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Cite this article: Nishide Y, Kageyama D, Yokoi K, Jouraku A, Tanaka H, Futahashi R, Fukatsu T. 2019 Functional crosstalk across IMD and Toll pathways: insight into the evolution of incomplete immune cascades. *Proc. R. Soc. B* **286**: 20182207.
http://dx.doi.org/10.1098/rspb.2018.2207

Received: 30 September 2018
Accepted: 5 December 2018

Subject Category:
Evolution

Subject Areas:
evolution, immunology

Keywords:
insect immunity, innate immunity, antimicrobial peptide, IMD pathway, *Plautia stali*

Authors for correspondence:
Yudai Nishide
e-mail: nishiyu0@affrc.go.jp
Daisuke Kageyama
e-mail: kagymad@affrc.go.jp
Takema Fukatsu
e-mail: t-fukatsu@aist.go.jp

Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.4391378.

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Functional crosstalk across IMD and Toll pathways: insight into the evolution of incomplete immune cascades

Yudai Nishide[1], Daisuke Kageyama[1], Kakeru Yokoi[1], Akiya Jouraku[1], Hiromitsu Tanaka[1], Ryo Futahashi[2] and Takema Fukatsu[2,3,4]

[1]Institute of Agrobiological Sciences, National Agriculture and Food Research Organization (NARO), Ohwashi, Tsukuba 305-8634, Japan
[2]National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan
[3]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Tokyo 113-0033, Japan
[4]Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan

YN, 0000-0002-5978-3367; DK, 0000-0002-9026-9825; RF, 0000-0003-4791-7054; TF, 0000-0001-5987-2602

In insects, antimicrobial humoral immunity is governed by two distinct gene cascades, IMD pathway mainly targeting Gram-negative bacteria and Toll pathway preferentially targeting Gram-positive bacteria, which are widely conserved among diverse metazoans. However, recent genomic studies uncovered that IMD pathway is exceptionally absent in some hemipteran lineages like aphids and assassin bugs. How the apparently incomplete immune pathways have evolved with functionality is of interest. Here we report the discovery that, in the hemipteran stinkbug *Plautia stali*, both IMD and Toll pathways are present but their functional differentiation is blurred. Injection of Gram-negative bacteria and Gram-positive bacteria upregulated effector genes of both pathways. Notably, RNAi experiments unveiled significant functional permeation and crosstalk between IMD and Toll pathways: RNAi of IMD pathway genes suppressed upregulation of effector molecules of both pathways, where the suppression was more remarkable for IMD effectors; and RNAi of Toll pathway genes reduced upregulation of effector molecules of both pathways, where the suppression was more conspicuous for Toll effectors. These results suggest the possibility that, in hemipterans and other arthropods, IMD and Toll pathways are intertwined to target wider and overlapping arrays of microbes, which might have predisposed and facilitated the evolution of incomplete immune pathways.

1. Introduction

The innate immune system is highly conserved among metazoans, which enables prompt defense against microbial intruders [1,2]. In insects, the innate immunity is often classified into three categories: physical, cellular and humoral [3]. The humoral immunity rapidly and transiently activates immune genes upon microbial infections and elicits the release of an array of antimicrobial peptides (AMPs) to haemolymph [4]. Humoral immunity is triggered by sensing of microbial infections via interactions of peptidoglycan recognition proteins (PGRP) and Gram-negative binding proteins (GNBP) with microbial surface molecules [2]. In general, each of the recognition proteins induces subsequent reactions of one of the two signalling pathways, namely immune deficiency (IMD) pathway, which is preferentially activated by Gram-negative bacteria, and Toll pathway, which is preferentially activated by Gram-positive bacteria and fungi. In the fruit fly *Drosophila melanogaster*, most of the AMPs are preferentially activated by either IMD pathway or Toll pathway, but exceptionally, some AMPs (such as Drosomycin) are activated by both IMD and Toll pathways [5,6]. Despite the presence of some minor crosstalk, the two immune pathways are generally recognized as independent.

