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RESEARCH REPORT

A putative insulin receptor involved in immune response of Chinese mitten crab *Eriocheir sinensis*

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Abstract

Insulin plays important roles in metabolic homeostasis during environmental challenges. The insulin receptor is a key molecule to receive and transduce insulin signals. In the present study, a novel insulin receptor was identified from the Chinese mitten crab *Eriocheir sinensis* (designated as *Es*IR). The coding region of *Es*IR gene was 3573 bp in length and encoded 1190 amino acids with all the functional domains of mammal insulin receptors, including furin-like domain, receptor L domain, transmembrane domain, and tyrosine kinase domain. Phylogenetic analysis showed that the *Es*IR shared the closest evolutionary relationship with the insulin receptor from *Macrobrachium rosenbergii*. Cell transfection experiments confirmed that *Es*IR proteins were localized on the cytomembrane. The mRNA transcripts of *EsIR* were widely distributed in various tissues with higher abundance in hepatopancreas and eyestalk of *E. sinensis*. After *Aeromonas hydrophila* stimulation, the expression level of *Es*IR mRNA decreased from 3 h to 6 h, and then increased at 12 h. The conserved structure and subcellular localization of *Es*IR together with its sensitivity to *A. hydrophila* stimulation implied that *Es*IR was probably involved in immune response of *E. sinensis.* The present study provided clues for the further investigation about the evolution and function of the insulin signaling pathway in invertebrates.

Key Words: *Aeromonas hydrophila* infection; Chinese mitten crab; immune response; insulin receptor

Introduction

Insulin plays important roles in metabolism, fecundity, growth, immunity, and aging (De Meyts, 2004). The modulation effects of insulin are mediated primarily via the insulin receptor. This receptor belongs to the superfamily of tyrosine kinase receptors, and it is always located on cytomembrane (White and Kahn, 1994). The binding of insulin to its receptor initiates a cascade of intracellular signal transduction, including autophosphorylation of tyrosine kinase domain and the interaction of multiple molecules with insulin receptor. The key molecules in the downstream pathway are the insulin receptor substrates (IRSs), which are protein substrates of the intrinsic tyrosine kinase activity of insulin receptor, transmitting the signal to downstream cascades (Taniguchi *et al.*, 2006).

Vertebrate insulin signaling pathway possesses single insulin and several insulin receptor family members, including insulin receptor, insulin-like growth factor receptor (IGFR) and insulin receptor-related receptor (IRR). However, the increasing evidences demonstrate that the insulin signaling pathway in invertebrates has unique characteristics. There are multiple copies of genes in their genome encoding insulin-like peptides (ILPs) but only one copy of receptor and IRS gene (Mao *et al.*, 2018b). The relative simplicity of the insulin signaling components, together with the diversification of ILP, implies the functional diversification of the insulin signaling pathway in invertebrates (Guirao-Rico and Aguade, 2011).

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Compared to higher animals, invertebrates face more severe environmental challenges, such as frequent food shortages and pathogen infection (Karpac and Jasper, 2009). The activation of immune system and maintenance of homeostasis are energetically costly. Therefore, the metabolic regulation to environmental stress is crucial for the long-term survival of invertebrate (Broughton and Partridge, 2009). As a crucial synthetic metabolic signaling pathway, insulin action is always inhibited in order to enhancing the resistance to environmental stress. For instance, bacterial infection leads to the activation of Toll signaling in *Drosophila melanogaster*, which suppresses the insulin signaling, extending the survival against bacterial pathogens (McCormack *et al.*, 2016). Loss-of-function for the insulin receptor homolog in *Caenorhabditis elegans* larval dramatically increases the oxidative stress tolerance and adult lifespans compared to the wild-type counterparts (Tatar, 2001). These studies collectively indicate that the insulin signaling pathway is critical for invertebrate survival during environmental stress.

The Chinese mitten crab *Eriocheir sinensis* is an important aquaculture crustacean in Asian areas (Sang *et al*., 2016). It was found that ILP in *E. sinensis* participated in the immune response against *Aeromonas hydrophila* infection by providing more glucose (Wang *et al.*, 2020). Investigation of the potential metabolism and immune related genes, such as insulin receptor in *E. sinensis*, is necessary to elucidate the homeostasis regulation during stress resistance, which might be helpful to develop strategy for economic and efficient aquaculture. The purposes of this study were to (1) identify the insulin receptor homologue from *E. sinensis* (designated as *Es*IR), (2) characterize the its expression at subcellular and tissue levels, and (3) investigate its response against *A. hydrophila* stimulation to better understand the homeostasis regulation role of *Es*IR during the immune response.

Materials and methods

Crab and bacteria stimulation

Adult chinses mitten crabs *Eriocheir sinensis* (about 50 ± 5 g) were obtained from a commercial farm in Qingdao, China and maintained in aerated freshwater at 25 °C for one week before the experiments.

