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ORIGINAL ARTICLE

Pelle and *Tube* contribute to the Toll pathway-dependent antimicrobial peptide production in the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Abstract. Insects are solely dependent on an innate immune system. Antimicrobial peptide production is the main immune response of insects. The molecular mechanisms underlying this reaction in *Drosophila melanogaster* involves the induction of antimicrobial peptide genes, which is regulated by the Toll and IMD pathways. The Toll pathway is mainly activated by fungi or Gram-positive bacteria and the IMD pathway by Gram-negative bacteria. In terms of comparative immunology, we investigated the antimicrobial peptide production system in the beetle, *Tribolium castaneum*, which differs from that in *D. melanogaster*. To obtain a more detailed understanding, we examined whether *Pelle* and *Tube*, orthologues of which in *D. melanogaster* are the Toll pathway components, contributed to antimicrobial peptide production and immune reactions. These two genes were not induced by challenges from any type of microbe, which in this case were Gram-positive bacteria, -negative bacteria and an eukaryote. Using *Pelle* and *Tube* knockdown pupae, it was demonstrated that *Pelle* and *Tube* are involved in the induction of *Cec2* as a representative Toll pathway-dependent gene in *T. castaneum* by Gram-positive and -negative bacteria and eukaryote challenges. Furthermore, neither *Pelle* nor *Tube* contributed to immune defences against two entomopathogenic bacteria. These results, taken together with our previous findings, led to the conclusion that the Toll pathway immune signaling reported in *D. melanogaster* indeed occurs in *T. castaneum*, and the gene sets involved in Toll signal transduction in *T. castaneum* did not differ significantly from those in *D. melanogaster*, but transduced immune signals to challenges from Gram-positive bacteria, -negative bacteria and an eukaryote, which differed from those in *D. melanogaster.*

INTRODUCTION

Insects do not possess an adaptive immune defense system; they rely solely on an innate immune system. Insect immune reactions consist of both cellular and humoral immune reactions. While the cellular reaction is mediated by haemocytes, the humoral reaction produces effector molecules, such as antimicrobial peptides (AMPs) and melanin, the functions of which are to protect the host from microbial attacks (Hultmark, 2003; Hillyer, 2016). The regulatory system of AMP production has been extensively examined in *Drosophila melanogaster* (Meigen) and mainly involves two signaling pathways, the IMD and Toll pathways (Ferrandon et al., 2007; Lemaitre & Hoffmann, 2007).

In *D. melanogaster*, the Toll pathway is activated by lysine-type peptidoglycans derived from Gram-positive bacteria or β-1,3 glucan from fungi or yeast, which are recognized by the Gram-negative binding protein 1 (GNBP1) and peptidoglycan recognition protein SA (PGRP-SA) complex and GNBP3, respectively (Gottar et al., 2006; Wang et al., 2006). Signals from sensor proteins are then transduced through a serine protease cascade, leading to the proteolytic activation of the Toll ligand, Spätzle (Spz) (Weber et al., 2003). Two Tolls with activated Spz in turn activate the intracellular signaling pathway, the Toll pathway. After signal transduction, the protein MyD88 is recruited through the TID domain, which then recruits another adapter protein, Tube and the kinase Pelle (Medzhitov et al., 1998; Horng & Medzhitov, 2001). These three proteins ultimately form heterotrimer complexes, which phosphorylate and degrade the inhibitor of NF-κB, cactus by an unknown mechanism (Horng & Medzhitov, 2001; Valanne et al., 2011). Consequently, the NF-κB transcription factor Dif or Dorsal translocate into the nucleus and induces a number of immune effector genes, including several AMPs (e.g., *Drosomycin*) (Ip et al., 1993; Lemaitre et al., 1996). The IMD pathway is mainly activated by *Meso*-diaminopimelic acid-type peptidoglycans derived from Gram-negative bacteria through PGRP-LC, and an immune

signal is transmitted (Choe et al., 2002, 2005; Kaneko et al., 2006). The signal transduces through IMD protein and intracellular signaling proteins consisting of the IMD pathway (Hu & Yang, 2000; Guntermann & Foley, 2011). After signal transduction to the end of the IMD pathway, another NF-κB class transcription factor, Relish, is processed and translocated into the nucleus, and Relish induces several effector genes, including some AMPs (e.g., *Diptericin*) (Stoven et al., 2003; Ferrandon et al., 2007; Lemaitre & Hoffmann, 2007; Kleino & Silverman, 2014).

The red flour beetle, *Tribolium castaneum* (Herbst), is a model coleopteran insect for which the whole genome sequence and gene set data are available (Zou et al., 2007; Tribolium Genome Sequencing Consortium et al., 2008). Furthermore, systemic RNA interference (RNAi) is induced in beetles by an injection of double-stranded RNA (dsRNA) (Tomoyasu et al., 2008). Therefore, this beetle is very useful for genomic and molecular biological research.

In terms of comparative immunology and entomology, a more detailed understanding of the immune systems of non-*Drosophila* species is required. Due to the importance and features of *T. castaneum* described above, we investigated its immune system, particularly the regulation of AMP production. Our findings revealed that AMP genes in *T. castaneum* are induced by several types of microbe, namely, Gram-positive and -negative bacteria as well as yeast (Yokoi et al., 2012b). By using knockdown pupae of *IMD* and *MyD88*, both of which are adapter genes in the IMD and Toll pathways, respectively, nine *T. castaneum* AMP genes were divided into four groups: group I (IMD pathway-dependent and high level induction 6 h after a challenge), group II (Toll and IMD pathway-dependent and high level induction 6 h after a challenge), group III (Toll pathway-dependent and sustained induction 6 to 24 h after a challenge), and group IV (neither Toll nor IMD pathway-dependent). We further investigated AMP induction systems using AMP genes as a read-out for the two signaling pathways. Our findings revealed that *MyD88-Dif* (Dorsal) and *IMD-Relish* transduced the same signals, while *PGRP-LA* and other PGRPs contributed to the recognition of both Gram-positive and -negative bacteria, which activated the IMD pathway (Yokoi et al., 2012a). We also demonstrated, using knockdown assessments of *MyD88*, *IMD* and the other IMD intracellular-signaling components, *DREDD* and *FADD*, that crosstalk in immune signals between the Toll and IMD pathways ["crosstalk" means that signals transducing a signaling pathway transduce or affect other signaling pathways. In this study, "crosstalk" means immune signals aroused from upstream of Toll or IMD pathway may affect or transduce another pathway, which is a feature of the IMD and Toll pathways in *T. castaneum* (Yokoi et al., 2022)]. In the present study, in order to obtain insights into the IMD and Toll pathways in *T. castaneum*, we investigated whether *Tube* and *Pelle*, which are Toll pathway components, are involved in the immune reaction mediated by the Toll or IMD pathway using RNAi. The results indicate that *Pelle* and *Tube* function as Toll pathway signal transducers and do not contribute to signal crosstalk, which is reported occurring in *FADD*, *DREDD* and *IMD* knockdown pupae (Yokoi et al., 2012b, 2022). Furthermore, neither *Pelle* nor *Tube* are involved in immune defences against two entomopathogenic bacteria, which is consistent with *MyD88*.

