well developed with lateral branches to receive the pheromone efficiently. However, how these structures have evolved remains elusive, because the mechanism of development of these antennae has not been studied at a molecular level. To elucidate the developmental mechanism of this type of antenna, we observed morphogenesis, cell proliferation, cell death and antennal patterning gene expression in the branched antenna of the silk moth, *Bombyx mori*. Region-specific cell proliferation and almost ubiquitous apoptosis occur during early pupal stages and appear to shape the lateral branch cooperatively. Antennal patterning genes are expressed in a pattern largely conserved among insects with branchless antennae until the late 5th larval instar but most of them change their expression dramatically to a pattern prefiguring the lateral branch during metamorphosis. These findings imply that although antennal primordium is patterned by conserved mechanisms before metamorphosis, most of the antennal patterning genes are reused to form the lateral branch during metamorphosis. We propose that the acquisition of a new regulatory circuit of antennal patterning genes may have been an important event during evolution of the sensory antenna with lateral branches in the Lepidoptera.

Introduction

Nocturnal and cave-dwelling animals often have highly sensitive sensory organs to receive information from their surroundings in a dark environment (Balkenius et al., 2006; Jeffery, 2001). Among the lepidopteran insects, moths have extremely sensitive pheromone-sensing systems that enable mate-searching behavior in such an environment. Their pheromone reception device, an antenna, is frequently equipped with many protruding lateral branches and has a gigantic feather-like morphology. It has been shown that the lateral branches enhance air-trapping efficiency and are important for the efficient reception of pheromone molecules (Bau et al., 2005; Vogel, 1983). This antennal morphology is thought to have been acquired independently in several families within the Lepidoptera, because most lepidopteran species – including primitive moths – have simple filiform or serrated antennae without lateral branches (Scoble, 1992). Therefore, studying antennal development of the Lepidoptera at a molecular level will provide important insights into the evolution of an ecologically significant appendage variant.

The insect antenna can be divided into three parts along the proximodistal (PD) axis according to function (reviewed in Angelini and Kaufman, 2005a). The proximal part is the first antennal segment (the a1 segment or scape) that supports the entire weight of the antenna. The intermediate part is the second antennal segment (the a2 segment or pedicel) containing Johnston's organ that senses the physical forces exerted on the antenna, such as sound, gravity and wind (Boekhoff-Falk, 2005; Kamikouchi et al., 2009; Sane et al., 2007; Tsujiuchi et al., 2007; Yack, 2004; Yorozu et al., 2009). The distal part making up most of the antenna is the third antennal segment (the a3 segment or flagellum), which has thousands of olfactory sensilla, including pheromone-receptive forms (Steinbrecht et al., 1995; Vosshall and Stocker, 2007). The a3 segment is usually constructed from the reiteration of sub-segments. Lateral branches on the antennae of lepidopteran insects protrude from each a3 subsegment and their morphologies differ from species to species, along with their number per subsegment: from one to four.

Antennal development has been studied in detail in the fruit fly, *Drosophila melanogaster* (Chu et al., 2002; Dong et al., 2000, 2001, 2002). In the developing antenna, several genes encoding transcription factors are expressed region-specifically and combinatorially subdivide the developing antenna into several domains along the PD axis (Fig. S1A). For example, the a1 segment is determined by the strong expression of *homothorax* (*hth*), the a2 segment by the strong expression of both *Distal-less* (*Dll*) and *hth*, and strong *Dll* expression with weak *hth* expression determines the a3 segment. Comparative analyses between species suggest that expression and function of these transcription

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factor genes are conserved in other insects such as the cricket *Gryllus bimaculatus* (Ronco et al., 2008), grasshopper *Schistocerca americana* (Jockusch et al., 2004), milkweed bug *Oncopeltus fasciatus* (Angelini and Kaufman, 2004) and flour beetle *Tribolium castaneum* (Angelini et al., 2009). In addition, *aristaless* (*al*) is expressed in the most distal region of the antenna and *Bar* is expressed in the region just proximal to it. Although these regions form a fly-specific structure, arista, *al* expression or *al* necessity in the most distal part of the antenna has been reported also in the cricket (Miyawaki et al., 2002) and flour beetle (Angelini et al., 2009), whose antennae do not have aristae. This implies that the underlying patterning mechanism is conserved among insects, even though the resulting structures differ.

The expression of antennal transcription factor genes such as *Dll* and *hth* is induced by the morphogen signals of Wnt and transforming growth factor-beta (TGF-β) (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997, Fig. S1B). In addition, based on the homology to leg development, a gradient of epidermal growth factor receptor (EGFR) signaling is thought to induce genes for several transcription factors such as *al* and *Bar* in the most distal part of the antenna (Fig. S1C). The ligands of Wnt and TGF-β signals are encoded by *wingless* (*wg*) and *decapentaplegic* (*dpp*), respectively. Two EGFR ligands, Vein and Spitz, are thought to function in antennal development. Vein is a neuregulin-type ligand and secreted simply from cells expressing its mRNA, while Spitz, a TGF-α-type ligand, is produced from cells expressing its activator, *rhomboid* (*rho*).

