RNA interference-based characterization of Caspar, DREDD and FADD genes in immune signaling pathways of the red flour beetle, Tribolium castaneum (Coleoptera: Tenebrionidae)

Kakeru Yokoi1,2,*, Wataru Ito5,*, Daiki Kato1 and Ken Miura1

1 Applied Entomology Laboratory, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya 464-8601, Japan; e-mails: yokoi123@affrc.go.jp, wataru-ito@agr.nagoya-u.ac.jp, daiki-kato@agr.nagoya-u.ac.jp, k-miura@agr.nagoya-u.ac.jp
2 Insect Design Technology Group, Division of Insect Advanced Technology, Institute of Agrobiological Sciences, NARO, Owashi, Tsukuba 305-8643, Japan

Key words. Anti-microbial peptide, IMD pathway, Toll pathway

Abstract. We previously demonstrated that two immune signaling pathways, Toll and IMD, were concomitantly activated in the model beetle Tribolium castaneum by challenges to their immune system by several species of microbes, including Gram-positive and -negative bacteria as well as yeast. This contrasts with the Drosophila immune system in which more specific pathway activation depending on the type of microbe is well established. We suggest that the activation of an indiscriminate immune pathway in T. castaneum is due in part to an unselective recognition of pathogen-associated molecular patterns by the extracellular sensing modules of the two pathways. In order to obtain a more detailed understanding of the T. castaneum immune pathway, we investigated whether potential components of the T. castaneum IMD pathway, Caspar, DREDD and FADD, are involved in immune reactions triggered by microbial challenges. A sequence analysis of these three genes with the orthologues of other species, including insects, mouse and human, indicated that T. castaneum Caspar, DREDD and FADD functioned as immune signal transducers, which are usually induced by microbial challenges. However, these genes were not induced by microbial challenges. To establish whether these genes are involved in immune reactions, we used RNA interference-mediated knockdown of these genes to assess the microbial induction levels of the representative read-out antimicrobial peptide genes of the respective classes. The results indicated that these genes encode the canonical constituents of the IMD pathway of this beetle. DREDD and FADD influenced the induction of Toll-dependent antimicrobial peptide genes, providing novel crosstalk points between the two immune pathways, which appears to support indiscriminate pathway activation in T. castaneum. Furthermore, the phenotypes of DREDD or FADD knockdown pupae challenged by the two model bacterial pathogens correlated with AMP gene induction in the respective knockdowns, indicating that these intracellular factors contributed to antibacterial host defenses.

INTRODUCTION

The insect immune system is solely composed of innate immune reactions that utilize germ line-encoded receptors to detect the invasion of several distinct pathogens (Hultmark, 2003; Ferrandon et al., 2007; Lemaitre & Hoffmann, 2007). When recognized, invading pathogens are attacked by a battery of innate immune responses. Insect immune responses as well as vertebrate immune responses may be conveniently divided into cellular and humoral immune responses as well as vertebrate immune responses may be conveniently divided into cellular and humoral immunity, the latter of which involves the systemic and robust production of antimicrobial peptides (AMPs) as a major and well-characterized response. Intensive studies on the model organism Drosophila melanogaster (Meigen) over the past two decades have delineated the pathways and factors that uphold the AMP production response as one of the hallmarks of insect immunity.

Extracellular pathogen recognition leads to the activation of two signaling pathways in Drosophila, the Toll and IMD pathways (Valanne et al., 2011; Kleino & Silverman, 2014). The Toll pathway is mainly activated by either Lys-type peptidoglycan associated with most Gram-positive bacteria or fungal β-1,3 glucan, and the recognition of these two pathogen-associated molecular patterns (PAMPs) in circulation is mainly executed by peptidoglycan recognition protein SA (PGRP-SA) and Gram-negative binding protein 3, respectively (Gobert et al., 2003; Gottar et al., 2006). PAMP binding to these receptors subsequently activates a downstream serine protease cascade, which leads to the proteolytic activation of the Toll ligand Spät-
zle (Spz) (Weber et al., 2003). Two Toll receptors, bound by two processed Spz dimers, undergo a conformational change and recruit the adaptor protein MyD88 through intracellular TID domains (Gangloff et al., 2008). This interaction also recruits another adaptor protein, Tube and the kinase Pelle via death domain (DD)-mediated interactions to form a heterotrimeric complex comprising MyD88, Tube and Pelle (Sun et al., 2002). The formation of heterotrimers leads to the phosphorylation and degradation of Cactus, a Drosophila inhibitor of NF-κB, through paths that are not yet identified, and the NF-κB class transcription factor Dif or Dorsal translocates into the nucleus and activates a set of targets, including several AMP genes (Wu & Anderson, 1998).

Gram-negative bacterial infections preferentially activate the IMD pathway (Kleino & Silverman, 2014). Meso-diaminopimelic acid (DAP)-type peptidoglycan associated with Gram-negative bacteria and Gram-positive bacilli are mainly sensed by the membrane-bound receptor, PGRP-LC (Choe et al., 2002, 2005). Ligand binding may promote the dimerization or multimerization of PGRP-LC, and its cytoplasmic portion, harbouring the receptor-interacting protein homeotypic interaction motif, interacts with the adaptor protein IMD (Kaneko et al., 2006). IMD recruits the initiator caspase-8 homolog DREDD via the adaptor function of FADD (Leulier et al., 2000; Naitz et al., 2002), and DREDD is activated by mechanisms that have yet to be completely defined, but include K63 polyubiquitination by the E3 ubiquitin ligase DIAP2 (Meinander et al., 2012). Activated DREDD cleaves IMD at Asp46, which exposes the interaction interface to DIAP2, and cleaved IMD is also subjected to K63 ubiquitination (Paquette et al., 2010). The polyubiquitin chain recruits and activates the kinase TAK1, which, in turn, activates the JNK and IKK branches of the IMD pathway (Rutschmann et al., 2000; Silverman et al., 2000, 2003). The Drosophila IKK complex comprises catalytic IKKβ and regulatory IKKγ subunits. The IKK complex is thought to phosphorylate another NF-κB class transcription factor, Relish (Stöven et al., 2000; Stöven et al., 2003). Phosphorylated Relish is then translocated into the nucleus after the proteolytic removal of its ankyrin portion by DREDD, ultimately activating its target genes.

