Gene 557 (2015) 98–102

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short communication

Two novel male-associated peroxinectin genes are downregulated by exposure to delousing drugs in *Caligus rogercresseyi*

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ARTICLE INFO

Article history:
Received 5 November 2014
Accepted 4 December 2014
Available online 5 December 2014

Keywords:
Peroxinectin
Caligus rogercresseyi
Deltamethrin
Azamethiphos
RNA-seq

ABSTRACT

Peroxinectin (PX) is a protein involved in cell adhesion, peroxidase activities, and the encapsulation of invaders in diverse species, including parasitic copepods. Recently, a transcript denoted *peroxinectin-like* was identified in the salmon louse *Lepeophtheirus salmonis*, and this was significantly correlated with the immune response of host fish. Thus, the PX gene is a potential candidate to evaluate host–parasite interactions, as well as to analyze responses to delousing drugs used in the salmon aquaculture industry worldwide. The objective of this study was to identify *Peroxinectin* transcripts in the Chilean salmon louse *Caligus rogercresseyi*, and to determine expression levels after exposition to the antiparasitics deltamethrin and azamethiphos. Two novel transcript homologs to peroxinectins were identified from a transcriptomic library of *C. rogercresseyi*. Moreover, in silico gene transcription levels were evaluated through RNA-seq analyses based on unique gene read levels in transcriptomic libraries that were constructed from sea lice exposed to delousing drugs. The identified transcripts were named *Peroxinectin-Cr1* and *Peroxinectin-Cr2*, which, respectively, had lengths of 2543 and 2555 base pairs. Both PX transcripts were highly associated with male adults, and transcription levels were significantly reduced by deltamethrin and azamethiphos. This result suggests a modulation of peroxinectin in response to delousing drugs.

1. Introduction

Peroxinectin (PX) is a protein with diverse biological functions in the immune system and prophenoloxidase activities, especially in crustacean species (Du et al., 2013). The first described biological processes for PX in crustaceans were cell adhesion and the degranulation of crayfish hemocytes (Johansson and Soderhall, 1988, 1989). Later, other functions were identified, such as the promotion of hemocyte encapsulation (Kobayashi et al., 1990), opsonization (Thornqvist et al., 1994), and peroxidation to control infectious agents (Johansson, 1999). Of the different homologs found in crustacean species, each PX contains at least one putative integrin-binding motif and a peroxidase domain (Liu et al., 2004, 2005). These structural features confer the biological processes mentioned and also allow PX to participate in the peroxidase activity of other antioxidant proteins such as superoxide dismutase (Holmblad and Soderhall, 1999). Furthermore, PX is an inducible gene in response to specific pathogen-associated molecular patterns and bacteria, therefore suggesting a role in the innate immune response of crustaceans (Liu et al., 2005; Dong et al., 2009, 2011).

The importance of studying PX genes in different sea lice species is based on a recently published work, which found a correlation between the gene transcription of a peroxinectin-like transcript of *Lepeophtheirus salmonis* and the immune innate response of the host species (*Salmo salar*) (Wotton et al., 2014). Herein, a correlation between the expression patterns of parasite PX and fish chemokines was found, suggesting that peroxinectin has a role in host–parasite interactions. In addition to this, there is concern that sea lice have developed resistance to commonly used antiparasitic drugs (Lees et al., 2008). As sea lice species cause great economic loses in the salmon industry (Costello, 2009), novel control strategies have to be developed, and in this context, understanding the host–parasite interaction is a promising possibility (Ingvarsdóttir et al., 2002).

The main objective of this study was to evaluate the response of PX transcripts in the Chilean salmon louse *Caligus rogercresseyi* to treatment with two delousing drugs currently applied in salmon farming, deltamethrin and azamethiphos. Subsequently, the expression levels of PX were measured in exposed sea lice. The results provided novel insights into the regulation of this gene, thus contributing to discussions regarding the effects that these chemicals have for PX gene transcription, including sex-specific bias.

