Original Article

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Tissue-dependent induction of antimicrobial peptide genes after body wall injury in house fly (*Musca domestica*) larvae

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Summary **Injury of the insect body wall, which enables environmental microorganisms to invade into insect tissues, induces innate immune responses including the induction of antimicrobial peptides (AMPs) in flies and silkworms. Here, house fly (*Musca domestica*) larvae and pupae were injured using a needle and the effects on the expression of genes encoding AMPs were examined. The expression of AMP genes including *defensin*, *attacin*, *diptericin*, and *sarcotoxin II* dramatically increased in both larvae and pupae after injury of the body wall, indicating that innate immune responses were induced. Furthermore, the injury-dependent expression of AMP genes was examined in larval tissues including fat bodies, hemocytes, salivary glands, and digestive tracts. Injury-dependent AMP gene expression was observed in salivary glands, hemocytes, and fat bodies, but not in digestive tracts. The degree of the transcriptional induction of each gene differed among tissues, suggesting that their expression is governed by complex regulatory machinery and that AMPs have tissue-specific functions. To further examine the properties of the AMPs, we examined the antimicrobial activities of partial synthetic peptides corresponding to portions of the predicted AMP proteins deduced from the AMP genes. A synthetic peptide exhibited antimicrobial activity, indicating that these injury-inducible genes are potential medicinal resources.**

Keywords: Antimicrobial peptide, innate immunity, house fly, larval tissue, insect

1. Introduction

Insects respond to microbial infections *via* the activation of an innate immune system consisting of germline-encoded sensor, signaling, and defense molecules including antimicrobial peptides (AMPs) (*1*). In insects including the flesh fly (*Sarcophaga peregrina*) and silkworm (*Bombyx mori*), innate immune responses including the induction of AMPs have been reported to be induced by injury of the body wall, which enables environmental microorganisms to invade into insect tissues (*2,3*). Additionally, we previously observed that antimicrobial activities were induced in the hemolymph of house fly (*Musca domestica*) larvae after injury (*4*). It has been shown that infectious inflammation signals induced by microbial components derived from microorganisms invaded through the injury sites and non-infectious inflammation signals induced by the physical damage due to the injury induce expression of AMPs in *Drosophila melanogaster* (*5-7*). In insects, the injury-dependent expression of AMPs has been reported to occur in the fat bodies, in which most of the intermediary metabolism takes place, including lipid and carbohydrate metabolism as in the mammalian liver (*8,9*). The recognition of invading microorganisms by sensor molecules induces the synthesis of AMPs, and the AMPs secreted into the hemolymph play an essential role in inhibiting the growth of invading microorganisms (*10*). Furthermore, the excretions or secretions of medicinal maggots of the blowfly (*Lucilia sericata*) contain AMPs in the absence of injury or bacterial invasion (*11*). These observations suggest that some flies express inducible and constitutive AMPs, and the defense system of flies can be established by various AMPs that are expressed constitutively or injury-dependently in a tissue-specific manner.

AMPs contain a region of positively-charged amino acids that specifically bind to negatively-

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charged microbial surface molecules such as bacterial lipopolysaccharide (*12-14*). This interaction disrupts the microbial membrane and leads to cell lysis and/or cell death (*15-17*). In addition to the general characteristics of AMPs, insect AMPs possess certain structural features, and they are classified into four families based on their structures. These four families are cysteine-rich peptides (such as defensin and drosomycin), glycine-rich peptides/proteins (such as attacin, sarcotoxin II, and diptericin), α-helical peptides (such as cecropin and sarcotoxin I) and proline-rich peptides (such as drosocin) (*18,19*).