MATERIALS AND METHODS

Insect rearing

T. castaneum was reared on whole wheat flour at 30°C in the dark. Pupae for experiments were staged as described in our previous study (Yokoi et al., 2012b).

Microbes and injections

Heat-killed *Escherichia coli* DH5a, *Micrococcus luteus* ATCC4698 and *Saccharomyces cerevisiae* S288C were prepared as elicitors of immune reactions, which are representative microbes of Gram-negative bacteria, Gram-positive bacteria and eukaryotes, respectively. The preparation of these microbes is previously described (Yokoi et al., 2012b). Fifty nanolitres of suspensions containing *E. coli*, *M. luteus* or *S. cerevisiae*, which are equivalent to 2.9×10^8, 2.9×10^7, and 6.3×10^6 cells/ml, respectively, were injected into untreated or previously treated with dsRNA 3 day-old pupae using Nanoject II (Drummond Scientific company, Broomall, PA, USA). In addition, living *Enterobacter cloacae* and *Bacillus subtilis* were used in the survival assay as described in our previous study (Yokoi et al., 2012a).

Gene sequences

T. castaneum gene sequences used in the present study from reference genome version Tcas2.0 were retrieved from Tcas2.0/annotation/TCGleanPrediction data (URL: ftp://ftp.hgsc.bcm.edu/Tcastaneum/) available at "BCM-HGSC data" in the "Red Flour Beetle Genome Project" site (URL: https://www.hgsc.bcm.edu/arthropods/red-flour-beetle-genome-project) (Zou et al., 2007; Tribolium Genome Sequencing Consortium, 2008): *Pelle* (GLEAN_15365); *Tube* (Gene ID in gene set data: GLEAN_11895); *Attacin 1* (*Att1*) (GLEAN_07737); (GLEAN_07739); *Cecropin 2* (*Cec2*) (GLEAN_00499); *Coleoptericin 1* (*Col1*) (GLEAN_05093 and GLEAN_10517: *Col1* was split into two genes in gene set data); *Defensin 3* (*Def3*) (GLEAN_12469); *IMD* (GLEAN_10851); and the normalizer gene for qRT-PCR analyses, *ribosomal protein L32* (*RPL32*) (GLEAN_06106).

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from whole *T. castaneum* pupae with TRIZOL reagent (Invitrogen, Waltham, MA, USA) and spectrophotometric scanning was used to assess the quality of the extracted RNA. According to manufacturer's instructions, 0.5 µg of total RNA was converted into first-strand cDNA using PrimeScript RT Reagent Kit with gDNA Eraser (TAKARA. Kusatsu, Shiga, Japan), and qRT-PCR was performed using first-strand cDNA and a primer pair designed for each target gene with the SYBR Premix Ex *Taq* Perfect Real Time Kit Tli RNAaseH Plus (TAKARA) and Thermal Cycler Dice Real Time System (Model TP800, TAKARA). The sequences of primer pairs used for qRT-PCR are shown in Supplementary Table 1. qRT-PCR conditions and methods for calculating levels of mRNA relative to *RPL32* are described in our previous study (Yokoi et al., 2012b).

RNAi

RNAi was performed as described in our previous study (Yokoi et al., 2012b). Briefly, the dsRNA of each gene was synthesized using the cDNA template with T7 RNA polymerase promoter sequences on both sides by the MEGAscript RNAi Kit (Ambion,

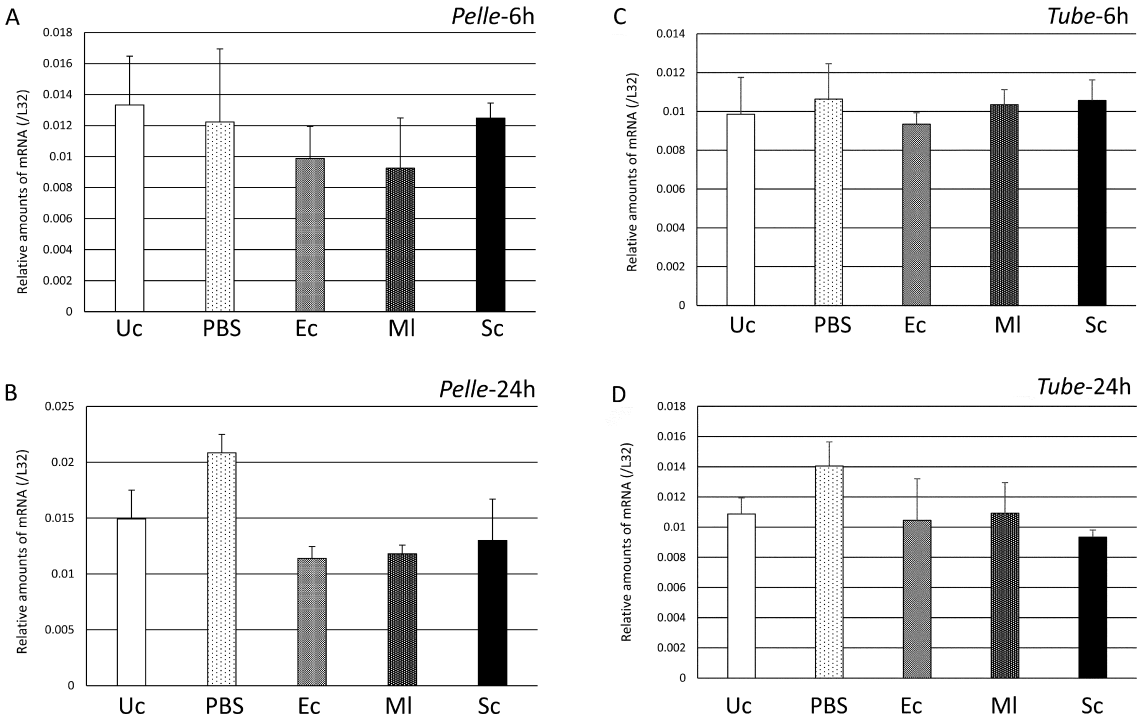

Fig. 1. Effects of microbial challenges on the relative amounts of mRNA of *Pelle* and *Tube*. Heat-killed cell suspensions of *E. coli* (Ec), *M. luteus* (Ml), or *S. cerevisiae* (Sc) were injected into day 3 pupae, and the amounts of mRNA of *Pelle* post challenge 6 h (A), 24 h (B), *Tube* post challenge 6 h (C) and 24 h (D) were measured using qRT-PCR 6 and 24 h after the challenges. Unchallenged (Uc) and PBS-injected day 3 pupae (PBS) were the negative controls. The amounts of mRNA of the two genes are shown relative to those of *RpL32* levels in the same samples. Experiments with 3 animals were independently repeated at least three times, and each column is the mean ± S.D. Student's *t*-tests of the *Pelle* and *Tube* mRNA values in Uc pupae versus PBS-, Ec-, Ml- Sc- injected pupae were done and none of the combinations were significant ($P > 0.05$).

