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Identification of a novel immune-related gene in the immunized black soldier fly, *Hermetia illucens* (L.)

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Abstract

The larvae of *Hermetia. illucens* have a high probability of coming into contact with microorganisms such as bacteria and fungi. Therefore, the survival of *H. illucens* is primarily the protection of their own against microbial infection. This effect depends on the development of the innate immune system. Antimicrobial Peptides (AMPs) exhibit antimicrobial activity against other bacterial strains and can provide important data to understand the basis of the innate immunity of *H. illucens*. In this study, we injected larvae with *Enterococcus. faecalis* (gram-positive bacteria) and *Serratia. marcescens* as (gram-negative bacteria) to test the hypothesis that *H. illucens* is protected from infection by its immune-related gene expression repertoire. To identify the inducible immune-related genes, we performed and cataloged the transcriptomes by RNA-Seq analysis. We compared the transcriptomes of whole larvae and obtained a DNA fragment of 465 bp including the poly (A) tail by RACE as a novel *H. illucens* immune-related gene against bacteria. A novel target mRNA expression was higher in immunized larvae with *E. faecalis* and *S. marcescens* groups than non-immunized group. We expect our study to provide evidence that the global RNA-Seq approach allowed for the identification of a gene of interest which was further analyzed by quantitative RT-PCR, together with genes chosen from the available literature.

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Introduction

The larvae of black soldier fly *Hermetia illucens* (L.) are known as ecological decomposers, and as a result they are often exposed to relatively high concentrations of harmful microorganisms, such as bacteria and fungi (Elhag *et al.*, 2017; Park *et al.*, 2014). Therefore, the survival of this insect mainly depends on the development of an effective innate immune system that confers them protection against microbial infections (Elhag *et al.*, 2017). Antimicrobial peptides (AMPs) are a key factor of the innate immune system of many organisms and play an important role in host-protecting mechanisms from pathogen invasion (Elhag *et al.*, 2017; Holaskova *et al.*, 2015; Wei *et al.*, 2016). Recent research has been actively underway to explore the biodefense mechanisms of insects in order to identify novel AMPs (Dang *et al.*, 2010; Park *et al.*,

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2015; Wei *et al.*, 2015). So far, more than 2,000 AMPs from vertebrates and invertebrates have been reported according to the antimicrobial peptide database (Elhag *et al.*, 2017; Lee *et al.*, 2016). Moreover, medicinal insects such as centipede, scorpions, leeches and silkworms have been studied extensively due to their pharmacological uses in traditional medicine (Calvino and Szczupak, 2008; Choi *et al.*, 2018; Feng *et al.*, 2007; Lee *et al.*, 2015; Singh *et al.*, 2013; Singh *et al.*, 2014; Xu *et al.*, 2010; Zhou *et al.*, 2011). Interestingly, the AMPs of these insects exhibit antimicrobial activity against other bacterial strains. Thus, the characterization of AMPs or their analogs can provide important data to help us understand the basis of the innate immunity of *H. illucens*. Furthermore these AMPs could potentially be considered a source of new antibiotic-like compounds for antibiotic-resistant strains (Elhag *et al.*, 2017).

To test the hypothesis that *H. illucens* is protected from infection by its immune-related gene expression repertoire, we injected larvae with *Enterococcus faecalis* (gram-positive bacteria) and *Serratia marcescens* (gram-negative bacteria). We then performed and cataloged the transcriptomes by high-throughput RNA sequencing (RNA-Seq) in order to identify the inducible immune-related genes. We compared the transcriptomes of whole larvae and identified the novel *H. illucens* immune-related gene against these bacteria. In this study, we induced and screened a novel AMP gene from *H. illucens* immunized with gram-positive and gram-negative bacteria in order to identify its functional characteristics and cDNA sequence. To examine the gene expression of the larvae, the global RNA-Seq approach was followed that allowed the identification of a gene of interest which was further analyzed using quantitative RT-PCR, together with genes chosen from the available literature.

Materials and Methods

Preparation and culture conditions of bacterial strains. *E. faecalis* and *S. marcescens* were used as pathogenic bacterial strains in this study, and were purchased from KCTC (Korean Collection for Type Culture, Korea). The bacteria were grown in 10 mL of Luria-Bertani (LB) broth at 37°C for 24 h. Fresh LB broth solution (10 mL) was inoculated with 10 μL of the bacterial culture, followed by further incubation at 37°C.