AMPs are a novel class of antibiotics because of their broad-spectrum antimicrobial activities and their low tendency to induce resistance in microbes (*20*). Chemically synthesized AMPs have been developed based on the amino acid sequences of insect AMPs. For example, the synthetic peptide KLKLLLLLKLK-NH_2, which was developed from the *S. peregrina* AMP sapecin B, exhibits broad-spectrum antimicrobial activities (*21*). Furthermore, the same synthetic peptide synthesized using D-amino acids displayed a higher antimicrobial activity than its L-form counterpart (*22,23*). The potential utility of AMPs as an adjuvant has been examined because of their activity to induce immune responses in mammals (*24-26*). These observations demonstrate the importance of natural AMPs as medicinal resources.

The house fly (*M. domestica*) genome encodes a larger number of AMPs than any other dipteran genome (*27*). Thus, we expected that the AMPs encoded by the *M. domestica* genome are potential medicinal resources. In this study, we examined the properties of AMP genes in *M. domestica*. Particularly, we focused on the larval tissue-dependent induction of AMP genes.

2. Materials and Methods

2.1. *Reagents*

Dimethyl sulfoxide and bovine serum albumin (fraction V) were purchased from Sigma-Aldrich (St. Louis, MO, USA). AMPs were commercially synthesized by Toray Research Center (Tokyo, Japan). The C-terminal of each synthetic peptide was modified by amidation. All peptides were initially solubilized in dimethyl sulfoxide and then used for the analysis.

2.2. *Injury of fly larvae and pupae, and collection of tissues from injured larvae*

Non-sterile third-instar larvae of *M. domestica* provided by E's, Inc. (Tokyo, Japan) were injured by inserting the tip of a stainless steel hypodermic needle (Dentronics, Tokyo, Japan) into the abdominal cavity. The injured larvae were incubated in contact with insect saline solution (130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$) in plastic containers at 30°C for the indicated times. As an experimental control, intact larvae were also incubated in the same manner as described above.

To analyze gene expression in the whole body, injured larvae were directly stored in a tube at −80°C. For the analysis of gene expression in tissues, the fat bodies, salivary glands, and digestive tracts were dissected from larval bodies using fine tweezers and a surgical knife under a binocular microscope following anesthesia on ice. In this study, we defined the digestive tract as the alimentary canal from mouth to anus with the caecum of ventriculus and Malpighian tubule. The dissection was performed based on a previously published anatomical drawing of *M. domestica* larvae (*28*). To collect hemocytes, hemolymph was collected in a tube on ice by cutting off the anterior tip of the larvae using fine scissors. Hemocytes were then collected by the centrifugation of hemolymph at 4°C for 10 min at 2,000 × g. The collected tissues were stored at −80°C until use.

To collect pupae, third-instar larvae were incubated with gauze in plastic containers containing insect saline solution at 30°C until pupation. At 20-24 h after pupation, the pupae were injured using a stainless steel hypodermic needle and incubated for a further 20-24 h at 30°C in plastic containers. As an experimental control, intact pupae were also incubated in the same manner. After incubation, the pupae were stored at –80°C.

2.3. *RNA extraction and synthesis of first-strand cDNA*

Total RNA was extracted from larval whole bodies, fat bodies, hemocytes, digestive tracts, salivary glands, and pupal whole bodies using a MagExtractor™ nucleic acid purification kit (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. Total RNA was then treated with DNase I (TaKaRa Bio, Kusatsu, Japan) as previously described (*29*) and was reverse-transcribed using a PrimeScript™ RT reagent kit (TaKaRa Bio) with oligo(dT) primers and random hexamer primers according to the manufacturer's protocol.

2.4. *Real-time polymerase chain reaction (PCR)*

Real-time PCR was performed with a LuminoCt™ SYBR® Green qPCR ready mix (Sigma-Aldrich) using an Eco™ real-time PCR system (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Primers were synthesized by FASMAC (Atsugi, Japan) or Nippon Gene (Tokyo, Japan). The primer sequences are listed in Table S1 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=36*). The optimized thermal cycler settings for the PCR reactions were as follows: initial denaturation at 95°C for 20 s, followed by 45 amplification cycles of denaturation (95°C for 5 s) and annealing/extension. The annealing/extension conditions used are indicated in Table S1. The amount

of transcript was normalized to that of *glyceraldehyde-3-phosphate dehydrogenase (gapdh) or ribosomal protein 18 (rps18)* (*30*).