Waltham, MA, USA). Sequences of primer pairs for the cDNA template are shown in Supplementary Table 2. One hundred nanograms of each dsRNA was injected into day 0 pupae, which were then kept in the dark at 30°C for 72 h. For preparation of negative controls, dsRNA of *maltose binding protein E* (*malE*) was injected into day 0 pupae. These pupae were used in microbe challenges or for qRT-PCR.

Survival assay

Fifty nanolitres of live *E. cloacae* and *B. subtilis* suspensions, the A_{600} values of which were 0.1 and 4.0, respectively, were injected into dsRNA-treated day 3 pupae using Nanoject II. Surviving pupae were counted every 24 h. Data are shown in Kaplan-Meier plots and P-values were calculated using the Gehan-Breslow-Wilcoxon test in R ("survival" and "MASS" library with RStudio Version 1.3.1073).

RESULTS

Pelle and *Tube* were not induced by microbial challenges

Some of the genes involved in immune reactions are generally induced by microbial challenges (De Gregorio et al., 2001, 2002) and several immune-related genes in *T. castaneum* are induced by microbial challenges (Yokoi et al., 2012a, b; Koyama et al., 2015). Therefore, we investigated whether *Pelle* and *Tube* in *T. castaneum* were induced 6 or 24 h after microbial challenges by *E. coli*, *M. luteus* or *S. cerevisiae*. As shown in Fig. 1, the amounts of *Pelle* and *Tube* mRNA in unchallenged pupae did not differ significantly from those in PBS- or any microbe-injected pupae, suggesting that neither *Pelle* nor *Tube* were induced by any microbial challenge.

Knockdown effects of *Pelle* and *Tube* by RNAi

To confirm the knockdown effects against *Pelle* and *Tube* by RNAi, the amounts of mRNA of *Pelle* and *Tube* in dsRNA-injected pupae were assessed using qRT-PCR (pupae treated with *malE* dsRNA were used as controls). The amounts of mRNA of *Pelle* after 72 h were markedly lower in pupae treated with *Pelle* dsRNA than in control pupae (Fig. 2A). Furthermore, the amounts of mRNA of *Tube* after 72 h were markedly lower in pupae treated with Tube dsRNA than in control pupae (Fig. 2B). These results indicate that dsRNA-treated pupae are suitable for use in assessments of *Pelle* and *Tube* functions.

Neither *Pelle* nor *Tube* were involved in Group I AMP gene induction (IMD pathway-dependent)

To assess whether *Pelle* and *Tube* contribute to IMD or Toll pathway-dependent immune signaling, the amounts of mRNA of several AMP genes as read-outs of the Toll or IMD pathway in *Pelle* or *Tube* knockdown pupae challenged by several microbes were measured. The amounts of mRNA of *Att1* and *Col1*, as representative IMD pathway-dependent AMP genes (group I AMP genes) (Yokoi et al., 2012b), were measured in *Pelle* or *Tube* knockdown plus control pupae (pre-treated with *malE* dsRNA) 6 and

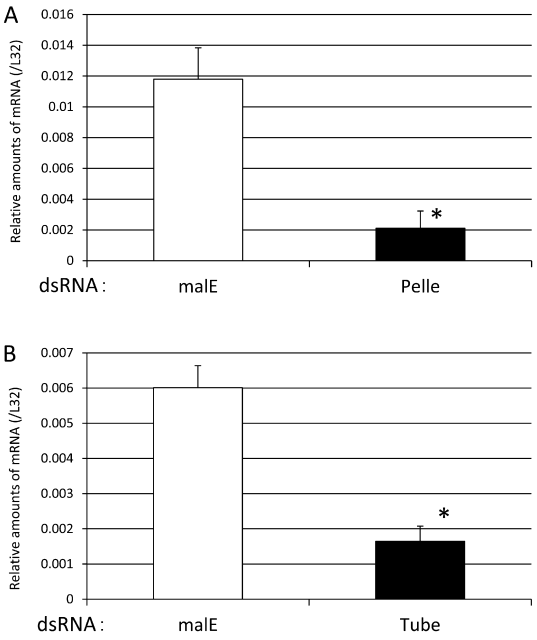

Fig. 2. Efficiency of RNAi-mediated gene knockdown on *Pelle* and *Tube*. Day 0 pupae were injected with 100 ng of *Pelle*, *Tube* or control *malE* dsRNA and the levels of mRNA of *Pelle* (A) and *Tube* (B) relative to those of *RpL32* were calculated after 72 h using qRT-PCR. Experiments were independently repeated at least three times with 3 animals, and the mean ± S.D. is shown. Asterisks indicate $P < 0.05$ versus controls based on Student's *t*-test.

24 h after being challenged by either *E. coli*, *M. luteus* or *S. cerevisiae* (Figs 3 and 4). The knockdown of *Pelle* or *Tube* only slightly affected the induction of *Att1* by *E. coli* after 6 h (Fig. 3A), while after 24 h it was not altered (Fig. 3B). Regarding the *M. luteus* challenge, the induction of *Att1* after 6 h was very slightly affected by the *Pelle* or *Tube* knockdown (Fig. 3C), whereas that after 24 h was not (Fig. 3D), which was similar to the results obtained after the *E. coli* challenge. Neither the *Pelle* nor *Tube* knockdown affected the induction of *Att1* by the *S. cerevisiae* challenge after 6 and 24 h (Fig. 3E and 3F).

The amounts of mRNA of *Col1* 6 h after the challenge by *E. coli* were significantly lower in *Pelle* knockdown pupae than in control pupae, whereas those in *Tube* knockdown pupae were not (Fig. 4A). The amounts of mRNA of *Col1* in *Pelle* or *Tube* knockdown pupae 24 h after the *E. coli* challenge were not significantly different from those in control pupae (Fig. 4B). In the case of the *M. luteus* challenge, no significant differences were recorded in the amounts of mRNA of *Col1* between *Pelle* or *Tube* knockdown pupae and control pupae 6 and 24 h after the challenges (Fig. 4C and 4D). The amount of mRNA of *Col1* in *Pelle* or *Tube* knockdown pupae challenged with *S. cerevisiae* did not significantly differ from those in control pupae (Fig. 4E and 4F). These results indicate that neither *Pelle* nor *Tube* contributed to Group I induction by any of the microbes used.

