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Peptidoglycan recognition protein genes and their roles in the innate immune pathways of the red flour beetle, *Tribolium castaneum*

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ABSTRACT

We have previously demonstrated that the functional Toll and IMD innate immune pathways indeed exist in the model beetle, *Tribolium castaneum* while the beetle's pathways have broader specificity in terms of microbial activation than that of *Drosophila*. To elucidate the molecular basis of this broad microbial activation, we here focused on potential upstream sensors of the *T. castaneum* innate immune pathways, peptidoglycan recognition proteins (PGRPs). Our phenotype analyses utilizing RNA interference-based comprehensive gene knockdown followed by bacterial challenge suggested: PGRP-LA functions as a pivotal sensor of the IMD pathway for both Gram-negative and Gram-positive bacteria; PGRP-LC acts as an IMD pathway-associated sensor mainly for Gram-negative bacteria; PGRP-LE also has some roles in Gram-negative bacterial recognition of the IMD pathway. On the other hand, we did not obtain clear phenotype changes by gene knockdown of short-type PGRP genes, probably because of highly inducible nature of these genes. Our results may collectively account for the promiscuous bacterial activation of the *T. castaneum* innate immune pathways at least in part.

1. Introduction

One of the hallmarks of insect innate immune response is a massive synthesis of antimicrobial peptides (AMPs) by the fat body, which is functionally homologous to mammalian liver, and the AMPs are secreted into the blood at high concentrations, where they kill invading microbes (Hultmark, 2003; Lemaitre and Hoffmann, 2007). In *Drosophila melanogaster*, this systemic response has been well-established to be mediated by two distinct, NF-κB transcription factor-dependent signaling pathways, namely the Toll and IMD pathways (Kleino and Silverman, 2014; Lemaitre and Hoffmann, 2007; Valanne et al., 2011). Pathogen-associated molecular patterns (PAMPs), for example peptidoglycans, are recognized by a pathway-specific set of pattern-recognition receptors known as peptidoglycan recognition proteins (PGRP) (Dziarski and Gupta, 2006; Royet et al., 2011; Werner et al., 2000).

PGRPs, which function as a sensor, signal transducer and effector molecule in immune systems, are characterized by the occurrence of at least one PGRP domain that is structurally related to bacteriophage and bacterial type 2 amidases (Dziarski and Gupta, 2006; Royet et al., 2011). PGRP genes are generally classified based on the length of corresponding mRNA into either PGRP-L (long-type) or PGRP-S (short-type) class, rather than on the polypeptide length, and *Drosophila* thereby has six PGRP-L class genes and seven PGRP-S class genes, which generate more protein variants through alternative splicing (Werner et al., 2000). Lys-type peptidoglycan found in most Gram-positive bacteria is detected in circulation primarily by PGRP-SA associated with another class protein, Gram-negative binding protein 1 (GNBP1) named so by mistake, as well as by PGRP-SD (Bischoff et al., 2004; Gobert et al., 2003; Wang et al., 2006), while fungal β-glucan is sensed by GNBP3 (Gottar et al., 2006). Recognition of Gram-positive Lys-type peptidoglycan as well as fungal β-glucan by these soluble receptor complexes activates the Toll pathway, ultimately regulating the expression of its target genes (De Gregorio et al., 2002). Meanwhile, *meso*-diaminopimelic acid (DAP)-type peptidoglycan associated with Gram-negative bacteria and Gram-positive bacilli is preferentially recognized by a membrane-bound receptor

Abbreviations: PGRP, peptidoglycan recognition protein; AMP, antimicrobial peptide; PAMP, pathogen-associated molecular patterns; GNBP, Gram-negative binding protein; DAP, *meso*-diaminopimelic acid; qRT-PCR, real-time quantitative RT-PCR; Att1, attacin1; Att2, attacin2; Att3, attacin3; Cec2, cecropin2; Cec3, cecropin3; Col1, coleoptericin1; Def1, defensin1; Def2, defensin2; Def3, defensin3; RPL32, ribosomal protein L32; RNAi, RNA interference; dsRNA, double strand RNA; malE, maltose binding protein E; RHIM, receptor-interacting protein homeotypic interaction motif.