2.5. *Droplet digital PCR (ddPCR)*

The ddPCR procedure was performed using a QX100™ droplet digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The reaction mixture (20 µL) containing 1 × ddPCR supermix for probes (Bio-Rad Laboratories), 900 nM primers, 250 nM hydrolysis probe, and sample cDNA was loaded into a DG8 cartridge (Bio-Rad Laboratories) together with 70 µL of droplet generator oil (Bio-Rad Laboratories). Then, a cartridge was loaded into the QX100™ droplet generator (Bio-Rad Laboratories) to generate PCR droplets. The droplets were then transferred to a 96-well PCR plate (Eppendorf, Hamburg, Germany). PCR thermal cycling was performed using a C1000 Touch™ thermal cycler (Bio-Rad Laboratories) as follows: initial incubation at 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 60 s, followed by a final incubation at 98°C for 10 min and holding at 12°C. After reaction, the droplets from each well of the plate were read using a QX100™ droplet reader (Bio-Rad Laboratories). The quantification data were analyzed with the QuantaSoft™ analysis software (Bio-Rad Laboratories). PCR primers and a hydrolysis probe, whose sequences are listed in Table S2 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=36*), were designed and synthesized by TaKaRa Bio. The hydrolysis probe was labeled with carboxyfluorescein at the 5′ end and Black Hole Quencher® 1 at the 3′ end.

2.6. *Primer design for real-time PCR and ddPCR*

Primers for the amplification of transcripts by real-time PCR or ddPCR were designed based on the house fly genome sequence in the National Center for Biotechnology Information (NCBI) database. The sequences *LOC101887540*, *LOC101887872*, and *LOC101887709* were designated as *M. domestica defensin* genes. Six cysteine residues that are characteristic of the mature defensin peptide were conserved in the amino acid sequences of the proteins encoded by these genes, and a common potential cleavage signal (Lys-Arg) was found in each sequence (*31-33*) (Figure S1) (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=36*). Primers for *defensin* were designed to amplify these genes (Table S1), since their nucleotide sequences were highly similar. Another three genes, namely *LOC101888225*, *LOC105261620*, and *LOC105261775*, were designated as *defensin-like 1*, *defensin-like 2*, and *defensin-like 3*, respectively. The conserved cleavage signal (Lys-Arg) was not found in their amino acid sequences, but the characteristic six cysteine residues were conserved in the genes (Figure S1). Primers were designed to simultaneously amplify both *defensin-like 1* and *defensin-like 2* (Table S1) because of their similar sequences. A further four genes, namely *LOC101893190*, *LOC101893350*, *LOC101893688*, and *LOC101893852* were designated as *M. domestica sarcotoxin II*. A short proline-rich domain and two glycine-rich domains that are characteristic of *sarcotoxin II* were found in their amino acid sequences (Figure S2) (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=36*) (*34-36*). Primers for *sarcotoxin II* were designed to amplify these four genes because of their close similarity. The two genes *LOC101901352* and *LOC101889632* were designated as *M. domestica sarcotoxin I-B*. A hydrophilic domain that was rich in charged residues and a hydrophobic domain that was rich in nonpolar amino acid residues, both of which are present in *S. peregrina sarcotoxin I-B*, were found in *M. domestica sarcotoxin I-B* (Figure S3) (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=36*) (*37*). Primers for *sarcotoxin I-B* were designed to amplify these two genes. The genes *LOC101889508* and *LOC101887777* were designated as *M. domestica attacin and attacin-like*, respectively (*27,38*). A signal peptide and two glycine-rich domains were found in the amino acid sequence of *LOC101889508* as well as that of *Drosophila melanogaster attacin*, while no signal peptide was found in the sequence of *LOC101887777* (*35*) (Figure S4) (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=36*). The genes *LOC101897067* and *LOC101896897* were designated as *diptericin* according to their annotations in the NCBI database, and primers for *diptericin* were designed to amplify these two genes (Table S1). The genes *LOC101900398*, *LOC101895233*, *LOC101892543*, and *LOC101896200* were designated as *M. domestica salivary gland-expressed bHLH* (*sage*), *cytochrome P450 4aa1* (*cyp4aa1*), *larval serum protein 2* (*lsp2*), and *eater*, respectively, because of their 43%, 69%, 59%, and 44% identities with the genes of the same names in *D. melanogaster*, respectively. The lengths of the PCR amplicons were confirmed to correspond to the predicted lengths by agarose gel electrophoresis to ensure the specificity of the designed primers.