Neither *Pelle* nor *Tube* were involved in Group II AMP gene induction (IMD and Toll pathway-dependent)

We assessed the effects of the knockdown of *Pelle* and *Tube*, as representative Toll and IMD pathway-dependent AMP gene (group II AMP gene), on the induction of *Def3* by microbe challenges in *T. castaneum* pupae (Yokoi et al., 2012b). The amounts of mRNA of *Def3* 6 and 24 h after the challenge with *E. coli* were significantly lower in *Pelle* knockdown pupae than in control pupae, but not in *Tube* knockdown pupae (Fig. 5A and 5B). The amounts of mRNA of *Def3* 6 and 24 h after the challenge with *M. luteus* did not differ significantly between *Pelle* or *Tube* knockdown pupae and control pupae (Fig. 5C and 5D). Furthermore, the abundant amounts of mRNA of *Def3* in *Pelle* or *Tube* knockdown pupae 6 and 24 h after the challenge with *S. cerevisiae* did not differ significantly from those in control pupae (Fig. 5E and 5F). These results indicate that *Pelle* was involved in the induction of *Def3*, but only when challenged by *E. coli*, whereas *Tube* did not contribute to its induction by microbe infections.

Pelle and *Tube* contributed to Group III AMP gene induction (Toll pathway-dependent)

To establish whether Pelle and Tube are involved in *Cec2* gene induction as representative Toll pathway-dependent AMP genes (group III AMP genes), which showed higher induction levels 24 h than 6 h after a challenge (Yokoi et al., 2012b), the amounts of mRNA of *Cec2* in *Pelle* or *Tube* knockdown pupae and control pupae (pre-treated with malE dsRNA) 6 and 24 h after challenge by *E. coli*, *M. luteus* or *S. cerevisiae* were measured with qRT-PCR, and the amounts of *Cec2* mRNA in these knockdown pupae were compared with those in control pupae (Fig. 6). The amounts of mRNA of *Cec2* in *Pelle* or *Tube* knockdown pupae 6 h after the challenge with *E. coli* were approximately 50% of that in the control pupae (Fig. 6A). Amounts of *Cec2* mRNA in *Pelle* and *Tube* knockdown pupae 24 h after the challenge with *E. coli* were approximately 30 and 50%, respectively, of that in the control pupae (Fig. 6B). Six hours after the *M. luteus* challenge, the amounts of *Cec2* mRNA in *Pelle* knockdown pupae were lower, but not significantly so, than those in control pupae, whereas those of *Tube* knockdown pupae were significantly lower (Fig. 6C). Levels of *Cec2* mRNA in *Pelle* and *Tube* knockdown pupae 24 h after the *M. luteus* challenge were markedly lower than those in control pupae, and the extent of the reductions in *Pelle* and *Tube* knockdown pupae were approximately 30 and 50%, respectively, which were similar to that recorded in pupae post *E. coli* challenge 24 h (Fig. 6D). Six hours after the challenge with *S. cerevisiae*, *Pelle* and *Tube* knockdown pupae both had smaller amounts of mRNA of *Cec2* than the control pupae (Fig. 6E). Twenty-four hours after the *S. cerevisiae* challenge, the amounts of mRNA of *Cec2* were significantly lower in *Pelle* and *Tube* knockdown pupae than in control pupae (Fig. 6F). These

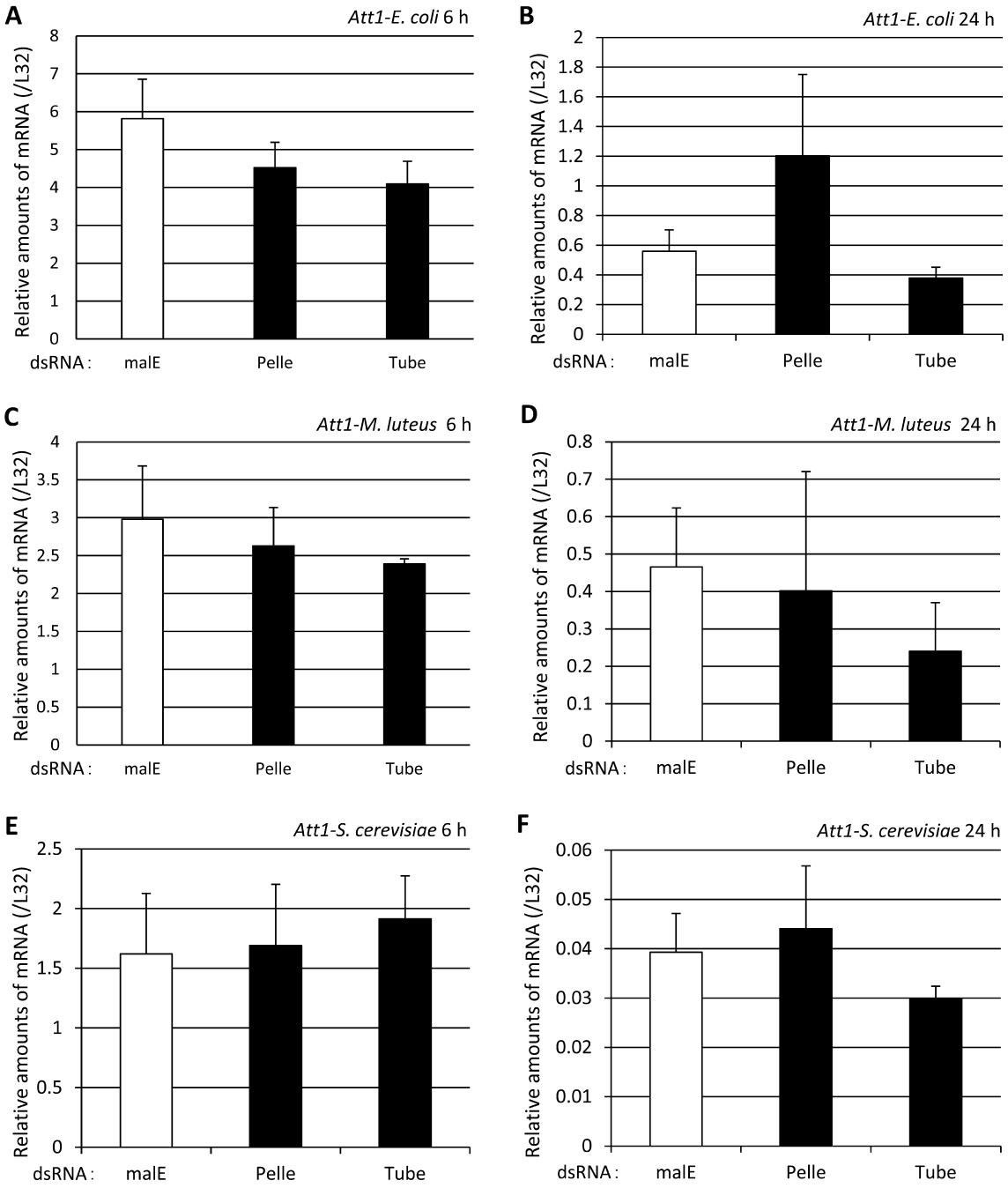

Fig. 3. Effects of the knockdown of *Pelle* and *Tube* on the microbial induction of the group I AMP gene, *Att1*. 100ng dsRNAs of *Pelle*, *Tube* and *malE* were injected into day 0 pupae. 72 h later, a heat-killed cell suspension of *E. coli* (A and B), *M. luteus* (C and D) or *S. cerevisiae* (E and F) was then injected into dsRNA-treated pupae. The relative amounts of mRNA of *Att1* to *RpL32* were assessed 6 h (A, C and E) and 24 h (B, D and F) after the microbial challenges. The results are the mean ± S.D. Experiments were independently repeated at least three times with three animals each.

results indicate that *Pelle* and *Tube* were both involved in *Cec2* (Toll pathway-dependent) gene induction by the three types of microbial challenges used.