A total of 30 crabs were randomly divided into two groups for *Aeromonas hydrophila* challenge experiment. The crabs in the control group received an injection of 50 μL PBS, while the crabs in bacteria stimulation group received an injection of 50 μL *A. hydrophila* suspension (3×10^6 CFU /mL, diluted in PBS). Three individuals from each group were randomly sampled at 0, 3, 6, 12, and 24 h post challenge. The hepatopancreas tissue was collected and stored in liquid nitrogen for total RNA extraction.

In addition, the hepatopancreas, eyestalks, gills, muscles, stomach, hemolymph and hematopoietic tissues were collected from three crabs in control group at 0 h for gene cloning and tissue expression analysis.

RNA isolation and cDNA synthesis

Total RNA was extracted from the tissues using Trizol Reagent (Invitrogen) according to the manufacture's protocol. The RNase-free DNase I (Promega) was used to digest the genomic DNA from the total RNA. First-strand cDNA synthesis was carried out based on M-MLV reverse transcriptase using the total RNA as template and oligo (dT)-adaptor as the primer (Table 1). The reactions were incubated at 42 °C for 1 h and terminated by heating at 95 °C for 5 min. The cDNA mixtures were diluted to 1:30 and stored at -80 °C for subsequent gene cloning and qRT- PCR (Qu *et al.*, 2018).

Gene cloning and sequence analysis

Blastp analysis of all crab protein sequences revealed that a sequence (VN_GLEAN_10002430, EsIR) was homologous to the insulin receptor

Table 1 Nucleotide sequences of primers used in this study

Primer	Sequence (5'-3')	Brief information
Adaptor primer	GGCCACGCGTCGACTAGTACT$_{17}$	Oligo (dT) for cDNA synthesizing
*Es*IR-F1	ATGCAGCGCTACAACCAGAT	Gene specific primer for CDS
*Es*IR-R1	ACACGGTTGTCTCACTGCGG	Gene specific primer for CDS
*Es*IR-F2	TACCGGACTCAGATCTCGAGATGCAGCGCTACAACCAGATC	Primer for vector constructing
*Es*IR-R2	TACCGTCGACTGCAGAATTCGCACGGTTGTCTCACTGCGGG	Primer for vector constructing
*Es*IR-F3	GGCAGAGTCGCCACAGAACC	Gene specific primer for qRT-PCR
*Es*IR-R3	AGTGGGTCGGAGCAGTAGCG	Gene specific primer for qRT-PCR
β-actin-F	GCATCCACGAGACCACTTAC	Internal control for qRT-PCR
β-actin-R	CTCCTGCTTGCTGATCCACATC	Internal control for qRT-PCR

Table 2 The insulin receptors used in multiple alignment and phylogenetic analysis

Species	Protein	Accession number
Homo sapiens	insulin receptor	AAA59452.1
Xenopus laevis	insulin receptor	CAB46565.1
Danio rerio	insulin receptor b	ACC77575.1
Ciona intestinalis	insulin receptor	XP_002125750.3
Aplysia californica	insulin receptor	2207309A
Drosophila melanogaster	insulin receptor	AAC47458.1
Anopheles gambiae	insulin receptor	XP_320130.3
Bombyx mori	insulin receptor	NP_001037011.1
Macrobrachium rosenbergii	insulin-like receptor	AKF17681.1
Sinonovacula constricta	insulin-like peptide receptor	AYV97262.1
Lymnaea stagnalis	insulin-like peptide receptor	CAA59353.1
Apostichopus japonicus	insulin-like peptide receptor	PIK45733.1
Acanthaster planci	insulin-like peptide receptor	XP_022110929.1

identified from other species (the threshold of e-value was 1 x 10^{-5}). A pair of specific primers (Table 1) was designed to amplify the full length cDNA of EsIR from cDNA library. The searches for protein sequences similarity of EsIR were conducted with BLAST algorithm at the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The Expert Protein Analysis System (https://www.expasy.org) was used to analyze the deduced amino acid sequence. The protein domain was predicted with SMART (http://smart.embl-heidelberg.de). Multiple sequence alignment of the *Es*IR with other insulin receptors was performed with the online multiple alignment program (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) and optimized manually. A phylogenetic tree was constructed by the maximum likelihood algorithm with the SeaView software based on the insulin receptors in different species (Table 2) (Gouy *et al.*, 2010). The reliability of the branching was tested by bootstrap resampling (100 pseudo-replicates).

Plasmid construction, HEK293T cell culture and transfection

To assess the subcellular location of *Es*IR protein, the target encoding region of EsIR was amplified by primers (Table 1) and inserted into p-EGFP-N1 expression vector (TransGene).

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (D-MEM, Gibco BRL, Gaithersburg, MD) supplemented with 15 % fetal bovine serum (FBS, TransGene) at 37 °C and 5 % CO_2.

The recombinant plasmid pEGFP-*Es*IR was transfected into HEK293T cells with Lipofectamine LTX™ and Plus™ Reagent (Invitrogen). The control group was transfected with the p-EGFP-N1 plasmid. After cultured at 37 °C for 48 h, the cells in the experimental group and the control group were washed, fixed with 4 % paraformaldehyde for 10 min, stained with the DiI staining solution, and photographed under a laser confocal microscope (Mao *et al*., 2018a).

