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Gene expression profiling of coelomic cells and discovery of immune-related genes in the earthworm, *Eisenia andrei*, using expressed sequence tags

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The coelomic cells of the earthworm consist of leukocytes, chlorogocytes, and coelomocytes, which play an important role in innate immunity reactions. To gain insight into the expression profiles of coelomic cells of the earthworm, *Eisenia andrei*, we analyzed 1151 expressed sequence tags (ESTs) derived from the cDNA library of the coelomic cells. Among the 1151 ESTs analyzed, 493 ESTs (42.8%) showed a significant similarity to known genes and represented 164 unique genes, of which 93 ESTs were singletons and 71 ESTs manifested as two or more ESTs. From the 164 unique genes sequenced, we found 24 immune-related and cell defense genes. Furthermore, real-time PCR analysis showed that levels of lysenin-related proteins mRNA in coelomic cells of *E. andrei* were upregulated after the injection of *Bacillus subtilis* bacteria. This EST data-set would provide a valuable resource for future researches of earthworm immune system.

Key words: earthworm; coelomic cells; expressed sequence tags; lysenin-related proteins; innate immunity

Since all animals are confronted with harsh environments that threaten their survival, they have various defensive mechanisms to help them to stay alive. Specifically, the ability of a multi-cellular organism to defend itself against pathogen invasion is called immune system. The crucial feature of the immune system is being able to discriminate between self and non-self, especially harmful things. Adaptive immune system is absent in invertebrates, but they have advanced and diverse innate immune systems that respond to molecules of the host and structure of microbes. The former are pattern recognition receptors that recognize microbial infection and the latter are pathogen-associated molecular patterns.[1)]

Earthworms live in an environment with abundant pathogens. These pathogens are primarily bacteria living in water and soil that are ingested during feeding, or invaded into the body of the earthworm following injury. Parasites, particularly larval forms representing the dissemination phase, are another important group of potentially pathogenic agents. Therefore, earthworms should be able to protect themselves against invading pathogens due to efficient innate defense mechanisms and have developed defense mechanisms.[2,3)]

Earthworms are protostomian animals endowed with a true coelom of mesenchymal origin. The coelomic cavity is filled with coelomic fluid containing free wandering and a variety of coelomic cells containing leukocytes, chlorogocytes, and coelomocytes. The nomenclature of coelomocytes is based on morphological and cytochemical criteria.[4,5)] Coelomic cells play a crucial role in innate immunity and defense strategies against pathogens, several of these being phagocytosis,[6)] encapsulation,[7)] as well as production of cytotoxic and microbial molecules.[8–10)] Cellular defense mechanisms are based mainly upon phagocytosis and leukocyte cell-to-cell recognition.[11)] The latter leads to cytotoxicity[12)], mixed lymphocyte stimulation-like reactions[13)] and cellular cooperation.[14)] In contrast, humoral defense molecules include lysozymes,[15)] synthesis and secretion of agglutinins,[16)] a phenoloxidase/peroxidase system,[17)] and synthesis and expression of the fetidins, first discovered lytic components in earthworm.[18)] In addition, several studies reported that some humoral factors included lysenin,[19)] hemolysin,[20)] coelomic cytolytic factor [1,21)] and lumbricin I.[22)]

Gene expression profile data of earthworms with *Lumbricus rubellus*,[23)] the midgut of *Eisenia andrei*,[24)] and *Eisenia fetida*[25)] were recently reported. In spite of the studies mentioned above, information regarding the gene expression of coelomic cells is limited. In this paper, we performed expressed sequence tags (ESTs) analysis and provided the expression profiling of earthworm coelomic cells. We also described the application of ESTs to the identification of immune-related genes and cell defense genes from earthworm coelomic cells.

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Abbreviations: EST, expressed sequence tag; NCBI, National Center for Biotechnology Information; CCF, cytolytic coelomic factor; PGRP, peptidoglycan recognition protein; nr, nonredundant.