Neither *Pelle* nor *Tube* contributed to the immune defense against entomopathogenic bacteria

To determine whether *Pelle* and *Tube* are involved in the immune defences of *T. castaneum* against invading microbes, we carried out survival assays using two model pathogenic bacterial species, *E. cloacae* and *B. subtilis*. Pupae pre-treated with *Pelle, Tube*, or *malE* dsRNA were injected with defined doses of live *E. cloacae* or *B. subtilis* and the proportions surviving were calculated every 24 h. When challenged with *E. cloacae*, the survival curves of *Pelle* and *Tube* knockdown pupae did not differ significantly from that of control pupae ($P > 0.5$) (Fig. 7A). When challenged with *B. subtilis,* more *Pelle* and *Tube* knockdown pupae survived than control pupae 24 to 72 h after the challenge (Fig. 7B). However, the survival curves of *Pelle* and *Tube* knockdown pupae did not differ significantly from that of control pupae.

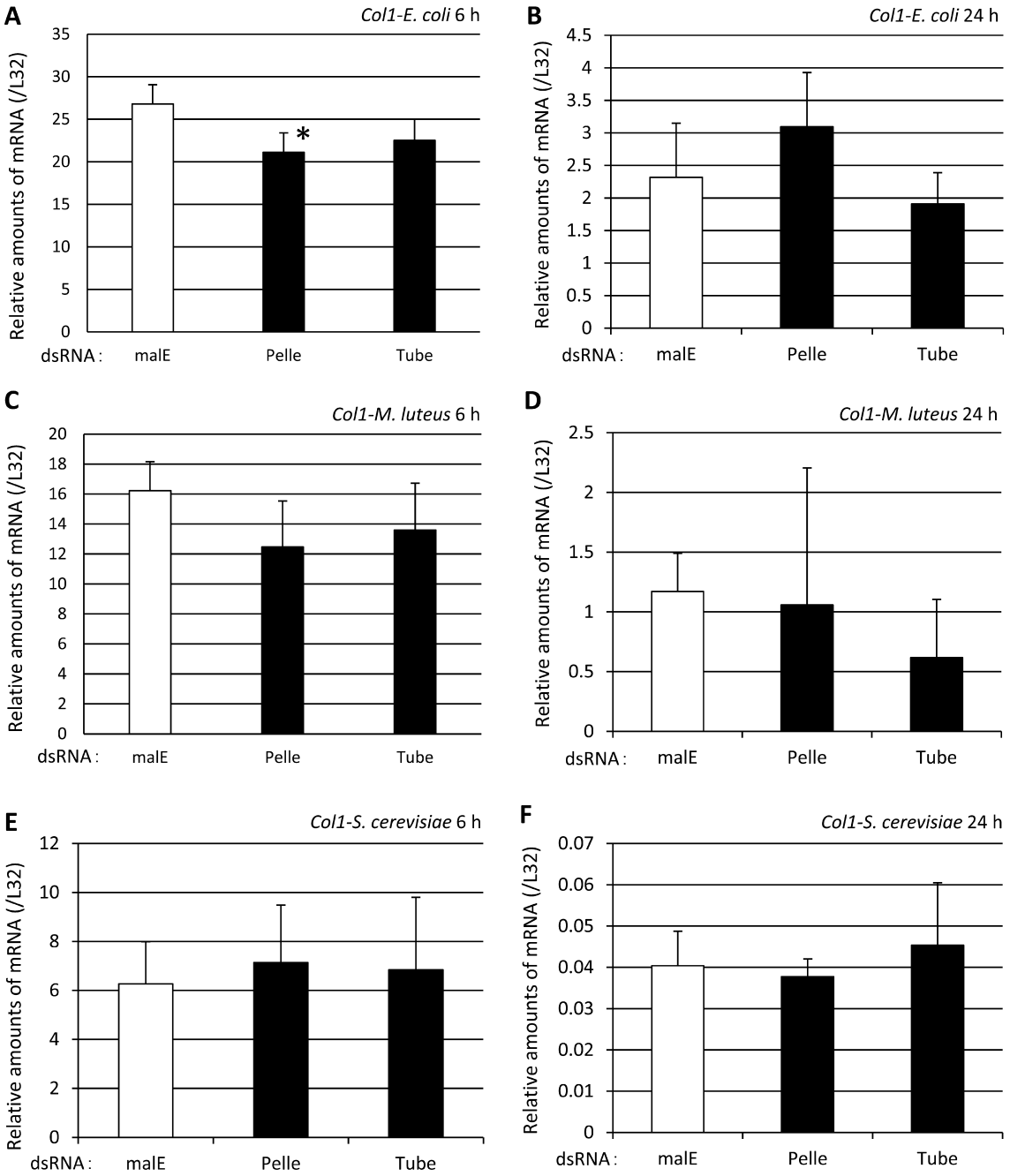

Fig. 4. Effects of the knockdown of *Pelle* and *Tube* on the microbial induction of the group I AMP gene, *Col1*. 100 ng dsRNAs of *Pelle*, *Tube*, and *malE* were injected into day 0 pupae. 72 h later, a heat-killed cell suspension of *E. coli* (A and B), *M. luteus* (C and D) or *S. cerevisiae* (E and F) was injected into dsRNA-treated pupae. The relative amounts of mRNA of *Col1* to *RpL32* were assessed 6 h (A and E) and 24 h (B, D and F) after the microbial challenges. The results are the mean ± S.D. Experiments were independently repeated at least three times with three animals each. Asterisks indicate $P < 0.05$ versus *malE* control pupae based on Student's *t*-tests.