Real-time fluorescence quantitative PCR (qRT-PCR)

The qRT-PCR was carried out in an ABI PRISM 7500 Sequence Detection System with a total volume of 10 μL. The primers used in the present study were listed in Table 1. The fragment of crab actin gene was employed as an internal control. All data were given in terms of relative mRNA expression using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Statistical analysis

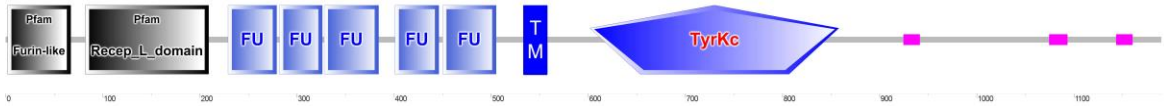
All data were given as means ± SD and subjected to one-way analysis of variance (one-way ANOVA) followed by a multiple comparison (LSD). Differences were considered significant (labeled with * or letters) at $p < 0.05$ or extremely significant (labeled with **) at $p < 0.01$.

Results

Molecular characteristics and multiple sequence alignments of EsIR

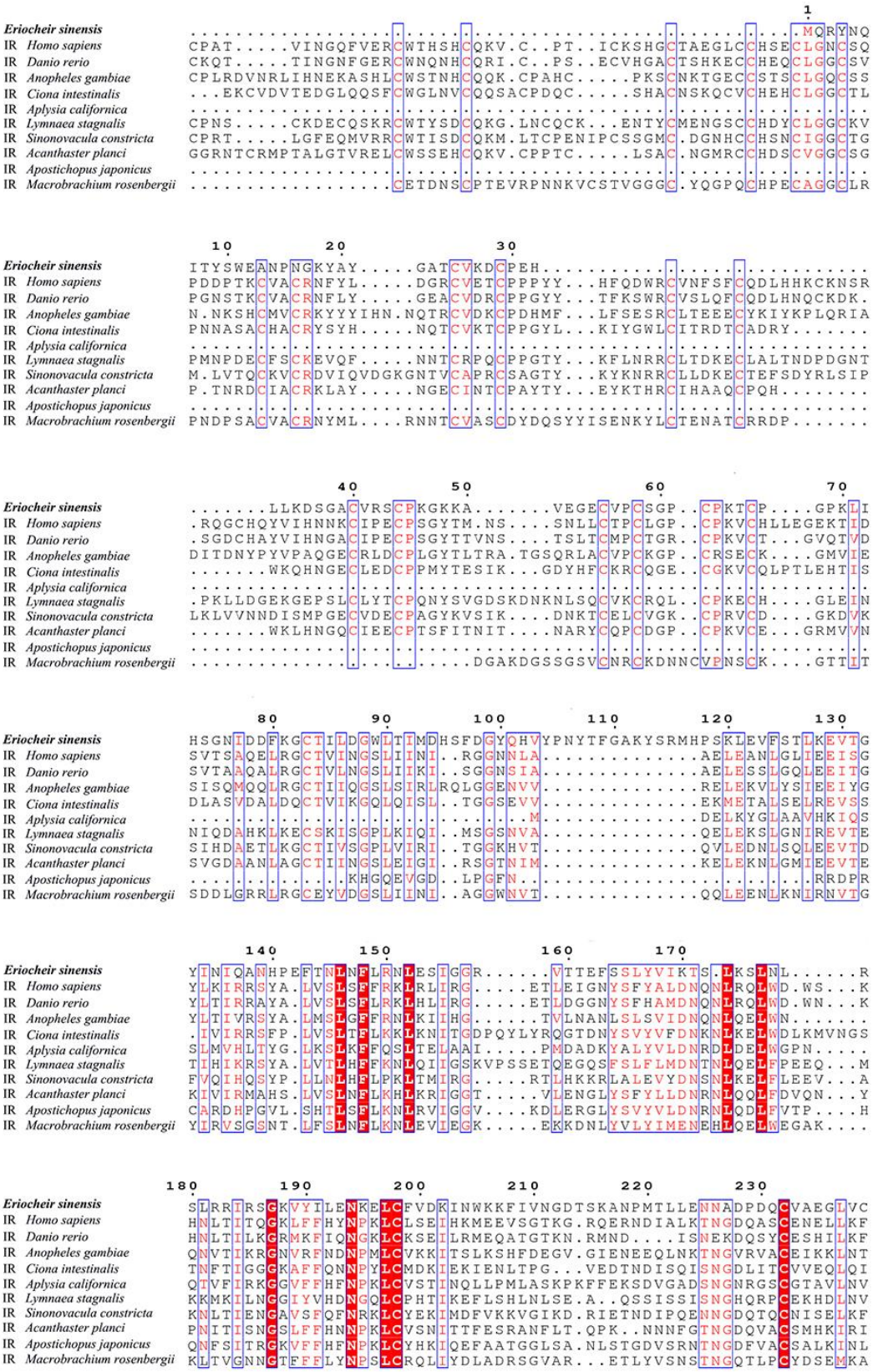
A potential insulin receptor in *E. sinensis* (*Es*IR) was revealed by bioinformatics analysis, which was deposited in GenBank under accession no. MN232176. The coding region of the *Es*IR was of 3573 bp and it encoding a peptide of 1190 amino acids. The predicted molecular size was 132.2 kDa and the theoretical isoelectric point was 6.43. SMART conserved domain analysis revealed that there were a furin-like domain (2-67 aa), a receptor L domain (82-209 aa), five FU domains (229-505 aa), a transmembrane domain (534-556 aa) and a tyrosine kinase domain (602-858 aa) in the deduced amino acid sequence of *Es*IR (Fig. 1A). Multiple