Materials and methods

Experimental animals and harvesting of coelomic cells. Sexually mature *E. andrei* were reared by the method of Cho et al.[26] Plastic container containing moistened culture soil was kept at room temperature in darkness, and powdered cow manure was supplied daily as a food material. Prior to the experimental procedure, earthworms were placed in petri dishes with wet filter paper for two days to purge the intestine materials. After purging in the petri dishes, earthworms were washed with DEPC-treated water, which was then removed with the filter paper. Then, the last quarter of the posterior end of the earthworm was massaged to expel excrement. Coelomic cells were obtained non-invasively as previously described by Eyambe et al.[27] In summary, the purged earthworms were placed into 50 mL tubes containing cold extrusion solution (5% ethanol, 2.5 mg/mL EDTA, and 10 mg/mL guaiacol glyceryl ether, pH 7.3), and the tubes were put on ice for 3 min. The extruded coelomic cells were washed twice with the Ca^{2+} free LBSS (1.5 mM NaCl, 4.8 mM KCl, 1.1 mM $MgSO_4 \cdot H_2O$, 0.4 mM KH_2PO_4, 0.3 mM NaH_2PO_4, and 4.2 mM $NaHCO_3$, pH 7.3), followed by centrifugal collection ($250 \times g$, 4 °C, 15 min).

mRNA isolation and construction of cDNA library. Total RNA of coelomocytes was isolated with the use of the TRIzol reagent (Sigma). $Poly(A)^+$ RNA was purified from total RNA using dynabeads (DYNAL) according to the manufacturer's instructions.

A directional cDNA library was constructed using the pCMV-script XR cDNA Library Construction Kit (Stratagene). A total 2000 bacterial clones were randomly picked up from the plates and grown in 1.5 mL LB medium overnight. The plasmid DNA was prepared by Plasmid Spin kits (RBC) and stored at −20 °C.

EST sequencing. Sequencing of the purified plasmids was performed with an ABI Prism Big-Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using a T3 primer by an ABI 3100 automated sequencer (Applied Biosystems).

Sequencing analysis. The vector sequences were trimmed from the raw sequence data using the VecScreen software in the National Center for Biotechnology Information (NCBI). The sequence of each EST was also manually edited to remove ambiguous bases, the poly (A) tracts, and poor-quality sequences (nucleotide sequences <150 bp). Contigs were assembled using the SeqMan II software (DNASTAR, USA). Each sequence was compared with the NCBI database using the BLASTX and BLASTN program on the BLAST network. Sequence similarities identified by the BLAST programs were considered statistically significant only if the *E*-value was less than 1.0×10^{-5}.

Obtaining full-length cDNA by RACE-PCR and alignment. To obtain the full-length sequences from the identified EST sequence, 5′ and 3′ -rapid amplification of cDNA ends (RACE) PCR were performed using the SMART™ RACE cDNA amplification kit (Clontech) according to the manufacturer's protocols. Gene-specific primers for the 5′ and 3′ RACE-PCR reactions were designed from the EST fragments. PCR primers used for *lysenin-related protein (EAlrp)* were as follows: 3′ RACE primer 5′- AGCAAATATGCTGAGTCTGCAAGGA -3′ and 5′ RACE primer 5′- TGCAGAACAATAGGGATGACAAATCG - 3′. The RACE-PCR products were then cloned into pGEM-T cloning vector (Promega) for sequencing.

The obtained sequences were compared with the GenBank Database via BLASTX. Alignments of the *lysenin-related proteins* were carried out using the Clustal W and GeneDoc program.

Bacterial challenge. *Escherichia coli* and *Bacillus subtilis* were cultured in LB broth at 37 °C overnight and collected by centrifugation (3000 rpm, 20 min). Collected cells were washed and resuspended with LBSS to $OD_{600} = 0.5$, and each 10 μL of LBSS (positive control), *E. coli* or *B. subtilis,* was injected to cultured earthworms. For real-time PCR analysis, coelomic cells were collected at the indicated times below.