Despite our comprehensive understanding of the developmental mechanisms of the antenna, all insects investigated so far at a molecular level have branchless antennae; therefore, the factors involved in the generation of the branched, feather-like antenna are largely unknown. This topic is important if we are to reveal the mechanism of antennal evolution from the simple, branchless form to the branched, feather-like form. Here, we studied in detail the development of the feather-like antenna of the silk moth, *Bombyx mori*, focusing on its morphogenesis, patterns of cell proliferation and cell death, and the expression patterns of genes known to function in the antennal patterning in other insects. We found that cell proliferation occurs in three distinct regions within each a3 subsegment that are closely related to lateral branch formation, whereas cell death occurs almost ubiquitously, indicating the importance of the balance between cell proliferation and cell death to form lateral branches. Gene expression analysis revealed that the antennal patterning genes are expressed at larval stages in a pattern largely conserved in other insects with branchless antennae. Intriguingly, almost all genes that we examined changed their expression dramatically to a pattern prefiguring the lateral branch in each a3 subsegment during the larva-to-pupa transition. Among them, *wg* and *rho* are the earliest to show the branch-related expression pattern. Because the expression of antennal patterning genes is largely unchanged during metamorphosis in insects with branchless antennae, such as *Drosophila* and *Tribolium*, we propose that the acquisition of a new regulatory circuit of antennal patterning genes may have been an important event during evolution of the branched, feather-like antenna in the Lepidoptera.

Materials and methods

Animals

Silkworms (N4 strain) were maintained in our laboratory and reared on artificial diet (Nihon Nousankou) under long day condition (L:D = 16 h:8 h) at 25 °C. Experiments were essentially carried out using male antennae.

Cloning of Bombyx orthologs of antennal patterning genes

Partial DNA fragments of *Bombyx* orthologs of antennal patterning genes, which were used as templates for RNA probes in *in situ* hybridization, were amplified by the reverse transcription-polymerase chain reaction (RT-PCR) from cDNAs derived of *Bombyx* antennal primordia in several developmental stages and cloned using the pGEM T-Easy vector system (Promega). Details are described in the supplemental text.

Scanning electronic microscopy

Adult antennae were dissected from heads and immediately attached to a detection stand with wood glue and observed with the Miniscope (Hitachi).

Histological analysis

Pupal antennae were dissected along with the head and thorax, fixed in 4% paraformaldehyde/Phosphate Buffered Saline (PBS) overnight at 4 °C and embedded in paraffin. The specimens were then sectioned at 5 μm and stained with Hematoxylin and Eosin.

Immunohistochemistory and in situ hybridization

Immunohisitochemistry and *In situ* hybridization were essentially carried out according to Sato et al. (1999). The guinea pig anti-Al (1:1000, Yasunaga et al., 2006), rabbit anti-horseradish peroxidase (HRP) (1:10,000, Sigma), rabbit anti-phospho-Histone H3 (Ser10) (pH3) (1:1000, Upstate), and Alexa Fluor 488, 555, or 647 conjugated secondary antibodies (1:100, Molecular Probes) were used. We stained both male and female antennae and remarkable sexually dimorphic expression was not found among the genes that we examined. The 5-bromo-2-deoxyuridine (BrdU) labeling was done basically as Franco et al. (2007) using the cell proliferation detection kit (GE healthcare). The terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay was done basically as Wang et al. (1999) using the *In Situ* Cell Death Detection kit, Fluorescein (Roche). Details of each procedure are described in the supplemental text.

Image processing

For the brightfield images, images from several planes were projected using Helicon focus (HeliconSoft) with Radius = 50 and Smoothing = 1. Images were processed with Photoshop Element (Adobe Systems).

Results

Morphology of the adult antenna and its morphogenesis during metamorphosis

The adult antenna of *B. mori* has a feather-like morphology (Figs. 1A, S2A), in which each a3 subsegment is equipped with a pair of ventrally curved lateral branches, one protruding anteriorly and the other posteriorly (Figs. 1B, C). The ventral sides of both lateral branches and the main shaft of the a3 segment are covered with numerous chemosensory sensilla, whereas most of the dorsal surface is smooth (Fig. 1B). It is known that although there is virtually no difference in the overall antennal morphology between male and female moths, male antennae have a denser distribution of sensilla (Steinbrecht et al., 1995) and pheromone receptors are expressed only in male olfactory neurons (Sakurai et al., 2004).

The silk moth is a holometabolous insect and the adult antenna is formed through metamorphosis from the antennal primordium, which has a hollow cylindrical morphology and is formed from the epithelium constituting the larval antenna in the 5th larval instar (Svácha, 1992; Figs. S2C–F, Details are described in the figure legend).

To understand the morphogenesis of the branch formation, we observed the a3 segment of the pupal antenna carefully at various