A



B

Ligand-binding domain in the extracellular region



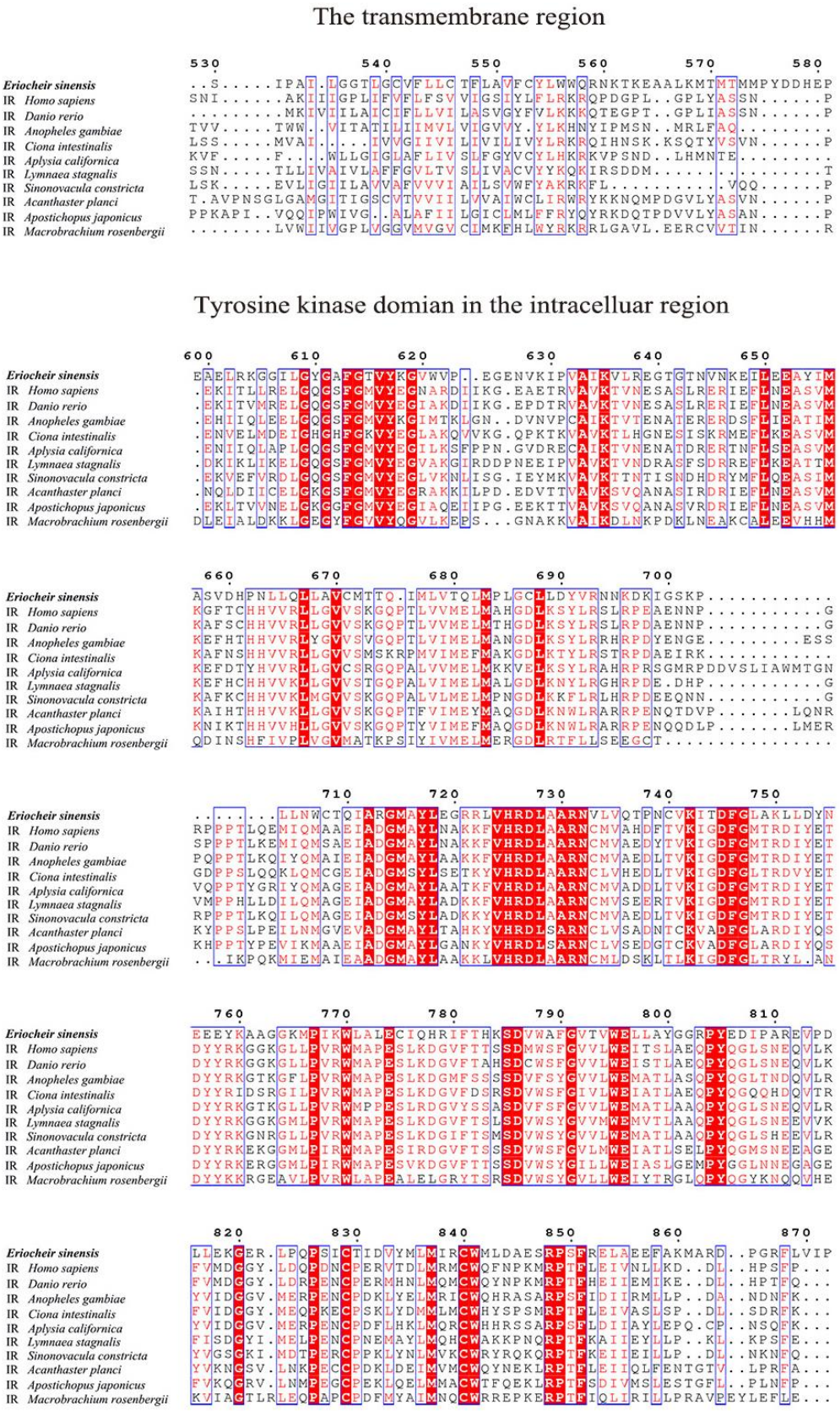


Fig. 1 Structure prediction and multi-sequence alignment of *Es*IR. (A) Structure prediction of *Es*IR by SMART analysis, which contained a Furin-like domain, a Receptor L domain, five FU domains, a Transmembrane domain (TM), and a Tyrosine kinase domain (TyrKc). (B) Multiple sequence alignment of *Es*IR extracellular region, transmembrane region and intracellular region with insulin receptors in other species. The red shadow region indicates all sequences share the same amino acid residue, and the blue box indicates the amino acids with similarity more than 50 %. Gaps are indicated by dots to improve the alignment