2.7. *Culture of bacteria*

Escherichia coli W3110 (NBRC12713) cells were grown at 37°C with aeration in Luria-Bertani (LB) medium (Nacalai Tesque, Kyoto, Japan). All experiments were conducted using bacterial cells in the logarithmic phase of growth.

2.8. *Determination of minimum inhibitory concentrations (MICs) of AMPs*

Bacterial suspensions in LB medium were adjusted to

an optical density at 600 nm = 0.016. Two-fold dilution series of peptide solutions were prepared in 10 mM phosphate buffer (pH 6) containing 130 mM sodium chloride, 0.2% bovine serum albumin, and 2% dimethyl sulfoxide. Each peptide solution (100 µL) was mixed with 100 µL of bacteria suspension. Bacterial cultures were incubated overnight at 37°C. The MIC was defined as the lowest concentration of antibiotic at which there was no visible growth of the organism (*39*).

2.9. *Determination of colony-forming units (CFUs)*

Bacterial suspensions were diluted appropriately with growth medium, and 500 µL of the suspension was mixed with 500 µL of peptide solution prepared as described above. The peptide/bacteria suspensions were incubated at 37°C for 1 h, then the suspensions were diluted and spread onto LB agar plates. After cultivation of the plates, the number of CFUs in each peptide/bacteria suspension was calculated based on the average of triplicate plates.

2.10. *Statistical analysis*

Statistical analysis were performed using one way ANOVA and Scheffe's test or Welch's *t*-test using Statcel3 (OMS publishing, Tokyo, Japan), and $p < 0.05$ was considered significant.

3. Results

3.1. *Needle injury induces the expression of AMP genes in both larvae and pupae*

In this study, we first examined whether the expression of *defensin*, *attacin*, *diptericin*, and *sarcotoxin II* would be induced by needle injury of the body wall in *M. domestica* larvae. Our analysis of gene expression by real-time PCR using transcripts from larval whole bodies revealed that the expression levels of the AMP genes in injured larvae were 24-300 times higher than those in intact larvae (Figure 1A). These observations indicated that needle injury induced the expression of AMP genes in *M. domestica* larvae. Furthermore, we examined whether needle injury would also induce the expression of AMP genes in pupae. As shown in Figure 1B, the expression levels of the AMP genes in injured pupae were 6-2,600 times higher than those in intact pupae. These observations indicate that the injury-dependent induction of AMP genes, which is a representative event of the innate immune response in some insects, occurred in pupae and larvae of the house fly (*M. domestica*).

