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# Activation of immune-associated phospholipase $A_2$ is functionally linked to Toll/Imd signal pathways in the red flour beetle, *Tribolium castaneum*

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ABSTRACT

Bacterial challenge enhances phospholipase $A_2$ ($PLA_2$) activity, which in turn induces biosynthesis of various eicosanoids that mediate non-self recognition signal to immune effectors in insects. But, there is little information on how $PLA_2$ activity is controlled after the non-self recognition. A recent genome analysis of the red flour beetle, *Tribolium castaneum*, has annotated both Toll and Imd signal pathways that are presumably considered to specifically respond to different microbial infections to express specific antimicrobial peptides (AMPs). This study set up a hypothesis that $PLA_2$ activation is linked to Toll and Imd pathways in *T. castaneum*. Bacterial challenge to the larvae of *T. castaneum* induced expressions of Toll and Imd genes. Different AMP genes were induced in larvae infected with Gram-positive or -negative bacteria. RNA interference using double-stranded RNAs (dsRNAs) specific to different Toll and Imd genes showed differential inhibition of these AMP expressions, indicating that the Toll and Imd pathways play critical roles in the production of AMPs by specifically responding to various bacterial challenges in *T. castaneum*. These Toll and Imd immune signals are also linked to the activation of $PLA_2$ in *T. castaneum*. Activation of $PLA_2$ was significantly induced in response to bacterial challenge, but was inhibited by dsRNAs specific to different Toll and Imd genes. The activation of $PLA_2$ via Toll and Imd pathways could be explained by induction of $PLA_2$ gene expression because the dsRNA treatments of Toll and Imd genes suppressed the gene expression of $PLA_2$ in response to bacterial challenge. The functional links were further supported by an immunofluorescence assay of $PLA_2$ intracellular translocation. Upon bacterial challenge, hemocytes from control larvae showed intracellular translocation of their $PLA_2$s near to cell membrane, but hemocytes from larvae treated with dsRNAs of the Toll and Imd genes did not show the translocation, at which the $PLA_2$s appeared to be evenly spread in the cytoplasm. These results suggest that recognition of bacterial challenge initiates Toll and Imd pathways in *T. castaneum*, which subsequently induces the activation of immune-associated $PLA_2$s as well as gene expression of various AMPs.



## 1. Introduction

Insect innate immunity comprises of both cellular and humoral immune responses [1]. Cellular immunity refers to hemocyte-mediated immune responses including phagocytosis, nodulation, and encapsulation processes [2]. By contrast, humoral immune responses include activity of antimicrobial peptides [3], toxic action of reactive intermediates of oxygen and nitrogen [4,5], and plasma melanization by phenoloxidase [6–8]. Innate immune responses initiate with recognition by pattern recognition receptors against pathogen-associated molecule patterns of invaded pathogens [9], such as lipopolysacharides (LPS), peptidoglycans, and β-1,3-glucans [10]. Peptidoglycan recognition proteins (PGRPs) recognizing bacterial cell wall are classified into PGRP-SA, PGRP-LC, and PGRP-LE in *Drosophila melanogaster* [11]. Gram-positive bacteria containing Lys-type peptidoglycans are recognized by PGRP-SA, which activates Toll receptor [12–14]. The intracellular Toll signal pathway induces expressions of specific antimicrobial peptides (AMPs) by its own specific NF-kBs [15,16]. By contrast, PGRP-LC and PGRP-LE are responsible for recognition of bacteria containing diaminopimelate-type peptidoglycan and activate Imd pathway [17–19]. Intracellular Imd signal pathway is further subdivided into activation of NF-kB and JNK [20–22].