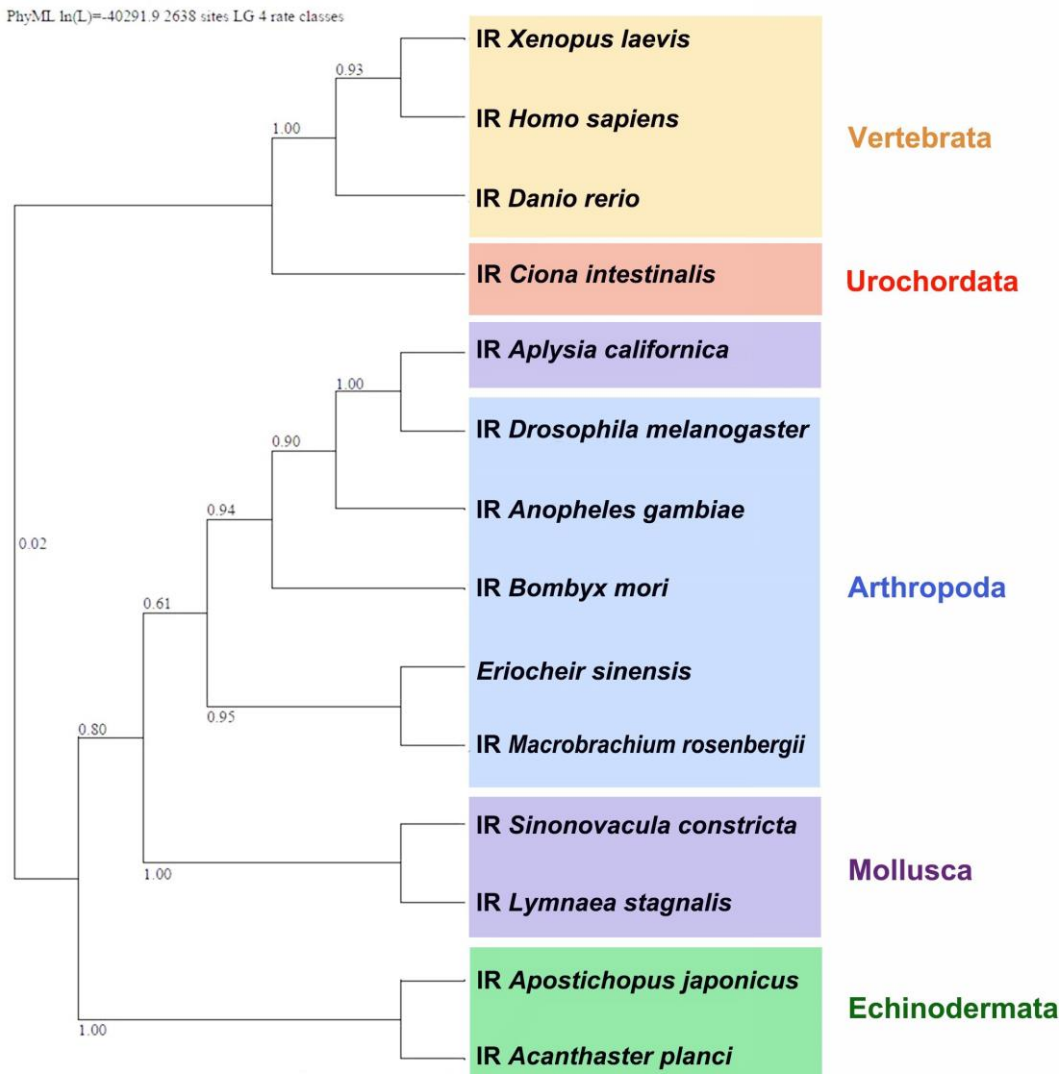


Fig. 2 Phylogenetic relationship of the insulin receptors in different species

alignments showed that *Es*IR exhibited relatively low similarity in the extracellular region, while shared high identity in intracellular region with other insulin receptors (Fig. 1B).

The phylogenetic analysis of EsIR

Phylogenic tree was constructed by the maximum likelihood method. All insulin receptors were clustered together according to phylum. *Es*IR was firstly clustered with the insulin receptor from *Macrobrachium rosenbergii*, constituting a sub-branch of crustacean insulin receptors. This branch was then clustered with other arthropods insulin receptors. In addition, insulin receptor from urochordata shared closer relationship with vertebrate insulin receptor (Fig. 2).

Subcellular localization of EsIR protein

A recombinant pEGFP-*Es*IR plasmid was constructed and transfected into well-growing HEK293T cells and observed under a laser confocal microscope. The recombinant vector was successfully transfected into HEK293T cells, and the signal of green fluorescent protein (green) was present throughout the cell. The positive signal of *Es*IR fusion protein with EGFP (in green) was co-localized with the DiI-stained cell membrane (in red) (Fig. 3).

