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Transcriptional response of *BmToll9-1* and RNAi machinery genes to exogenous dsRNA in the midgut of *Bombyx mori*

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ABSTRACT

Injection of dsRNA is widely applied to silence endogenous genes and study gene function in insects. However, it is not yet clear to what extent it can also exert non-specific effects, for instance by interference with the innate immune response. In this study, we report on the transcriptional response of *BmToll9-1* to lipopolysaccharide (LPS) and dsRNA in the silkmoth, *Bombyx mori*. *BmToll9-1* encodes a Toll receptor highly expressed in midgut tissue and that shows limited similarity to the mammalian TLR3 endolysosome receptor for dsRNA; while *Dcr2* and *Ago2* encode two key components of the RNAi machinery.

An expression pattern study of all 14 Toll receptors in *B. mori* showed that *BmToll9-1* was expressed in different larval and pupal tissues with the highest expression level detected in the midgut, indicating a possible function in immunity against pathogens taken up by the food. In order to investigate the response of *BmToll9-1*, different ways to deliver dsRNA, specific for GFP (dsGFP), and LPS were applied in *Bombyx* 5th instar larvae. The feeding experiments suggested that dsGFP did not suppress the expression of *BmToll9-1* significantly, while LPS could suppress the expression of *BmToll9-1* after 3 h of feeding. On the other hand, the injection experiments showed that dsGFP, as well as LPS, could significantly inhibit the expression of *BmToll9-1* in 3 h. Bacteria that constantly expressed dsGFP could also down-regulate the expression of *BmToll9-1* to a greater extent than bacteria that do not express dsGFP. The failure of dsGFP by feeding to affect the expression of *BmToll9-1* was correlated with the rapid degradation of dsGFP by dsRNase in the midgut juice. Expression of the RNAi machinery genes *Dcr2* and *Ago2*, as well as *dsRNase*, was also affected by injection of dsRNA and not by feeding, but in these cases an induction was observed instead of a down-regulation.

Because LPS is a well-known pathogen-associated molecular pattern (PAMP), it suggested that the decrease in *BmToll9-1* expression is a consequence of the activation of the innate immune response by LPS. The similar response of *BmToll9-1* between the two triggers, LPS and dsRNA, suggests that dsRNA can also act as a PAMP in the midgut of *Bombyx*. Furthermore, induction of the genes *Dcr2*, *Ago2* and *dsRNase* may also constitute a defense mechanism against invading dsRNA.

1. Introduction

RNA interference (RNAi) is a cellular process in which exogenous double-stranded RNA (dsRNA) molecules can recognize and degrade the complementary endogenous messenger RNA (mRNA), resulting in specific gene silencing. This mechanism is an evolutionary conserved defense response against virus infection or dsRNA structure molecules, but virus or dsRNA could also trigger other pathways in the immune response besides RNAi (Merkling and van Rij, 2012).

The innate immune system is the first and only host defense line in insects due to the lack of the adaptive immune system (Cheng et al., 2008). In this process, Toll receptors play a key role in the innate immunity (Takeda and Akira, 2005). However, it remains unknown whether dsRNA could also be recognized as 'foreign' and interact with immunity-related Toll receptors in insects.

In the insect Toll signaling pathway, Spätzle is the only ligand for the Toll receptors that has been identified so far (LeMosy et al., 1999). Spätzle is present in *Drosophila* (six genes), *Anopheles* (six genes), *Apis* (two genes) and *Bombyx* (three genes) (Tanaka et al., 2008). In *Drosophila*, the Toll signaling pathway was activated by Gram-positive bacteria or fungi (Merkling and van Rij, 2012). Then Spätzle binds to the extracellular domain of the Toll receptor (Arnot et al., 2010). The activated Toll receptor binds to

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the adaptor protein Myeloid differentiation factor 88 (MyD88) via intracellular TIR domains (Horng and Medzhitov, 2001). Upon this interaction, Tube and Pelle are recruited to form a complex through the death domain (Moncrieffe et al., 2008), which in turn leads to the phosphorylation of the IκB factor, Cactus (Wu and Anderson, 1998). After degradation of Cactus, the translocation of the NF-κB transcription factors, Dorsal and Dif, from cytoplasm into the nucleus leads to activation of antimicrobial peptide genes (AMPs), which are highly expressed in fat body and secreted into the hemolymph (Reichhart et al., 1993).

In mammalian genomes, at least 11 Toll-like receptors (TLRs) have been identified (Takeda and Akira, 2005). These mammalian TLRs are typical pattern recognition receptors (PRRs) which are able to recognize their specific pathogen-associated molecular patterns (PAMPs) in the immune responses. In contrast to insect Toll receptors, different pathogen-associated ligands could be identified for mammalian TLRs (Takeda and Akira, 2004), for instance TLR3 is an endolysosome receptor for dsRNA (Alexopoulou et al., 2001) and it has been reported that it can interfere with siRNA mediating gene suppression (Kariko et al., 2004).

In the silkmoth *Bombyx mori*, 14 Toll-related genes were identified (Tanaka et al., 2008). Of these 14 Toll-related genes, *BmToll9-1* and *BmToll9-2* are most closely related to *HsTLR3* (Fig. S1A), raising the question whether these Toll9 receptors could also interact with dsRNA. Because of the dominant expression of *BmToll9-1* in the midgut (Fig. 1) and the more close evolutionary relationship between *BmToll9-1* and *HsTLR3* than other *Bombyx* Toll receptors (Fig. S1B), we decided to focus on *BmToll9-1*.

