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ORIGINAL ARTICLE

The expression pattern of immunity-related genes in the immunized black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae)

Youngwoo Cho[1,2] and Saeyoull Cho[1,2,*]

[1] Department of Applied Biology, College of Agriculture and Life Science, Kangwon National University, Chuncheon, Kangwon-do 200-701, South Korea; e-mails: saeyoullcho@kangwon.ac.kr, 5120057@kangwon.ac.kr

[2] Department of Interdisciplinary Program in Smart Agriculture, College of Agriculture and Life Science, Environment Friendly Agriculture Center, Kangwon National University, Chuncheon, South Korea

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Abstract. After immunizing black soldier fly (*Hermetia illucens*) larvae using Gram-negative (*Escherichia coli*) and Gram-positive (*Bacillus thuringiensis*, Bt) bacteria, we compared expression patterns across the whole genome. Compared to a control group treated with PBS buffer, the group immunized using Bt showed significant differences in the expression patterns of 2,312 genes. Similarly, the group immunized using *E. coli* showed significant differences in the expression patterns of 2,251 genes compared to the control. The groups immunized with *E. coli* and Bt both showed overexpression of genes involved in the extracellular region, serine-type endopeptidase activity, and neuropeptide signaling pathways, including genes related to the Toll pathway. In other words, the immune response of black soldier flies involves the simultaneous expression of innate immunity-related genes, irrespective of the type of bacteria. This is determined to be because, rather than showing a specific immune response, the flies depend on responding rapidly based on high expression levels.

INTRODUCTION

Black soldier flies (*Hermetia illucens*) are a commercial insect with very high economic value. Their uses include bioremediation of food waste, production of growth-promoting fertilizer for crops using manure, and feed for chicken, pigs, and salmon (Stamer, 2015; Tomberlin & Huis, 2020; Park et al., 2021; Jensen et al., 2021). Commercial insects can also cause enormous economic losses if they become infected by any of a variety of pathogenic microbes during mass breeding, leading to large-scale natural mortality or extermination to control the spread of the pathogen(s) (Wang & Shelomi, 2017). However, all organisms, including insects, possess refined immune mechanisms to defend against invasive pathogens and maintain their populations (Vogel et al., 2018). Generally, insect immunity differs from mammals due to the absence of T and B cells, meaning that they do not exhibit immune mechanisms based on antigen-antibody interactions. However, insects have two types of immune response that have allowed them to survive in the face of various microbes (Cooper & Eleftherianos, 2017; Eleftherianos et al., 2021; Gomes et al., 2022).

Immune mechanisms in insects can be divided into the cellular immune system and the humoral immune system. Cellular immunity refers to immunity mediated by hemocytes in the blood of insects. In general, seven types of hemocyte have been reported in the blood of insects (prohemocytes, plasmatocytes, granulocytes, adipocytes, oenocytoids, spherulocytes, and coagulocytes), some of which only become active during invasion by pathogens, eliminating the pathogens through processes of phagocytosis, encapsulation, and nodulation (Lavine & Strand, 2002; Strand, 2008; Dubovskiy, 2016). Insects are highly diverse, and different types of insect exhibit differences in their hemocyte composition and immune cells. For example, insects in the order Diptera typically only have 3–4 types of hemocyte, and have been reported to possess unique hemocytes that are not found in other insects, such as crystal cells and lamellocytes (Hoffmann & Reichhart, 2002; Hoffmann, 2003).

The second immune system in insects is humoral immunity, in which antimicrobial peptides are produced to eliminate invasive pathogens (Janeway & Medzhitov, 2002; Tasakas & Marmaras, 2010). These antimicrobial peptides bind to the cell membrane/cell wall of the pathogen to form holes, causing release of the cell contents. The expression of diverse antimicrobial peptides is affected by the invading pathogen. For example, in the vinegar fly *Drosophila melanogaster*, the IMD pathway is activated upon invasion

* Correspondig author; e-mail: saeyoullcho@kangwon.ac.kr

by gram-negative bacteria and fungi, ultimately leading to the production of broad-spectrum antimicrobial peptides, including drosomycin, diptericin, and attacin. In contrast, when invaded by gram-positive bacteria and fungi, the Toll pathway is activated, leading to the production of various antimicrobial peptides that include drosomycin, metchnikowin, and defensin (Janeway & Medzhitov, 2002; Tasakas & Marmaras, 2010).

Currently, next-generation sequencing (NGS) is being used to investigate the immune response at the whole genome scale and to identify immunity-related genes (Zhang et al., 2023). By comparing the transcriptome before and after pathogen exposure, it is possible to identify genes that are up- or down-regulated during the immune response. For example, the overall immune response in insects can be understood by analyzing overexpression of Toll, IMD, antimicrobial peptide, and immune cell-related genes with changes in the expression of pattern recognition receptors (PRRs; Bang et al., 2013). In this study, we artificially infected *Hermetia illucens*, a very important commercial species, with gram-positive or gram-negative bacteria, and used NGS to analyze immunity-related genes and the differences in the immune responses to different types of bacteria.

MATERIALS AND METHODS

Insects

In this study, black soldier fly (*H. illucens*) larvae were obtained from the Korea Beneficial Insects Lab. Ltd. in Guri, South Korea. Plastic breeding containers (50 cm × 40 cm × 60 cm) were prepared with a rectangular opening cut in the lid, and a microscopic mesh screen secured over the opening to allow ventilation. A mixture of poultry feed, compost, manure, and sawdust was soaked in water until it became the texture of damp sponge, before being laid on the floor of the rearing containers. Adult black soldier flies (30 females and 20 males) were added to each container. We reared over 2,000 insects in a climate chamber (MIR-553; Sanyo Electric Biomedical, Japan), maintaining a temperature of 27 ± 1°C, light cycle of 16L : 8D), and relative humidity of 60 ± 10%. The insects used in the study were all reared in a sterile environment.

Bacteria

We used live representative gram-negative (*Escherichia coli* k12) and gram-positive (*Bacillus thuringiensis*, Bt) bacteria to immunize *H. illucens*. We inoculated a small ~2 ul of 1×10^5 of *E. coli* culture medium to LB Broth (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH = 7.0) and incubated the mixture for 12 h. For Bt (*B. thuringiensis*), ~2ul of 1×10^5 of Bt culture medium was inoculated to NA broth (Nutrient powder 1 g/L, peptone 5 g/L, yeast extract 2 g/L, NaCl 10 g/L, pH = 7.0) and the mixture was incubated for 18 h. The *E. coli* and Bt cultures were centrifuged at 5°C, 4,000 rpm for 5 min, washed three times with physiological saline (PBS buffer; NaH_2PO_4, Na_2HPO_4 and NaCl, pH = 7.2), and adjusted to an optical density (OD, 600 nm) of 1.3.

Immunization

We divided the insects into a group treated with PBS (control), a group treated with *E. coli* k12 and a group treated with Bt. The *H. illucens* in all groups were 5th instar larvae, measuring approximately ~ 10 mm in total length and weighing approximately 0.3 g each. The dorsal region was washed clean with 70% ethanol, and a Pasteur pipette was used to inoculate each insect with ~ 3 µl. The peak of immune activation was 12 h, which is typical for most insects (Kwon et al., 2014; Cho & Cho, 2019, 2021; Go et al., 2022). In other words, RNA was extracted for use 12 h after inoculation. The experiment was repeated in triplicate with 10 insects per group, meaning that a total of 30 insects were analyzed for each of the three groups.