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PGRP-LC, which leads to the activation of the IMD pathway and its target genes (Choe et al., 2005, 2002; De Gregorio et al., 2002; Kleino and Silverman, 2014). In addition, PGRP-LE has been shown to be involved in DAP-type peptidoglycan recognition. The full-length version senses intracellular occurrence of monomeric peptidoglycan, and the truncated, extracellular version enhances the detection of polymeric peptidoglycan by PGRP-LC (Kaneko et al., 2006; Yano et al., 2008). Whilst these 'sensor' PGRPs have lost enzymatic activities, some other PGRPs such as PGRP-SC1 and PGRP-LB retain *N*-acetylmuramoyl-L-alanine amidase activities, thereby supposed to function as scavengers to prevent excessive immune responses (Kim et al., 2003; Mellroth et al., 2003).

PGRP was first identified and purified from the hemolymph of silkworm *Bombyx mori* as a sensor protein upstream of the prophenoloxidase activating cascade (Yoshida et al., 1996). This discovery eventually led to the identification and characterization of *Drosophila* PGRP genes (Werner et al., 2000) as well as to the flowering of studies on the *Drosophila* Toll and IMD pathways mentioned above, whereas the functions of PGRPs in non-*Drosophila* insects innate immune pathways have not yet been fully defined partly because the two immune pathways have yet to be characterized clearly in non-*Drosophila* insect systems. In the meantime, Lee and his coworkers, mainly by utilizing reconstitution systems of coleopteran *Tenebrio molitor* immune-related components, have demonstrated the possible mechanisms for extracellular microbial recognition by short-type PGRPs, which is followed by the activation of both the Toll ligand spätzle and prophenoloxidase (Kan et al., 2008; Kim et al., 2008; Park et al., 2007). They have also reported that the Toll signaling activation occurs in *T. molitor* by both Lys-type and DAP-type peptidoglycan (Yu et al., 2010). Contrastingly, the IMD pathway in *T. molitor* has not yet been defined clearly.

The information on roles of PGRP in the Toll or IMD pathway activation in non-*Drosophila* insect species is in this way relatively scarce, but such knowledge is indeed needed from the viewpoints of comparative immunology as well as insect control. In recent years, RNA interference (RNAi)-based technologies for controlling herbivorous insect pests by using transgenic plants expressing double strand RNA (dsRNA) as well as auxiliary factors have seemed to become promising (Baum et al., 2007; Mao et al., 2007, 2013). In this context, therefore, well-characterized immune-related insect genes might provide good targets for such approaches in conjunction with the use of entomopathogenic microbes. The model beetle *Tribolium castaneum* is a serious, world-wide pest damaging grain and stored food (Campbell et al., 2010). The immunity-related genes of *T. castaneum* have been annotated (Zou et al., 2007), and its RNAi-friendly nature provides an excellent system to study the function of genes of interest (Tomoyasu et al., 2008). In a previous paper, we have characterized all the nine of AMP genes in the genome of *T. castaneum* and classified them into IMD-dependent group I, Toll-dependent group III and co-dependent group II besides non-inducible group IV by employing microbial challenge combined with knockdown of the intracellular adaptor protein gene representative of either the Toll or IMD pathway (Yokoi et al., 2012b). In addition to the microbial activation, some *Tribolium* AMP genes are reportedly induced by environmental stress, such as starvation, while some of its stress-related genes are up-regulated conversely by immune challenge (Altincicek et al., 2008; Freitak et al., 2012). We have subsequently demonstrated that the linkage of NF-κB transcription factor classes, namely Dif and Rel, to the Toll and IMD pathways found in *Drosophila* holds true as well in *T. castaneum*, and that potential Dif- and Rel-response elements occur in promoter regions of Toll- and Rel-target AMP genes, respectively (Yokoi et al., 2012a). In these studies, we have noted that the two immune pathways of the beetle are concomitantly activated by all the microbial species that we used and that this promiscuous activation may represent one of distinctive features of the *Tribolium* innate immune pathways when compared to those in *Drosophila*. The promiscuous activation might be mediated through promiscuous pathogen recognition by upstream sensing modules of the *T. castaneum* Toll and IMD pathways. According to the annotation work (Zou et al., 2007), *T. castaneum* has five long-type and two short-type PGRP genes in the genome. Here, we focused on *T. castaneum* PGRP genes and examined the respective knockdown phenotypes in terms of the bacterial activation of the innate immune pathways.