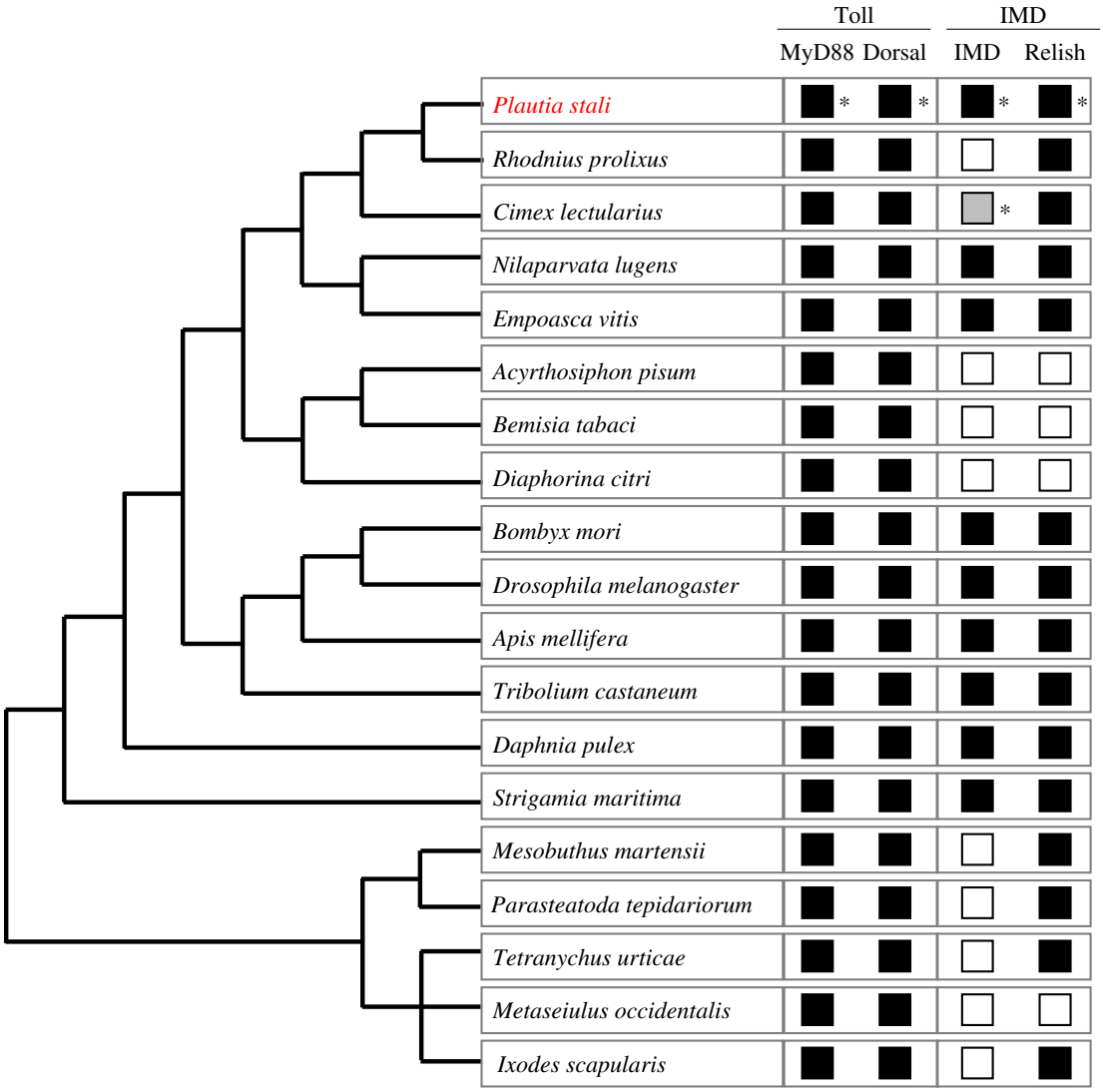

Figure 1. A schematic phylogenetic tree of arthropods on which presence/absence of humoral immunity-related genes is mapped. Black and white squares indicate the presence and absence of humoral immunity-related genes, respectively. There is a grey square that indicates uncertainty (see text for details). The phylogeny was drawn on the basis of Misof *et al*. [23], Kolokotronis *et al*. [24], Sharma *et al*. [25] and Palmer & Jiggins [20]. Presence/absence of the genes were based on Mesquita *et al*. [15] for *R. prolixus*, Benoit *et al*. [16] and Zumaya-Estrada *et al*. [17] for *C. lectularius*, Bao *et al*. [18] for *N. lugens*, Shao *et al*. [26] for *E. vitis*, Gerardo *et al*. [14] for *A. pisum*, Zhang *et al*. [27] for *B. tabaci*, Arp *et al*. [28] for *D. citri*, Sackton *et al*. [9] for *D. melanogaster*, Tanaka *et al*. [11] for *B. mori*, Evans *et al*. [12] for *A. mellifera*, Zou *et al*. [13] for *T. castaneum*, McTaggart *et al*. [19] for *D. pulex*, Palmer & Jiggins [20] for *S. maritima*, *P. tepidariorum* and *M. occidentalis*, Cao *et al*. [21] for *M. martensii*, Grbíc *et al*. [29] for *T. urticae* and Smith & Pal [22] for *I. scapularis*. Squares with asterisks indicate the results of this study (see Results and discussion). (Online version in colour.)

Genome-wide analyses have shown that IMD pathway and Toll pathway are both highly conserved in holometabolous insects including the fruit flies *Drosophila* spp. [7–9], the mosquito *Anopheles gambiae* [10], the silkmoth *Bombyx mori* [11], the honeybee *Apis mellifera* [12] and the flour beetle *Tribolium castaneum* [13]. These immune pathways correspond to the mammalian signalling pathways of interleukin-1 receptor and tumour necrosis factor receptor [14]. Hence, it is presumed that primitive forms of IMD pathway and Toll pathway must have arisen in the common ancestor of arthropods and vertebrates.

However, in some insects belonging to the Hemiptera, recent studies uncovered the absence or incompleteness of IMD pathway. The pea aphid *Acyrthosiphon pisum* lacks all IMD pathway genes including PGRP-encoding gene, *Imd* and *Relish* [14] and the assassin bug *Rhodnius prolixus* lacks key components of IMD pathway-like *Imd* but retains *Relish* [15]. Lack of *Imd* was also reported from the bedbug *Cimex lectularius* and other assassin bugs *Triatoma pallidipennis*, *T. dimidiata* and *T. infestans* [16,17]. On the other hand, the brown planthopper *Nilaparvata lugens* possesses a conserved IMD pathway [18]. The conserved IMD pathway was also found in non-insect arthropods like the water flea *Daphnia pulex* [19] and the coastal centipede *Strigamia maritima* [20], but the Chinese scorpion *Mesobuthus martensii* and the deer tick *Ixodes scapularis* do not possess *Imd* [21,22] and the western orchard predatory mite *Metaseiulus occidentalis* lacks almost all IMD pathway components including *Imd* and *Relish* [20]. Hence, phylogenetic mapping suggests repeated and independent losses of IMD pathway components in the evolutionary course of arthropods (figure 1), but it has been poorly understood how the erosion of IMD pathway has proceeded.

In this study, we demonstrate that a hemipteran insect, the brown-winged green stinkbug *Plautia stali*, retains both IMD pathway and Toll pathway but their functional differentiation has been blurred, which provides insight into the evolutionary process of incomplete immune pathways in hemipteran insects and other arthropods.

2. Material and methods

(a) Insects

Adult insects of *P. stali*, collected at the forest edge in the National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, were used to establish an inbred strain. A mass-reared colony of the strain was used as the source of the experimental insects. The insects were reared in plastic containers (150 mm in diameter, 60 mm high) supplied with raw peanuts, dry soya beans and water supplemented with 0.05% ascorbic acid at 25°C ± 1°C under a long-day regime of 16 L : 8 D dark following the previous study [30].

(b) RNA-seq analysis

Total RNAs were extracted from fat body, midgut or whole body of adult females approximately 5 days after ecdysis using RNeasy Mini Kit (Qiagen, Hilden, Germany). To efficiently obtain humoral immunity-related genes, RNAs were also extracted from fat body of adult insects injected with Gram-negative *Escherichia coli* or Gram-positive *Micrococcus luteus*. The cDNAs were sequenced by Illumina HiSeq 2500 with paired-end 101 bp (Macrogen Japan Corp., Kyoto, Japan), and the generated raw reads (Accession nos. DRR118501–DRR118507; electronic supplementary material, table S1) were cleaned by trimming low-quality regions and adapter sequences using Trimmomatic version 0.36 with minimum length setting as 50 bp [31]. Reference contigs were constructed by *de novo* assembly of all the cleaned paired-end reads using Trinity v. 2.4.0 [32]. After automatic assembly, we manually checked and corrected the target gene sequences using the Integrative Genomics Viewer [33]. For annotation of the reference contigs, each contig sequence was compared with NCBI-nr protein database by blastx (*e*-value less than $<1 \times 10^{-3}$), and a summary of the top-hit result was added to each contig. In addition, CDS region of each contig was predicted by TransDecoder v. r20140704 [32], and conserved domains in each predicted amino acid sequence were searched by HMMER v. 3.1b2 [34] using Pfam protein families database [35]. For searching orthologous genes related to Toll and IMD pathways, blastp search using *Drosophila* Toll and IMD genes as query sequences were performed against a database of the predicted amino acid sequences of *P. stali*. For transcript quantification of the reference contigs for each fat body sample, align_and_estimate_abundance.pl script bundled with Trinity v. r20140707 was used for the cleaned reads of each fat body sample with –est_method RSEM, –aln_method bowtie2 and –trinity_mode options, and the calculated expression levels of the reference contigs were normalized to fragments per kilobase per million mapped fragments (FPKM) values with Trimmed mean of *M* values (TMM) normalization among all the fat body samples by abundance_estimate_to_matrix.pl script bundled with Trinity v. r20140707. The normalized FRKM values were used for comparing gene (contig) expression level between control and bacteria-treated fat body samples.