RNA extraction, library construction, and Illumina sequencing

We used the RNasey Plus Mini Kit (Qiagen) and applied a bead-based method to enhance RNA extraction efficiency. We extracted and mixed RNA from three insects in each group. In simple terms, RNA extraction was achieved by dissecting the insect, extracting the fat body tissue, and mixing 1 ml of TRIzol per 100 mg of tissue. Plastic beads 0.4–0.6 mm, 2 mm, and 7 mm in diameter (Daihan Agent, Wonju, South Korea) were added to the tissue in a 50 ml tube, and the mixture was vortexed for 5–10 min. After centrifuging at 4°C and 14,000 rpm for 3 min, 1 ml of supernatant was collected. After adding 200 µl chloroform to the supernatant and shaking it gently, the mixture was left to react for 20 min at 4°C. After centrifugation at 4°C and 14,000 rpm for 15 min, the clear supernatant (~ 600 µl) was loaded into a gDNA elimination column to remove DNA. After the extract was centrifuged again at 4°C at 14,000 rpm for 1 min, after which 600 µl of 70% cold isopropanol/ethanol (maintained at –20°C) was added to precipitate the RNA. Using an RNeasy Mini Column, 700 µl of sample was centrifuged at 4°C and 14,000 rpm for 1 min, 700 µl of RW1 buffer (10 mM Tris-Hcl, 1 mM EDTA, 50 mM NaCl, 0.5% Tween-20, pH = 8.5) was added, and the mixture was centrifuged for another 1 min at 4°C and 14,000 rpm. After adding 500 µl of RPE buffer (10 mM Tris-Hcl, 80% ethanol, 0.5 mM EDTA pH = 7.5) to the sample, the mixture was centrifuged under the same conditions, and ~ 50 µl of RNase-free water was added to a new, sterilized RNase-free tube to extract the RNA.

Total RNA was isolated from three samples and DNA contamination was eliminated using DNase treatment. mRNA with poly-A tail was purified using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina). The purified mRNA was randomly fragmented and reverse transcribed into cDNA. Adapters were ligated onto both ends of the cDNA fragments and amplified by PCR. Fragments with insert sizes between 200–400 bp were selected for paired-end sequencing on an Illumina platform. All analyses were performed using RNA combined from three independent experiments for each group, followed by RNA-seq.

Date pre-processing

The raw reads underwent quality checking and trimming to eliminate low-quality reads, adapter sequences, contaminant DNA, or PCR duplicates. This process was carried out using FastQC v0.11.7 and Trimmomatic 0.38. The trimmed reads were mapped to the reference genome of H. illucens (available at https://www.ncbi.nlm.nih.gov/datasets/taxonomy/343691/) to produce Binary Alignment Map (BAM) files. The alignment success rate demonstrated a satisfactory level, approximately around 85%. From the BAM files, gene-specific read counts were extracted using FeatureCounts() of Rsubread v2.14.2 and integrated with gene annotation information for *H. illucens*. Utilizing this integrated dataset, an estimated total of 14,004 protein-coding genes were detected. Filtered protein-coding genes were used to construct a read count matrix, along with authored meta-information. The DESeq2 v1.40.2 was then applied for normalization and transformation, including log transformation and variance stabilization.

Expression analysis and differential expression gene (DEG) identification

Differentially expressed genes (DEGs) were identified using edgeR v3.38.4 with the P-value (adjusted by Benjamini-Hochberg method) < 0.001 and | Log2 (fold change) | > 2 threshold for significance under the assumption that biological coefficient of variance is 0.2. The gene symbols of his were converted into that of dme according to the match. For functional analysis, the gene information of *H. illucens* (his) was combined with that of *D. melanogaster* (dme) based on the Gene Group Identifier from NCBI Ortholog. Gene ontology (GO) analysis was performed using enrichGO() of clusterProfiler v4.9.0 based on org.Dm.eg.db v3.17.0. GO terms significantly enriched (P-value adjusted by Benjamini-Hochberg method < 0.05) on the significant DEGs were identified using clusterProfiler (version 4.4.4). Volcano plots and dot plots were generated using ggplot2 (version 3.4.0). All statistical analysis and visualizations were performed under R (version 4.2.2) and R studio (Build 576) envelopment.

RESULTS

The transcriptomic profiles of the group immunized with *E. coli* and Bt

After immunizing *H. illucens* larvae using *E. coli* and Bt, we compared expression patterns across the whole genome. We conducted the experiment three times, comparing it with a control group treated with PBS. Differences in genome-wide expression patterns in each group were analyzed using two-dimensional principal component analysis (PCA), which visualizes the transcriptomic distance (Fig. 1). The control samples are positioned on the left, while the gram-positive bacteria (Bt) and gram-negative bacteria (*E. coli*) samples are positioned on the right. It became evident that the administration of pathogens is the most significant factor determining the differences in the transcriptome (Fig. 1).

Differentially expressed genes (DEG)

Based on the above results, we analyzed the genes that showed significant differences between the groups (Fig. 2). For determining the statistical significance of Differentially Expressed Genes (DEGs), criteria were set with an absolute Log2FC > 2, a p-value < 0.05 and a fold change of at least 4. The results of the DEG analysis were visualized using a Volcano Plot and Bar Plot (Fig. 2). Compared to the control group treated with PBS buffer, the group immunized with Bt showed significant differences in expression patters for 2,312 genes. Specifically, the group immunized with Bt showed overexpression of 808 genes and underexpression of 1,504 genes. Similarly, the group immunized with *E. coli* showed significant differences in gene expression patterns compared to the control group for 2,251 genes in total (634 overexpressed genes, 1,617 underexpressed genes). These differences are thought to reflect differences in the expression patterns of genes induced by infections by these two types of bacteria. The most significantly differentially expressed genes, both up-regulated and down-regulated, were organized into bar plots, with the top 25 genes represented for each category (Fig. 2C, D). Furthermore, it was observed that common antimicropeptide genes such as *attacin* and *cecropin* were positioned among the top genes.

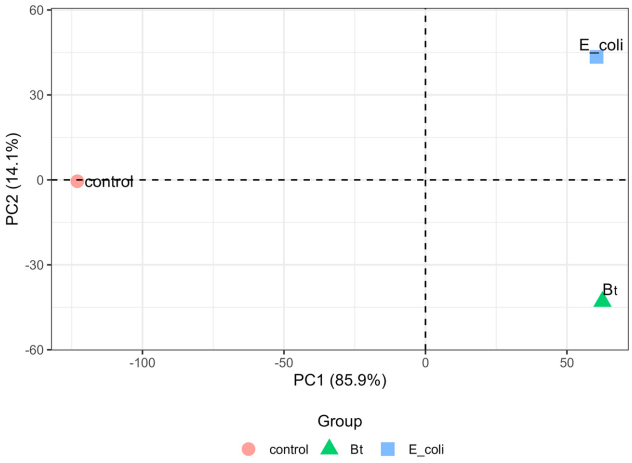

Fig. 1. Principal component analysis (PCA) performed on the full transcriptomes of black soldier flies, which were divided into three groups: a control group (injected with PBS), Bt group injected with gram-positive bacteria (*B. thuringiensis*), and *E. coli* group injected with gram-negative bacteria (*E. coli*). Regarding PC1, which predominantly encapsulates spatial information, the control is positioned on the left, while Bt and *E. coli* are distantly located on the right. In PC2, *E. coli* and Bt are vertically separated with control as the reference point, positioned in the middle. This indicates that the administration status and type of pathogen play a significant role in determining the differences in the transcriptome.