Immunization of H. illucens larvae with E. faecalis and S. marcescens. Only fifth-instar larvae of *H. illucens* were used for this study. Colonies of *H. illucens* were maintained under a lighting regime of 16:8 h (Ligh : Dark) to mimic photoperiod at room temperature (RT, 25 ± 2°C). Light was supplied by full spectrum light bulbs controlled by a plug-in timer. The larvae were maintained in electric propagators with plug-in thermostats at RT. To induce the immunization of *H. illucens* larvae, the larvae were washed three times with distilled water at RT, and excess water was removed using a clean piece of paper. The larvae were immunized with a fine needle dipped into a suspension (1×10^9 cfu/ml) of *E. faecalis* and *S. marcescens* on a clean bench, and reared at room temperature for 24 h. Immunized *H. illucens* larvae were collected and stored at -80°C until use.

RNA Isolation. Immunized *H. illucens* larvae were used for total RNAs isolation. Total RNA was extracted from each of the pooled larval samples and tissues described above using 1 mL of TRIzol Reagent (Life Technologies, Waltham, MA, USA). Genomic DNA was removed during the RNA extraction using the RNA-free DNase set (Turbo DNase; Ambion Life Technologies, Darmstadt, Germany) and the DNase was removed before further RNA purification using the Phenol-Chloroform Isoamyl Alcohol (PCI) DNA extraction. RNA was quantified by spectrophotometry using the ND-1000 (Nanodrop).

RNA-Seq analysis. Three different RNA samples were extracted for each experimental group from pools of *H. illucens* larvae, immunized with *E. faecalis* and *S. marcescens*. RNA quality and concentration were checked using a RNA 6000 Nano Chip on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Sequencing libraries were constructed using Illumina TruSeq RNA preparation kits following the manufacturer's instructions. Libraries were checked and quantified using Agilent Bioanalyzer 2100 (Agilent Technologies). Libraries were sequenced using an Illumina HiSeq 2000 platform, and 100 bp paired-end reads were generated. Data analysis and base calling were performed by Illumina instrument software.

cDNA synthesis and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNAs were treated with *DNase* I (Life Technologies) for 15 min at 37°C to remove genomic DNA and were purified from larvae using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using 1 μg of total RNA transcribed to cDNA using a reverse-transcription system with a TPII primer (Table 1) according to the manufacturer's instructions (Applied

Table 1. Primer pair sequences information.

Primer Name	Sequences (5` → 3`)
TPII	CGGGCAAGTTGTCGAGACAAATCAGCACC AGCAGAGCGATAGACGTTTTTTTTTTTTTTT
SPII	CGGGCAAGTTGTCGAGACAA
SP	ATCAGCACCAGCAGAGCGATAGACG
GOI(F)	CCACGGATACGAACACGGAC
5`-RACE (RT-primer)	(P)TCTCTTCGCACACACTC
5`-RACE (S1)	GTGGCACATGCAGCGGTGATA
5`-RACE (A1)	TGTGTCAGGATCCTCATATC
5`-RACE (S2)	CCAATTTTGAGAGCCGCTAAATA
5`-RACE (A2)	CCAGAGTCCGTGTTCGTATC

Biosystems, Foster City, CA, USA). The sequences for gene-specific primers (Bioneer, Daejeon, Korea) are listed in Table 1. RT-qPCR was performed on a StepOnePlus real-time PCR system with Power SYBR Green PCR Master Mix (Applied Biosystems) with 1 µl cDNA in 20-µl reaction mixtures comprised of 10 µl *RNase*H SYBR Green PCR Master Mix, 2 µl primers and 7 µl PCR-grade water (Takara, Mountain View, CA, USA). Reactions were performed with a denaturation step at 95˚C for 10 min, 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The crossing point of the target genes with β-actin was calculated using formula $2^{-(\text{target gene-β-actin})}$ and the relative amounts were quantified.

Sequence analysis of full-length cDNA. The full-length sequence of the target gene was revealed by rapid amplification of cDNA ends (RACE). First-strand cDNA was synthesized from 1 mg of total RNA using a modified oligo-d(T) primer (Table 1). For 3′-RACE, a cDNA fragment encoding a portion of the target gene was amplified by Polymerase Chain Reaction (PCR) with gene-specific forward primers (Table 1) and an internal primer for the reverse primers. For 5′-RACE, a gene-specific primer was designed from the internal sequence obtained fromthe previous 3′-RACE-PCR (Table 1) and performed with a Full RACE Core set (Takara). The RACE-PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI) and the nucleotide sequence was analyzed by Bioneer Inc. (Republic of Korea). Alignment of the sequences was carried out using the Clustal W multiple sequence alignment program (Thompson *et al.*, 1994).