DISCUSSION

In the present study, we investigated whether *Pelle* or *Tube* in *T. castaneum* play a role in the production of AMP, one of the main immune reactions in insects, using RNAi. The results indicate that neither *Pelle* nor *Tube* was induced by challenges from three microbes, namely, heat-killed *E. coli* (Gram-negative bacteria) *M. luteus* (Gram-positive bacteria) and *S. cerevisiae* (eukaryote), and *Pelle* and *Tube* mainly contributed to the induction of *Cec2* by these microbe challenges, which was mainly regulated by the Toll pathway (Yokoi et al., 2012b). Furthermore, we showed that neither *Pelle* nor *Tube* were involved in host immune defenses against two entomopathogenic bacteria, *E. cloacae* and *B. subtilis*.

In previous studies on *Drosophila*, several immune-related genes are reported induced by microbial challenges and contribute to immune reactions (De Gregorio et al., 2001, 2002). In *T. castaneum*, *Dif2* and *Rel*, which are NF-κB transcription factors, are mainly involved in Toll pathway- and IMD pathway-dependent AMP gene inductions, which are induced by challenges by the microbes: *E.*

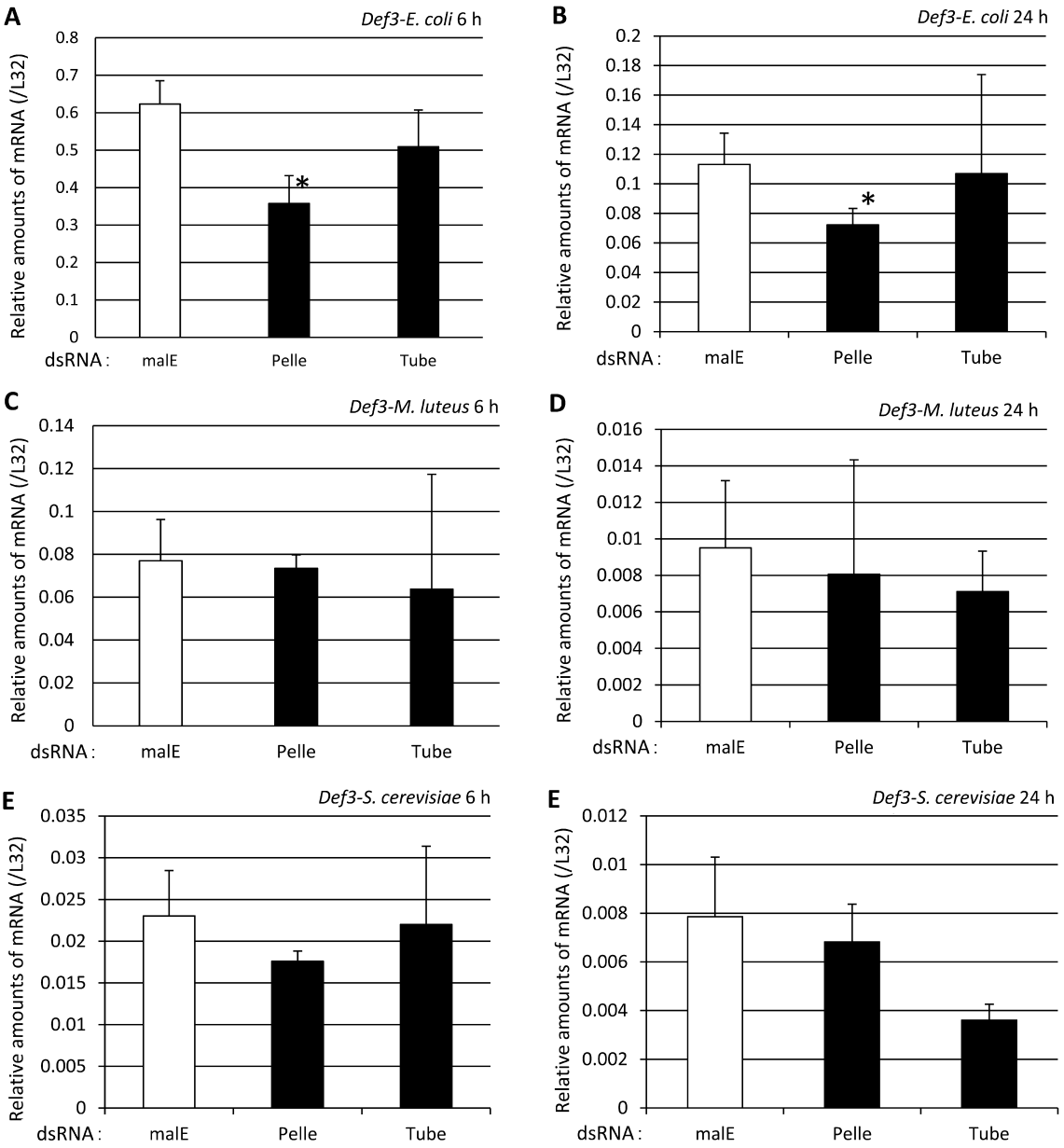

Fig. 5. Effects of the knockdown of *Pelle* and *Tube* on the microbial induction of the group II AMP gene, *Def3*. 100ng dsRNAs of *Pelle*, *Tube* and *malE* were injected into day 0 pupae. 72 h later, a heat-killed cell suspension of *E. coli* (A and B), *M. luteus* (C and D) or *S. cerevisiae* (E and F) was injected into dsRNA-treated pupae. The relative amounts of mRNA of *Col1* to *RpL32* were assessed 6 h (A, C and E) and 24 h (B, D and F) after the microbial challenges. The results are the mean ± S.D. Experiments were independently repeated at least three times with three animals each. Asterisks indicate $P < 0.05$ versus *malE* control pupae based on Student's *t*-tests.

coli, *M. luteus* and *S. cerevisiae* challenges (Yokoi et al., 2012a, b). Among PGRP family of genes, *PGRP-LA*, *-LC* and *-LE*, which function as sensors for the IMD pathway, are induced by *E. coli* and *M. luteus* challenges (Koyama et al., 2015). Although *PGRP-SA* and *-SB* are induced, their functions in AMP gene induction remain unknown. *DREDD* and *FADD,* which are components of intracellular-signaling part of the IMD pathway that are mainly involved in IMD pathway-dependent AMP gene induction, are not induced by any of the three microbes described above (Yokoi et al., 2022). In the present study, we showed that neither *Pelle* nor *Tube* was induced by any of the three microbes. Taken together these results, Immune-related genes located in the sensor and transcriptional factor layers were induced by microbial challenges, while genes comprising the intracellular-signalling parts of Toll and IMD pathways were not. These differences in induction may be due to the genes in the sensor and transcriptional factor layers only functioning when immune reactions occurred, whereas those located in intracellular-signalling parts might constantly function as non-immune signal transducers. For instance, a previous study indicates that the Toll pathway is also involved in the embryonic dorsoventral pattern signalling pathway in *D. melanogaster* (Morisato & Anderson, 1995). Thus, it is likely that both Toll and IMD pathways may have roles in immune reactions as well as unknown roles, leading to results that *Pelle* and *Tube* are not only induced by microbial challenges.