The gene ontology (GO) of genes that showed significant differences of expression in DEG analysis

Next, the enrichGO function in the specialized clusterProfiler (v4.4.4) R package was used to investigate the gene ontology (GO) of genes that showed significant differences of expression in DEG analysis. We selected the biological process (BP) terms that could represent the function of genes that were significantly up- or down-regulated in the DEG analysis, and performed a GO cluster map analysis based on the results (Fig. 3; significant representativeness was defined as a Benjamini-Hochberg adjusted p-value < 0.05). Using the emapplot function of ClusterProfiler, we visualized the correlations of the top 10 BP terms, with each dot corresponding to BP terms that were significantly representative (Fig. 2). Herein, the size of each dot represents the number of genes related to the selected BP term that showed a significant increase or decrease. The color of each dot represents the adjusted p-value of the corresponding BP term. The size of the points represents the number of genes. The lines between dots represent the number of genes shared between each pair of BP terms. The distance between dots represents the distance between BP terms, calculated based on the genes related to each BP term. Based on this distance, the BP terms were grouped and assigned a number from 1 to 3 (Fig. 3). We investigated each cluster and their GO terms. The genes in Cluster 1 for the group immunized with Bt were related to the defense response to pathogens, the innate immune response, and positive regulation of the Toll signaling pathway. For the group immunized with *E. coli*, the genes in this cluster were related to the defense response to gram-positive bacteria, the innate immune response, the peptidoglycan catabolic

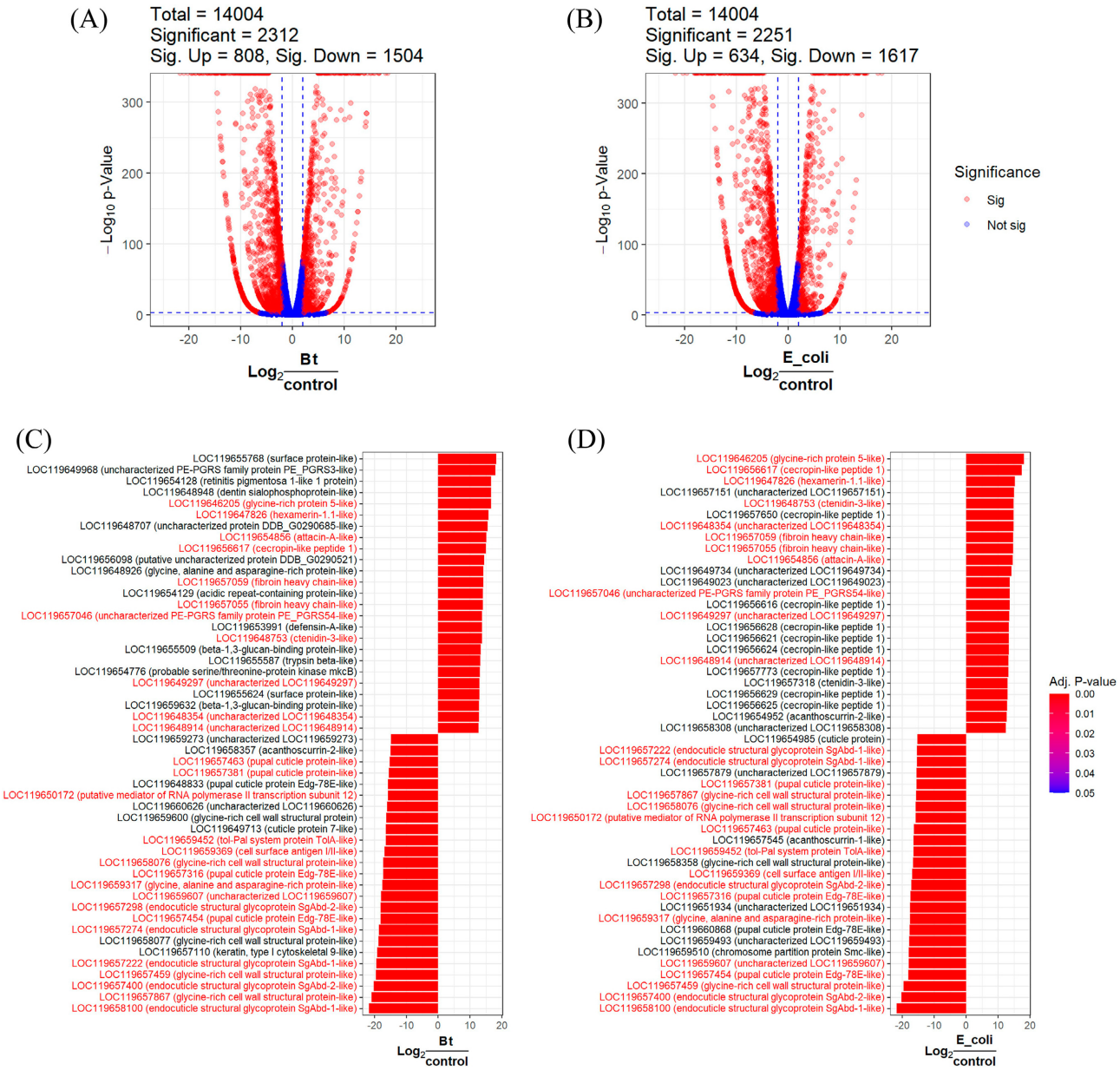

Fig. 2. Transcriptomic analysis. A volcano plot showing differences in expression between the groups depending on the treatment applied (A and B). The group treated with Bt manifested the overexpression of 808 genes and the underexpression of 1,504 genes compared to the control (A). Similarly, the group treated with *E. coli* manifested the overexpression of 634 genes and the underexpression of 1,617 genes compared to the control (B). The top 25 genes with the most significant differences, both up- and down-regulated, were organized into a barplot (C and D). The absolute value of Log_2FC was > 2 and the p-value was < 0.001.

process, and positive regulation of Toll signaling pathway. Compared to the control group, the first cluster included representative genes related to defense against pathogenic bacteria in insects (Toll and innate immune genes) in both the immunized groups (Fig. 3A, C, Tables 1, 3).

Compared to the control group, in the group immunized with Bt, the second cluster included genes related to the extracellular region, the extracellular space, and extracellular ligand-gated monoatomic ion channel activity (Fig. 3A, Table 1). In the group immunized with *E. coli*, the second cluster included genes related to extracellular ligand-gated monoatomic ion channel activity, the extracellular space, and iron ion binding (Fig. 3C, Table 3). The third cluster included genes related to serine-type endopeptidase activity, proteolysis, neuropeptide hormone activity, and neuropeptide signaling pathways in the group immunized by Bt, and genes related to the extracellular region, proteolysis, and serine-type endopeptidase activity in the group immunized with *E. coli*. Thus, genes related to serine-type endopeptidase activity and proteolysis were observed in this cluster in both groups (Fig. 3A, C, Table 1, 3). The down-regulated cluster exhibited genes related to the extracellular matrix, chitin-based cuticle development, and structural constituents of the cuticle. Genes related to the extracellular region, the chitin metabolic process, and chitin binding were grouped together, and genes related to heme binding, the extracellular space, and extracellular matrix structural constituents also formed a cluster (Fig. 3B, Table 2). In the

