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Identification and analysis of Toll-related genes in the domesticated silkworm, *Bombyx mori*

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Abstract
Silkworm (*Bombyx mori*), a model system for Lepidoptera, has contributed enormously to the study of insect immunology especially in humoral immunity. But little is known about the molecular mechanism of immune response in the silkworm. Toll receptors are a group of evolutionarily ancient proteins, which play a crucial role in the innate immunity of both insects and vertebrates. In human, Toll-like receptors (TLRs) are the typical pattern recognition receptors for different kinds of pathogen molecules. Toll-related receptors in *Drosophila*, however, were thought to function as cytokine receptors in immune response and embryogenesis. We have identified 11 putative Toll-related receptors and two Toll analogs in the silkworm genome. Phylogenetic analysis of insect Toll family and human TLRs showed that BmTolls is grouped with *Drosophila* Tolls and *Anopheles* Tolls. These putative proteins are typical transmembrane receptors flanked by the extracellular leucine-rich repeat (LRR) domain and the cytoplasmic TIR domain. Structural prediction of the TIR domain alignment found five stranded sheets and five helices, which are alternatingly joined. Microarray data indicated that *BmToll* and *BmToll*-2 were expressed with remarkable enrichment in the ovary, suggesting that they might play a role in the embryogenesis. However, the enriched expression of *BmToll*-2 and -4 in the midgut suggested that the proteins they encode may be involved in immune defense. Testis-specific expression of *BmToll*-10 and -11 and *BmToLK*-2 implies that these may be involved in sex-specific biological functions. The RT-PCR results indicated that 10 genes were induced or suppressed with different degrees after their immune system was challenged by different invaders. Expression profiles of *BmTolls* and *BmToLKs* reported here provide insight into their role in innate immunity and development.

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1. Introduction

Innate immunity is the first line of defense by animals against invading microorganisms such as bacteria, fungi and viruses. Despite the lack of an adaptive immune system, invertebrates are able to rapidly recognize non-self antigens and amplify the infected signal. In the process, signal transduction pathways such as the Toll pathway and the Imd pathway are triggered and the transcription of downstream genes are activated afterwards. Effector molecules, especially antimicrobial peptides, will be highly expressed in fat body and secreted into the hemolymph. In this process, the pattern recognition receptors known as Toll-like receptors (TLRs) play a key role in the induction of innate immunity and in the inflammatory responses.

Since Medzhitov and his colleagues successfully cloned a human homolog of *Drosophila Toll* [1], at least 10 TLRs (TLR1-10) have been found in the human genome. In contrast to *Drosophila* Toll proteins, the mammalian TLRs as pattern-recognition receptors (PRRs) are able to recognize directly their specific pathogen-associated molecular patterns (PAMPs) in the innate and adaptive immune responses [2]. So far, we know that: (i) TLR-2 is primarily involved in recognition of peptidoglycan and bacterial lipopeptides [3], (ii) TLR-3 is a cell-surface receptor for double-stranded RNA [4], (iii) the recognition of lipopolysaccharide (LPS) by TLR-4 is mediated by a complex (CD14, MD2, and TLR-4) [5], (iv) TLR-5 recognizes the flagellin that forms bacterial flagella [6], (v) TLR-6 cooperates with TLR2 for the recognition of mycoplasmal lipopeptides [7], (vi) TLR-9 functions as a receptor for unmethylated CpG motifs that are abundant in bacterial genome [8].

Indeed, the *Drosophila* genome encodes a family of nine Toll-related receptors. Most *Tolls* are highly expressed in normal embryos and pupae and probably have important developmental functions [9]. The *Drosophila Toll* functions not only in the establishment of dorsal–ventral polarity in the early embryo, but in the innate humoral and cellular immune response of larvae and adults [10]. *Drosophila Toll*-6–8 are expressed at high levels in embryos and pupae, suggesting that the proteins they encode function in embryogenesis and molting stages [9]. *Drosophila* Toll-9 similar to gain-of-function *Drosophila* Toll-1 activates strongly the expression of *Drosomycin* and antifungal genes by similar signaling components to *Drosophila* Toll-1. *Drosophila* Toll-1 has been implicated in the regulation of immune responses [11]. During *Drosophila's* immune response, the broad-spectrum microbial recognition pattern is reflected by PRRs such as peptidoglycan recognition proteins (PGRPs) and Gram-negative bacteria-binding proteins (GNBPs). Toll, a cytokine receptor, is activated by an endogenous ligand, Spätzle [12]. In human, however, TLRs directly interact with PAMPs acted as typical PRRs.