Distribution of EsIR mRNA in different tissues

qRT-PCR was performed to detect the distribution of *Es*IR mRNA in different tissues of *E. sinensis.* The mRNA transcripts of *Es*IR were detected in all the tested tissues, including hematopoietic tissue, stomach, muscle, gills, eyestalks and hepatopancreas, and hemocytes with the highest expression level in hepatopancreas, which was 94.00-fold ($p < 0.05$) of that in hematopoietic tissue. Higher expression levels of *Es*IR mRNA were also observed in eyestalks and

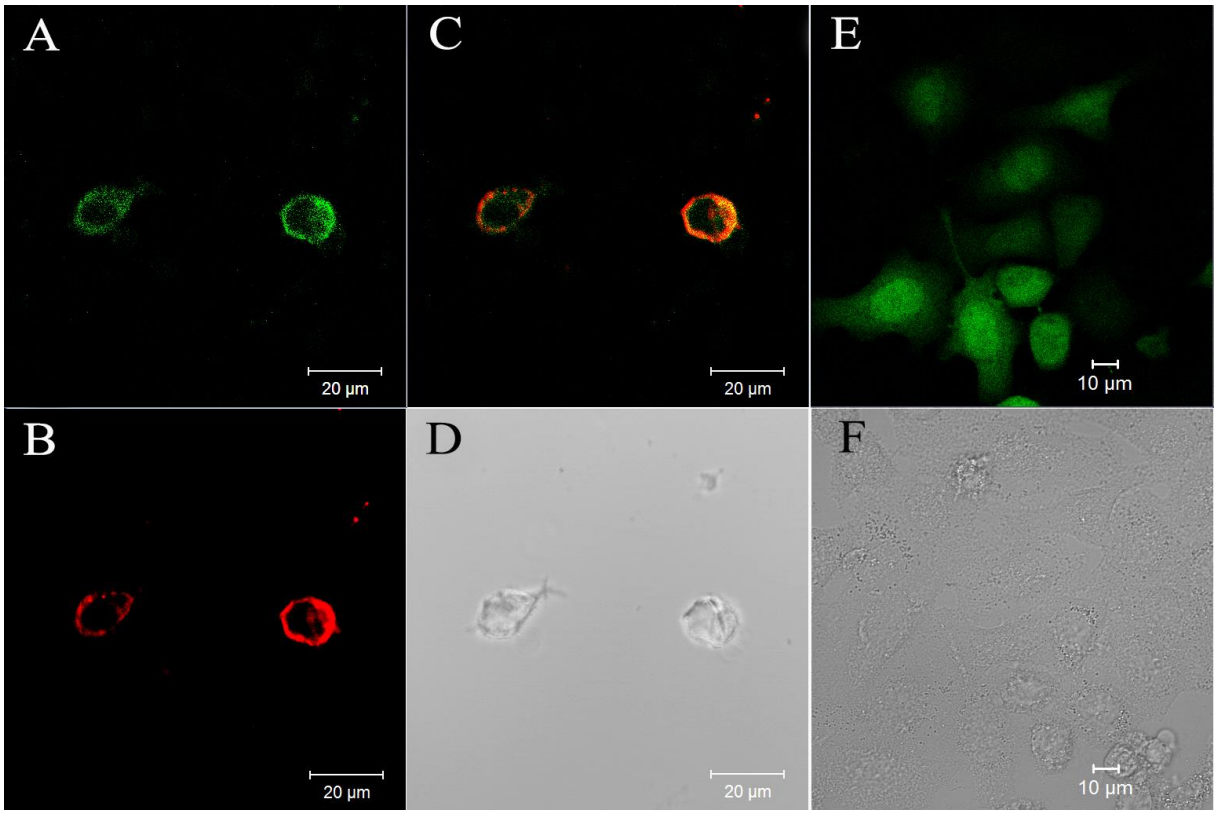


Fig. 3 Subcellular localization of *Es*IR in HEK293T cells. (A) *Es*IR protein (green signal) was expressed on cell membrane. (B) DiI (red signal) stained cells. (C) *Es*IR protein was co-located with DiI stained cell membrane. (D) The transfected cells showed normal morphology. (E) Control group EGFP (green signal) expression in the whole cell. (F) The control group cells showed normal morphology

gills, which were 43.70 and 41.15-fold ($p < 0.05$) of that in hematopoietic tissue, respectively. The expression levels of *Es*IR mRNA in muscle, stomach and hemocyte were 27.10, 20.16 and 1.43-fold ($p < 0.05$) of that in hematopoietic tissue, respectively (Fig. 4).

Temporal expression of EsIR mRNA in hepatopancreas after A. hydrophila infection

The expression of *Es*IR mRNA in hepatopancreas changed significantly after *A. hydrophila* infection. It decreased firstly from 3 h (0.09-fold of that in control group, $p < 0.01$) to 6 h (0.52-fold of that in the control group, $p < 0.05$), then increased to 1.62-fold ($p < 0.05$) that of the control group at 12 h, and finally returned to normal level at 24 h (Fig. 5).