Real-time PCR. Total RNA (1 μg) was isolated at indicated times (3, 6, 12, 24, 48, 72, and 120 h) from the coelomic cells of earthworms injected with LBSS, *E. coli* or *B. subtilis*. Each total RNA was reversely transcribed using a first-strand cDNA synthesis kit (Promega, USA). Quantitative real-time PCR was performed by 7500/7500 fast Real-Time PCR system (Applied Biosystems, USA) with specific primers EAlrpF (5′- AGCAAATATGCTGAGTCTGCAAGGA -3′) and EAlrpR (5′- TGCAGAACAATAGGGATGACAAATCG - 3′) using SYBR Green real-time PCR Master Mix (Applied Biosystems). The real-time PCR parameters were an initial denaturation at 95 °C for 15 min, denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 5 min. Forty cycles of amplification were performed. Quantitative measurements were normalized using *E. andrei β*-actin (forward: 5′- CATCCATCGTCCACAGGAAGTG -3′, reverse: 5′- CGTGTTCATCTCAGGAGGCAGA -3′) level as a housekeeping gene.

Results and discussion

Overall of ESTs from the coelomic cells of the earthworm, E. andrei

Approximately 1600 clones were picked up from the cDNA library of coelomic cells and sequenced. A total of 1151 ESTs were obtained with an average length of 384 bps after excluding sequences <150 bps and not clearly sequenced. Table 1 indicates the summary of EST analysis in the earthworm coelomic cells. A total of 1151 ESTs consisted of 501 singletons and 158 clusters,

Table 1. Composition of ESTs analyzed by BLAST search.

EST categories	Number of ESTs (%)	Number of clusters (number of comprised ESTs)	Number of singletons
(A) Sequences matched significantly[a]	493(42.8)	71 (400)	93
BLASTX (nr) matched	476(41.4)	68 (386)	90
BLASTN (nr) matched	17(1.4)	3 (14)	3
(B) Sequences not matched or matched in-significantly[b]	658(57.2)	87 (250)	408
Total	1151(100.0)	158 (650)	501
Total of unique ESTs		659	

[a]*E*-value < 10^{-5}.
[b]*E*-value > 10^{-5}.

indicating the presence of about 659 unique genes in our data-set. Approximately 42.8% (493 ESTs) of the total ESTs were found to match known genes with *E*-values lower than 10^{-5}. Of the 493 ESTs, 476 (41.4%) and 17 (1.4%) sequences were blasted against non-redundant protein and nucleotide databases, respectively. The remaining 658 ESTs (57.2%) were not similar to any known sequences with $E < 10^{-5}$ value, which exhibited a little higher rate than that of midgut EST analysis showed less than 50% previously.[24)]

Expression profiles in the earthworm coelomic cells

Fig. 1 represents the expression profiles of the unique genes identified from the earthworm coelomic cells. Among the 659 unique genes, 501 ESTs (43.5%) were sequenced only once, 346 ESTs (30.1%) were sequenced two to five times, and 304 ESTs (26.4%) were sequenced more than five times. Whereas almost 44% of the ESTs appeared once, the 12 most highly expressed genes accounted for 20.8% of all transcripts analyzed in this study (Supplemental Table 1), suggesting that the gene expression profile in the earthworm coelomic cells is polarized. Twelve clusters appeared to have more than 10 ESTs, and the largest cluster contained 42 ESTs. With the most abundantly expressed gene, NADH dehydrogenase subunit 3, accounted for 3.6% of overall expression, the expression of the top eight genes related to energy metabolism appeared to account for about 17% of the overall gene expression analyzed in this study. In addition, an innate immune gene, *lysenin-related protein* (*EAlrp*) gene, appeared with high frequency of 0.87%. Such expression polarity toward energy metabolism showed higher as twice as that of the midgut in the same earthworm species.[24)] Considered that in the gene expression profiling analysis using EST, the highly expressed genes would reflect the prime functions of the analyzed tissues or cell types,[28–31)] the primary function of coelomic cells in the earthworm might be associated with high energy-consuming functions including immune responses. In particular, among the 12 most highly expressed genes, the high frequency (2.87%) of no-matched genes possibly representing novel genes could address the new functions of earthworm coelomic cells.