2. Materials and methods

2.1. Insect rearing

T. castaneum was reared in whole wheat flour in the dark at 30 °C, and staged pupae prepared as in a previous paper (Yokoi et al., 2012b).

2.2. Bacteria and injection into insects

Escherichia coli DH5α (Gram-negative), *Micrococcus luteus* ATCC4698 (Gram-positive), *Enterobacter cloacae* (Gram-negative) and *Bacillus subtilis* (a Gram-positive bacillus bearing DAP-type peptidoglycan) were used in this study. Suspensions of heat-killed *E. coli* and *M. luteus* as well as those of live *E. cloacae* and *B. subtilis* were prepared as described in Yokoi et al. (2012a). Fifty nanoliters of heat-killed *E. coli* or *M. luteus* suspensions were injected into wild-type or dsRNA-treated day 3 pupae using a Nanoject II (Drummond Scientific Company) to evoke immune responses. Suspensions of live *E. cloacae* and *B. subtilis* were injected in a similar fashion, and the following survival assays performed (see Section 2.6). *E. cloacae* and *B. subtilis* are the generous gifts from Dr. Y. Yagi of Nagoya University. *M. luteus* ATCC4698 was provided by RIKEN Bioresource Center in Japan.

2.3. Sequences of genes and primers

The following *T. castaneum* gene sequences were retrieved from the Beetlebase (http://www.beetelebase.org) and utilized in this study: *PGRP-LA* (GLEAN_02789); *PGRP-LB* (GLEAN_15689); *PGRP-LC* (GLEAN_02790); *PGRP-LD* (GLEAN_02546); *PGRP-LE* (GLEAN_10508); *PGRP-SA* (GLEAN_10611); *PGRP-SB* (GLEAN_13620). The sequences of primer pairs used for real-time quantitative RT-PCR (qRT-PCR) of PGRP genes are listed in Table 1. The primer sequences for the following genes can be found in Yokoi et al. (2012b): *Attacin1* (*Att1*) (GLEAN_07737); *Attacin2* (*Att2*) (GLEAN_07738); *Attacin3* (*Att3*) (GLEAN_07739); *Cecropin2* (*Cec2*) (GLEAN_00499); *Cecropin3* (*Cec3*) (GLEAN_00500); *Coleoptericin1* (*Col1*) (GLEAN_05093); *Defensin1* (*Def1*) (GLEAN_06250); *Defensin2* (*Def2*) (GLEAN_10517); *Defensin3* (*Def3*) (GLEAN_12469); a normalizer gene for qRT-PCR analyses, *ribosomal protein L32* (*RPL32*) (GLEAN_06106).

2.4. RNA extraction and qRT-PCR

Total RNA was extracted from the whole body of *T. castaneum* using TRIZOL reagent (Invitrogen). Spectrophotometric scanning of RNA preparations showed that the ratios of A260/A280 and A260/A230 were always above 1.7 and 2.0, respectively. Since only a portion of primer pairs used for the qRT-PCR analyses, namely those of *Att1*, *Att3*, *Cec2*, *Def3* and *RPL32*, span exon-intron boundaries, we used a PrimeScript RT Reagent Kit with gDNA Eraser