Discussion

The insulin receptors have been well studied deeply since the protein fragments on the cell membrane was first discovered to specifically bind insulin in 1970 (De Meyts, 2004; House and Weidemann, 1970). These evidences confirm that the insulin receptors regulate metabolic homeostasis in a systemic manner and reallocate energy during stress response. However, only a few insulin receptors have been described in crustacean species, and their roles in maintenance of homeostasis are far from well understood. In the present study, a homologue of insulin receptor (*Es*IR) was identified from the Chinese mitten crab *E. sinensis*. The extracellular portion of *Es*IR protein contained a cysteine rich region with a Furin-like domain, a receptor L domain and five FU domains, which were cysteine rich repeats (Fig. 1A). This domain architecture in the extracellular portion has also been reported in many other invertebrates, such as *M. rosenbergii* and *Daphnia pulex* (Boucher *et al*., 2010). In vertebrate, the extracellular portion of IR consists of two L-domains, a cysteine rich region，and three fibronectin type III (FnIII) domains (Hernandez-Sanchez *et al*., 2008). Most invertebrates possess more ILPs, but only one insulin receptor (Mao *et al*., 2018b). The unique domain composition in the extracellular region suggests that the ligand-receptor contact can be diverse in invertebrate. The intracellular portion is responsible for ligand-induced signal transduction and phosphorylation of second-messenger proteins inside cells (Shu and Steiner, 2000). The architecture of functional domains in this region of *Es*IR is same as that in other vertebrates. Alignment of the *Es*IR with the other insulin receptors from invertebrates and vertebrates revealed that the

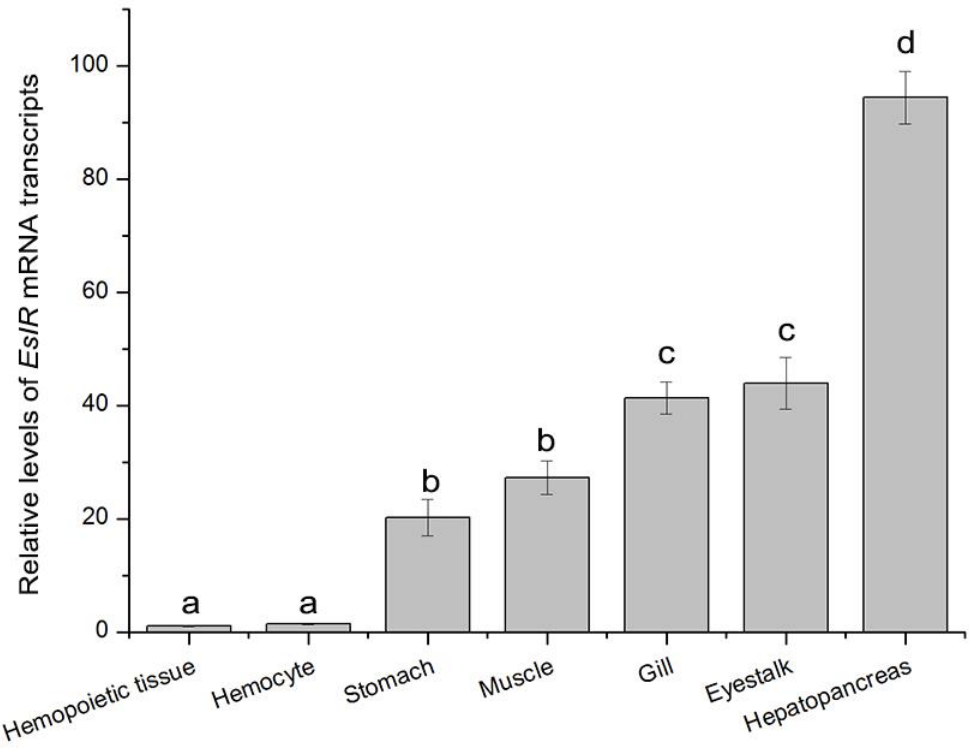


Fig. 4 The expression of *EsIR* mRNA transcripts in different tissues of *E. sinensis* detected by quantitative RT-PCR. Different letters (a, b, c, d) represent statically significant differences ($p < 0.05$)

intracellular components were less variable than the extracellular parts, indicating that the insulin signal transduction was conserved (Fig. 1B). Further evolutionary analysis showed that insulin receptors from different species were clustered together according to the phylogenetic relationship of the species. There was an independent replication event between chordate and invertebrate insulin receptors. In invertebrates, *Es*IR shared the closest homology with the insulin receptor in *M. rosenbergii*, and constituted a sub-branch with that of other arthropods (Fig. 2). These results indicated the highly conservation of insulin receptors throughout evolution.

The insulin receptors distribute in nearly all cells surface, where they specifically bind to insulin to activate intracellular signaling cascades and cause a series of physiological reactions, and no insulin receptor has been found in the cytoplasm (Hernandez-Sanchez *et al.*, 2008). In the present study, the recombinant pEGFP-*Es*IR plasmids were transfected into HEK293T cells, and the *Es*IR protein was found to be localized on the cytomembrane of HEK293T cells, which supported our assumption that the *Es*IR protein was an insulin-like membrane-bound receptor (Fig. 3). Together with the prediction of *Es*IR domain, it was speculated that *Es*IR was anchored to cytomembrane by the transmembrane domain.