3.2. *Expression of AMP genes in larval tissues upon injury*

We examined the expression of AMP genes in larval fat

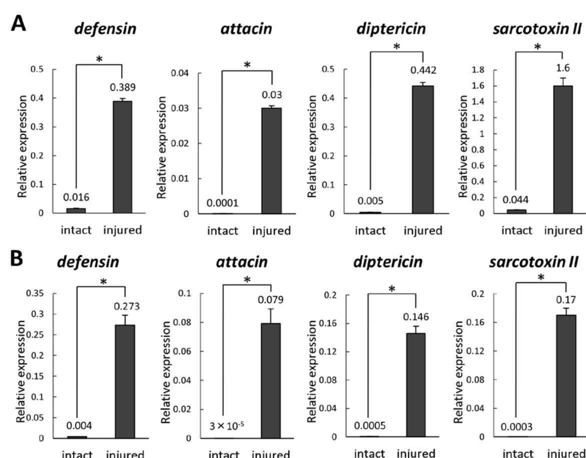

Figure 1. Needle injury induces the expression of antimicrobial peptide genes in *Musca domestica* larvae and pupae. The expression levels of *defensin*, *attacin*, *diptericin*, and *sarcotoxin II* in injured and intact *M. domestica* larvae **(A)** and pupae **(B)** were examined by real-time polymerase chain reaction (PCR). Larvae and pupae were used for mRNA extraction at 20-24 h after injury, and 10 larvae or 20 pupae were used as one batch. The expression level of each gene is given as a ratio to that of the internal control gene *gapdh*. The bars represent the mean relative expression level of triplicate assay of a batch and the error bars represent the standard deviation. An average values are shown above the bars. Data were analyzed by the Welch's *t*-test. *p < 0.05. The results shown were representative of two independent experiments.

bodies to examine whether the fat body in *M. domestica* larvae is a tissue in which innate immune responses are induced by needle injury of the body wall. The expression levels of AMP genes in the fat bodies of injured larvae were much higher than those in the fat bodies of intact larvae at 6, and 24 h after injury (Figure 2), and the expression levels peaked at 6 h after injury (Figure 2). These observations indicate that the injury-dependent expression of AMP genes was transient in fat bodies, peaking at approximately 6 h after injury and remaining significantly elevated at 24 h after injury.

To further explore the injury-dependent expression of AMP genes in larval tissues, we examined their expression in the salivary glands, digestive tracts, and hemocytes in addition to fat bodies. In these experiments, we examined various structural types of AMPs, including cysteine-rich peptides (*defensin*, *defensin-like 1*/*defensin-like 2*, and *defensin-like 3*), glycine-rich peptides (*attacin*, *attacin-like*, *sarcotoxin II*, and *diptericin*), and an α-helical peptide (*sarcotoxin I-B*). The structural features of the AMPs are shown in Figures S1-S5 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=36*). Additionally, *LOC101888779*, which was previously classified as a *diptericin* gene based on its sequence (*27*), was analyzed, although *LOC101888779* was annotated as an uncharacterized protein in the NCBI database.

Before we analyzed the expression of AMP genes in the larval tissues, we examined whether the tissues were appropriately collected using the following tissue-specific marker genes: a salivary gland marker gene,

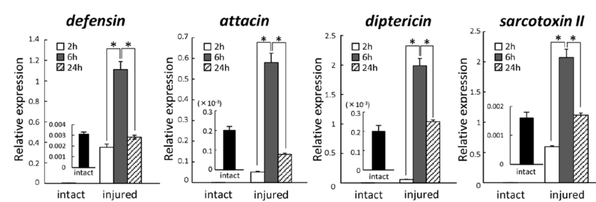

Figure 2. Transient expression of antimicrobial peptide genes in fat bodies after injury. The expression levels of *defensin*, *attacin*, *diptericin*, and *sarcotoxin II* in fat bodies were examined by real-time PCR. Fat bodies were prepared from larvae at 2, 6, and 24 h after injury or from intact larvae. Fat bodies were collected from 40 larvae as one batch at each time point. The expression level of each gene is given as a ratio to that of *gapdh*. Values represent the mean relative expression level of triplicate assay of a batch and the error bars represent the standard deviation. Data were analyzed by the one way ANOVA and Scheffe's test. *p < 0.05. Insets are enlarged views of parts of the panels. The results shown were representative of two independent experiments.

sage; a digestive tract (hindgut) marker gene, *cyp4aa1*; a fat body marker gene, *lsp2*; and a hemocyte marker gene, *eater*. It is well established that these four genes are expressed in a tissue-specific manner in *D. melanogaster* larvae (*40-43*). These genes also showed tissue-specific expression in *M. domestica* larvae, indicating the validity of the tissue collection (Figure S6) (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=36*).