Abbreviations: PX, peroxinectin; UTR, untranslated region; bp, base pairs; mRNA, messenger RNA; RNA-seq, RNA sequencing; ORF, open reading frame; RACE, rapid amplification of cDNA ends; GO, Gene Ontology.

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http://dx.doi.org/10.1016/j.gene.2014.12.006
0378-1119/

Table 1
Primers used in this study to obtain full sequences of two peroxinectin mRNAs in *Caligus rogercresseyi*.

Primer name	Sequence (5′ to 3′)	Specification
PX-Cr1_5i	TTCACATCTCCAAAGGCCAA	5′-RACE
PX-Cr1_5o	CTAATTCGTTCTTCTATGCTC	5′-RACE
PX-Cr1_3i	ATGTCACTGGAGGTTTCAT	3′-RACE
PX-Cr1_3o	GACCAATTCGCTAGGCTTAAGA	3′-RACE
PX-Cr2_5i	GTGTCCCTAAATAGATTACTC	5′-RACE
PX-Cr2_5o	AGTTTCACATCATTTTGG	5′-RACE
PX-Cr2_3i	GTATCATCAGCGATCAATTCG	3′-RACE
PX-Cr2_3o	AGTTCTTTTACACCTGATCAAT	3′-RACE

2. Materials and methods

2.1. Peroxinectin gene discovery

Two transcripts annotated as *Peroxinectin* (*PX*) were obtained from a RNA-seq library constructed for *C. rogercresseyi* (Gallardo-Escárate et al., 2014). Briefly, a RNA-seq library was generated through high-throughput sequencing of the developmental stage of the *C. rogercresseyi* lifecycle in the MiSeq platform (Illumina®, San Diego, CA, USA). Then, *PX* sequences from marine invertebrates were downloaded from the GenBank database in order to perform a MultiBlast analysis against all contigs using the CLC Genomic Workbench software (version 6.0, CLC Bio, Aarhus, Denmark). Two contigs annotated as *peroxinectin* genes were extracted from the transcriptomic library in order to obtain the partial sequences.

2.2. Full PX cDNA characterization

For each contig, a pair of primers was designed to amplify a sequence fragment of the PCR (Table 1) by using the Geneious 6.0.4 software (Biomatters, Auckland, New Zealand). PCR amplification was conducted using 200 ng of cDNA obtained from adult sea lice, 10 μM of each primer, 1.5 mM $MgCl_2$, and 0.06 U taq DNA polymerase (Thermo Scientific). PCR cycles consisted of initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 60 s, annealing at 60 °C for 30 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. PCR products were visualized through electrophoresis in agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA) and then directly sequenced in an ABI 3730xl capillary sequencer.

Full-length cDNA of each *PX* transcript was obtained by the rapid amplification of cDNA ends (RACE) technique using the SMARTer™ RACE cDNA Amplification kit (Clontech Laboratories Inc., Mountain View, CA, USA). Primers for amplifying the 5′UTR and 3′UTR ends were designed according to the manufacturer's instructions (Table 1). Amplicons were purified and cloned using the TOPO TA cloning kit (Invitrogen, Life Technologies), followed by transformation into *Escherichia coli* JM109 in LB/amp/IPTG/Xgal. Positive clones were selected by galactosidase reaction visualization, and resulting plasmids were purified using the E.Z.N.A.® Plasmid DNA Mini kit II (Omega Bio-tek, Doraville, GA, USA). Purified plasmids were sequenced in both forward and reverse directions using M13 universal primers. Sequence analyses were carried out in the Geneious software and consisted of quality visualizations, assembly, and BLASTn against the GenBank non-redundant database.

2.3. PX gene transcription analysis in sea lice developmental stages

Total RNA extractions were performed from the following developmental stages of *C. rogercresseyi* (N = 10): nauplius I, nauplius II, copepodid, chalimus, and female and male adults. Extractions were performed using the Trizol Reagent® (Invitrogen, USA) following the manufacturer's instructions.