The 493 matched ESTs could be categorized into seven groups according to their putative functions (Supplemental Fig. 1): (1) cell signaling and cell–cell communication, (2) protein synthesis and ribosomal proteins, (3) energy metabolism, (4) immunity, cell defense, and detoxification, (5) cell structure and mobility, (6) cell replication, and (7) unknown function. Among the recognized protein-coding genes, the gene category associated with energy metabolism appeared to be the largest, accounting for 48% of the expression in the coelomic cells, followed by protein synthesis and ribosomal proteins (34%), immunity, cell defense, and detoxification (8%), cell signaling and cell–cell communication (3%), cell replication (2%), and cell structure and mobility (1%). The

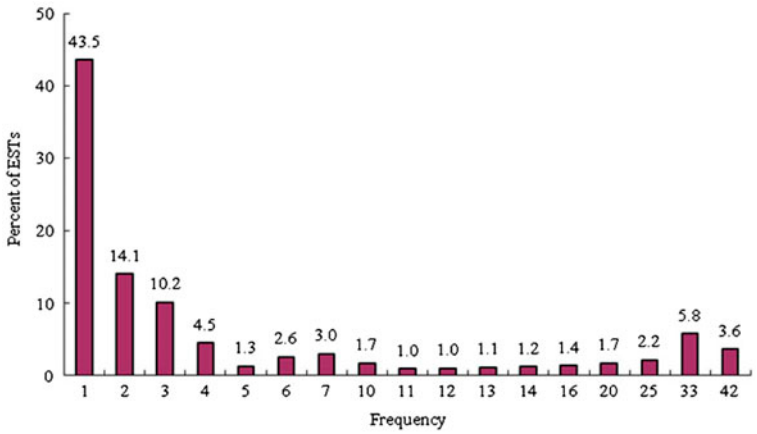

Fig. 1. Expression profiles and sequencing redundancy in the analysis of ESTs from the coelomic cells of the earthworm *E. anderi.*
Note: Of the 1151 ESTs analyzed, while 501 ESTs (43.5%) were sequenced only once, 346 ESTs (30.1%) appeared two to five times, and 304 ESTs (26.4%) were sequenced more than five times.