Statistical analysis. Values are expressed as mean ± SD. Student's t-test was used to evaluate differences between control and *E. faecalis* or *S. marcescens* treated samples. Values of $*p < 0.05$ and $**p < 0.01$ were considered to indicate statistical significance.

Results and Discussion

In order to extract AMPs from *H. illucens*, larvae were immunized and clustered into three different groups as follows: a group of larvae immunized with *E. faecalis* during the last instar stage (E), a group without immunization (N), and a group of larvae immunized with *S. marcescens* during the last instar stage (S). Larvae were extracted from all groups in order to compare

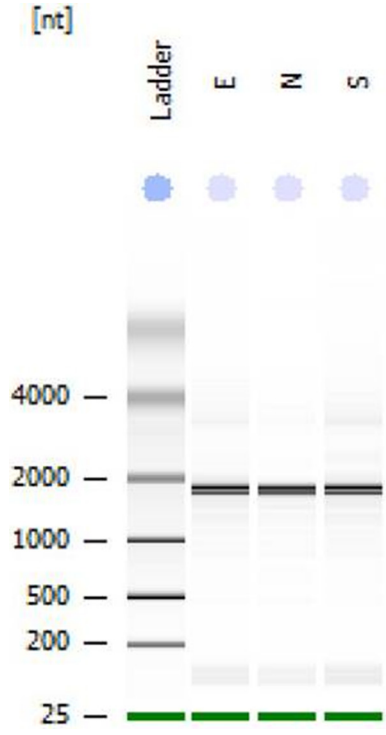
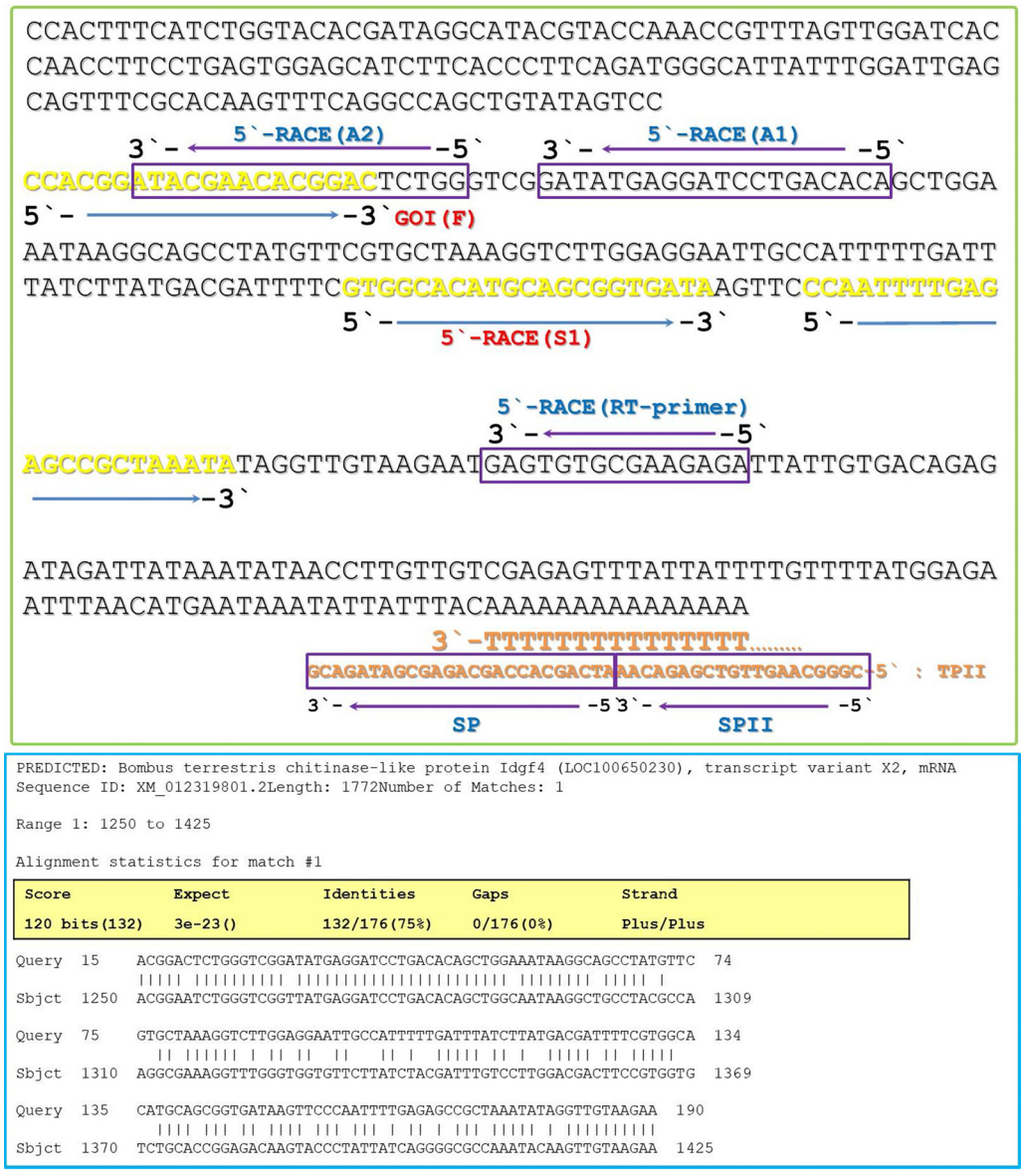