As important molecules in metabolic process, the insulin receptors are widely distributed in various tissues. *Es*IR mRNA transcripts were detected in all examined tissues, indicating its basic physiological function (Fig. 4). In crustacean, hepatopancreas functions crucially in carbohydrates metabolism while eyestalk plays an important role in synthesizing and secreting the endocrine hormones (Roszer, 2014; Nguyen *et al.*, 2016). The higher expression levels of *Es*IR mRNA in hepatopancreas and eyestalk implied the potential roles of *Es*IR in metabolism and endocrine.

Previous studies showed that the activation of Toll-like signaling triggered by infection interfered with insulin signaling pathway in rat liver. The survival rate of *D. melanogaster* carrying loss-of-function for the insulin receptor increased after bacterial infection (Karpac and Jasper, 2009). These results implied that the insulin signaling pathway played important roles in antibacterial immune responses. In the present study, the expression of *Es*IR mRNA in hepatopancreas decreased significantly from 3 h to 6 h post *A. hydrophila* stimulation (Fig. 5). It was speculated that the activated immune response inhibited *Es*IR expression during this time, thereby limiting glycogen synthesis in hepatopancreas. These results were consistent with previous report that the mRNA expression level of *Es*ILP decreased significantly in hepatopancreas of *E. sinensis* after *A. hydrophila* stimulation (Wang *et al.*, 2020). Meanwhile, the decreased *Es*IR expression might also be involved in immune modulation during bacterial infection. It has been reported that *A. hydrophila* stimulation could significantly elevate the

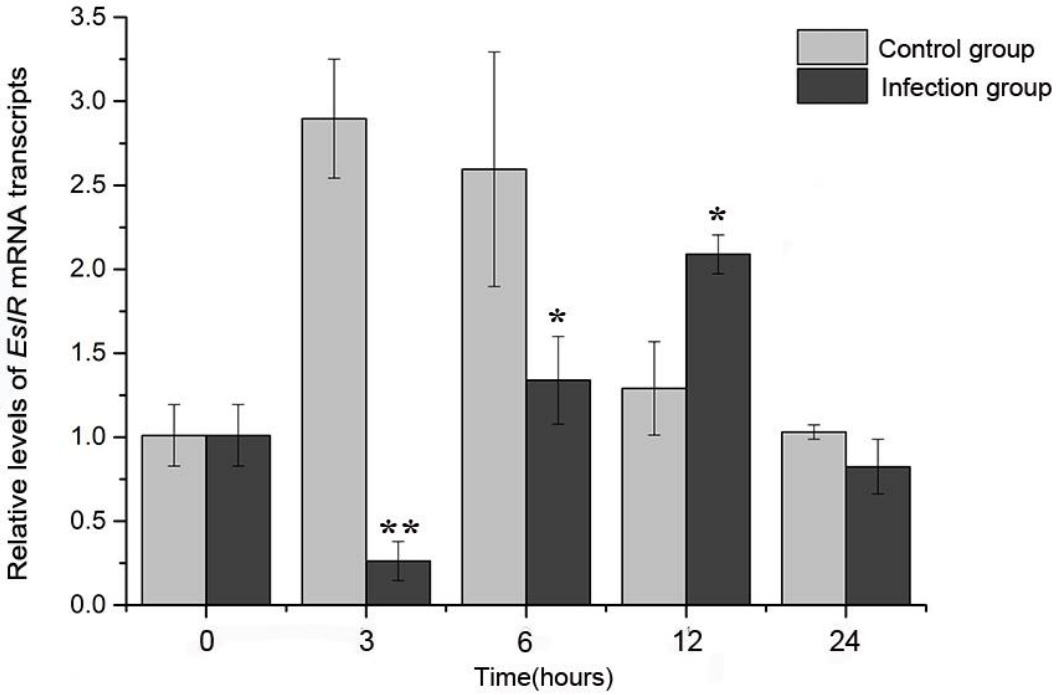


Fig. 5 The expression of *EsIR* mRNA transcripts in hepatopancreas after *A. hydrophila* stimulation. Statistical significance is indicated by single ($p < 0.05$) or double ($p < 0.01$) asterisks

activity of phenoloxidase in *E. sinensis* (Jia *et al.*, 2018). The loss-of-function of insulin receptor was also found to promote melanization and phenoloxidase activity in *Drosophila* (McCormack *et al.*, 2016). It has been reported that the metabolic statuses (glycolysis/TCA cycle) varied greatly in crustacean during the early or late stage of infection (Su *et al*., 2014). Compared to glycolysis, TCA cycle costs less glucose for ATP production. Therefore, the upregulated *Es*IR at 12 h indicated a metabolic shift to promote the glucose transport and glycogen synthesis in hepatopancreas of the challenged crabs. These results collectively suggested that the insulin receptor (*Es*IR) played important roles in both metabolic and immune modulation during immune response.

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