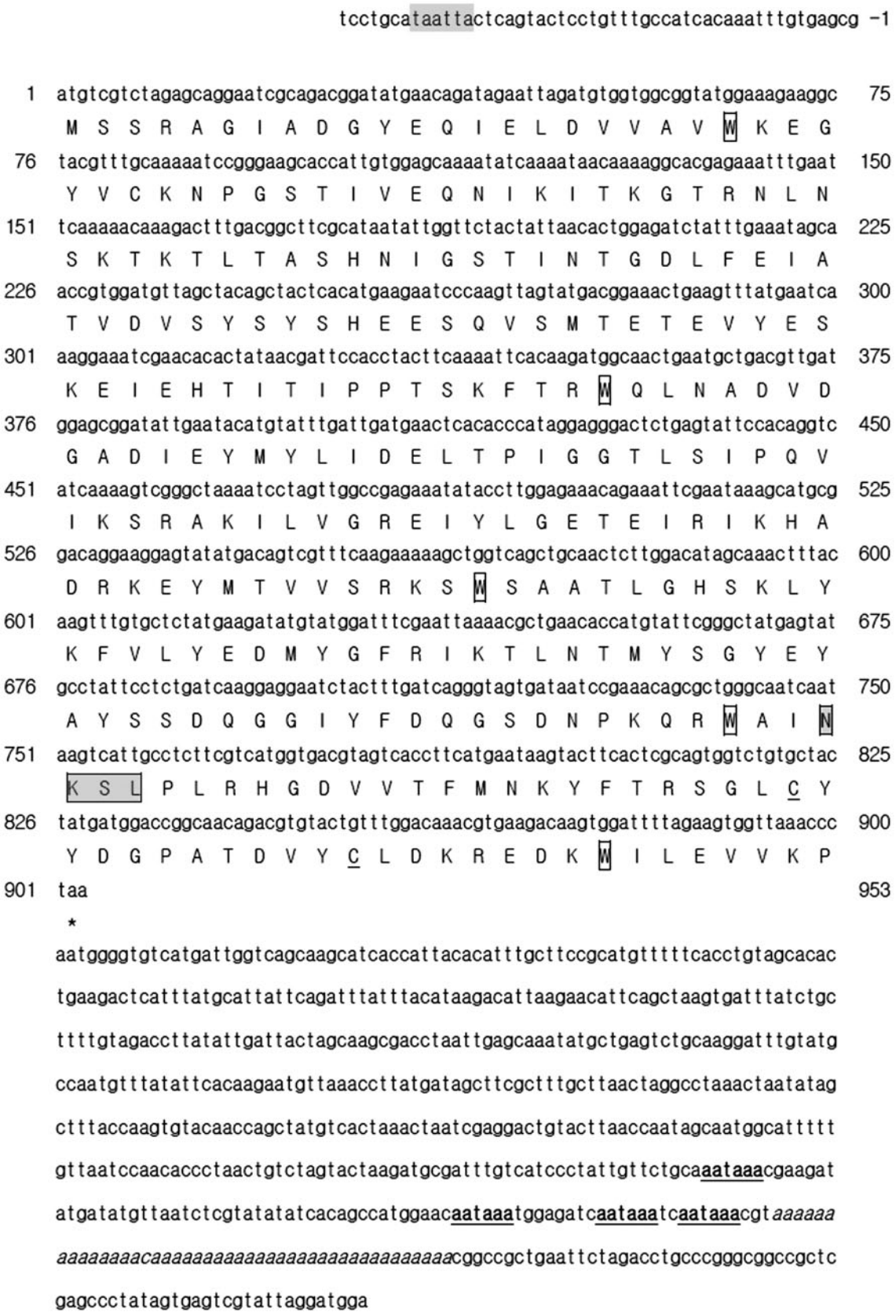

Fig. 2. The nucleotide and deduced amino acid sequences of the *lysenin-related protein (EAlrp)* from the coelomic cells of *E. andrei*.

Notes: The first nucleotide of the start codon (ATG) was designated as nucleotide 1, and the stop codon was marked by an asterisk. A N-glycosylation sequence is indicated by a closed box, and shade region at 5′-end indicated the TATA box. The boxes represent conserved tryptophan residues. The polyadenylation signal AATAAA is in bold, and poly (A) tail sequences were denoted in italics.

distinguished difference from midgut EST analysis using the same species[24] is that the appearance of immune- and cell defense-related genes is almost ten times higher in coelomic cells, indicating that these cells would be potent in self-defensing activity of the earthworm.