The expression of *defensin* was increased in fat bodies and hemocytes after injury (Figure 3A). Interestingly, significant *defensin* expression was also observed in hemocytes from intact larvae at a higher level than that in salivary glands, digestive tracts and fat bodies from intact larvae. The expression of *defensin-like 1* and *2* was also observed in hemocytes from intact larvae to some extent, and their expression was apparently increased after injury (Figure 3B). By contrast, the expression of *defensin-like 1* and *2* did not increase after injury in other tissues including fat bodies (Figure 3B). The injury-dependent induction of *defensin-like 3* expression was also apparent in hemocytes (Figure 3C). These results indicated that defensin and defensin-related peptides were induced after injury, but their induction levels or patterns differed among tissues. Furthermore, some defensin peptides were constitutively expressed in hemocytes. These observations imply that defensin and defensin-related peptides might have some tissue-specific functions in *M. domestica* larvae.

The expression of *attacin* increased in hemocytes and fat bodies after injury, whereas that of *attacin-like* increased in hemocytes but not in fat bodies (Figures 3D and E). These observations suggest that attacin-related peptides also have tissue-specific functions in *M. domestica* larvae.

The expression of *diptericin* was strongly induced in fat bodies and hemocytes (Figure 3F). By contrast, the expression of *LOC101888779* was observed in all tissues examined in intact larvae to some extent and its

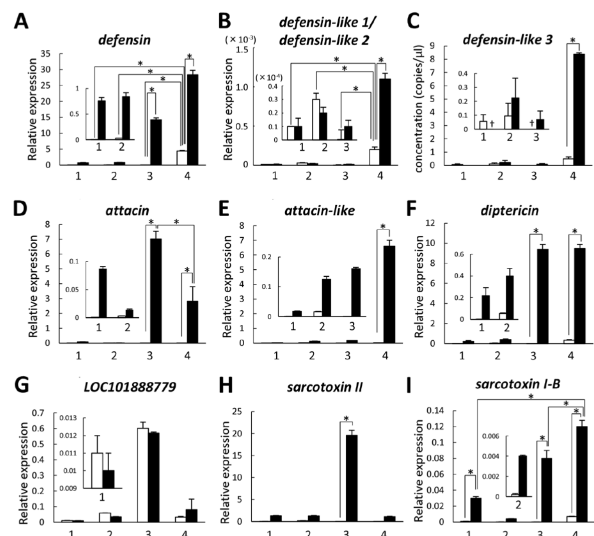

Figure 3. Expression of antimicrobial peptide genes in larval tissues after injury. The expression levels of *defensin* **(A)**, *defensin-like 1/defensin-like 2* **(B)**, *defensin-like 3* **(C)**, *attacin* **(D)**, *attacin-like* **(E)**, *diptericin* **(F)**, *LOC101888779* **(G)**, *sarcotoxin-II* **(H)**, and *sarcotoxin I-B* **(I)** in the salivary gland (1), digestive tract (2), fat body (3), and hemocyte (4) were measured by real-time PCR **(A, B, D-I)** or droplet digital PCR **(C)**. Tissue samples were prepared from larvae at 20-24 h after injury or from intact larvae. Salivary glands, digestive tracts, and fat bodies were collected from 5-15 larvae as one batch. Hemocytes were collected from approximately 100-400 larvae as one batch. For real-time PCR analysis, the expression level of each gene is given as a ratio to that of *rps18*. For droplet digital PCR analysis, gene expression data are represented as the copy number per µL of first-strand cDNA solution. In both analyses, values represent the means of triplicate assay of a batch from intact larvae (white bars) or injured larvae (black bars) and the error bars represent the standard deviations. Dagger (†) indicates that the expression was not detected. Insets in panels A, B, C, D, E, F, G, and I are enlarged views of parts of the panels. Data were analyzed by the one way ANOVA and Scheffe's test. *$p < 0.05$. The results from another batch were shown in Figure S7.