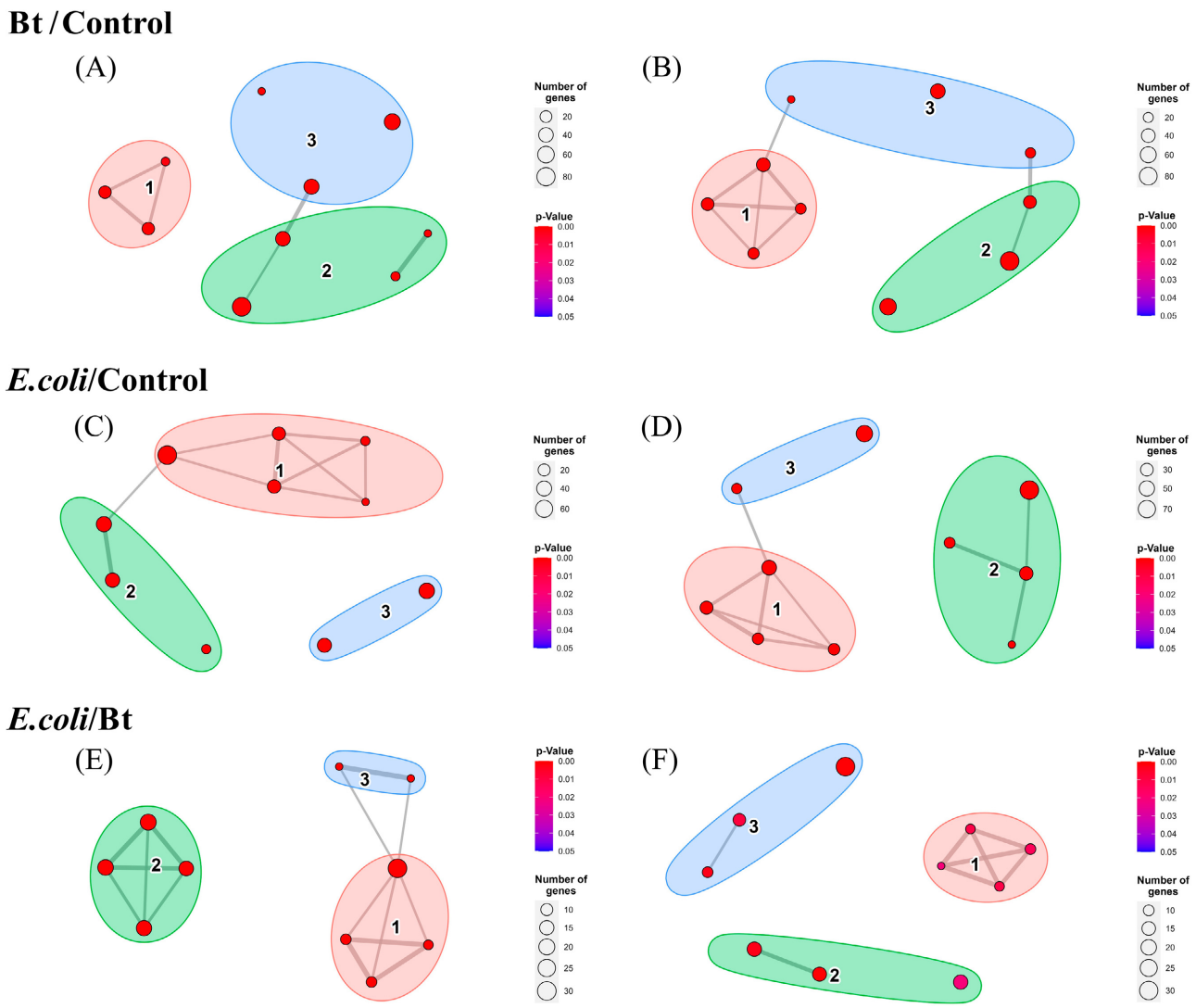

Fig. 3. Scores plot of principal component analysis (PCA) of a control group (injected with PBS), Bt group injected with gram-positive bacteria (*B. thuringiensis*), and *E. coli* group injected with gram-negative bacteria (*E. coli*). Using enrich-GO in cluster-Profiler (v4.4.4), an R package specialized for gene ontology (GO), we conducted a search for biological process (BP) terms that could represent the functions of genes exhibiting significantly increased or decreased expression in the results of the DEG analysis. We selected 10 BP terms based on their significance, and used the emap-plot function in cluster-Profiler to visualize their correlations (the criterion for determining significance was a Benjamini-Hochberg adjusted p-value < 0.05). The color of each dot represents the adjusted p-value of the corresponding BP term (p-value). The size of the points represents the number of genes. The lines between the dots represent the number of genes shared between each pair of BP terms. The distance between dots represents the distance between BP terms, calculated based on the genes related to each BP term; based on this distance, the BP terms were grouped and assigned a number (1–3).

comparison of the group treated with *E. coli* and the group treated with Bt, no noticeable immunity-related genes were detected in the up-regulation, but it was observed that the gene related to serine-type endopeptidase activity was more down-regulated in the group treated with *E. coli* (Fig. 6E, F, Table 5, 6).

Table 1. A list of up-regulated clusters of Bt compared to the control.

Cluster	Name	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Count
1	defense response to Gram-positive bacterium	24/712	67/9065	7.91E-11	1.48E-08	1.39E-08	24
1	innate immune response	24/712	101/9065	6.5E-07	6.94E-05	6.53E-05	24
1	positive regulation of Toll signaling pathway	9/712	22/9065	2.11E-05	0.00158	0.001487	9
2	extracellular region	86/712	352/9065	1.45E-22	1.08E-19	1.02E-19	86
2	extracellular space	55/712	282/9065	1.55E-10	2.31E-08	2.17E-08	55
2	extracellular ligand-gated monoatomic ion channel activity	8/712	15/9065	5.45E-06	0.000453	0.000426	8
3	serine-type endopeptidase activity	40/712	108/9065	8.74E-18	3.26E-15	3.07E-15	40
3	proteolysis	45/712	149/9065	6.27E-16	1.56E-13	1.47E-13	45
3	neuropeptide hormone activity	8/712	11/9065	1.86E-07	2.32E-05	2.18E-05	8
3	neuropeptide signaling pathway	10/712	21/9065	1.33E-06	0.000125	0.000117	10

Fig. 4. Gene Ontology (GO) enrichment analysis. For each comparison group, we selected the top 20 BP terms based on significance and visualized them in a dotplot. The size of each dot represents the number of genes (count) related to the corresponding BP term that showed a significant increase or decrease in the DEG analysis. The color of each dot represents the adjusted p-value of the corresponding BP term (p.adjust).

To summarize the results above, the genes that were overexpressed compared to the control group were grouped into 3 clusters, but immunity-related genes showed high relevance in each group. Moreover, given that the group immunized with gram-negative bacteria (*E. coli* k12) also showed a high percentage of genes related to defense against such bacteria, black soldier flies were shown to defend against bacteria by expressing genes related to innate immunity. Next, we performed a dotplot analysis to summarize our results.

Dotplot by selecting the BP terms for each comparison group

We created a summary dotplot by selecting the top 20 BP terms, in terms of significance, for each comparison group, and visualizing them in a dotplot. The size of each dot represents the number of genes (count) related to the corresponding BP term that showed a significant increase or decrease in the DEG analysis. The color of each dot represents the adjusted p-value of the corresponding BP term (p.adjust). There were very similar expression patterns for

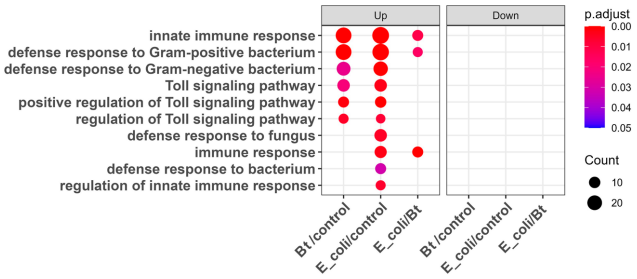

Fig. 5. Gene Ontology (GO) biological process (BP) terms associated with insect immune related response. For each comparison group, we selected the BP terms that satisfied the significance criteria and included immune, defense, T cell, B cell, or Toll, and we visualized these in a dot-plot. The size of each dot represents the number of genes (count) related to the corresponding BP term that showed a significant increase or decrease in the DEG analysis.

genes that were overexpressed, compared to the control group, in both the group immunized with gram-positive and gram-negative bacteria (Fig. 4). Next, we summarized the above results using immune system-related GOs. We made a dotplot by selecting the BP terms for each comparison group that satisfied the significance criteria and included immune defenses, T cell, B cell, or Toll. The size of each dot represents the number of genes (count) related to the corresponding BP term that showed a significant increase or decrease in the DEG analysis. The color of each dot represents the adjusted p-value of the corresponding BP term (p.adjust). Compared to the control group, the groups treated with Bt and *E. coli* k12 both showed significant overexpression of genes related to the innate immune response. In other words, there was significant overexpression of genes related to defense against both gram-negative and gram-positive bacteria (Fig. 5).

Normalization count of overexpressed gene

Subsequently, normalization counts were conducted for the detected genes, and their expression levels were illustrated with a bar plot. Toll pathway-related genes were observed to be overexpressed in the immunostimulated groups with Bt and *E. coli* compared to the control group (Fig. 6A, B). The Toll-like receptor-related genes were found to be most highly expressed in response to Bt (Fig. 6A). Furthermore, genes associated with the Toll pathway, such as *Pelle*, *Tube*, and *MyD88*, were also observed to be most highly expressed in the group treated with Bt (Fig.