While Drosophila immune signaling events leading to the activation of humoral responses have been examined in detail, comprehensive information on other insect species, particularly non-dipteran insects, is limited. This may be attributed to the unavailability of genomic information, except for some model insects, and also to the incompatibility of genetic manipulation techniques, including RNA interference (RNAi) in some experimental systems. Therefore, some of the information obtained on immune responses in non-Drosophila insects may be annotated on the basis of the Drosophila framework.

We started research on insect immune signaling using the model beetle Tribolium castaneum (Herbst) because information on its genome is available and this beetle exhibits systemic RNAi responses, and our aim was to provide another framework for an insect immune system (Tomoyasu et al., 2008; Tribolium Genome Sequencing Consortium, 2008). In the initial phase, we characterized all nine AMP genes in the genome of T. castaneum and categorized them into IMD pathway-dependent group I, Toll pathway-dependent group III, and co-dependent group II as well as non-inducible group IV genes by utilizing the RNAi of the pathway-specific intracellular adaptor protein genes, IMD and MyD88 (Yokoi et al., 2012b). We subsequently demonstrated that epistatic relationships between the pathways and subclasses of NF-κB transcription factors in Drosophila were also present in T. castaneum (Yokoi et al., 2012a). Collectively, these findings indicated that the Toll and IMD pathways exist in this beetle, and we also noted that the specificity of pathway activation was distinct from that in Drosophila because the target AMP genes of both pathways were concomitantly activated by any of the five microbial species used, including Gram-negative and -positive bacteria as well as budding yeast. We considered this indiscriminate pathway activation to be a distinct feature of T. castaneum immune signaling, which differs from that of Drosophila, and we have been investigating the molecular mechanisms that support this phenomenon. We demonstrated that PGRP-LA encodes the canonical receptor of the IMD pathway in this beetle, and also that this isoform recognizes both Gram-positive and -negative bacteria (Koyama et al., 2015). The finding of extracellular pathogen recognition may account in part for the aforementioned immune pathway activation with lower specificity. In the present study, to extend our understanding of the T. castaneum immune system, we focused on potential receptor-proximal intracellular components in the IMD pathways of T. castaneum, DREDD and FADD, as well as the DREDD inhibitor Caspar (Kim et al., 2006), and investigated whether these factors provide bifurcating points of immune signaling and are involved in immune reactions against bacterial infections.

**MATERIALS AND METHODS**

**Insect rearing**

T. castaneum was reared on whole wheat flour at 30°C in the dark and their pupae were prepared as previously described (Yokoi et al., 2012b).

**Microbes and injections into insects**

Escherichia coli DH5α, Micrococcus luteus ATCC4698, and the budding yeast, Saccharomyces cerevisiae S288C were used as heat-killed cell suspensions with defined O.D. values, as described in Yokoi et al. (2012a). Prepared suspensions of E. coli, M. luteus and S. cerevisiae were 2.9 × 10⁹, 2.9 × 10⁷ and 5 × 10⁶ cells/ml, respectively. Fifty nanolitres of heat-killed suspensions was injected into naive or double strand RNA (dsRNA)-treated day 3 pupae using Nanoject II (Drummond Scientific Company) to elicit immune responses. Suspensions of live Enterobacter cloacae and Bacillus subtilis were also prepared and used in survival assays, as previously described in Yokoi et al. (2012b). E. cloacae and B. subtilis were generous gifts from Dr. Y. Yagi of Nagoya University. S. cerevisiae S288C was from Dr. T. Ushinomaru of Shizuoka University. M. luteus ATCC4698 was provided by the RIKEN Bioresource Center in Japan.
Genes examined in the present study

The following *T. castaneum* gene sequences from reference genome version Tcas2.0 were retrieved from Tcas2.0 (annotation/TCGleanPrediction data (URL: ftp://ftp.hgsbc.bcm.edu/Tcastaneum/)) available at “BCM-HGSC data” in the “Red Flour Beetle Genome Project” site (URL: https://www.hgsbc.bcm.edu/arthropods/red-flour-beetle-genome-project) and utilized in the present study (Zou et al., 2007; Tribolium Genome Sequencing Consortium, 2008): *Caspar* (Gene ID in gene set data: GLEAN_009985); *DREDD* (GLEAN_14026); *FADD* (GLEAN_14042); *Attacl 1* (At1) (GLEAN_07737); *Cecropin 2* (Cec2) (GLEAN_00499); *Cecropin 3* (Cec3) (GLEAN_00500); *Co-leptopericin 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).

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the whole body of *T. castaneum* pupae using TRIZOL reagent (Invitrogen). After spectrophotometric scanning and assessments, 0.5 μg of total RNA was converted to first-strand cDNA utilizing a PrimeScript RT Reagent Kit with gDNA Eraser (TAKARA), and first-strand cDNA was analysed by qRT-PCR using the SYBR Premix Ex Taq Perfect Real Time Kit Tli RNAsEase Plus (TAKARA) and Thermal Cycler Dice Real Time System (Model TP800, TAKARA). The sequences of primer pairs used for qRT-PCR of *Caspar*, *DREDD* and *FADD* are shown in Supplementary Table 1. The sequences of qRT-PCR primers for the other genes and PCR conditions are described in our previous studies (Yokoi et al., 2012a, b).

RNAi

RNAi-mediated gene knockdown was done as described in our previous studies (Yokoi et al., 2012a, b). Briefly, T7 RNA polymerase promoter sequence-tagged cDNA generated by conventional PCR was used as a template for dsRNA synthesis with the MEGAscript RNAi Kit (Ambion). One hundred nanograms of dsRNA, typically in a volume of 33 nl, for each target gene was injected into day 0 pupae using Nanoject II. Pupae were examined 72 h later for knockdown efficiency or challenged with microbes. dsRNA with a partial maltose binding protein E (malE) sequence served as a negative control. The sequences of primer pairs used for the production of qRT-PCR of *Caspar*, *DREDD* and *FADD* were shown in the Supplementary Table 1. The sequences of qRT-PCR primers for the other genes and PCR conditions are described in our previous studies (Yokoi et al., 2012a, b).