injury-dependent induction was not apparent (Figure 3G). These observations indicate that *diptericin* is injury-inducible but *LOC101888779* is not. The amino acid sequences of diptericins in *Phormia terraenovae* and *D. melanogaster* possess a short proline-rich domain and a long glycine-rich domain, both of which are characteristic features of diptericin (*34*). The *diptericin* encoded by *LOC101897067* and *LOC101896897* in *M. domestica* possesses both these domains, indicating that it is a typical diptericin (Figure S5). However, no sequence similar to the proline-rich domain or the glycine-rich domain was found in the deduced amino acid sequence of *LOC101888779* (Figure S5). Combined with the fact that the expression of *LOC101888779* was not injury-inducible, *LOC101888779* might not belong to the diptericin family or function as an AMP.

The expression of *sarcotoxin II* increased strongly in fat bodies after injury (Figure 3H). The expression of *sarcotoxin I-B* increased in salivary glands, fat bodies and hemocytes after injury (Figure 3I).

The reproducibility of the results described above was confirmed (Figure S7) (*http://www.ddtjournal.com/*

Table 1. MICs of synthetic peptide fragments derived from defensin-related genes in *M. domestica* against *E. coli*

Gene designation	Sequence	MIC (μg/mL)
sapecin B	RSLCLLHCRLK	150
defensin	HSACAAHCLLRGNR	> 300
defensin-like 1	KDSVCAAHCLLIGKS	> 300
defensin-like 2	HSVCAAHCLLLGKS	> 300
defensin-like 3	KVSCQAHCLLLKKR	300

The experiments were performed two times independently and obtained same results.

action/getSupplementalData.php?ID=36). In Figure S7, injury-dependent inductions of *defensin*, *attacin*, *sarcotoxin II*, and *sarcotoxin I-B* were observed in salivary glands. Since the reproducibility of significant inductions of *defensin*, *attacin*, and *sarcotoxin II* were not confirmed in salivary glands (Figure 3), precise analysis is required for the AMP genes inductions in the salivary glands. These results, taken together, indicated that AMP genes were induced in the fat body, hemocyte, and/or salivary gland in response to injury. However, the induction pattern differed among tissues. These findings suggest that AMPs have tissue-specific functions and that their expression is governed by complex regulatory machinery.

3.3. *A peptide fragment from a defensin-related peptide shows antimicrobial activity*

The peptide fragment RSLCLLHCRLK-NH_2 from sapecin B, an AMP belonging to the defensin family in the flesh fly (*S. peregrina*), exhibits an antimicrobial activity against *Staphylococcus aureus*, *E. coli*, and *Candida albicans* (*21*). Since *M. domestica* defensin and defensin-related peptides possess a region that is similar to the peptide RSLCLLHCRLK-NH_2, we examined the antimicrobial activities of four peptide fragments corresponding to that region of four predicted *M. domestica* AMPs against *E. coli* (Table 1).

The MICs of these peptide fragments against *E. coli* were determined (Table 1). The peptide fragment derived from *defensin-like 3*, namely KVSCQAHCLLLKKR-NH_2, showed a significant antimicrobial activity (Table 1). However, other peptide fragments derived from *defensin*, *defensin-like 1*, and *defensin-like 2* did not show any antimicrobial activity (Table 1). Furthermore, a CFU assay revealed that KVSCQAHCLLLKKR-NH_2 exhibited its antimicrobial activity (Figure 4). These results indicated that the peptide region from *defensin-like 3* possessed an antimicrobial activity. These observations indicate the potential of the injury-inducible AMPs of *M. domestica* as medicinal resources.

4. Discussion

In this paper, we have demonstrated that the expression of genes encoding AMPs in the house fly (*M. domestica*) was induced by needle injury. The needle injury can induce infectious inflammation signal by microbial components derived from environmental microorganisms invaded through the wound, and it can also induce non-infectious inflammation signals by physical damage. The inductions of AMP genes were observed in both larvae and pupae. Our analysis of larval tissues revealed that the induction of AMP genes occurred in hemocytes, fat bodies, and salivary glands, but not in digestive tracts. The degree of induction for each gene differed among tissues, suggesting that complex regulatory machinery governs their expression.