Table 2. A list of down-regulated clusters of Bt compared to the control.

Cluster	Name	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Count
1	extracellular matrix	40/843	90/9065	1.48E-18	6.3E-16	5.89E-16	40
1	chitin-based cuticle development	33/843	73/9065	8.44E-16	1.8E-13	1.68E-13	33
1	structural constituent of cuticle	25/843	48/9065	4.49E-14	7.63E-12	7.13E-12	25
1	structural constituent of chitin-based larval cuticle	23/843	41/9065	5.63E-14	7.98E-12	7.46E-12	23
2	extracellular region	92/843	352/9065	4.75E-21	4.04E-18	3.78E-18	92
2	chitin binding	34/843	67/9065	3.23E-18	9.17E-16	8.56E-16	34
2	chitin metabolic process	21/843	39/9065	2.11E-12	2.25E-10	2.1E-10	21
3	extracellular space	67/843	282/9065	2.22E-13	2.7E-11	2.53E-11	67
3	heme binding	48/843	207/9065	1.52E-09	1.43E-07	1.34E-07	48
3	extracellular matrix structural constituent	16/843	36/9065	3.34E-08	2.84E-06	2.65E-06	16

Table 3. A list of up-regulated clusters of *E. coli* compared to the control.

Cluster	Name	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Count
1	defense response to Gram-positive bacterium	27/594	67/9065	2.13E-15	3.36E-13	3.01E-13	27
1	innate immune response	28/594	101/9065	2.87E-11	3.62E-09	3.24E-09	28
1	peptidoglycan catabolic process	8/594	12/9065	1.27E-07	1E-05	8.96E-06	8
1	positive regulation of Toll signaling pathway	10/594	22/9065	4.26E-07	2.98E-05	2.67E-05	10
2	extracellular ligand-gated monoatomic ion channel activity	10/594	15/9065	3.02E-09	3.17E-07	2.84E-07	10
2	extracellular space	45/594	282/9065	1.75E-08	1.57E-06	1.41E-06	45
2	iron ion binding	33/594	206/9065	1.35E-06	7.92E-05	7.1E-05	33
3	extracellular region	78/594	352/9065	1.12E-22	7.06E-20	6.32E-20	78
3	proteolysis	42/594	149/9065	1.43E-16	4.52E-14	4.05E-14	42
3	serine-type endopeptidase activity	35/594	108/9065	4.42E-16	9.28E-14	8.31E-14	35

Table 4. A list of down-regulated clusters of *E. coli* compared to the control.

Cluster	Name	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Count
1	extracellular matrix	45/957	90/9065	4.26E-21	3.89E-18	3.75E-18	45
1	chitin-based cuticle development	32/957	73/9065	2.47E-13	5.66E-11	5.45E-11	32
1	structural constituent of chitin-based larval cuticle	23/957	41/9065	8.5E-13	1.56E-10	1.5E-10	23
1	structural constituent of cuticle	24/957	48/9065	7.48E-12	1.14E-09	1.1E-09	24
2	chitin binding	35/957	67/9065	1.79E-17	8.2E-15	7.9E-15	35
2	extracellular region	87/957	352/9065	8.18E-15	2.5E-12	2.4E-12	87
2	chitin metabolic process	21/957	39/9065	2.46E-11	3.22E-09	3.1E-09	21
3	chitin catabolic process	14/957	27/9065	1.04E-07	8.68E-06	8.36E-06	14
3	extracellular matrix structural constituent	19/957	36/9065	3.53E-10	4.03E-08	3.88E-08	19

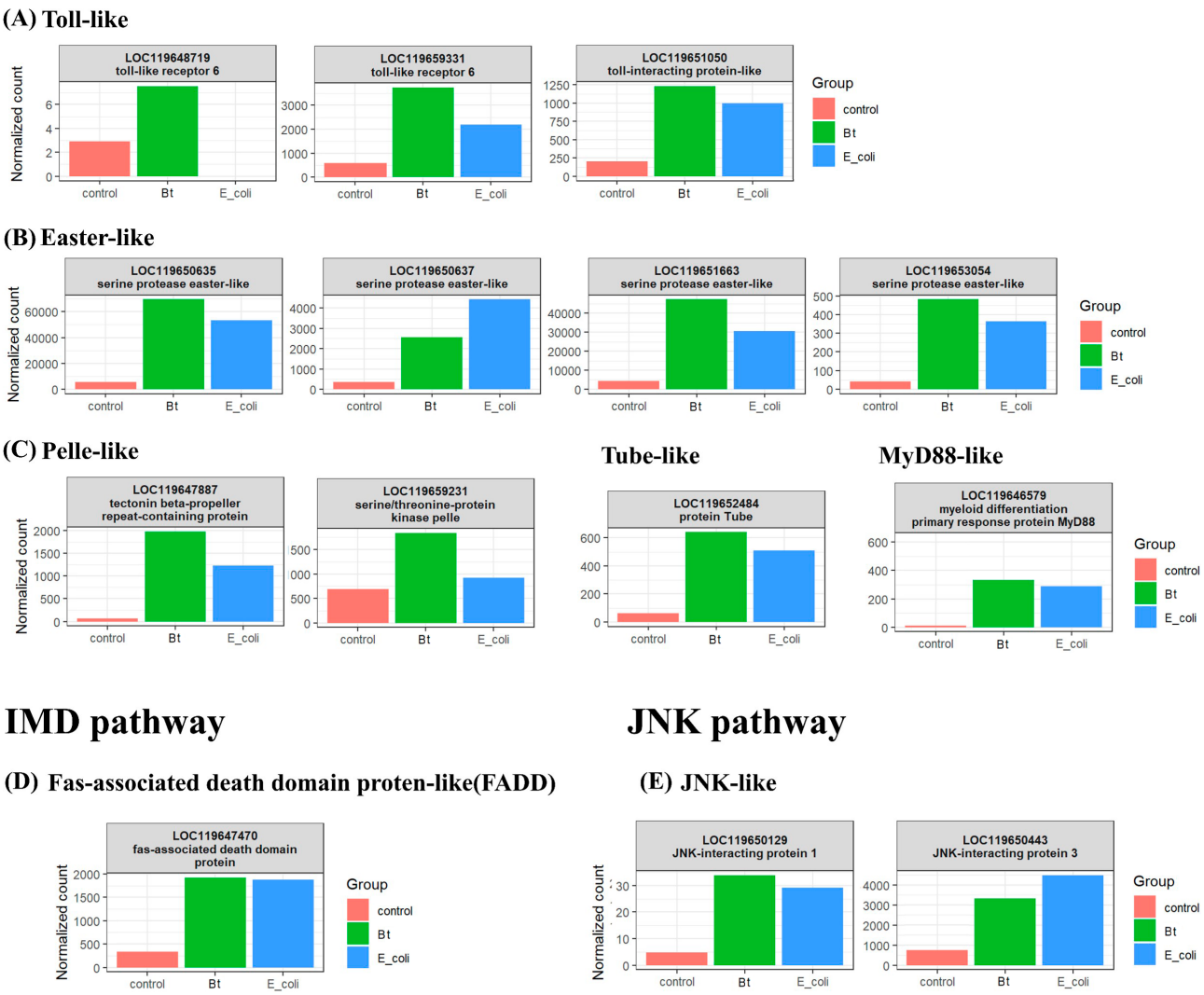

Fig. 6. Normalization counts were performed for the detected genes, and their expression levels were depicted using a bar plot. Toll pathway-related genes are overexpressed in the groups treated with Bt and *E. coli* compared to the control (A–C). Interestingly, it was observed that IMD pathway-related genes were expressed similarly in the groups treated with Bt and *E. coli* (D).