(c) Septic shock experiments

Adult female *P. stali* approximately 5 days after ecdysis were injected by glass capillary tubes (size: 100 µl; Drummond, Alabama, USA). The females (approximately 7.0 mm in thorax width and 0.13 mg in body weight) were injected with *ca* 5 µl of 0.9% saline (Otsuka Pharmaceutical, Tokyo, Japan) or 10^8 µl^{-1} heat-killed bacteria (*E. coli* or *M. luteus*), into the ventral septum between thoracic and abdominal segments. As controls, non-treated females and mock-injected (wounded by glass capillary tubes only) females were used. The fat body was excised 1, 4, 8 or 24 h after injection and total RNA was extracted (see below; electronic supplementary material, figure S1a).

(d) Quantitative RT-PCR

Total RNAs extracted from abdominal fat bodies using TRIzol (Life Technologies Japan Ltd, Tokyo, Japan) were reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies Japan Ltd, Tokyo, Japan). Quantitative RT-PCR of humoral immunity-related genes (PGRP-encoding genes, *GNBPs*, *Imd*, *Relish*, myeloid differentiation protein-88 (*MyD88*), *Dorsal*, *Defensin*, *Hemipterricin* and *Lysozyme*) was conducted with primer pairs designed on the basis of RNA-seq data (electronic supplementary material, table S2) using LightCycler 480 and LightCycler 480 SYBR Green Master (Roche Diagnostics, Tokyo, Japan).

(e) RNAi experiments

To knockdown the mRNA levels of humoral immunity-related genes, adult females approximately 5 days after ecdysis were injected with roughly 5 µl of double-stranded RNA (dsRNA) solution (100 ng µl^{-1}) into the ventral septum between thoracic and abdominal segments. dsRNA was synthesized from a PCR product using primers listed in electronic supplementary material, table S3, and MEGAscript RNAi Kit (Thermo Fisher Inc., Kanagawa, Japan). Three days after dsRNA injection, several individuals were subjected to RNA extraction to examine the effects of RNAi (electronic supplementary material, figure S1b). Other individuals were injected with heat-killed *E. coli* or *M. luteus* 3 days after dsRNA injection, and then, subjected to RNA extraction on the following day (electronic supplementary material, figure S1c). The extracted RNAs were subjected to quantitative RT-PCR (as above) to infer the expression levels of AMPs.

(f) Phylogenetic analysis

The amino acid sequences inferred from PGRP, GNBP and lysozyme genes were aligned by ClustalW [36] and subjected to phylogenetic analyses by the maximum-likelihood method using MEGA7 program [37,38].

3. Results

(a) Genes of *Plautia stali* upregulated by septic shock

Compared with non-treated control, 650 RNA-seq contigs showed 10-fold or higher expression upon injection of heat-killed *E. coli* (a representative of Gram-negative bacteria), whereas 554 contigs showed 10-fold or higher expression upon injection of heat-killed *M. luteus* (a representative of Gram-positive bacteria). Notably, 274 contigs were commonly identified between them (i.e. upregulated by both *E. coli* and *M. luteus*) (electronic supplementary material, table S4).

(b) Immune pathway genes of *Plautia stali*

Blastp searches on transcriptomes of *P. stali* (both septic shocked and non-treated ones) using immunity-related genes of *D. melanogaster* as queries suggested that *P. stali* possesses nearly complete IMD pathway and Toll pathway (figure 2; electronic supplementary material, figure S2).

(i) Pattern recognition molecules

Three *P. stali* orthologues of a peptidoglycan recognition protein (i.e. *PsPGRP-L1a*, *PsPGRP-L1b* and *PsPGRP-L2*) and an orthologue of a lysin motif protein (*PsLysM*), a member of peptidoglycan recognition proteins known to bind to peptidoglycans in plants [39], were found. The three PGRPs in *P. stali* were named as *PsPGRP-L1a*, *PsPGRP-L1b* and *PsPGRP-L2*, respectively, because all of them were