Toll receptors are part of an ancient receptor family involved in immune defense, conserved from lower metazoans to higher vertebrates. A typical TLR contains generally extracellular leucine-rich repeats (LRRs) connected to a cysteine-rich domain and an intracytoplasmic Toll-interleukin homolog domain (TIR) [13,14]. Toll-related proteins have been predicted and identified from several genomes of model species in addition to human, including *Drosophila melanogaster* [9], *Anopheles gambiae* [15], and *Danio rerio* [16], etc. In *Bombyx mori*, a *BmToll* has also been cloned and characterized [17]. It is interesting to analyze phylogenetic relationships of Toll-related receptors in insects.

Recent completion of the genome sequencing of *B. mori* provides a unique opportunity to analyze Toll-related genes [18,19]. Due to its broad experimental potential as molecular and genetic tools, *B. mori* is an important model system for studying insect innate immunity and development biology. The silkworm has contributed enormously to the study of insect pathology and insect immunology, particularly humoral immunity. In this paper, we have (1) identified *B. mori* Toll-related genes and their TIR domains from the silkworm genome using bioinformatics, (2) compared the evolutionary relationship of TIR domains with those found in dipteran insects, and (3) analyzed their temporal and spatial expression patterns. The study on Toll-related genes may help us better understand the immune-response mechanism in silkworm.

2. Materials and methods

2.1. Identification of Toll-related genes in the silkworm genome

Complete Toll-related protein sequences of *Drosophila* retrieved from GenBank (http://www.ncbi.nih.gov/Genbank/) were used as queries to search for Toll-related genes in the 9x coverage silkworm genome map assembled by the China and Japan silkworm genome projects. The following protein sequences and accession numbers were used as queries: *D. melenogaster*: DmToll (AAA28941), Dm18w (AAF57509), DmToll-3 (AAF54021), DmToll-4 (AAF52747), DmToll-5 (AAF53306), DmToll-6 (AAF49645), DmToll-7 (AAF57514), DmTollo (AAF49650), DmToll-9 (AAF51581); *A. gambiae*: AgToll (EAA45376), AgToll-1 (EAA07066), AgToll-5 (EAA04891), AgToll-6 (EAA00379), AgToll-7 (EAA00348), AgTrex (AAL37904), AgToll-9 (EAA04650), AgToll-10 (EAA05150), AgToll-11 (EAA05071). A PSI-BLAST search of the silkworm genome database was performed using the TIR conserved domain from BmToll (BAB85498) as a query. To predict the exon/intron boundaries, the assembled genomic sequences were then analyzed using *FGENESH* program (http://sun1.softberry.com/berry.phtml). The results were further manually annotated by comparing with insect Toll-related receptors using BLASTX (http://www.ncbi.nlm.nih.gov/BLAST). In addition, to test whether or not these *BmTolls* are conceptual translations of pseudogenes, a BLAST search of the all-available ESTs was performed and the matched sequences can be retrieved from NCBI or the web (http://papilio.ab.a.u-tokyo.ac.jp/Bombyx_EST/). The extracellular, transmembrane, and cytoplasmic domains of protein sequences were predicted using SMART (http://smart.embl.de/) [20]. The secondary and tertiary structures were predicted by the neural network program PHD [21] and by comparative modeling with SWISS-MODEL [22], respectively.

2.2. Alignment and phylogenetic analysis of BmTolls

Multiple sequence alignments of TIR domains were performed using Clustal X [23]. In order to compare equivalent regions, the TIR domains were retrieved using their