Eicosanoids are the oxygenated metabolites of biologically active C20 polyunsaturated fatty acids from arachidonic acid, a catalytic product of phospholipid by phospholipase $A_2$ ($PLA_2$) [23]. Eicosanoids play critical roles in several physiological functions including immune responses [24]. In insects, eicosanoids mediate both cellular and humoral immune responses [25,26] against bacteria, fungi, viruses, and protozoa [27–30]. In response to bacterial infection, eicosanoids mediate microaggregation [31], spreading behavior [32], phagocytosis [33,34], and nodulation

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[35,36]. They also mediate production of AMPs [37,38]. Recently, four immune-associated $PLA_2$ genes are identified in the red flour beetle, *Tribolium castaneum*, and exhibit significant increase in their activities in response to bacterial challenge [39]. Although it is well understood that non-self recognition in response to bacterial challenge activates Toll or Imd pathway, there is no information to link the recognition signal to $PLA_2$ activation. This study demonstrated that Toll and Imd pathways play critical roles in mediating immune signals in *T. castaneum* and addressed a hypothesis that these signal pathways are functionally linked to activation of $PLA_2$ to produce eicosanoid, which would propagate the immune signal locally or systematically to immune effectors.

## 2. Materials and methods

### 2.1. Insect rearing and bacteria culture

*T. castaneum* was reared in a dry and dark condition (a relative humidity 60 ± 5%) at room temperature (25 ± 1 °C) with wheat flour (Pareve, USA). Fully grown late instar larvae (≥5 mm) were used in this study. *Xenorhabdus nematophila* K1, *Bacillus subtilis*, *Escherichia coli*, and *Flavobacterium* sp. were cultured in Luria–Bertani (LB) broth at 28 °C with shaking at 200 rpm.

### 2.2. Chemicals

Tris-buffered saline (TBS) was composed of 50 mM Tris–HCl, 100 mM dextrose, 5 mM KCl, 2.5 mM $MgCl_2$, and 50 mM NaCl. For preparing anti-coagulating buffer (ACB), 4 mg of L-cysteine was added to 5 ml of TBS and adjusted to pH 7.5 using 0.1N NaOH. Phosphate-buffered saline (PBS) was composed of 100 mM $Na_2HPO_4{\cdot}12H_2O$, 18 mM $KH_2PO_4$, 138 mM NaCl, and 28 mM KCl, and then adjusted to pH 7.4. Arachidonic acid [5,8,11,14-eicosatetraenoic acid] and $PGE_2$ [[5Z,11α,13E,15S]-11,15-dihydroxy-9-oxoprosta-5,13-dienoic acid] were purchased from Sigma–Aldrich Korea (Seoul, Korea).

### 2.3. Bacterial challenge and RNA extraction

Overnight cultured bacteria were washed and resuspended with sterile PBS in $5 \times 10^9$ cells/ml. The bacterial cells were injected into full grown larvae of *T. castaneum* with a nanoliter injector (WPI, Sarasota, USA) under a stereomicroscope (S730, Olympus, Japan). After incubation at room temperature for 8 h, the larvae were sterilized with 70% ethanol and used for RNA extraction. Total RNA was extracted using Trizol reagent (MRC, OH, USA) according to manufacturer's instruction.

### 2.4. Immune-associated genes and RT-PCR

A previous study [40] has annotated genes associated with immune responses of *T. castaneum*, in which four Toll genes (Toll 1, GLEAN00176; Toll 2, GLEAN04452; Toll 3, GLEAN04438; and Toll 4, GLEAN04439), one Imd gene (GLEAN10851), and thirteen genes of putative AMPs (cecropin 2, GLEAN00499; cecropin 3, GLEAN00500; attacin 1, GLEAN07737; attacin 2, GLEAN07738; attacin 3, GLEAN07739; lysozyme 1, GLEAN10349; lysozyme 2, GLEAN10350; lysozyme 3, GLEAN10351; lysozyme 4, GLEAN10352; defensin 1, GLEAN06250; defensin 2, GLEAN10517; defensin 3, GLEAN12469 and coleoptericin 1, GLEAN05093) were retrieved from the beetlebase (http://www.beetlebase.org).