Survival assay

Day 0 pupae were treated with dsRNA. Pupae were challenged 72 h after the dsRNA treatment with live *E. cloaca* (50 nl, A600 = 0.1) or *B. subtilis* (100 nl, A600 = 3.0), as described in Yokoi et al. (2012a). The survival of pupae was thereafter recorded every 24 h. Data were presented as Kaplan-Meier plots and *p*-values calculated using the Gehan-Breslow-Wilcoxon test by using a software package (Eksens-Toukei 2010 ver. 1.13, Social Survey Research Information Co., Ltd.).

Sequence analyses

Multiple amino acid sequence alignments were performed using Clustal Omega (version 1.2.4) (Sievers & Higgins, 2021). Newick files were constructed from Clustal Omega output files (aln files) using FastTree program version 2.1.10 (Price et al., 2009), and approximate maximum-likelihood phylogenetic trees constructed using newick files with MEGAX version 10.1.7 (Kumar et al., 2018). All sequence analyses were done with default settings. To search for functional domains in the *Caspar*, *DREDD* and *FADD* of *T. castaneum*, the predicted amino acid sequences of these genes were analysed using InterProScan with InterPro version 86.0 (Blum et al., 2021, URL: https://www.ebi.ac.uk/interpro/search/sequence). All raw data from these analyses, which are fasta, aln, newick and gff files, are available in figshare (see Supplementary Data 1–3).

RESULTS

Phylogenetic analyses of *Caspar*, *DREDD* and *FADD*, and search for functional domains in *T. castaneum* *Caspar*, *DREDD* and *FADD*

The sequences of *T. castaneum* orthologues for *Caspar*, *DREDD* and *FADD* (Tc-Casar, Tc-DREDD, and Tc-FADD, respectively) were obtained from gene set data based on *T. castaneum* genome data (Zou et al., 2007; Tribolium Genome Sequencing Consortium, 2008), and their deduced amino acid sequences were subjected to phylogenetic analyses (Supplementary Fig. 1) together with the related sequences of other organisms [*Aedes aegypti* (Linnaeus), *Anopheles gambiae* (Giles), *D. melanogaster*, *Bombyx mori* (Linnaeus), *Apis mellifera* (Linnaeus), *Nilaparvata lugens* (Stål), mouse (Linnaeus) and human] (Supplementary Fig. 1). Furthermore, functional domains in the predicted amino acid sequences of Tc-Casar, Tc-DREDD and Tc-FADD were searched for by InterProScan (Supplementary Fig. 2). Supplementary Fig. 1A shows the phylogenetic tree of *Caspar*-related proteins. Tc-Casar forms a clade with *B. mori* Caspar (Bm-Caspar), indicating that Bm-Caspar is the closest protein to Tc-Casar in the phylogenetic tree. The results of InterProScan showed that Tc-Casar has functionally important domains, which are the FAF1_UBA domain (functions as a Fas-associated domain), ubiquitin-associated (UAS) domain and ubiquitin-like regulatory X domain (UBX) (Supplementary Fig. 2A). The death effector domain-interacting domain (DEDID) is not predicted but the ubiquitin-like motif is predicted (IPR029071) (Chu et al., 1995; Park et al., 2004; Kim et al., 2006; He et al., 2013). However, the ubiquitin-like motif is found in the Ras-binding domain, suggesting that this motif functions as DEDID (Nassar et al., 1995). Similar analyses were performed on DREDD/Caspase 8 proteins and FADD proteins (the FADD orthologue is not present in the *N. lugens* genome). In the phylogenetic trees of *DREDD* and *FADD*, Tc-DREDD is located near a clade including *D. melanogaster*, *A. aegypti* and *A. gambiae* DEREDDs (Supplementary Fig. 1B), while Tc-FADD is located in a clade comprising *D. melanogaster* and *B. mori* FADDs (Supplementary Fig. 1C). The results of domain searches in Tc-DREDD InterProScan showed that Tc-DREDD has two caspase domains and a single death effector domain (DED) (Supplementary Fig. 2B), which were previously reported in *D. melanogaster* DEREED and shown to be functionally important (Luelier et al., 2000). Tc-FADD has a single death domain and another death domain in the homologous superfamily result, which might correspond to the death domain and death-interacting domains, respectively in *D. melanogaster* FADD (Supplementary Fig. 2C).
counterparts of proteins in other species. DREDD encode the functional in T. castaneum and FADD. Microbial induction of (Hu & Yang, 2000). These results suggest that Caspar, DREDD and FADD in T. castaneum encode the functional counterparts of proteins in other species.

Microbial induction of Caspar, DREDD and FADD

The levels of expression of insect immune-related genes are frequently influenced by microbial invasion in several organisms, such as, D. melanogaster and T. castaneum (De Gregorio et al., 2001, 2002; Yokoi et al., 2012a, b). To confirm these findings, we investigated whether T. castaneum Caspar, DREDD and FADD were induced by an injection of heat-killed microbes (Fig. 1). The results indicate that the mRNA amounts of Caspar (Fig. 1A), DREDD (Fig. 1B) and FADD (Fig. 1C) were not or only weakly affected 6 and 24 h after the challenges with E. coli, M. luteus and S. cerevisiae.