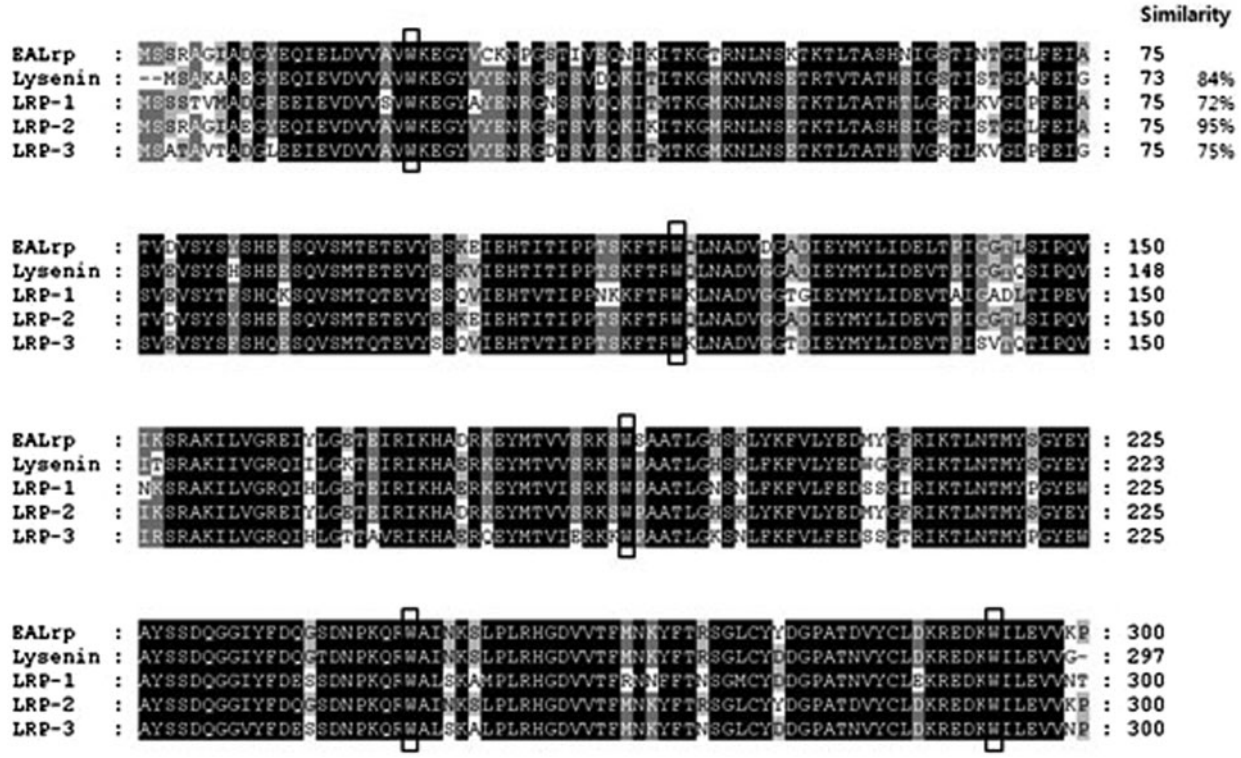

Fig. 3. Multiple alignments of the deduced amino acid sequence of lysenin-related protein of *E. andrei* (EAlrp) with previously reported lysenin (Accession No. O18423), LRP-1 (Accession No. O18424), LRP-2 (Accession No. O18425), and LRP-3 (Accession No. Q3LX99).

Note: The conserved tryptophan residues are boxed, probably essential for binding sphingomyelin and important for inducing hemolysis.

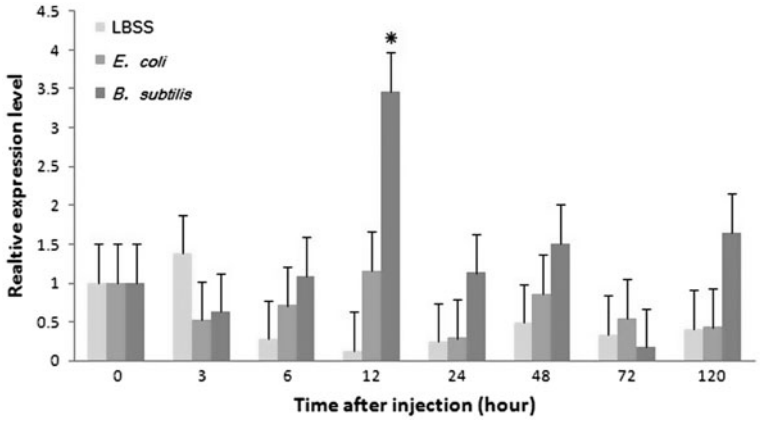

Fig. 4. Changes in mRNA levels of *lysenin-related protein* (*EAlrp*) in the earthworm, *E. andrei,* by bacterial challenge.

Notes: The changes of the mRNA level of *lysenin-related protein* (*EAlrp*) in *E. andrei* injected with *E. coli* and *B. subtilis* were determined by real-time PCR and normalized by a housekeeping gene, *β-actin*. Fold changes in gene expression (relative expression level) are relative to *EAlrp* level in non-stimulated earthworms. Each value represents the means of three experiments (±SD). $*p < 0.05$

Sequence analysis of lysenin-related protein (EAlrp)

Since the pioneering works of Metchnikoff,[32] invertebrate immunology has become an intensive field of study, seeking not only to understand the evolutionary significance and the diversity of invertebrate immunity, but also to identify new molecules with possible therapeutic uses. In particular, the earthworm coelomic cells are considered to be an immune-competent cell, as well as a highly possible source for molecules that might have some industrial and medical applications. We were able to identify ESTs related to the earthworm's innate immune system including several novel genes.