**Table 1**
Primer sequences for RT-PCR analysis of one Imd, four Toll and thirteen antimicrobial peptide genes of *Tribolium castaneum*.

| Genes[a] | Primer sequences | Annealing temperature (°C) | Expected size (bp) |
|---|---|---|---|
| Toll 1 | 5′-CGTTGGTACCCAGATTGGAATCAG-3′<br>5′-TACGTCCAGTTTCCGATGAGGT-3′ | 46.0 | 364 |
| Toll 2 | 5′-GGATGTTTAGTCGTCGCG-3′<br>5′-CACTATTAACATATCTTCCTCCTCTTG-3′ | 48.0 | 301 |
| Toll 3 | 5′-ACACGCAAGCCATGACCGAA-3′<br>5′-GAGACCAGTCGAAACCAGCAA-3′ | 58.0 | 408 |
| Toll 4 | 5′-AAGCCATGACCGAAGGACGG-3′<br>5′-AAAACCAGTCGAGCCCCAA-3′ | 58.0 | 389 |
| Imd | 5′-ATGTCAGACCAGAATAACTGTGAT-3′<br>5′-GCTGGTTTGTGTCCCACAAC-3′ | 47.0 | 550 |
| Cecropin-2 | 5′-ATGTCTACCAAAATTTTCGTTCT-3′<br>5′-GTATCCATAGTTGTGTACGGG-3′ | 46.0 | 279 |
| Cecropin-3 | 5′-ATGAGCACCAAACTTTTTGTTCTT-3′<br>5′-CTCGATTTCTTCGATATTGTTCG-3′ | 51.4 | 270 |
| Attacin-1 | 5′-ATGAACAAAATCATCACCTTTACA-3′<br>5′-GAAGCGGTGGCTAAACTGGACGCCT-3′ | 56.0 | 495 |
| Attacin-2 | 5′-ATGCAAAAAATCGTAATTTTTGCAC-3′<br>5′-AAACCTTCCTCGGTCGAAGCCTCC-3′ | 60.0 | 435 |
| Attacin-3 | 5′-ATGAAGCTTTTCCTCATTTTACTG-3′<br>5′-GAGCGTTGTTTTGACCACTGCACCC-3′ | 51.0 | 444 |
| Lysozyme-1 | 5′-ATGGCAGAACTCTTCGTGCT-3′<br>5′-ATTCAGACTGGTCTGCACCG-3′ | 50.0 | 780 |
| Lysozyme-2 | 5′-ATGAGTCCTTTGAGCAAAATAC-3′<br>5′-AAAGCATCCTTTGATGAAATG-3′ | 46.0 | 441 |
| Lysozyme-3 | 5′-ATGAATCCTTTGAGCAAATTACTT-3′<br>5′-GAAGCATCCTTTAATGAACTTG-3′ | 47.5 | 444 |
| Lysozyme-4 | 5′-ATGAAACTTCTCGCTGTGGTTGC-3′<br>5′-ACATCCAGCAATCCACCC-3′ | 49.4 | 426 |
| Defensin-1 | 5′-GCAAGTCACGACCCCCTTATC-3′<br>5′-TTTACGACAAACACAGACAGC-3′ | 54.0 | 320 |
| Defensin-2 | 5′-ATGAAGCTACTCATTGTCGCTCT-3′<br>5′-ATTGCGACAAACACAGATGGC-3′ | 51.4 | 237 |
| Defensin-3 | 5′-ATGAAGTTTATCGTAATTTTTGT-3′<br>5′-ATTCCTGCAGACACAAATTCTTC-3′ | 47.0 | 249 |
| Coleotericin-1 | 5′-ATGTTCAGAACCAGTTTCTATTTGG-3′<br>5′-CCACCTGTAGGTTCCACCC-3′ | 47.5 | 423 |

[a] Annonated by Zou et al. [40].