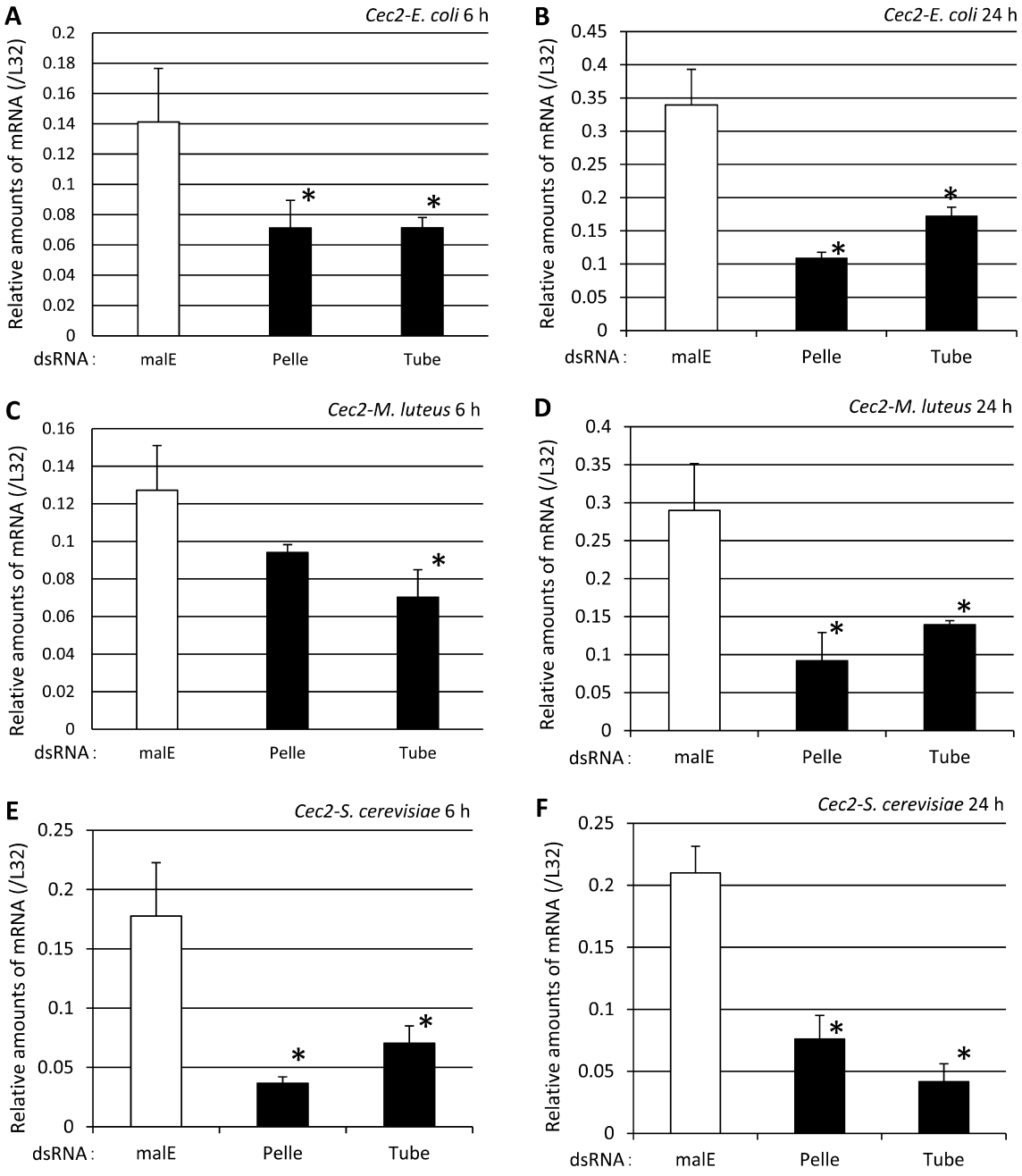

Fig. 6. Effects of the knockdown of *Pelle* and *Tube* on the microbial induction of the group III AMP gene, *Cec2*. 100 ng dsRNAs of *Pelle*, *Tube* and *malE* were injected into day 0 pupae. 72 h later, a heat-killed cell suspension of *E. coli* (A and B), *M. luteus* (C and D) or *S. cerevisiae* (E and F) was injected into dsRNA-treated pupae. The relative amounts of mRNA of *Col1* to *RpL32* were assessed 6 h (A, C and E) and 24 h (B, D and F) after the microbial challenges. The results are the means ± S.D. Experiments were independently repeated at least three times with three animals each. Asterisks indicate $P < 0.05$ versus malE control pupae based on Student's *t*-tests.

The knockdown of *Pelle* or *Tube* altered the levels of *Cec2* induced 6 and 24 h after the microbial challenges by *E. coli*, *M. luteus* and *S. cerevisiae*, while neither the *Pelle* nor *Tube* knockdown affected the levels of *Col1*, *Att1* or *Def3* induced by the three microbial challenges, except in some combinations (*Col1* and *Def3* in *Pelle* knockdown pupae by *E. coli*). The significantly lower level of *Col1* expression in *Pelle* knockdown pupae in response to *E. coli* challenges might be partially due to individual variants of the strength of *Col1* induction in pupae sampled. If *Pelle* mainly involved *Col1* induction by *E. coli* challenges, over 40% reduction in the expression levels in knockdown pupae compared with controls is expected, however, the actual reduction in the percentage of *Col1* is much smaller (see values in Fig. 4A and Fig. 6). Only *Def3* induction by *E. coli* injections, both post 6 h and 24 h, were altered by *Pelle* knockdown. One reason for this might be that *Def3* inductions by *E. coli* are stronger than by *M. luteus* and *S. cerevisiae*. A previous study showed that *Def3* is regulated by both IMD and Toll pathway, and *Def3* is more dependent on the IMD pathway than the Toll pathway (Yokoi et al., 2012a, b). Considering the above and the results presented in this study, the levels of induction of *Def3* might be to some extent regulated mainly by the IMD pathway