Fig. 1. Agilent bioanalyzer gel-like image of total RNA. The image shows a total RNAs gel like-image produced by the Bioanalyzer. Three samples used in the RNA-Seq experiment are shown as no more than three samples can be run at once. Lane Ladder: Size markers. Lane E: total RNAs from *H. illucens* larvae immunized with a fine needle dipped into a suspension of *E. faecalis* on a clean bench. Lane N: total RNAs from *H. illucens* larvae un-immunized. Lane S: total RNAs from *H. illucens* larvae immunized with a fine needle dipped into a suspension of *S. marcescens* on a clean bench. The distinctive 28S and 18S ribosomal RNA bands are observed for all samples.



PREDICTED: Bombus terrestris chitinase-like protein Idgf4 (LOC100650230), transcript variant X2, mRNA
Sequence ID: XM_012319801.2Length: 1772Number of Matches: 1

Range 1: 1250 to 1425

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
120 bits(132)	3e-23()	132/176(75%)	0/176(0%)	Plus/Plus

```
Query  15    ACGGACTCTGGGTCGGATATGAGGATCCTGACACAGCTGGAAATAAGGCAGCCTATGTTC  74
             ||||| |||||||||| ||||||||||||||||||||||| |||||||| ||||| |
Sbjct  1250  ACGGAATCTGGGTCGGTTATGAGGATCCTGACACAGCTGGCAATAAGGCTGCCTACGCCA  1309

Query  75    GTGCTAAAGGTCTTGGAGGAATTGCCATTTTTGATTTATCTTATGACGATTTTCGTGGCA  134
               || |||||| | || ||  ||   || |  ||||| || |  ||||| || |||||
Sbjct  1310  AGGCGAAAGGTTTGGGTGGTGTTCTTATCTACGATTTGTCCTTGGACGACTTCCGTGGTG  1369

Query  135   CATGCAGCGGTGATAAGTTCCCAATTTTGAGAGCCGCTAAATATAGGTTGTAAGAA  190
               |||| ||| || |||| ||| ||| | || | ||| ||||| | ||||||||||
Sbjct  1370  TCTGCACCGGAGACAAGTACCCTATTATCAGGGGCGCCAAATACAAGTTGTAAGAA  1425
```

Fig. 2. Sequence analysis of the novel immune-related target cDNA and multiple sequence alignment. (A) DNA sequence of the full-length 465 bp of a novel immune-related cDNA from immunized *H. illucens*. Yellow letters indicate forward primers, and purple boxes indicate reverse primers. (B) The novel immune-related gene was compared by multiple sequence alignment using the ClustalW program.

the antimicrobial activity against microorganisms. Figure 1 shows a total RNA gel like-image produced by the Bioanalyzer. Three samples were used in the RNA-Seq analysis are shown as no more than three samples can be run at once. On average 1.3 μg of total RNA was isolated from pools of three different *H. illucens* larvae samples. The RNA integrity ranged from 0 to 0.1 based on the ratio between the 28S and 18S ribosomal RNA bands from the Bioanalyzer gel-like image (Fig. 1). RNAs from the three different samples of *H. illucens* larvae were sequenced using an Illumina HiSeq 2000 platform. The clean reads were mapped to the *H. illucens* reference genomic database (Flybase: http://flybase.org) and gene annotation using Bowtie2 software. The uniquely mapped reads were normalized and calculated using the reads per Kilobase Per Million Reads (RPKM) method and the following formula was used to estimate the abundance of transcripts of each immune related gene.