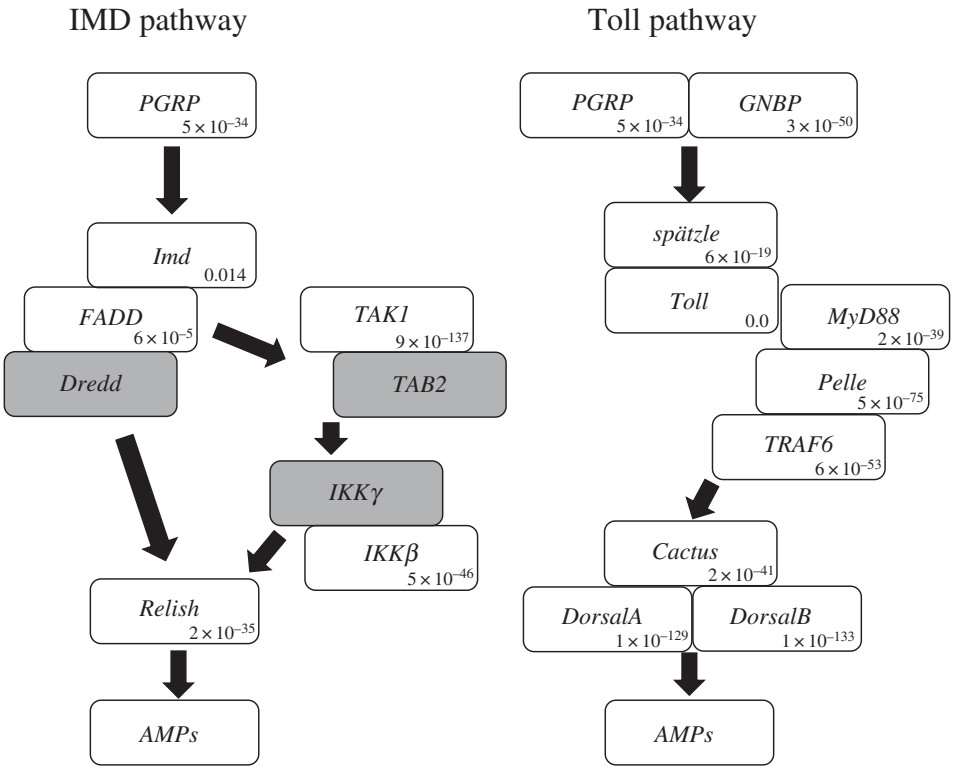

Figure 2. Genes cascades of IMD and Toll pathways. White and grey boxes show presence and presumable absence of the genes in *P. stali*, respectively. The number in each white box is blastp *e*-value of the orthologue in *P. stali* using the amino acid sequence of *D. melanogaster* as a query. For PGRP and GNBP family members, those with lowest *e*-value are shown (For PGRP, *PsPGRP-L1b* and *DmPGRP-LE* exhibit lowest *e*-value (5×10^{-34}); for GNBP, *PsGNBP1* and *DmGNBP1* exhibit lowest *e*-value (3×10^{-50})).

categorized as long type and the two PGRPs (*PsPGRP-L1a* and *PsPGRP-L1b*) were considered as isoforms. Predicted amino acid sequences of *PsPGRP-L1a*, *PsPGRP-L1b* and *PsPGRP-L2* exhibited transmembrane domains and were presumably without amidase activities for scavenging digested peptidoglycan (electronic supplementary material, figures S3–S5). Two orthologues of GNBPs (*PsGNBP1* and *PsGNBP2*) were also found (electronic supplementary material, figure S6).

(ii) Signalling pathway components

Orthologues of all major IMD pathway and Toll pathway components were found in the RNA-seq data (figure 2). However, we failed to detect several IMD component genes such as death-related ced-3/Nedd2-like caspase (Dredd), TAK1-binding protein (TAB2) and IEC-intrinsic IκB kinase (IKKγ), although full genomic sequencing of *P. stali* is needed to verify the absence of these genes. *PsImd* and Fas-associated protein with death domain (*PsFADD*) exhibited relatively low homology values to *DmImd* and *DmFADD*, respectively (*e*-values by blastp were 0.014 and 6×10^{-5}, respectively; note that *PsImd* appeared to be functional in IMD pathway; see below).

(iii) Immune-responsive effectors

As immune-responsive effector genes, two orthologues of defensin (*PsDefensin1* and *PsDefensin2*), one orthologue of hemiptericin (*PsHemiptericin*) and six orthologues of lysozyme (*PsLysozyme b-1*, *PsLysozyme b-2*, *PsLysozyme c-1*, *PsLysozyme c-2*, *PsLysozyme c-3* and *PsLysozyme i-1*) were identified in the RNA-seq data (electronic supplementary material, figure S7).

(c) Expression of immune pathway genes upon septic shock

(i) Pattern recognition molecules

PsPGRP-L1a was significantly upregulated by *M. luteus* 8 h after injection but was not by *E. coli* (×3.4) (figure 3; electronic supplementary material, figure S8). *PsPGRP-L1b* was significantly upregulated not only by *E. coli* (×3.4) and *M. luteus* (×2.7) but also by mock (×2.4) and saline (×3.3) 8 h after injection (figure 3; electronic supplementary material, figure S8). By contrast, *PsPGRP-L2* and *PsLysM* were not affected by the septic shock treatments (figure 3; electronic supplementary material, figure S8). *PsGNBP1* was upregulated by both *E. coli* (×16.0–22.3) and *M. luteus* (×14.2–27.1) 4 and 8 h after injection, whereas *PsGNBP2* was not affected by the septic shock treatments (figure 3; electronic supplementary material, figure S8).

(ii) Signalling pathway components

Among five pathway component genes, only *PsRelish* (an IMD pathway component) was upregulated by both *E. coli* (×4.6–24.5) and *M. luteus* (×3.6–15.5) 1, 4, 8 and 24 h after injection, while the others (i.e. *PsImd*, *PsMyD88*, *PsDorsalA* and *PsDorsalB*) were not affected by the septic shock treatments (figure 3; electronic supplementary material, figure S9).

(iii) Immune-responsive effectors

PsDefensin1 and *PsHemiptericin* were upregulated by both *E. coli* (×8.1–134.5 and ×6.2–166.6) and *M. luteus* (×6.1–169.1 and ×6.9–168.1) 1 h after injection and on, while *Defensin2* was upregulated by *E. coli* (×14.2–96.8) and *M. luteus* (×14.2–60.4) 4 h after injection and on (figure 3; electronic