Fig. 1. Microbial challenge and effects on mRNA levels of Caspar, DREDD and FADD. Day 3 pupae were challenged with a heat-killed cell suspension of E. coli (Ec), M. luteus (Ml) or S. cerevisiae (Sc) and the mRNA levels of Caspar (A), DREDD (B) and FADD (C) were assessed using qRT-PCR, 6 and 24 h after a challenge. Unchallenged (Uc) and PBS-injected day 3 pupae (PBS) served as controls. mRNA values are shown relative to those of RpL32, which were approximately 0.01 and 0.05 respectively (Yokoi et al., 2012b). Knockdown pupae and pupae treated with maleE dsRNA as controls were challenged with the heat-killed cells of E. coli, M. luteus or S. cerevisiae, and the levels of mRNA in these treated pupae were measured 6 and 24 h after the challenges. As previously reported the amounts of maleE mRNA in unchallenged pupae were approximately 0.01 and 0.05 respectively (Yokoi et al., 2012a), which are clearly lower than those in maleE dsRNA-treated control pupae, 6 and 24 h post challenge with three microbes, except for Att1 and Col1 post challenge 24 h by S. cerevisiae. Thus, the results in Figs 3 and 4 can be considered to show the effects of the knockdown of Caspar, DREDD and FADD on Col1 inductions by the three microbial challenges, except for Fig. 4F. The mRNA amounts of Att1 in DREDD, FADD and Caspar dsRNA pre-treated pupae 6 h after the E. coli challenge were not significantly different from those in control pupae, whereas they were significantly lower in DREDD and FADD knockdown pupae than in control pupae 24 h after a challenge (Fig. 3A and 3B). Pupae treated with Caspar dsRNA, 6 and 24 h after the E. coli challenge had slightly higher mRNA amounts of Att1 than control pupae. Comparisons

Efficiency of the infection of dsRNA in the knockdown of Caspar, DREDD and FADD

Prior to the functional assay of these three genes, we determined whether they were effectively knocked by RNAi. Day 0 pupae were injected with the dsRNA for the respective targets along with control maleE dsRNA and the amounts of mRNA were measured 72 h later. The results are presented in Fig. 2, which show that gene knockdown resulted in 80 to 85% reductions in the amounts of mRNA for all three genes.

Effects of the knockdown of Caspar, DREDD and FADD on the microbial induction of group I AMP genes

Using Caspar, DREDD and FADD knockdown pupae, the effects of the knockdown of these genes on the expressions of the group I genes, Att1 (Fig. 3) and Col1 (Fig. 4), are largely dependent on the IMD pathway (Yokoi et al., 2012b). Knockdown pupae and pupae treated with maleE dsRNA as controls were challenged with the heat-killed cells of E. coli, M. luteus or S. cerevisiae, and the levels of mRNA in these treated pupae were measured 6 and 24 h after the challenges. As previously reported the amounts of Att1 and Col1 mRNA in unchallenged pupae were approximately 0.01 and 0.05 respectively (Yokoi et al., 2012a), which are clearly lower than those in maleE dsRNA-treated control pupae, 6 and 24 h post challenge with three microbes, except for Att1 and Col1 post challenge 24 h by S. cerevisiae. Thus, the results in Figs 3 and 4 can be considered to show the effects of the knockdown of Caspar, DREDD and FADD on Col1 inductions by the three microbial challenges, except for Fig. 4F. The mRNA amounts of Att1 in DREDD, FADD and Caspar dsRNA pre-treated pupae 6 h after the E. coli challenge were not significantly different from those in control pupae, whereas they were significantly lower in DREDD and FADD knockdown pupae than in control pupae 24 h after a challenge (Fig. 3A and 3B). Pupae treated with Caspar dsRNA, 6 and 24 h after the E. coli challenge had slightly higher mRNA amounts of Att1 than control pupae. Comparisons

Fig. 2. Efficiency evaluation of RNAi-mediated gene knockdown. Day 0 pupa were injected with 100 ng of Caspar, DREDD, FADD or control maleE dsRNA, and the levels of mRNA relative to those of Rpl32 were measured 72 h later. Experiments were independently carried out at least three times, and the mean ± S.D is shown. Double asterisks indicate P < 0.01 versus controls based on Student’s t-test.
of the mRNA amounts of \textit{Att1} in control pupae challenged with \textit{E. coli} and \textit{M. luteus} revealed that the levels of activation of \textit{Att1} induced by the \textit{M. luteus} challenge were lower than those of the \textit{E. coli} challenge, which is consistent with previous findings (Yokoi et al., 2012a). The effects of the knockdown of \textit{DREDD}, \textit{FADD}, or \textit{Caspar} on gene induction did not significantly alter the levels of \textit{Att1} mRNA 6 h after the \textit{M. luteus} challenge (Fig. 3C). Pupae treated with \textit{DREDD} or \textit{FADD} dsRNA had lower mRNA levels of \textit{Att1} 24 h after the \textit{M. luteus} challenge than control pupae (\textit{Att1} mRNA levels of \textit{DREDD} knockdown and control pupae were clearly different but not significantly so), while the pupae with \textit{Caspar} dsRNA showed modest enhancements 24 h after the \textit{M. luteus} challenge (Fig. 3D). Six hours after the \textit{S. cerevisiae} challenge, no significant differences were observed in \textit{Att1} mRNA levels between any dsRNA-treated and control pupae (Fig. 3E). Significantly lower mRNA levels of \textit{Att1} 24 h after the \textit{S. cerevisiae} challenge were recorded in \textit{DREDD}, \textit{FADD} and \textit{Caspar} dsRNA-treated pupae than in control pupae (Fig. 3F).

Similar knockdown and microbial induction experiments were performed for the group I gene, \textit{Col1} (Fig. 4). Overall results were similar to those for \textit{Att1}. Six hours after the \textit{E. coli} challenge, \textit{Col1} mRNA amounts were significantly lower in \textit{DREDD} knockdown pupae than in control pupae, but did not differ significantly between \textit{FADD} knockdown and control pupae (Fig. 4A). \textit{Col1} mRNA amounts were significantly higher in \textit{Caspar} knockdown pupae than in control pupae. Twenty-four hours after the \textit{E. coli} challenge, \textit{Col1} mRNA amounts were significantly lower in \textit{DREDD} and \textit{FADD} knockdown pupae than in control pupae, while those in \textit{Caspar} knockdown pupae were slightly higher (Fig. 4B). \textit{Col1} mRNA amounts in \textit{DREDD}, \textit{FADD} and \textit{Caspar} knockdown pupae 24 h after the \textit{M. luteus} challenge did not differ significantly from those in the control pupae (Fig. 4C). Twenty-four hours after the \textit{M. luteus} challenge, \textit{Col1} mRNA amounts were slightly higher and lower in \textit{Caspar} and \textit{DREDD} knockdown pupae, respectively, than in control pupae, but were significantly lower in \textit{FADD} knockdown pupae than in
control pupae (Fig. 4D). Similar to 6 h after the *M. luteus* challenge, no significant differences were recorded in *Col1* mRNA amounts between *DREDD*, *FADD* and Caspar knockdown pupae and control pupae 6 h after the *S. cerevisiae* challenge (Fig. 5). *Col1* mRNA amounts varied among *DREDD*, *FADD* and Caspar knockdown pupae and control pupae 24 h after the *S. cerevisiae* challenge, but were not significantly different (Fig. 4F).