We elucidated a candidate gene as an immune-related gene. To identify gene sequences, we obtained DNA fragment of 319 bp including the poly (A) tail by 3`- and 5`-RACE (Fig. 2A). Alignment of candidate gene sequences was identified in

this study and summarizes the screened gene sequences, gene alignment results (Fig. 2B). Alignment of the sequences by the Clustal W multiple sequence alignment programs showed this fragment to be somewhat similar to the chitinase-like protein ldgf4 found in the buff-tailed bumblebee *Bombus terrestris*.

An initial control RT-PCR was performed using the internal control primers to test for genomic DNA contamination in the RNA isolated from three different tissues and to assess the quality of the synthesized cDNA. We confirmed that the isolated RNA contained no genomic DNA and that the synthesized cDNA was suitable for RT-PCR analysis of the target genes. RT-PCR was used to examine target gene expression in total RNAs isolated from different larvae from the N, E, and S groups. To determine levels of novel target mRNA expression in three different groups, the qPCR method was used. The expression of target gene was normalized to the levels of the *H. illucens* Actin gene, which was utilized as an internal reference. The C_T values obtained indicated differential expression depending on whether the larvae were immunized with *E. faecalis* or *S. marcescens* during the last instar stage (Fig. 3). Interestingly, novel target mRNA expression was higher in E and S groups than in group N. Additionally, the expression level in group S was higher than the one for group E. Next, high-throughput RNA sequencing and quantitative real-time PCR were performed to examine the expression patterns of the AMP genes in different larvae, with and/or without immunization. The novel target gene expression hardly occurred throughout the whole body with immunization. According to several previous studies, the black army larvae have several immune related functions. First, significant reductions were observed in *E. coli* and *S. enterica* when black soldier fly larvae fed chicken manure (Elhag *et al.*, 2017; Erickson *et al.*, 2004). Next, it was reported that black soldier fly larvae could remove *E. coli* from dairy manure (Elhag *et al.*, 2017; Liu *et al.*, 2008). Lastly, a recent study identified a new 40 amino acid AMP and named it peptide4, like defensing (Elhag *et al.*, 2017; Park *et al.*, 2015). This AMP was originally derived and purified exclusively from the immunized body fluids of black army larvae.

In summary, in this study, novel immune-related gene sequences (named Isolde) were identified from immunized *H. illucens* larvae. Novel target gene sequences derived from immunized larvae were found to be overexpressed in comparison with those from non-immunized larvae. These results clearly show that the immunization of larvae can increase the expression of immune-related genes through activation of humoral

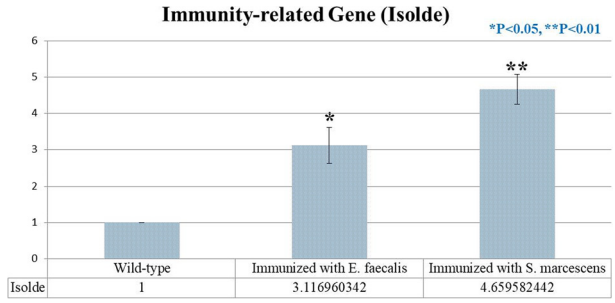


Fig. 3. Relative expression of the novel immune-related target gene in the *H. illucens* larvae. The expression of the novel immune-related target gene was normalized to the expression levels of the Actin gene in *H. illucens* larvae as an internal reference. The transcription level of immune-related target gene of two groups of immunized *H. illucens* larvae were compared with the rates in un-immunized *H. illucens* larvae. N: total RNAs from *H. illucens* larvae un-immunized; S: total RNAs from *H. illucens* larvae immunized with a fine needle dipped into a suspension of *S. marcescens* on a clean bench; E: total RNAs from *H. illucens* larvae immunized with a fine needle dipped into a suspension of *E. faecalis* on a clean bench.

immunity induced in the body of insects. To the best of our knowledge, this is the first report of a novel immune-related gene in immunized *H. illucens* larvae. Further studies are required to address the antimicrobial activity against gram-positive and gram-negative bacteria and the functional role of immunity in wild *H. illucens*. We expect that our study provides comparative genomics between immunized and wild *H. illucens* showed different insect defense systems as immune-related genes expression during artificial feeding.

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