Lysenin is a type of representative proteins for antimicrobial defense in the earthworm, exhibiting lytic effects on erythrocytes or foreign organisms.[19,21,33] In this study, we have detected a lysenin-related protein *(EAlrp)* gene in coelomic cells from the earthworm, *E. andrei*. ESTs identified as *EAlrp* gene in our study showed 10 times (0.87%) higher frequency (Supplemental Table 1) with low *E*-values (1e-179) as compared with the lysenin-related proteins previously reported in the earthworm, *E. fetida*, possibly suggesting the presence of new forms of lysenin. Lysenin, lysenin-related protein, and fetidin have a high sequence homology, suggesting that these molecules

would be isoforms.[21,29,34,35] It has been also reported that the fetidin mRNA expression was not significantly modulated in coelomic cells by microbial stimulation.[35]

In addition, we found out 24 immune-related and cell defense genes from earthworm coelomic cells (Supplemental Table 2). Among them, we obtained the full-length cDNA of *lysenin-related protein* (*EAlrp*) gene, of which predicted protein is composed of 300 amino acids (Fig. 2). Its molecular mass and theoretical isoelectric point were estimated to be 34.12 kDa and 5.77, respectively, for the molecule without the signal peptide. The lysenin conserved six tryptophan residues at the position of 20, 116, 187, 206, 245, and 291. They shared five conserved amino acids (20, 116, 187, 245, and 291) specific to the LRP-1 and LRP-2. It had been previously suggested that the conserved tryptophan residue would be demanded both for the recognition of sphingomyelin and for the hemolytic activity of lysenin.[36]

The EAlrp appeared to contain the conserved five trytophan residues, crucial for binding sphingomyelin and important for inducing hemolysis (Fig. 3). We compared these results with the lysenin and other lysenin-related proteins previously established in earthworm, *E. fetida*, by multiple alignments (Fig. 3). The EAlrp was found to exhibit high homology with lysenin (84%), lysenin-related proteins 1 (LRP-1, 75%), lysenin-related proteins 2 (LRP-2, 95%), and lysenin-related proteins 3 (LRP-3, 72%) (Fig. 3). In general, LRP-2, namely fetidin, as well as lysenin[37] could induce hemolysis. The fact that EAlrp shows a high amino acid sequence homology with LRP-2 (Fig. 3) possibly suggests that EAlrp may induce hemolytic activity as lysenin and LRP-2 could. Also, based on the results of ESTs analysis from earthworm coelomic cells, we could first discover other immune-related genes with deduced amino sequences from NCBI database. They include oncoprotein NM23 (NDPK) and myeloid leukemia-associated SET translocation protein, which are composed of 153 and 245 amino acids, respectively (data not shown).

Changes in mRNA levels of lysenin-related protein (EAlrp) in the earthworm E. andrei *following bacterial challenge*

Innate immunity is an important defense mechanism against numerous diseases, including infections, inflammatory diseases, and cancers. There are many molecules and mechanisms involved in the innate immune system which include antimicrobial peptides and programmed cell death. We carried out real-time PCR to recognize the effect of bacteria challenge on the expression level of *EAlrp* mRNA. Real-time PCR results showed an increase in *EAlrp* gene expression level in the earthworm, *E. andrei,* by challenging with *B. subtilis* but not with *E.coli*. It appeared that the mRNA expression of *EAlrp* reached the maximum level at 12 h after stimulation with *B. subtilis,* while with *E. coli* stimulation, no significant change was detected (Fig. 4). Although more detail investigation should be promised to elucidate the action mechanism of this molecule, it would be likely that the formation of cell membrane pore occurs to gram-positive bacteria by cell wall component different from gram-negative bacteria.

In this study, we have newly discovered several immune- and stress-related genes, particularly *lysenin-related protein* named *EAlrp*, which exhibited higher than 70% amino acid sequence similarity with other lysenin and lysenin-related proteins reported earlier from *E. fetida*. In addition, the high occurrence (57.2%) of unknown genes not able to be annotated using the NCBI database may reflect that the innate immune system of the earthworm is more complicated with many unknown mechanisms. It could be also noted that the present sequence data would serve as useful and potent information on the better understanding of invertebrate immune system.

Supplemental material

The supplemental material for this paper is available at http://dx.doi.org/10.1080/09168451.2014.988677.

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