**Effects of the knockdown of Caspar, DREDD and FADD on the microbial induction of the group II AMP gene**

The results recorded for the group II gene *Def3* were distinct from those for group I genes (Fig. 5). The level of expression of *Def3* in unchallenged pupae is approximately 0.003 (Yokoi et al., 2012a), which is clearly a lower value than that recorded in pupae treated with *malE* dsRNA and post challenged 6 and 24 h later with three microbes, except for post challenge 24 h with *M. luteus* and *S. cerevisiae*. Thus, results in Fig. 5A, B, C and E are considered to show effects of the knockdown of Caspar, DREDD and FADD on *Def3* inductions by the three microbial challenges. Levels of *Def3* expression 6 h after the *E. coli* challenge were significantly lower in *DREDD* and *FADD* knockdown pupae than in control pupae, while those in Caspar knockdown pupae were significantly higher (Fig. 5A). Levels of expression of *Def3* were slightly lower in *FADD* knockdown pupae. Similar results were obtained for pupae 24 h after the *E. coli* challenge (Fig. 5B). Levels of expression of *Def3* were similar in pupae 6 h after the *M. luteus* and *E. coli* challenges, with the lack of significant differences attributed to large variations in *malE* control values (Fig. 5C). Twenty-four hours after the *M. luteus* challenge, the levels of expression of *Def3* were significantly lower in *DREDD* and *FADD* knockdown pupae than in control pupae, but were higher in Caspar knockdown pupae (Fig. 5D). Regarding the induction by *S. cerevisiae* 6 h after the challenge, the knockdown of *DREDD* and *FADD* appeared to suppress the induction of *Def3* (Fig. 5E). The induction profiles of *Def3* by *M. luteus* and *S. cerevisiae* at 24 h post challenge

---

*Fig. 4. Effects of the knockdown of Caspar, DREDD and FADD on the expression of the group I AMP gene, Col1, in pupae challenged by a microbe.* Day 0 pupae were injected with preparations of dsRNA. Seventy-two hours later, the dsRNA-treated pupae were injected with a heat-killed cell suspension of *E. coli* (A and B), *M. luteus* (C and D) or *S. cerevisiae* (E and F). The relative levels of mRNA of *Att1* to *RpL32* were assessed 6 (A, C and E) and 24 h (B, D and E) after a microbial challenge and shown as the mean ± S.D. Experiments were independently repeated at least three times with three animals each. Single asterisks indicate *P* < 0.05 versus *malE* controls based on Student’s *t*-tests, while double asterisks denote *P* < 0.01.
apparently exhibits some knockdown effects, but in both cases the induction will have ceased by this time according to our previous results (Yokoi et al., 2012a, b) and is well supported by the low Def3 mRNA levels (Fig. 5D and 5F).

**Effects of the knockdown of Caspar, DREDD and FADD on the microbial induction of group III AMP genes**

Two group III genes, Cec2 and Cec3, the expression of which is largely dependent on Toll signaling, were investigated (Figs 6 and 7) (Yokoi et al., 2012b). The values of the expressions of Cec2 and Cec3 in unchallenged pupae is approximately 0.02 and 0.1, respectively (Yokoi et al., 2012a, b), which are clearly lower than those recorded for malE dsRNA-treated controls depicted in Figs 6 and 7. Thus, the results in Figs 6 and 7 can be considered to show the effects of the knockdown of Caspar, DREDD and FADD on the induction of Cec2 and Cec3, respectively, by the three microbial challenges. The level of expression of Cec2 in Caspar and DREDD knockdown pupae 6 h after the challenge with E. coli did not differ significantly from those in control pupae, while modest up-regulation and suppression were only recorded in FADD knockdown pupae 6 and 24 h, respectively, after the challenge (Fig. 6A and 6B). Six hours after the challenge with M. luteus, the amounts of Cec2 mRNA were significantly and slightly lower in DREDD and FADD knockdown pupae, respectively, than in control pupae (Fig. 6C). Twenty-four hours after the challenge, the amounts of Cec2 mRNA in DREDD and FADD knockdown pupae did not differ significantly from those in control pupae (Fig. 6D). The knockdown of Caspar appeared to activate effects that became more prominent (also significantly different) in the later phase of induction (Fig. 6C and 6D). No significant differences were recorded between Caspar, DREDD and FADD knockdown
The challenge with knockdown and control pupae (Fig. 7B). Six hours after were slightly more abundant in M. luteus 6 h after the challenge controls (Fig. 7C). Amounts of Cec3 F ADD and DREDD fitness and the challenge with S. cerevisiae were lower in DREDD and FADD knockdown pupae than in control pupae, while no significant differences were recorded 24 h after the challenge (Fig. 7E and 7F).

**Survival assays**

To clarify the involvement of these IMD pathway-associated intracellular factors in the antimicrobial host defenses of T. castaneum, survival was assayed using two model pathogenic bacterial strains. Pupae pre-treated with dsRNA were inoculated intrahaemocytically with defined doses of live E. cloacae or B. subtilis, and percentage survival was then recorded every 24 h. maleE dsRNA-treated pupae served as negative controls, while those treated with IMD dsRNA were positive controls. Results are shown in Kaplan-Meier plots (Fig. 8). In comparisons with the negative control, the knockdown of IMD severely impaired host defenses against E. cloacae and B. subtilis (Fig. 8A to 8D). DREDD and FADD knockdown pupae represented intermediate phenotypes. The knockdown of FADD had similar effects to that of IMD in terms of defense against the two bacteria, while those of the knockdown of DREDD appeared to be weaker. DREDD knockdown pupae succumbed to E. cloacae infection slightly faster than maleE controls ($P = 0.0536$, Fig. 8B). In addition, the knockdown of DREDD significantly weakened host defense against B. subtilis, and the $P$-value was higher than in the case of IMD knockdown (Fig. 8D). The knockdown of Caspar resulted in slightly higher levels of mRNA of some AMP genes than in the maleE control (Figs 3 to 7). Nevertheless, it did not increase the resistance to these two bacterial pathogens.