6C). Fas-associated death domain protein (FADD), a gene associated with the IMD pathway, was notably expressed similarly in the groups treated with Bt and *E. coli* (Fig. 6D). JNK pathway-related genes were also detected, and it was noted that they were most highly expressed in the group treated with Bt and the group treated with *E. coli*, respectively (Fig. 6E). The JNK pathway is known to regulate hemolymph phenoloxidase (PO) activity, hydrogen peroxide concentration, and hemocyte phagocytosis. In Fig. 7, it was observed that genes coding for antimicrobial proteins (AMPs) belonging to *defensin*, *attacin*, and *cecropin* families were highly overexpressed. Especially noteworthy, in

Table 5. A list of up-regulated clusters of *E. coli* compared to Bt.

Cluster	Name	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Count
1	extracellular region	30/218	352/9065	1.21E-09	3.87E-07	3.34E-07	30
1	response to pheromone	7/218	12/9065	3.02E-09	4.84E-07	4.18E-07	7
1	odorant binding	8/218	20/9065	9.67E-09	1.03E-06	8.92E-07	8
1	sensory perception of chemical stimulus	8/218	27/9065	1.47E-07	7.89E-06	6.83E-06	8
2	iron ion binding	20/218	206/9065	1.02E-07	7.89E-06	6.83E-06	20
2	heme binding	19/218	207/9065	5.19E-07	2.37E-05	2.05E-05	19
2	oxidoreductase activity	18/218	193/9065	8.29E-07	2.95E-05	2.55E-05	18
3	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	16/218	160/9065	1.37E-06	4.39E-05	3.79E-05	16
3	peptidoglycan catabolic process	6/218	12/9065	1.48E-07	7.89E-06	6.83E-06	6

AMPs

Fig. 7. Normalization counts were performed for the detected antimicrobial peptides (AMPs) of the *defensin*, *attacin*, and *cecropin* families, revealing high expression levels. It was observed that *attacin-A* in the group treated with Bt increased significantly, reaching approximately 170,000 times higher levels (A). *Defensin* showed a significant increase, reaching approximately 150,000 times in the Bt-treated group and about 70,000 times in the *E. coli*-treated group (B). Genes related to cecropin included both the highest expressed gene in the *E. coli*-treated group and the highest expressed gene in the Bt-treated group (C).

the group treated with Bt, *attacin-A* was detected to be increased by approximately 170,000 times (Fig. 7A). Some of the genes related to *defensin* family were found to increase by nearly 150,000 times in the group treated with Bt and approximately 70,000 times in the group treated with *E. coli* (Fig. 7B). Genes related to *cecropin* family included the gene that was most highly expressed, surpassing 25,000 times, in the *E. coli*-treated group and a gene that was highly expressed only in the Bt-treated group (Fig. 7C). In Fig. 8, immunity-related genes were examined. The genes related to lysozyme family gene showed minimal expression in the control group, while it was highly expressed in both the Bt-treated and *E. coli*-treated groups (Figs 8A, S1). Genes coding for inactive serine protease and serine protease inhibitor were highly expressed only in the control group, while serine protease was highly expressed in both the Bt-treated and *E. coli*-treated groups (Fig. 8B). Similarly, diverse serine protease outcomes with comparable trends have been confirmed (Fig. S2). These activities are recognized as part of the insect's immune system, contributing to defense against pathogens and maintaining overall immune homeostasis. Additionally, it was confirmed that *peroxidase* and *peritrophin* related genes were predominantly highly expressed in Bt-treated samples (Fig. 8C, D). The functions of peroxidase include detoxification, stabilization of extracellular matrix, and potential involvement in insect immunity. Furthermore, peritrophin is known to be a protein involved in forming the membrane of the insect gut, contributing to innate immunity that protects against pathogenic infections. In the group treated with Bt, it is likely to be highly expressed because Bt toxin destroys insect gut cells. The *proPO* gene exhibited the highest ex-

Table 6. A list of down-regulated clusters of *E. coli* compared to Bt.

Cluster	Name	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Count
1	single-stranded DNA endodeoxyribonuclease activity	9/324	55/9065	0.000127	0.010859	0.009999	9
1	DNA topoisomerase binding	9/324	56/9065	0.000146	0.010859	0.009999	9
1	double-strand break repair via nonhomologous end joining	10/324	72/9065	0.000221	0.014364	0.013226	10
1	DNA double-strand break processing	8/324	53/9065	0.000531	0.020926	0.019269	8
1	negative regulation of chromosome organization	8/324	53/9065	0.000531	0.020926	0.019269	8
2	serine-type endopeptidase activity	16/324	108/9065	1.26E-06	0.000654	0.000602	16
2	proteolysis	17/324	149/9065	2.19E-05	0.003166	0.002915	17
3	extracellular region	31/324	352/9065	2.92E-06	0.000757	0.000697	31
3	transmembrane transporter activity	10/324	56/9065	2.44E-05	0.003166	0.002915	10
3	transmembrane transport	13/324	105/9065	8.79E-05	0.009125	0.008402	13