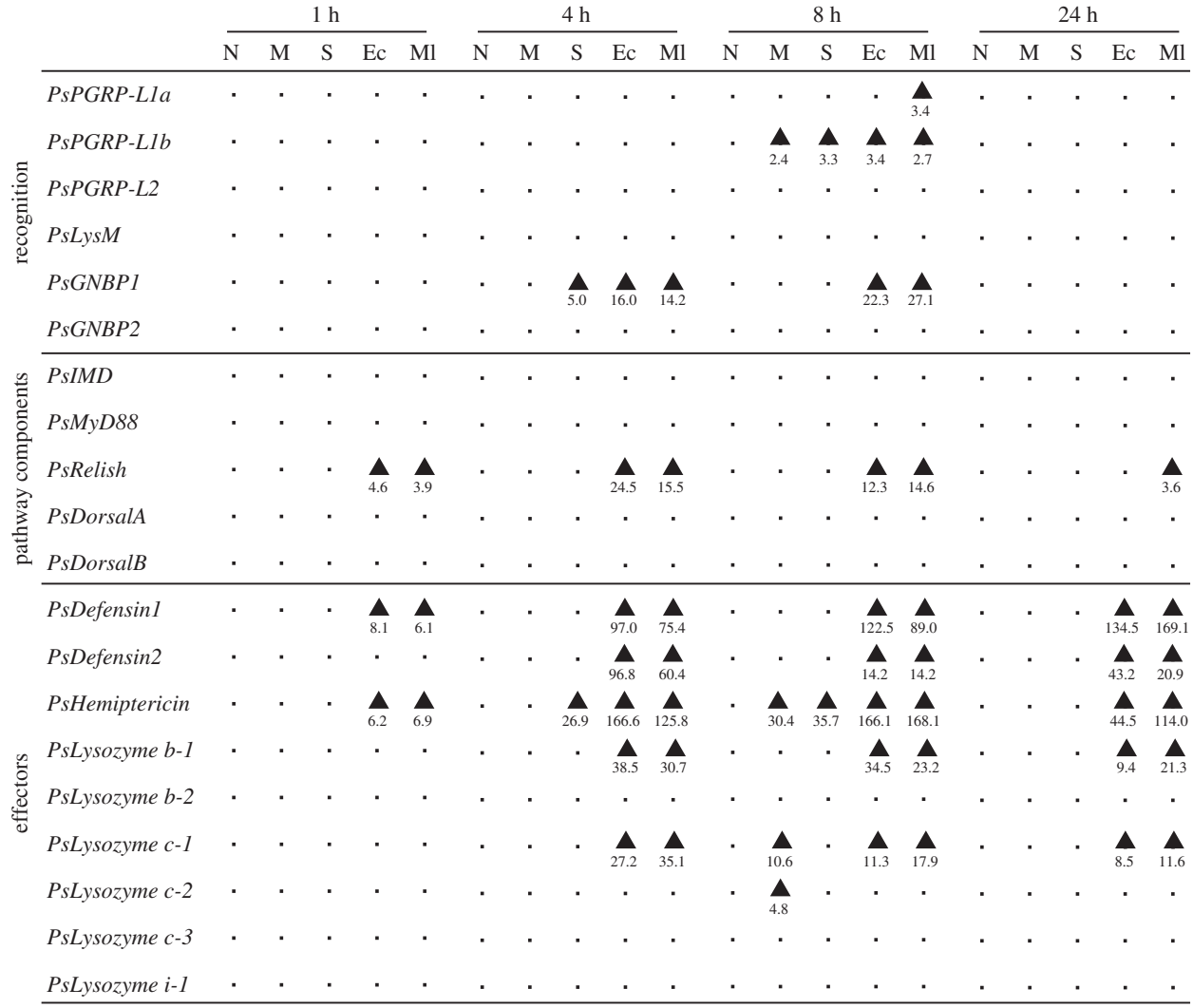

Figure 3. Effects of septic shock treatments on the expression levels of immune pathway genes in *P. stali*. Adult females were subjected to the following treatments: N, no treatment; M, mock injection; S, saline injection; Ec, *E. coli* injection; Ml, *M. luteus* injection. The abdominal fat body was excised 1, 4, 8 and 24 h after injection ($n = 5-8$) and quantitative RT-PCR was performed for the samples. Significantly upregulated cohorts in comparison to non-treated cohorts were shown by arrowheads (Steel-Dwass test, $p < 0.05$). Numbers below the arrowheads indicate the fold-change value for the upregulation compared with non-treated cohorts. Detailed data are shown in electronic supplementary material, figures S7–S10.

supplementary material, figure S10). Among six lysozyme genes, *Lysozyme b-1* and *Lysozyme c-1* were upregulated by both *E. coli* (×9.4–38.5 and ×8.5–27.2) and *M. luteus* (×21.3–30.7 and ×11.6–35.1) 4 h after injection and on, while the remaining four lysozyme genes were not upregulated by the septic shock treatments (figure 3; electronic supplementary material, figure S11).

(d) RNAi-mediated knockdown of immune pathway genes and expression levels of effector genes

In *P. stali*, RNAi generally worked efficiently and stably by injection of dsRNA. For example, injection of *dsPsRelish* into adult insects suppressed the expression levels of *PsRelish* to approximately one-tenth in comparison with the *dsEGFP*-injected control insects during at least 14 days after injection (electronic supplementary material, figure S12). All the other immune component genes we examined, namely *PsImd*, *PsMyD88*, *PsDorsal*, *PsPGRP-L1a*, *PsPGRP-L1b*, *PsPGRP-L2*, *PsLysM*, *PsGNBP1* and *PsGNBP2*, were also suppressed efficiently by RNAi (electronic supplementary material, figures S13–S16).

(i) Which immune pathway controls AMPs and lysozymes?

Blocking of IMD pathway by RNAi of *PsImd* or *PsRelish* resulted in suppressed upregulation of *PsDefensin1*, *PsHemiptericin*, *PsLysozyme b-1* and *PsLysozyme c-1* (figure 4*a*,*b*,*e*–*j*), indicating that these immune effector molecules are mainly under the control of IMD pathway. By contrast, blocking of Toll pathway by RNAi of *PsMyD88* or *PsDorsal* caused suppressed upregulation of *PsDefensin2* (figure 4*c*,*d*), indicating that this effector is mainly under the control of Toll pathway. Here it should be noted that the RNAi blocking of IMD pathway also resulted in, though less remarkable, suppressed upregulation of *PsDefensin2* (figure 4*c*,*d*). Likewise, the RNAi blocking of Toll pathway caused, though less remarkable, suppressed upregulation of *PsDefensin1*, *PsHemiptericin*, *PsLysozyme b-1* and *PsLysozyme c-1* (figure 4*a*,*b*,*e*–*j*). Simultaneous blocking of IMD pathway and Toll pathway by RNAi of *PsRelish* and *PsDorsal* was most effective in all effector genes (figure 4*a*–*j*).

(ii) Which recognition protein triggers the immune pathways?

RNAi of *PsPGRP-L1a* resulted in suppressed upregulation of *PsDefensin1*, *PsHemiptericin* and *PsLysozyme c-1* when