In this article, the expression profiles of the 14 Toll-related genes were determined in silkmoth 5th instar larvae and pupae as well as the silkmoth-derived Bm5 cell line. Different ways of dsRNA delivery were applied in the *Bombyx* larvae, studying the possible effects of dsRNA on *BmToll9-1* expression. Because lipopolysaccharide (LPS) was proven to affect expression of other Toll receptors, such as *BmToll7-2* (Imamura and Yamakawa, 2002), application of LPS was also performed. The persistence of dsGFP was tested in hemolymph and midgut extracts to explain the absence of effects of dsRNA on *BmToll9-1* expression by feeding in the larvae. The expression of two RNAi machinery genes, *BmDcr2* and *BmAgo2*, were also tested after dsRNA treatment.

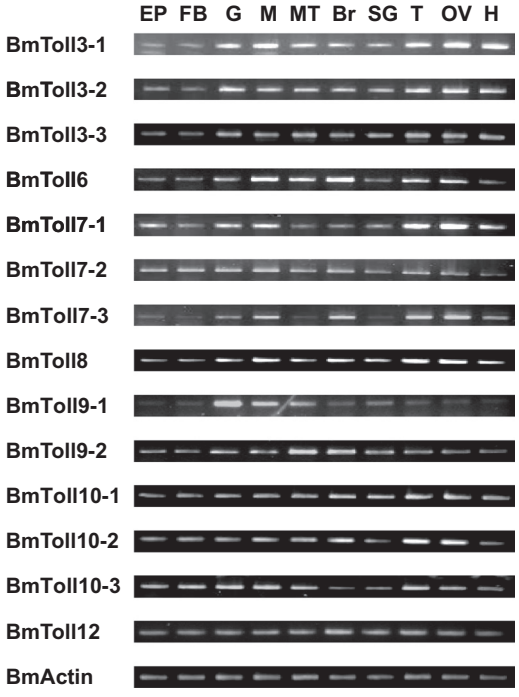

Fig. 1. *In vivo* expression pattern study of *BmToll* mRNAs in different larval tissues. Fragments were obtained after 35 cycles of PCR. Larval tissues, epidermis (EP), fat body (FB), midgut (G), muscle (M), Malpighian tubules (MT), brain (BR), silk gland (SG), testis (T), ovaries (OV) and hemocytes (H), were collected from 5th instar larvae at day 4–5 after the molt.

2. Materials and methods

2.1. Experimental animals

The larvae of *B. mori*, Daizo strain, were reared on artificial diet (Yakuruto Co., Japan) at 25 °C under a photoperiod of 12 h light and 12 h dark. Larval tissues were dissected from 5th instar larvae at day 4-5 after the molt. Tissues from 3 to 10 larvae were collected in eppendorf tubes on ice and samples were frozen at −70 °C until further processing for RNA extraction. To isolate hemocytes, hemolymph was collected on ice after cutting the first proleg and subsequently centrifuged at low speed (800×*g*) at 4 °C to collect the cell pellet. The following tissues were used in the subsequent experiments: epidermis, fat body, midgut, thoracic muscles, Malpighian tubules, brain, silk glands, testis, ovaries and hemocytes.

2.2. RNA extraction and reverse-transcription PCR (RT-PCR)

Frozen tissues were homogenized in TRI Reagent (Sigma) and total RNA was extracted according to the manufacturer's protocol. The quantity of extracted RNA was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and/or by electrophoresis on 1% (w/v) agarose gels. First-strand complementary DNA (cDNA) synthesis was performed using a SuperScript II reverse transcriptase (Invitrogen). Detailed RNA extraction and RT-PCR reactions were described by (Machado et al., 2007).

Primers initially used for detection of mRNA of *BmToll* receptors by PCR are listed in Table 1. Template cDNA was denatured at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 40 s for each cycle. Control PCRs employed primers to

Table 1
Primer sets used for mRNA amplification of *BmToll* receptors.

Genes	Primer pair	Product size (bp)
BmToll3-1	CGAGCACCTCTTGCGACTTGACC (forward) CGATAACCATCTACGGGGGGGAG (reverse)	457
BmToll3-2	GCAGTGATGGTCGCAGTTAC (forward) GTTCCAGAGCAGGTAGGTGTT (reverse)	483
BmToll3-3	CGTGGCTGCATCACTCTGTTT (forward) GTCGATCCTGGCGTCGTTAGT (reverse)	466
BmToll6	CGTTACTGTTGATAGCCCTGTG (forward) AACTGTCTATCACCCCATTCG (reverse)	541
BmToll7-1	ACAACCTTCCGTTATTGGCGT (forward) CCGTTTCTCCTTCCATGTTATCTC (reverse)	536
BmToll7-2	TTCCCATGATGGTTTCAACTC (forward) TATATACCTGAGACGCTCCCAG (reverse)	572
BmToll7-3	TGCCTATGATGGTAACGACTC (forward) TTTCAAGTACGGCCTTAAATC (reverse)	515
BmToll8	CATACCTCTTCTTATCGCAACAC (forward) GTGCAAACTTCAACTTCTCCC (reverse)	554
BmToll9-1	TTGTGCGTCGTTTGCTTCGG (forward) TGGAGGCAGACGCTGATGTT (reverse)	375
BmToll9-2	CGTTGCGATGCCTGATG (forward) CACCATTGGGATTTAGCA (reverse)	430
BmToll10-1	CGCCTTCGGACTTTCTTTGC (forward) ATGAAGCCCGTATCTTTGGT (reverse)	672
BmToll10-2	TCAGCGTTTACTTTGCCTCA (forward) GAAAGCAAGAACAGCCCTCA (reverse)	331
BmToll10-3	CTGTAGATGGAAACAACTGG (forward) GCACTCACTAAACTATCACCC (reverse)	556
BmToll12	GACGACAGCCTACAGCAA (forward) GGTGAGTCCCTAAGTAACAG (reverse)	308