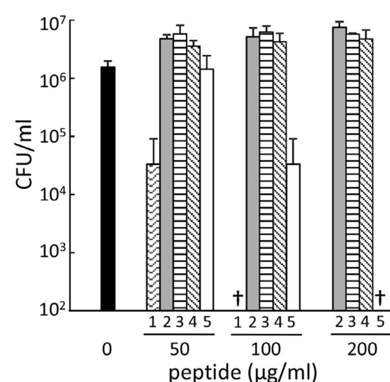

Figure 4. Antimicrobial activities of synthetic fragment peptides derived from defensin-related genes in *M. domestica*. The antimicrobial activities of synthetic fragment peptides from *M. domestica* antimicrobial peptides against *E. coli* were examined by determining the number of colony-forming units in the assay mixture containing synthetic fragment peptides derived from *sapecin B* (1), *defensin* (2), *defensin-like 1* (3), *defensin-like 2* (4), or *defensin-like 3* (5). The sequences of the peptide fragments are indicated in Table 1. Values represent the mean of triplicate samples and the error bars represent the standard deviation. Dagger (†) indicates that no bacteria were detected.

Previously, Tang *et al*. showed that the expression of AMP genes in *M. domestica* larvae was induced by the injection of bacteria (*44*). Our observation that the expression of AMP genes was induced by injury is consistent with their result. The injury-dependent expression of AMP genes was revealed to also occur in pupae. Furthermore, our analysis of larval tissues revealed that the induction of the genes was regulated in a complex manner. For example, *attacin* was induced in both fat bodies and hemocytes by injury, but *attacin-like* was induced only in hemocytes. At present, the molecular machinery and physiological functions of the localized induction of AMPs remains unknown, but this phenomenon implies that the AMPs have tissue-specific functions. Additionally, our results revealed that some defensin-related peptides were constitutively expressed in hemocytes and their expression increased further after injury. In addition to their acute induction in response to injury, the constitutive expression of AMPs in hemocytes might have some functions in intact larvae.

It is noteworthy that almost all the studied AMP genes, except *sarcotoxin II*, were induced in hemocytes after injury and that some AMP genes were not induced

in the fat body, which was previously characterized as the dominant organ of injury-dependent AMPs synthesis (*8*). Our findings suggest that hemocytes are the most sensitive tissue to the induction of innate immune responses after injury.

The *M. domestica* genome encodes a larger number of AMPs than any other Dipteran genome (*27*). Our results demonstrate that the *M. domestica* AMP genes show tissue-specific and injury-inducible expression, and their complex induction machinery might imply that they have tissue-specific functions against infection. The merit of multiple expressions of AMPs in larval tissues is the combinatorial activity of AMP against invaded microorganisms. The larvae of *L. sericata*, which are known as medicinal maggots, expressed attacins, cecropins, diptericins, and sarcotoxins in addition to lucifencin, and the AMPs showed additive as well as synergetic activity (*11,45*). Also, insect AMP complexes, in contrast to individual AMP, has been shown to be well protected from resistance development in bacteria (*46*). Examinations of AMP combinations in natural products, including insect hemolymph and insect tissues, are valuable for development of combinatorial products of AMP, because the AMP combinations in natural products may be suitable for the merits described above.

In this study, we showed that a synthetic peptide fragment from the injury-inducible AMP gene exhibit antimicrobial activity. This observation implies that injury-inducible AMP genes in house fly were potential resource for some applications including medicine. AMP, such as polymyxin B and colistin, is clinically used as an antimicrobial agent (*47*). Interestingly, some AMPs showed biological activities, such as immunomodulation, angiogenesis, and wound healing, against mammalian cells and tissues (*48-50*). The usefulness of injury-inducible AMP genes in house fly is limited now, and the further study of the AMP is necessary for the formation of grounds of the practical use.

Acknowledgements

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