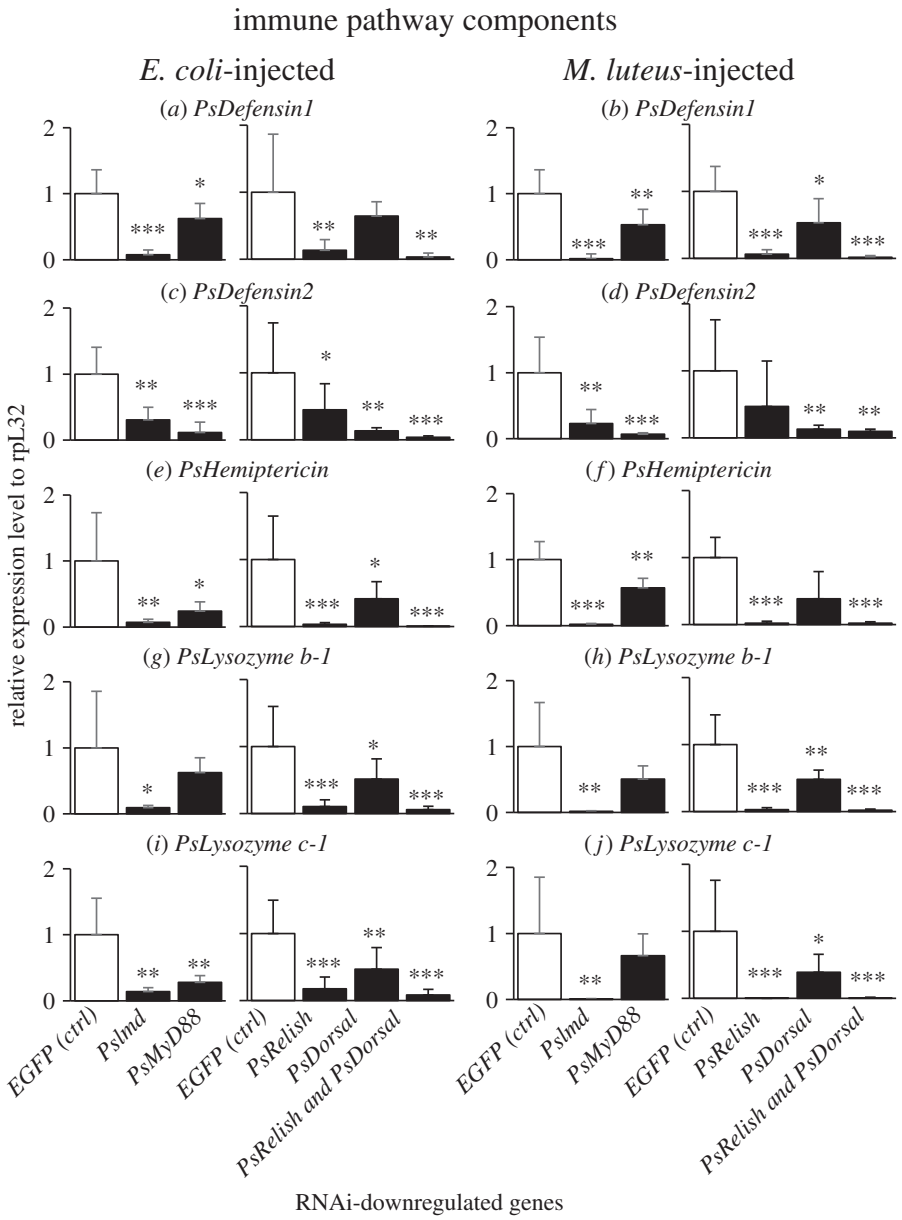

Figure 4. Effects of RNAi knockdown of immune pathway component genes on septic shock-induced upregulation of immune-responsive effector genes in *P. stali*. The expression levels of the immune effector genes were standardized to the expression levels of ribosomal protein L32 (rpL32). Adult females were injected with heat-killed *E. coli* or *M. luteus* 3 days after dsRNA injection and subjected to RNA extraction from fat bodies on the following day. Significant downregulation compared with *EGFP* injection is shown by asterisks (*t*-test: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). The error bar indicates the standard deviation (s.d.).

challenged by Gram-negative *E. coli* (figure 5*a*,*e*,*i*). For the effectors that are mainly under the control of IMD pathway (figure 4), the suppression was not observed when challenged by Gram-positive *M. luteus* (figure 5*b*,*f*,*j*).

Meanwhile, the suppression patterns observed with the effector *PsDefensin2*, which is mainly under the control of Toll pathway (figure 4), were observed by RNAi of *PsPGRP-L1a*, *PsPGRP-L2* and *PsLysM* (and marginally *PsPGRP-L1b*), irrespective of bacterial challenges by Gram-negative *E. coli* or Gram-positive *M. luteus* (figure 5*c*,*d*).

4. Discussion

(a) Immune pathway genes of *Plautia stali*

Our RNA-seq analysis showed that *P. stali* possesses a repertoire of well-conserved genes of IMD pathway and Toll pathway, but some components of IMD pathway (*Dredd*, *TAB2* and *IKKγ*) had not been found. The lack of *Dredd*, *TAB2* and *IKKγ* was also reported in the bedbug *C. lectularius* and the assassin bug *R. prolixus*, in which *Imd* was also assumed to be absent [15,17]. Using *PsImd* as a query, however, we found a homologous sequence in *C. lectularius* (uncharacterized protein, XP_014246002; e-values were 4e-5 by blastp and 0.084 by tblastx) but not in *R. prolixus* (lowest *e*-values were 0.15 to mapmodulin-like protein [AY340275] by blastp and 0.19 to Krüppel gene [JN092576] by tblastx). It is likely that the barely homologous sequence in *C. lectularius* may function as *Imd*. We speculate that the *Imd* sequence is so divergent that it is extremely difficult to find by homology search in some hemipteran species, mites and ticks (figure 1) [20].

(b) Induction of immune pathway genes in response to Gram-negative and Gram-positive bacteria

Almost all immune pathway genes upregulated by septic shock treatments in *P. stali*, including *PsPGRP-L1b*,