**DISCUSSION**

In the present study, we examined the genes coding for potential receptor-proximal components of the IMD pathway in the model beetle T. castaneum, namely, Caspar, DREDD and FADD, the products of which are constituents of the IMD pathway of Drosophila (Klein & Silverman, 2014). We previously reported that the IMD and Toll pathways of T. castaneum exhibit a nearly non-specific manner of activation in terms of microbial species used as an elicitor, while the magnitude of activation differed depending on the type of microbe (Yokoi et al., 2012a, b). In the present study, we investigated the involvement of these genes in the activation of this indiscriminate immune pathway, which indicates a marked difference in the immune signaling in this model beetle from the Drosophila system.

Caspar, DREDD and FADD in T. castaneum encode proteins bearing structural features that are typical of the respective family members, including domain configurations, suggesting that these are orthologous genes of the functionally defined counterparts in other organisms. We then showed that these genes were not activated by im-
We characterized these genes by estimating their contribution to the microbial induction of two group I genes that represent the IMD pathway read-out. While the results obtained 6 h after the microbial challenge were obscure, the knockdown of DREDD and FADD resulted in the unequivocal suppression of these two IMD pathway-dependent genes after 24 h. Based on these results, we conclude that DREDD and FADD functioned as canonical constituents of the IMD pathway in this beetle. We previously conducted similar experiments that targeted IMD, and found that the knockdown of IMD took effect by 6 h after the microbial challenge, with an approximately 50% reduction in group I gene induction (Yokoi et al., 2012b). Based on similar reductions in the mRNA levels of these factors upon the dsRNA treatment in our present and previous studies, the different phenotypes in the earlier phase of microbial gene induction may be attributed to differences in signal strengths, which may be attributed to differences in sensitivities to reductions in group I AMP genes by an approximately 1.5-fold to 4-fold more potent than M. luteus (the present study, Yokoi et al., 2012b, a). The double knockdown of PGRP-LA plus PGRP-LC, PGRP-LD, or PGRP-LE or the triple knockdown of the PGRP-LA, PGRP-LC and PGRP-LE proteins, however, suppress the induction of group I and II AMP genes by an E. coli or M. luteus challenge. These findings indicate that PGRP-LA bound by an E. coli-derived DAP-type peptidoglycan or M. luteus-derived Lys-type peptidoglycan undergo strong conformational changes that intensively recruit intracellular components, suggesting the lack of a clear difference in the strength of the recognition of both types of peptidoglycans (Koyama et al., 2015). Differences in sensitivities to reductions in group I AMP genes may be attributed to differences in signal strengths, which activate the IMD and Toll pathways during the E. coli and M. luteus challenges; M. luteus may activate the Toll pathway more strongly than E. coli.

We noted that the early-phase responses of the group I genes Att1 and Col1 appeared to be slightly more sensitive to the gene knockdown of these intracellular factors when challenged with E. coli than with M. luteus. In terms of group I gene induction, E. coli is approximately 1.5-fold to 4-fold more potent than M. luteus (the present study, Yokoi et al., 2012b, a). The double knockdown of PGRP-LA plus PGRP-LC, PGRP-LD, or PGRP-LE or the triple knockdown of the PGRP-LA, PGRP-LC and PGRP-LE proteins, however, suppress the induction of group I and II AMP genes by an E. coli or M. luteus challenge. These findings indicate that PGRP-LA bound by an E. coli-derived DAP-type peptidoglycan or M. luteus-derived Lys-type peptidoglycan undergo strong conformational changes that intensively recruit intracellular components, suggesting the lack of a clear difference in the strength of the recognition of both types of peptidoglycans (Koyama et al., 2015). Differences in sensitivities to reductions in group I AMP genes may be attributed to differences in signal strengths, which activate the IMD and Toll pathways during the E. coli and M. luteus challenges; M. luteus may activate the Toll pathway more strongly than E. coli.

Group I gene induction by heat-killed S. cerevisiae 6 h after the challenge was not affected by the knockdown of DREDD or FADD.

These knockdown phenotypes of DREDD and FADD, and previously obtained IMD knockdown phenotypes in terms of group I gene induction, along with the results of group II and group III genes, appear to be consistent with the results of the survival assays in the present study with the following order of severe impairments in host defenses: IMD knockdown > FADD knockdown > DREDD knockdown.

Fig. 8. Effects of the knockdown of Caspar, DREDD and FADD on host defenses against two model bacterial pathogens. Day 3 pupae that were treated with respective preparations of dsRNA on day 0 were challenged by either E. cloacae (A and B) or B. subtilis (C and D), and the survival of pupae thereafter was monitored and recorded every 24 h. Results are shown in Kaplan-Meier plots. The first set of experiments was carried out using malE, IMD, Caspar and FADD dsRNA-treated pupae (A and C), and the second set using malE, IMD and DREDD dsRNA-treated pupae (B and D). malE dsRNA served as negative controls, while IMD dsRNA provided positive controls. Significant differences from malE-treated controls based on the Gehan-Breslow-Wilcoxon test are denoted by * (P < 0.05) or ** (P < 0.01).
Caspar, DREDD or FADD. Twenty-four hours after the challenge, mRNA levels of Att1 in Caspar, DREDD and FADD knockdown pupae were lower than in control pupae, while Col1 expression was not affected by any of the three knockdows. The induction of group I genes, including Att1 by heat-killed S. cerevisiae cells, has ceased by this time (Yokoi et al., 2012a). Actually, Att1 mRNA levels in control pupae 24 h post challenges (approximately 0.05 in Fig. 3F) were not so different from in unchallenged pupae (approximately 0.01 (Yokoi et al., 2012a)) compared to that in the other conditioned pupae. (Att1 levels in control pupae 24h post E. coli and M. luteus challenges was approximately 0.6 and 0.4 respectively). These results indicate that the observed differences reflect variations in the status of the final phase. Furthermore, based on these results, we conclude that DREDD and FADD constitute the canonical IMD pathway of T. castaneum, as in Drosophila, and are not mainly involved in immune signaling induced by the S. cerevisiae challenge. Regarding Caspar, its inhibitory effects on the activation of the IMD pathway appeared to be weak or negative and counteracted DREDD in some cases.