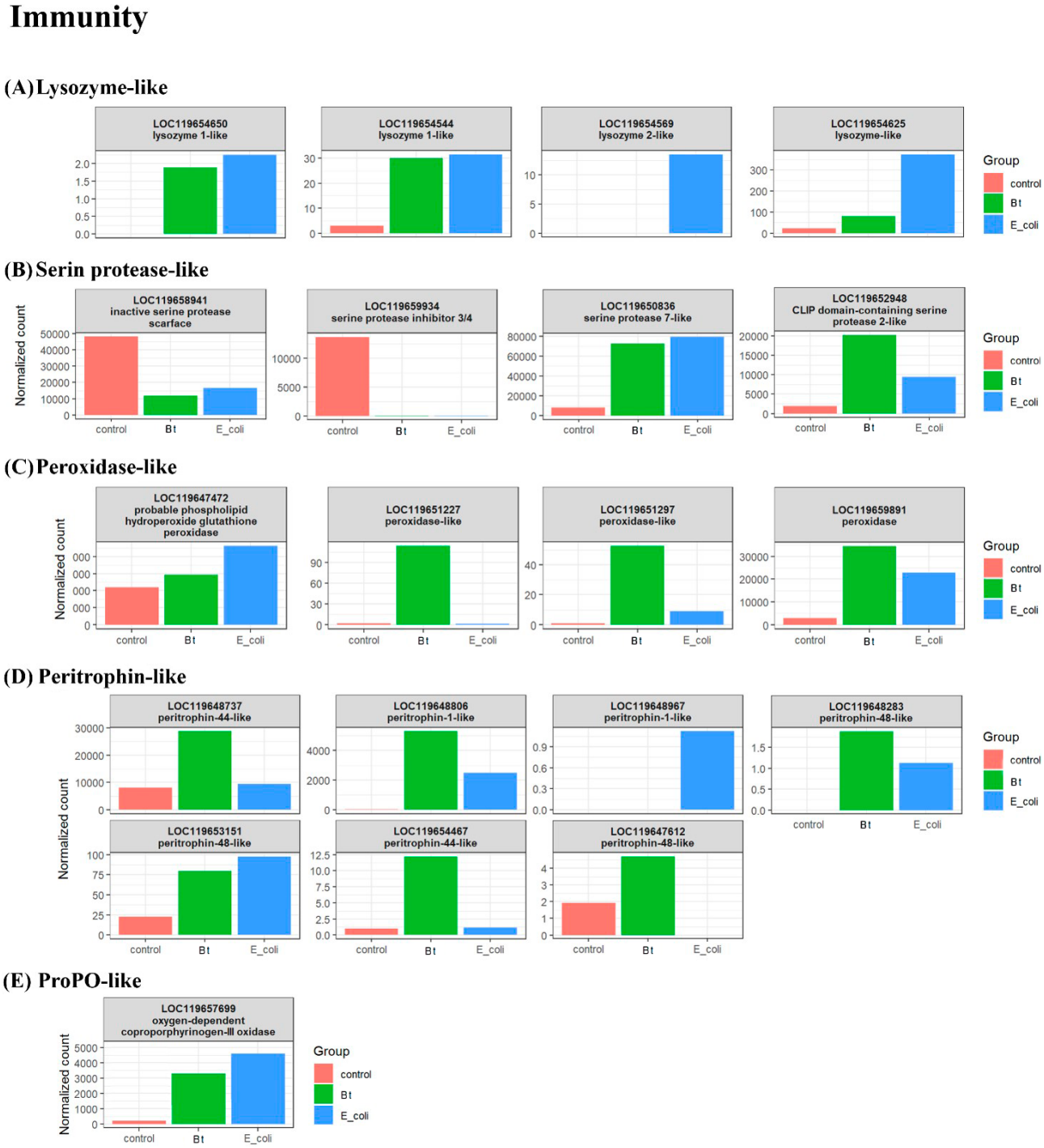

Fig. 8. The results of normalization count for immunity-related genes indicate that genes associated with *lysozyme* were highly expressed either exclusively in the groups treated with both Bt and *E. coli* or solely in the group treated with *E. coli* (A). Inactive serine protease and serine protease inhibitor were highly expressed only in the control group, while serine protease was highly expressed in both the groups treated with Bt and *E. coli* (B). Peroxidase-related genes were highly expressed in both the Bt and *E. coli* groups, but predominantly in the Bt group (C). *Peritrophin* genes were mostly highly expressed in the Bt group (D). The *proPO* gene was most highly expressed in the group treated with *E. coli*, followed by elevated expression in the group treated with Bt (E).

pression in the group treated with *E. coli*, followed by elevated expression in the group treated with Bt (Fig. 8E). The *proPO* gene is known to play a crucial role in the immune response of melanization in insects. Therefore, its elevated expression in the group treated with pathogens suggests its potential involvement in the immune response to pathogen treatment.

DISCUSSION

Our results indicate that there are distinct groups of genes expressed in black soldier flies after injection with gram-positive bacteria (Bt), gram-negative bacteria (*E. coli*), and PBS. Infections in insects have been reported to exhibit differences in gene expression depending on the type of

pathogen, as documented in numerous research papers (Greenwood et al., 2017; Ma et al., 2020; Mondotte et al., 2020). For example, the Toll pathway is mostly activated to defend against gram-positive bacteria and molds. Activation of the Toll pathway begins when pathogen-associated molecular patterns (PAMPs) bind to pattern recognition receptors (PRRs) on insect immune cells, and ultimately acts to eliminate the pathogen via the production of various antimicrobial peptides. At the same time, the Toll pathway has also been reported to be involved in the activation of plasmatocytes and granulocytes, which are major immunity-related hemocytes in insects (Anderson, 2000; Valanne et al., 2011; Alejandro et al., 2022). Additionally, the IMD pathway has been reported to start an immune response when gram-negative bacteria-binding protein (GNBP) in insects binds to invading gram-negative bacteria (Yokoi et al., 2022). Like the Toll pathway, the IMD pathway can also be initiated when various PRRs detect invading pathogens. In the case of *D. melanogaster*, it has been reported that the immune pathways initiated upon invasion by gram-positive bacteria and gram-negative bacteria are distinct (Hoffmann & Reichhart, 2002; Aggarwal, 2008; Mahanta et al., 2023).

As shown in Fig. 3, even though immunization was induced by either gram-positive or gram-negative bacteria, it was not possible to clearly distinguish the genes related to the IMD and Toll pathways, or to observe over- or underexpression. This can be explained by a report that, in many insects, the IMD pathway acts in parallel to the Toll pathway, which is the other essential immune pathway in insects (De Gregorio et al., 2002). One possible interpretation is that, in black soldier flies, whether immunized by gram-positive bacteria (Bt) or gram-negative bacteria (*E. coli*), there is no clear difference in the immune response, and that insects respond with simultaneous expression of innate immunity-related genes. Unlike *D. melanogaster*, in which the Toll and IMD pathways are clearly distinguished, *H. illucens* appeared to show cross-activation of the two pathways; this has also been reported in a previous study (Tanji et al., 2007; Aggarwal et al., 2008; Kleino & Silverman, 2014; Myllymäki et al., 2014; Alejandro et al., 2022). Similar to our findings, in a previous study of *Plautia stali*, overexpression of the Toll and IMD pathway effector genes was regulated by gram-positive and gram-negative bacteria, and the two pathways could not be distinguished, but were expressed together (Nishide et al., 2019). In other words, these species are thought to depend less on a specific immune response and more on a rapid response, based on expression levels. It was speculated that PRRs on insect immune cells may act on both gram-positive and gram-negative bacteria, simultaneously regulating the Toll and IMD pathways. Furthermore, we noted a high expression of IMD-related Fas-associated death domain protein (FADD), serine-type endopeptidases, and JNK pathway-related genes in the groups treated with both Bt and *E. coli*. The Clip-Domain serine protease is known to regulate various innate immune responses in insects, including processes such as melanization, cell death, blood clotting, and the synthesis of antimicrobial peptides (Patel, 2017; El Moussawi et al., 2019; Sousa et al., 2020; Wang et al., 2021). It has been reported that JNK pathway-related genes are linked to hemolymph phenoloxidase (PO) activity, hydrogen peroxide concentration, and hemocyte phagocytosis (Marmaras et al., 1996; Zhao et al., 2001; González-Santoyo & Córdoba-Aguilar, 2012; Ma et al., 2020; Yokoi et al., 2022). Attacin-A, which was expressed in high levels in the group treated with Bt, is an AMP that is activated in response to both gram-positive and gram-negative bacteria, while defensin is an AMP that specifically responds to gram-positive bacteria (Buonocore et al., 2021). In contrast, cecropin, which was expressed in high levels in the group treated with *E. coli*, is activated in response to fungi, gram-positive, and gram-negative bacteria (Buonocore et al., 2021). Generally, the AMPs expressed in response to Bt and *E. coli* showed a trend for cross-activation against both gram-negative and gram-positive bacteria, which is similar to our findings regarding expression pathways (Figs 6 and 7). Based on these results, we can conclude that black soldier flies show a rapid, efficient immune response through cross-activated immune systems, rather than a specific response. As shown in Fig. 2, we also observed differences in gene expression patterns between the groups immunized with Bt and *E. coli*. These differences seem to involve genes unrelated to the Toll and IMD pathway effector genes. For example, we observed that peritrophin was predominantly highly expressed in Bt-treated samples. Peritrophin is recognized as a protein involved in the formation of the insect gut membrane, and its high expression is likely due to the destruction of insect gut cells by the Bt toxin (Kuraishi et al., 2011; Palma et al., 2014).

The genes that are underexpressed when insects are infected by pathogens come from various groups, but especially include metabolic genes and developmental genes (Kryukov et al., 2022). For example, in a DEG analysis of *D. melanogaster* that had been infected by *Photorhabdus*, which lives symbiotically with entomopathogenic nematodes, genes related to chitin metabolism, cell surface receptor signal transduction, and lipid metabolism were reported to be down-regulated as part of the immune response (Castillo et al., 2015). Our results also showed underexpression of developmental genes and genes related to chitin metabolism. It can be speculated that, when infected by a pathogen, black soldier flies down-regulate metabolism-related genes to divert more resources to the immune response.

Insects are some of the most diverse and abundant organisms on the planet, and they play many important ecological roles. In particular, black soldier flies are a commercial insect species with very high economic value, which are used in food waste degradation, production of fertilizer using manure, and as a source of food for domestic animals. Understanding the immune systems that protect insects from pathogens and parasites is, therefore, crucial for understanding the health and stability of ecosystems.

We believe that our research will help to rear and maintain healthy *H. illucens* populations.

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Supplementary Figs S1, S2 follow on next page.