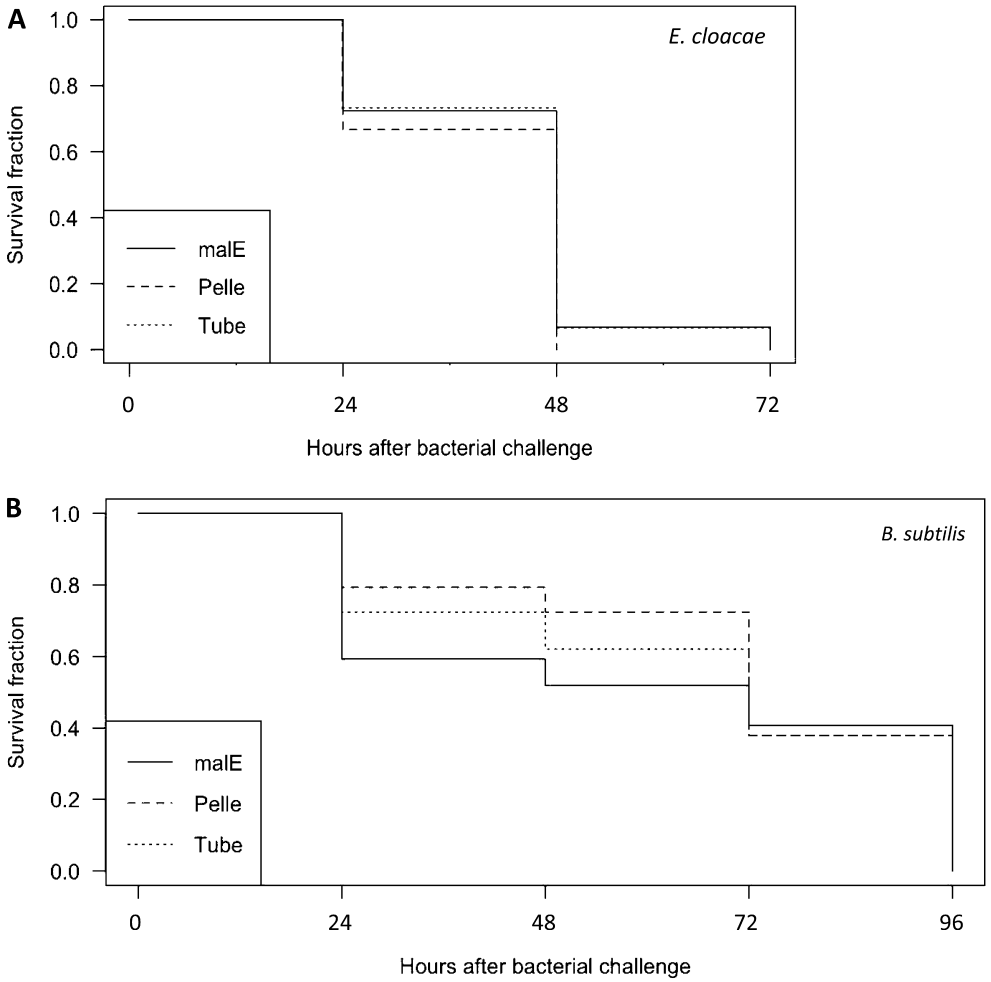

Fig. 7. Effects of the knockdown of *Pelle* and *Tube* on host defences against two model bacterial pathogens. Day 3 pupae that were pre-injected with the respective dsRNAs (*malE*-treated pupae as controls) on day 0 were challenged by *E. cloacae* (A) or *B. subtilis* (B) and the survival of pupae was monitored and recorded every 24 h. Results are shown as Kaplan-Meier plots.

and greater inductions of *Def3* could be regulated by both Toll and IMD pathways. It is not clear why the variation in the induction of *Def3* were recorded only for *Pelle* knockdown pupae. There might be a gene, which is redundant in the functioning of *Tube* when great levels of *Def3* were induced, whereas there is no such gene with redundant functions in *Pelle*. The results shown in Figs 3–6 indicate that *Pelle* and *Tube* mainly contributed to the immune signal transduction via the Toll pathway as *MyD88*, *Dif1* and *Dif2* (Yokoi et al., 2012a, b). Also, these results indicate that clear immune signal crosstalk between Toll and IMD pathways via *Pelle* or *Tube* did not occur. In contrast, knockdown of the *FADD* and *DREDD* components of the IMD pathway, affected both IMD- and Toll-dependent AMP inductions, indicating that immune signal crosstalk occurred via *DREDD* and *FADD* (Yokoi et al., 2022). Thus overall, it is likely that the immune signaling via the Toll pathway reported in *D. melanogaster* also occurs in *T. castaneum,* and the gene sets involved in Toll signal transduction in *T. castaneum* do not differ significantly from those in *D. melanogaster* (Valanne et al., 2011). As described above, differences in the immune systems in terms of AMP production in *T. castaneum* and *D. melanogaster* involved the mechanisms underlying the activation of the Toll and IMD pathways. The Toll and IMD pathways were both activated by the three types of microbe challenges in *T. castaneum*, whereas the Toll pathway in *D. melanogaster* is mainly activated by prokaryote and Gram-positive bacterial challenges and the IMD pathway by a Gram-negative bacterial challenge. The present results support these findings, suggesting that the Toll pathway functioned as an immune signal transduction pathway induced by three types of microbial challenges transduced via the Toll pathway in *T. castaneum*.

The results of survival assays revealed that neither the *Pelle* nor *Tube* knockdown affected percentage survival following challenges with the two entomopathogenic bacteria: *E. cloacae* and *B. subtilis*. In previous reports the *MyD88, Dif1* or *Dif2i* knockdown pupae with a Toll pathway, did not succumb earlier to *E. cloacae* and *B. subtilis* infections than control pupae, whereas either *IMD, FADD, DREDD* or *Relish* knockdown pupae, with an IMD pathway, succumbed earlier to infections by *E. cloacae* and *B. subtilis*. Furthermore, we also showed that although Group I, II- and III-AMP genes were induced by *E. cloacae* and *B. subtilis* injections, the induction of Group III AMP

genes were not affected by *Relish* knockdown (Yokoi et al., 2012a, b, 2022). Results from this study and previous reports indicate that genes related to the Toll pathway are not involved in immune reactions against these bacterial infections, whereas the genes related to the IMD pathway are involved. In *D. melanogaster*, adult flies with Toll pathway-related gene mutants succumbed earlier to fungi or Gram-positive bacteria (Rutschmann et al., 2002; Gottar et al., 2006), while those with IMD pathway-related gene mutants succumbed earlier to Gram-negative bacteria (Lemaitre et al., 1995; Leulier et al., 2000). These findings reflect the differences in the immune systems of *T. castaneum* and *D. melanogaster* and indicate that the IMD pathway plays a pivotal role in immune defences against bacterial infections in *T. castaneum.* Therefore, further studies to elucidate the immune roles of the Toll pathway in *T. castaneum* are warranted.

The present results showed that *Pelle* and *Tube* in *T. castaneum* were mainly involved in Toll-dependent AMP gene induction by three microbial challenges, but not in host bacterial defense reactions against two entomopathogenic bacteria. This study provides an insight into the role of the Toll pathway in this species immune system.

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Supplementary tables

Two supplementary tables are available in figshare (DOI: 10.6084/m9.figshare.c.5529771).

Supplementary Table 1

Primer sequences for qRT-PCR (DOI: 10.6084/m9.figshare.15059349).

Supplementary Table 2

Primer sequences for synthesizing T7-tagged cDNA (DOI: 10.6084/m9.figshare.15059367).