The kinetics of group II gene Def3 differed from those of the group I gene upon gene knockdown of these intracellular factors. The suppressive effects of the knockdown of DREDD and FADD became apparent 6 h after an E. coli or M. luteus challenge. These results indicate that the group II gene is more sensitive to a decrease in IMD pathway components than group I genes, which is consistent with our previous findings on IMD (Yokoi et al., 2012b) PGRP-LA (Koyama et al., 2015). As described above, PGRP-LA functions as the canonical receptor of the T. castaneum IMD pathway, and this isoform recognizes Gram-negative DAP-type peptidoglycans and Gram-positive Lys-type peptidoglycans. In addition, analyses of the 3’ regions of these AMP genes reveal that each group I gene has a single copy of the κB motif potentially bound by Relish, while group II Def3 has two inverted copies of potential κB motifs (Yokoi et al., 2012a). Collectively, these findings indicate that the expression of group I genes is readily driven by one Relish molecule, while other classes of transcription factors, such as tissue- or developmental stage-specific factors, synergistically support their high levels of expression (Rus et al., 2013). On the other hand, the full activation of group II Def3 may at least require the occupation of two copies of κB sites in its proximal promoter region by two molecules of the activated Relish protein. These findings account for the nature of Def3, in that it responds more sensitively to the decline in IMD pathway constituents. The degree of Def3 suppression upon knockdown of DREDD and FADD 24 h after the E. coli challenge appeared to be less conspicuous than those for group I genes and the results of the M. luteus or S. cerevisiae challenge after 24 h was not considered because the induction of Def3 had ceased before then and also because lower levels of expression of Def3 were recorded in control pupae post challenge 24 h with M. luteus and S. cerevisiae than in the conditioned pupae as described in the previous paragraph (Yokoi et al., 2012a). This finding may be attributed to the Toll pathway-dependent nature of Def3, which supports the later phase of the induction of Def3.

The group III genes, Cec2 and Cec3, are largely dependent on the Toll pathway for their microbial induction (Yokoi et al., 2012a, b). We determined the involvement of DREDD and FADD in group III gene regulation and found that the knockdown of DREDD significantly suppressed gene induction 6 h after the M. luteus challenge, while the effects of the knockdown of FADD in terms of the suppression of induction by E. coli were recorded 24 h after the challenge. Since this was the case for Cec2 and Cec3, it is reasonable to speculate that these factors function at points common to the regulatory cascade of these group III genes, namely, the Toll pathway, and not due to a decrease in the terminal NF-κB transcription factor of the IMD pathway, Relish. The low sensitivity of group III genes to the knockdown of Relish provides support for this idea (Yokoi et al., 2012a). Six hours after the M. luteus challenge, the knockdown of FADD had a similar outcome to that of DREDD on the induction of both Cec2 and Cec3, indicating that these two Toll-dependent group III genes are also regulated by FADD and DREDD via common mechanisms and that the products of these two genes cooperate in this putative path similar to the multimeric molecular assembly activation of the IMD pathway (Kleino & Silverman, 2014). Since the knockdown of IMD did not suppress the induction of the group III gene by M. luteus after 6 h (Yokoi et al., 2012b), DREDD and FADD appear to function independently of the IMD protein under these conditions. Twenty-four hours after the E. coli challenge, the knockdown of DREDD and FADD resulted in distinct phenotypes; only the knockdown of FADD negatively affected the induction of the group III gene. This result indicates that the FADD protein has different roles under these two distinct conditions.

Caspar was initially identified as a suppressor of IMD signaling based on the genetic screening of a set of Drosophila mutants (Kim et al., 2006). Caspar, which is homologous to mammalian Fas-associating factor 1 (Chu et al., 1995), targets the proteolytic activation of Relish by DREDD. Its loss-of-function phenotype involves the constitutive expression of AMP genes associated with the IMD pathway and increased resistance to Gram-negative infection. In the present study on T. castaneum, the knockdown of Caspar generally resulted in an opposite effect on the induction of the microbial AMP gene when that of DREDD had a negative effect. This result appears to support the functions of DREDD recorded in the present study, which depends on its caspase activity. The basal levels of mRNA of AMP genes were not elevated in unchallenged Caspar knockdown pupae (data not shown). This result, taken together with the mild phenotypes of challenged Caspar knockdown pupae for the induction of the AMP gene, appear to be consistent with the results of survival assays showing that the knockdown of Caspar did not increase the percentage survival over that of maleE-treated controls.

Of the three microbes used as elicitors in the present study, we did not obtain clear results with S. cerevisiae.
The transcriptional activation of T. castaneum AMP genes by S. cerevisiae is very dependent on Toll signaling, even for group I genes (Yokoi et al., 2012b), and in a subsequent study on NF-kB transcription factor genes involved in the immune challenge from S. cerevisiae they may evoke unidentified activating signals other than IMD or Toll signaling (Yokoi et al., 2012a). Therefore, we attribute the equivocal results obtained with S. cerevisiae in the present study to the nature of S. cerevisiae as an elicitor.