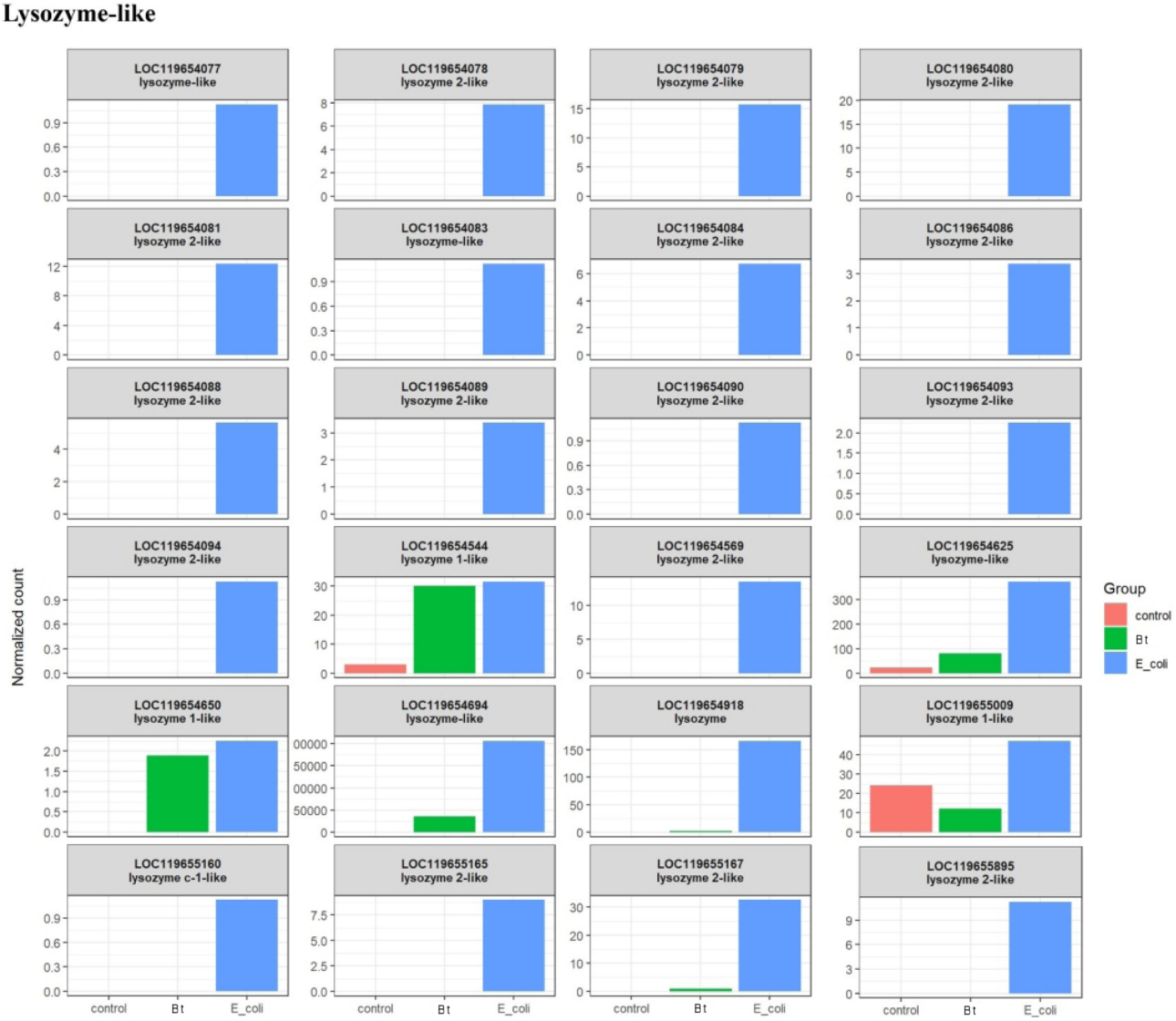

Fig. S1. The expression of lysozyme-like genes in the control, Bt-treated, and *E. coli*-treated groups. Various lysozyme-like genes were expressed in the *E. coli*-treated group. The lysozyme 1-like gene in particular exhibited high expression in both the Bt-treated and *E. coli*-treated groups.

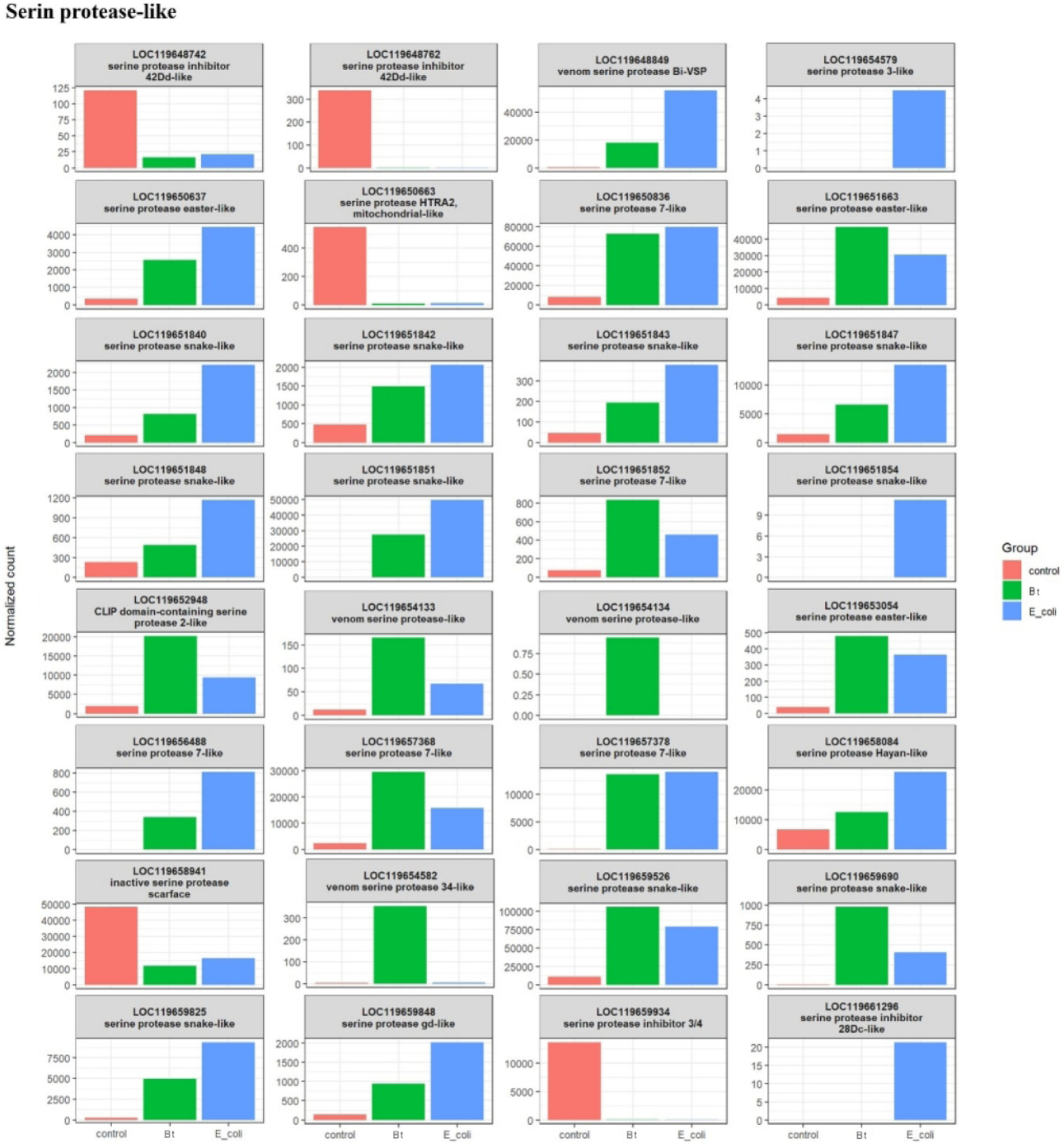

Fig. S2. The expression of serine protease-like genes in the control, Bt-treated, and *E. coli*-treated groups. Various serine protease-like genes were expressed in the Bt-treated or *E. coli*-treated groups. In particular, the serine protease 7-like and serine protease snake-like genes exhibited high expression in both the Bt-treated and *E. coli*-treated groups.