Primers for qPCR analysis were designed by the Geneious software (Table 1). Dynamic range analysis was conducted to obtain qPCR efficiencies and optimal conditions for qPCR runs. For this, five serial dilutions of cDNA were prepared starting with 80 ng and with a serial factor of 1:5 in order to generate an amplification curve with both pairs of primers designed for qPCR analysis. The qPCR runs were assayed in an ABI StepOnePlus™ Mastercycler (Applied Biosystems®, Life Technologies) with the Maxima Kit® SYBR Green/ROX qPCR Master Mix (Thermo Scientific®) following manufacturer's instructions. The run method comprised of a holding stage at 95 °C for 10 min to activate the enzyme, then 40 cycles of 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s. After the amplification stage, a melting curve was performed from 57 °C to 95 °C, and data was collected every 0.3 °C to determine the existence of a sole amplification product and to verify the inexistence of contaminations and primer dimers. Efficiencies were calculated

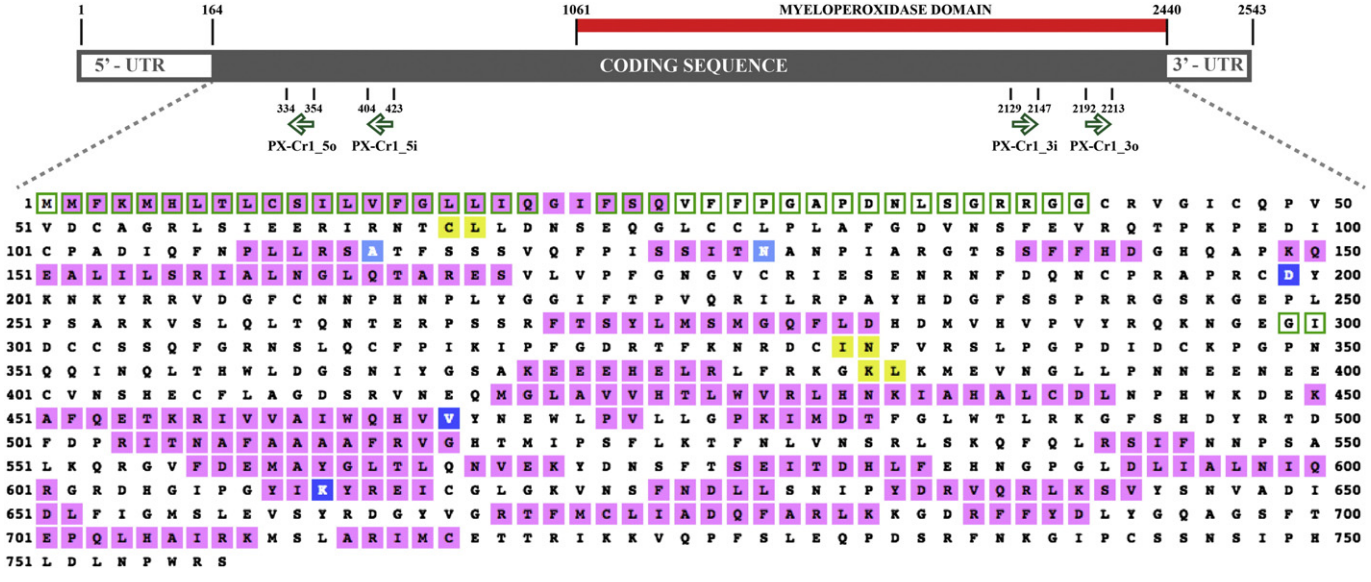

Fig. 1. Full mRNA sequence and amino acid prediction of *Peroxinectin-Cr1*. The schematic structure of mRNA is shown in the gray rectangle, and a characteristic myeloperoxidase domain is shown in red. The secondary structure of the predicted amino acid sequence is shown as pink = helix; yellow = sheet; blue = boundaries; and green = disordered protein binding sites. Additionally, green arrows show the position of primers designed for RACE.