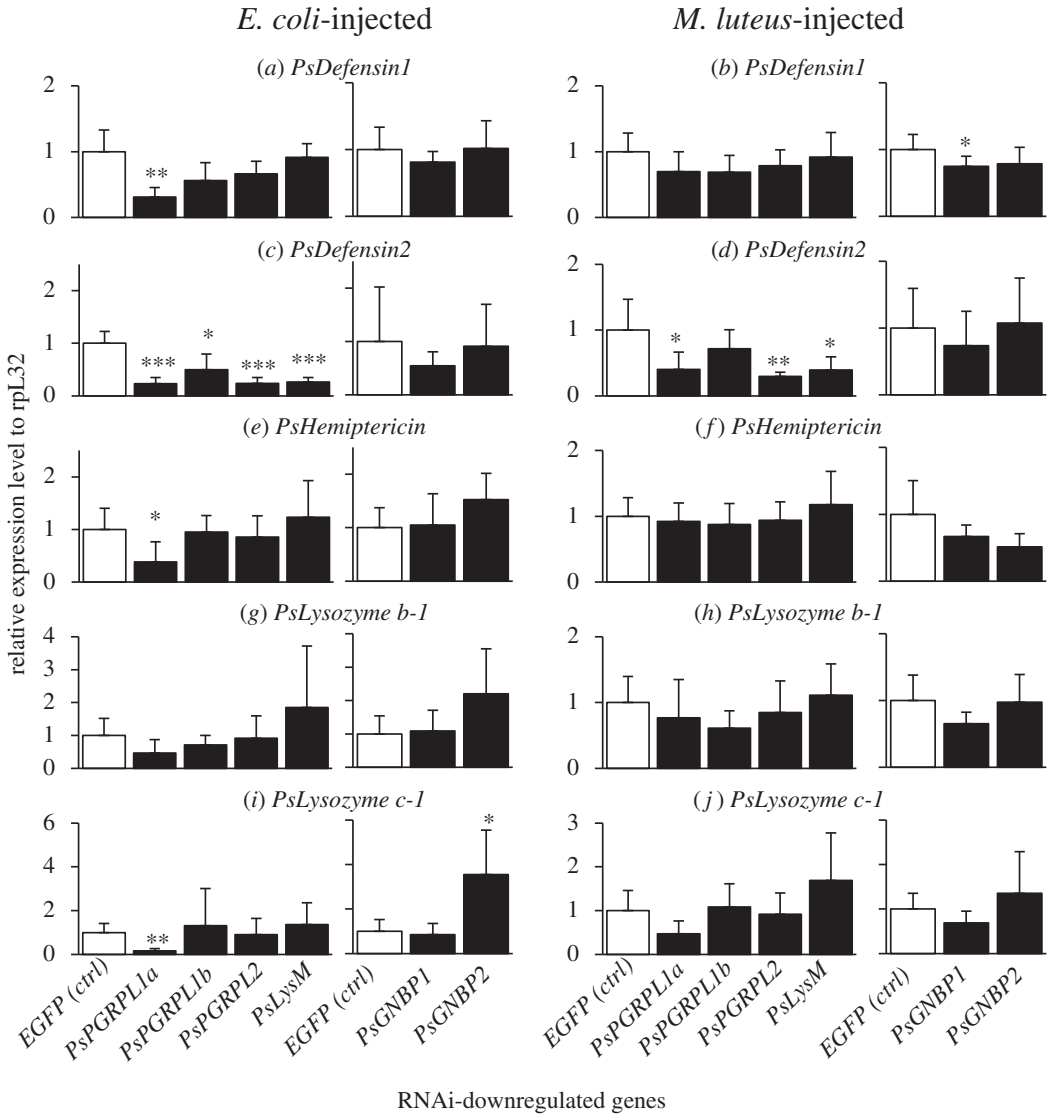

Figure 5. Effects of RNAi knockdown of immune recognition genes on the septic shock-induced upregulation of immune-responsive effector genes. The expression levels of the immune effector genes were standardized to the expression levels of ribosomal protein L32 (rpL32). Adult females were injected with heat-killed *E. coli* or *M. luteus* 3 days after dsRNA injection and subjected to RNA extraction from fat bodies on the following day. Significant downregulation compared with *EGFP* injection is shown by asterisks (*t*-test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The error bar indicates the standard deviation (s.d.).

PsGNBP1, *PsRelish*, *PsDefensin1*, *PsDefensin2*, *PsHemiptericin*, *PsLysozyme b-1* and *PsLysozyme c-1*, responded to Gram-negative *E. coli* and Gram-positive *M. luteus* indiscriminately, although *E. coli* and *M. luteus* represent different cell wall components on their surface, namely *E. coli* with DAP-type peptidoglycan and *M. luteus* with Lys-type peptidoglycan. These upregulation patterns are distinct from the well-known patterns established for *D. melanogaster*: most of the AMPs are preferentially activated by either IMD pathway or Toll pathway (but with lesser degree for Drosomycin [6]), whereas IMD pathway and Toll pathway are preferentially activated by Gram-negative bacteria and Gram-positive bacteria, respectively [40–42]. In *T. castaneum* [43] and *A. mellifera* [44], it has been reported that some AMPs ambiguously respond to various bacteria while other AMPs respond to specific bacteria in a canonical manner. Our results in *P. stali* is peculiar in that, in all the affected genes, the degree of response was similar between the insects challenged by Gram-negative bacteria and those challenged by Gram-positive bacteria.

Notably, upregulation of these responded effectors was maintained for at least 24 h, which was in contrast to most AMPs in *D. melanogaster* that exhibit transient monomodal upregulation within 12 h after bacterial challenge [40,41,45]. The long-lasting expression of AMPs might be relevant to the presumable lack of amidase activities in the PGRPs of *P. stali*. Because the amino acid sequences of three PGRP orthologues in *P. stali* assumed to have no amidase activity, three PGRP orthologues were considered presumably to play some roles in recognizing invading bacteria. PGRPs with amidase activity (amidase PGRPs), such as PGRP-LB and PGRP-SC in *D. melanogaster*, play important roles in downregulation of IMD pathway by degrading bacterial peptidoglycans [46,47].

(c) Functional crosstalk across IMD and Toll pathways in *Plautia stali*

The knockdown of IMD pathway components reduced the upregulation of effectors of both pathways (but more

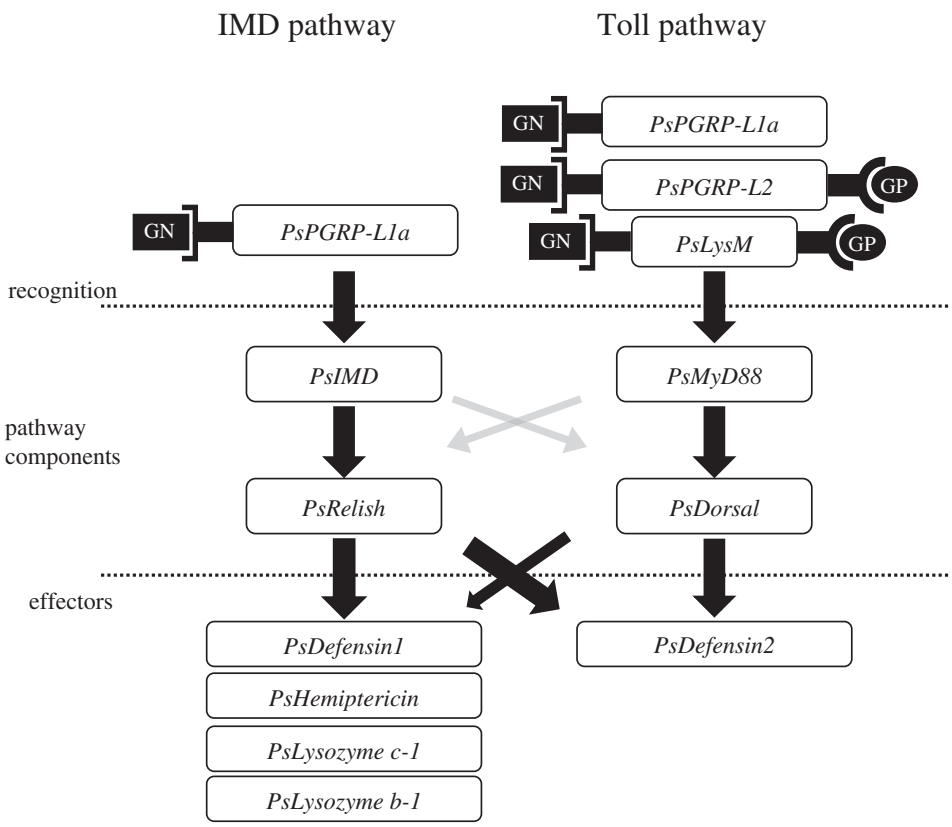

Figure 6. Schematic overview of IMD and Toll pathways in *P. stali*. Although the two conserved pathways are present, considerable crosstalk occurs across the two pathways. The left and right arms on the recognition proteins depict receptors for Gram-negative (GN) bacteria and Gram-positive (GP) bacteria, respectively.