We compared the immune reactions related to IMD, Toll pathway and induction of the AMP gene with our results on T. castaneum and previous findings on D. melanogaster. Data recorded for T. castaneum were from pupae, whereas those for D. melanogaster were mainly for adult flies. In D. melanogaster, the induction levels of several AMP genes, including Dipetecin, CecropinA, Drosocin and AttacinA, differ in third instar larvae and adults (Verma & Tagadia, 2012). Therefore, the levels of induction of AMP may differ between pupae and other life stages in T. castaneum. Moreover, comparison of the present results for T. castaneum pupae with previous findings on Drosophila, which were mainly obtained from adults, may be inappropriate. Although the levels of induction of AMP may differ between life stages, both in D. melanogaster and T. castaneum, the basic immune regulation of AMP genes by Toll and IMD pathways did not markedly differ between the life stages. Therefore, a discussion on the differences in immune signaling via the Toll and IMD pathways between T. castaneum and D. melanogaster based on comparisons of the present results with previous findings on D. melanogaster, as described above, is appropriate for the interpretation of immune signaling.

In the present study, we determined the involvement of the Caspar, DREDD and FADD genes of T. castaneum in two immune signaling pathways, namely, the IMD and Toll pathways. The results indicate that the intracellular factors encoded by these genes represent the canonical constituents of the IMD pathway in this beetle, as in Drosophila. Furthermore, the present study demonstrated that DREDD and FADD influenced the activation of Toll-dependent group III AMP genes, presumably through their IMD-independent functions to some extent. This intracellular component shared by the two immune signaling pathways provides possible points of signal crosstalk between the two pathways and will facilitate further research on the molecular background of the indiscriminate microbial pathway activation characteristics of T. castaneum’s antimicrobial immunity.

ACKNOWLEDGEMENTS. This work was supported by JSPS KAKENHI Grant Numbers JP21H03831 to K. Yokoi and 20K06050 to K. Miura. We also thank Y. Yagi, T. Ushimaru, A. Miyanoshita, and M. Murata for providing research material.

REFERENCES


doi: 10.14411/eje.2022.003


Received July 19, 2021; revised and accepted December 17, 2021

Published online January 12, 2022

Supplementary figures, supplementary tables and supplementary data

All supplementary figures, supplementary table and supplementary data were uploaded to figshare. DOI: 10.6084/m9.figshare.c.5429688

Supplementary Fig. 1

Approximately-maximum-likelihood phylogenetic trees of (A) Caspar, (B) DREDD and (C) FADD. Numbers near branch points and along the branches are bootstrap values and lengths of branches, respectively. DOI: 10.6084/m9.figshare.14618898

(A) Phylogenetic tree results using the Caspar genes of T. castaneum (Tc-caspar), A. aegypti (Ac-caspar, GenBank ID: XP_021697938.1), A. gambiae (Ag-caspar, GenBank ID: XP_316513.1), D. melanogaster (Dm-caspar, GenBank ID: NP_611080.1), B. mori (isoform X1) (Bm-caspar, GenBank ID: XP_037836765.1), A. mellifera (isoform X1) (Am-caspar, GenBank ID: XP_392750.1), N. lugens (Nl-caspar, GenBank ID: XP_022206141), mouse orthologue FAF1 (Mouse FAF1, GenBank ID: NP_032009.2) and the human orthologue, FAF1 (Human-FAF1, GenBank ID: CAB67705.1). The fasta file of the predicted amino acid sequences is in Supplementary Data 1 (Caspar-AA-seqs.fa),
(B) Phylogenetic tree results using the DREDD genes of
_T. castaneum_ (Tc-Dredd), _A. aegypti_ (Ae-DREDD, Gen-
Bank ID: ABI74776.1), _A. gambiae_ (Ag-Dredd, GenBank
ID: XP_552560.2), _D. melanogaster_ (Dm-DREDD (isoform
gamma), GenBank ID: AAC33118.1), _B. mori_ (Bm-DREDD,
GenBank ID: NP_001108337), _A. mellifera_ (Am-DREDD, Gen-
Bank ID: XP_006570976), _N. lugens_ (Ni-DREDD, GenBank
ID: XP_039280806), mouse orthologue Caspase-8 (Mouse-Cas-
pase-8, GenBank ID: NP_032009.2) and the human orthologue,
Caspase-8 (Hm-Casp8, GenBank ID: AAD24962.1). The fasta
file of the predicted amino acid sequences is in Supplemen-
tary Data 1 (DREDD-AA-seqs.fa).

(C) Phylogenetic tree results using the FADD genes of
_T. castaneum_ (Tc-FADD), _A. aegypti_ (Ae-FADD, Gen-
Bank ID: EAT46931.1), _A. gambiae_ (Ag-FADD, GenBank
ID: XP_308597.4), _D. melanogaster_ (Dm-FADD, Gen-
Bank ID: AAF55950.1), _B. mori_ (Bm-FADD, GenBank ID:
NP_001189465), _A. mellifera_ (Am-FADD, GenBank ID:
XP_006572135), mouse FADD (Mouse-FADD, GenBank ID:
NP_034305) and human FADD (Hm-FADD, GenBank ID:
CAG33019.1). The _N. lugens_ orthologue was not found in the
_N. lugens_ genome. The fasta file of the predicted amino acid se-
quencies is in Supplementary Data 1 (FADD-AA-seqs.fa).

Supplementary Fig. 2

InterPro scan results of (A) Tc-Caspar, (B) Tc-DREDD and (C)
Tc-FADD. The numbers in the upper part of each figure indicate
the sequence numbers of amino acid residues counted from the
N terminal end. The gff result file of each amino acid sequence
is uploaded in Supplementary Data 3. Detailed results are on the
InterPro site (URL: https://www.ebi.ac.uk/interpro/about/inter-
proscan/). DOI: 10.6084/m9.figshare.14625522

Supplementary Table 1

Primer sequence list in the present study. DOI: 10.6084/
m9.figshare.14625510

Supplementary Data 1

Fasta files of predicted amino acid sequences of Caspar,
DREDD and FADD used for phylogenetic trees. DOI: 10.6084/
m9.figshare.16918201

Supplementary Data 2

Aln files (Clustal omega output files) and newick files (Fast-
Tree output files) of Caspar, DREDD and FADD. DOI: 10.6084/
m9.figshare.14618706

Supplementary Data 3

Gff files of InterProScan results of Tc-Caspar, Tc-DREDD and
Tc-FADD. DOI: 10.6084/m9.figshare.16918210