remarkable for those of IMD pathway), and the knockdown of Toll pathway components also reduced the upregulation of effectors of both pathways (but more remarkable for those of Toll pathways). These results suggest that IMD pathway and Toll pathway are not distinct but intertwined in *P. stali*. Figure 6 shows a hypothetical model of innate immune pathways in *P. stali*. The crosstalk between IMD pathway and Toll pathway is mainly mediated by *PsRelish* and *PsDorsal*, although the possibility of crosstalk mediated by upstream genes cannot be excluded. The conspicuous crosstalk between IMD pathway and Toll pathway in *P. stali* counter the well-established notion in *D. melanogaster* that IMD pathway and Toll pathway are preferentially activated by and acting on Gram-negative bacteria and Gram-positive bacteria, respectively [40,41]. Meanwhile, several studies reported slight interactive effects between the immune pathways in *D. melanogaster*, which are mediated by NF-κB family transcription factors that bind to promoters of immune effector genes [48,49]. The NF-κB-mediated interactive effects were also found in the honeybee *A. mellifera* [44]. Because Toll pathway component *MyD88* and *Imd* share a death domain, *MyD88* might activate IMD pathway in *D. melanogaster* [50]. It was also reported that, in the flour beetle *T. castaneum*, IMD pathway and Toll pathway are activated and used more promiscuously than in *D. melanogaster* [43].

(d) Which recognition protein triggers the immune pathways?

RNAi of *PsPGRP-L1a* resulted in suppressed upregulation of effectors that are mainly under the control of IMD pathway when challenged by Gram-negative *E. coli* but not by the challenge of Gram-positive *M. luteus*. Meanwhile, the suppression patterns observed with the effector that is mainly under the control of Toll pathway was observed by RNAi of *PsPGRP-L1a*, *PsPGRP-L2* and *PsLysM* (and marginally *PsPGRP-L1b*). These results indicate that *PsPGRP-L1a* recognizes Gram-negative *E. coli*, thereby upregulating the downstream effector molecules *PsDefensin1*, *PsHemiptericin* and *PsLysozyme c-1*. *PsPGRP-L2* and *PsLysM* (and possibly *PsPGRP-L1b*) are located at the upstream of the pathway(s) leading to upregulation of *PsDefensin2* and might be capable of recognizing not only Gram-positive *M. luteus* but also Gram-negative *E. coli*. On the other hand, the suppressive effect of *PsPGRP-L1a* RNAi on upregulation of *PsDefensin2* when challenged by Gram-positive *M. luteus* ($p = 0.032$) is confusing and difficult to interpret, deserving detailed analyses in future studies. Taken together, these results suggest that the specificity of the recognition proteins to Gram-positive and Gram-negative bacteria may be also blurred in *P. stali*.

This blurred recognition of Gram-negative and Gram-positive bacteria might cause intertwining across IMD pathway and Toll pathway. While IMD pathway is mainly induced by Gram-negative bacteria via *PsPGRP-L1a*, Toll pathway is stimulated not only by Gram-positive bacteria but also by Gram-negative bacteria via *PsPGRP-L2* and *PsLysM* (and possibly *PsPGRP-L1b*).

(e) *PsLysM* as a presumable immune recognition protein

Our observation that RNAi of *PsLysM* suppressed the septic shock-induced upregulation of *PsDefensin2* (figure 5*c*,*d*) suggests that *PsLysM* may function as an immune recognition

protein in *P. stali*. *PsLysM* contains a lysin motif (LysM), which is a ubiquitous protein module identified in many prokaryotes and eukaryotes [40]. In some plants, LysM-containing genes recognize bacteria, especially symbiotic ones [51]. To our knowledge, this study is the first to show an immunity-related role of a LysM-containing gene in insects. It is notable that LysM-containing genes have been identified in other hemipteran insects such as *Riptortus pedestris* [52] and *Diaphorina citri* [28], but the function has not been examined.

(f) Evolutionary insight into the loss of IMD pathway genes in some arthropods

In previous studies on the genomics of the pea aphid *A. pisum*, the following aspects were argued in relation to the loss of IMD pathway genes [14,53]. First, the aphid feeds on plant phloem sap that is substantially sterile [54] and thus may entail the relatively low risk of encountering microbial pathogens. Second, the aphid often harbours facultative bacterial symbionts (including *Regiella*, *Hamiltonella*, *Rickettsia*, *Rickettsiella* and *Spiroplasma*) that exhibit defensive activities against microbial pathogens and insect parasitoids [55–57], which may at least partially compensate for the host's innate immune functions. Notably, *P. stali* have obligate symbionts but its defensive function had not been explored [30,58,59]. Finally, upon injury, the aphid was reported to increase terminal reproductive investment, presumably at the expense of humoral immunity [60], which may to some extent attenuate dependence on its own innate immune system. In this study, we propose a hypothesis that the functional redundancy between IMD pathway and Toll pathway may have predisposed and facilitated the loss of some or entire IMD pathway genes. It should be noted that this hypothesis is, although more generally applicable, not mutually exclusive with the above-mentioned hypotheses and might be strengthened by functional analysis of IMD pathway and Toll pathway in other hemipteran insects and non-insect arthropods that have incomplete IMD pathway. The functional crosstalk across IMD pathway and Toll pathway may be more common than generally recognized, and it might be the evolutionarily intermediate step towards the loss of IMD-related genes.

Data accessibility. The supporting datasets are available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.t50q216 [61].

Authors' contributions. Y.N. performed all experiments, analysed the data and wrote the draft manuscript. Y.N. and H.T. conceived of and designed the experiments. K.Y., A.J. and R.F. analysed RNA-seq data. D.K., R.F and T.F. revised the manuscript. All authors discussed the results and contributed to writing the article.

Competing interests. We have no competing interests.

Funding. This study was supported by the JSPS KAKENHI grant nos. JP16K21613 to Y.N. and JP25221107 and JP17H06388 to T.F.

Acknowledgements. We thank Masae Takashima for technical assistance.

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