Gene 697 (2019) 35–39

Contents lists available at ScienceDirect

Gene

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

Research paper

Silencing of ionotropic receptor 25a decreases chemosensory activity in the salmon louse *Lepeophtheirus salmonis* during the infective stage

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ARTICLE INFO

Keywords:
Salmon louse
IR25a
Olfactory receptor
Host recognition
RNA interference

ABSTRACT

Chemoreception is critical for marine ectoparasites - such as salmon lice (*Lepeophtheirus salmonis*) - to identify and locate salmonid hosts. The molecular receptors that parasites employ to detect host-specific chemical stimuli from hosts (kairomones) have not been well characterised. In the present study, transcription of the sea louse *Ionotropic receptor 25a* (*IR25a*) was blocked to evaluate whether it functions as a chemical-perception related gene for a specific chemical cue from the Atlantic salmon host. Double-strand RNA interference (dsRNA) oligonucleotides were applied to salmon lice by *in vitro* transcription and then exposing salmon lice nauplii to dsRNA by soaking overnight. Silencing of the *IR25a* gene was confirmed by qPCR in experimental groups of knock-down copepodids (dsIR25a). Behavioural responses associated with host recognition were evaluated in dsIR25a sea lice after exposure to a peptide produced by the salmon host (Cath-2). The dsIR25a group decreased expression levels of *IR25a* by > 7-fold with respect to the control group. This group was also 26% slower than the control group (control swimming speed was 69 mm/s, while the treated group was 51 mm/s). Since the swimming activity of salmon lice copepodids is associated with the activation of the chemosensory system, these results indicate that the *L. salmonis* chemosensory perception system was not fully activated due to gene silencing. The results of this study demonstrate the role of ionotropic receptor 25a during host recognition by sea lice.

1. Introduction

Myriad chemicals are released by marine organisms and form the basis for complex chemically mediated interactions between conspecifics, predators and prey, and parasites and their hosts (Lindsey and Lasker, 1974; Hay, 2009; Steiger et al., 2011). In marine parasitic copepods, such as *Lepeophtheirus salmonis* (hereafter referred to as salmon lice), the non-feeding, free-living early life stages must find a suitable host before their energy reserves are exhausted. The salmon lice relies on a diverse suite of sensory mechanisms to locate hosts, including chemical signalling (Fields et al., 2007; Mordue Luntz and Birkett, 2009; Heuschele and Selander, 2014; Fields et al., 2018). The detection of chemical signals relies on the presence of membrane-bound receptors to which chemical stimulants bind and, in turn, activate sensory transduction pathways.

We investigated a chemoreceptor, belonging to the ionotropic receptors group (IRs), which increases its expression depending on the concentration of host-derived chemical compounds, and decreases expression in the presence of semiochemical-blocking compounds (Núñez-Acuña et al., 2016b). Ionotropic receptors are a non-conserved group derived from the ionotropic glutamate receptors (IGluRs), with specific roles in chemical perception (Benton et al., 2009). In marine arthropods, the first description was in the spiny lobster, in which the *Ionotropic receptor 25 a* gene (*IR25a*) is tightly coupled to the olfactory neurons (Corey et al., 2013). This gene corresponds to a co-receptor that functions together with specific chemosensory receptors by ligand

Abbreviations: IR25a, Ionotropic receptor 25a; mRNA, messenger RNA; cDNA, copy DNA; RNAi, RNA interference; dsRNA, double-strand RNA; qPCR, quantitative polymerase chain reaction; Cath-2, cathelicidin-2; AMP, antimicrobial peptide

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https://doi.org/10.1016/j.gene.2019.02.012
Received 12 October 2018; Received in revised form 4 January 2019; Accepted 6 February 2019
Available online 19 February 2019
0378-1119/

Table 1
Primers used in this study for the salmon louse (*Lepeophtheirus salmonis*).

Primer name	Gene name	Use	Sequence	Product size	Source
Ls-IR25a-F1	Ionotropic receptor 25a	qPCR	TTCGCTTTTCTAGGGGATGC	112 bp	LiceBase (EMLSAG00000004146)
Ls-IR25a-R1			GTTGAACCGCCAAAGCATAC		
Ls_EF1_2F	Elongation factor 1	qPCR	TTAAGGAAAAGGTCGACAGACGTA	77 bp	Genbank (EF490880) (Frost and Nilsen, 2003)
Ls_EF1_2R			GCCGGCATCACCAGACTT		
dsRNA_IR25_1F	Ionotropic receptor 25a	dsRNA	GCTGCCTATTTTGCAACCAG	794 bp	LiceBase (EMLSAG00000004146)
dsRNA_IR25_1R			CAGCGTTCCTTGAGAGTCTC		
dsRNA_EGFP_1F	Enhanced green fluorescent protein	dsRNA	TGGTCGAGCTGGACGGCGACG	720 bp	This study
dsRNA_EGFP_1R			ACGAACTCCAGCAGGACCATG		

binding, and it is also the most conserved and well-studied IR co-receptor in invertebrates (Benton et al., 2009; Croset et al., 2010; Abuin et al., 2011; Wicher, 2012; Rytz et al., 2013). This gene has also been characterised in the fish ectoparasite *Caligus rogercresseyi* (hereafter referred to as sea lice), where it was highly expressed during the infective copepodid stage (Núñez-Acuña et al., 2014). *IR25a* was also involved in the response of sea lice to the presence of the antimicrobial peptide cathelicidin-2 (Cath-2). Cath-2 is a small peptide produced by salmon (the host fish) (Núñez-Acuña et al., 2016a) that has been identified as a molecular fingerprint that salmon lice use as a chemical activation cue during the host-recognition process (Núñez-Acuña et al., 2018). This activation was described by behavioural, physiological and molecular analyses in salmon lice copepodids, in which various chemosensory receptors were over-expressed when exposed to Cath-2 (Núñez-Acuña et al., 2018). Furthermore, when this gene was silenced in salmon lice parasites, the host preference shifted from a species-specific parasite to more generalist parasite (Komisarczuk et al., 2017).

In this study, the swimming behaviour of salmon lice in response to increasing concentrations of the Cath-2 peptide was investigated. The copepods were stimulated using an overhead shadow that triggers a repeatable behavioural response (Fields et al., 2018). Using gene silencing techniques targeting the *IR25a* gene, we investigated the impact of disrupting this chemosensory pathway as a possible mechanism to diminish the efficacy of host detection by the salmon lice. These data provide novel insight into the intricate connection between parasites and hosts, as well as having possible practical significance in addressing the significant economic losses on salmon farms due to infestations by these parasites (Costello, 2009; Torrissen et al., 2013; Igboeli et al., 2014).

2. Materials and methods

2.1. Sea lice culture

Gravid salmon lice females were collected from an experimental salmon farm located at the Austevoll Research Station of the Institute of Marine Research (IMR), Norway. Egg strings were separated from the female louse using a scalpel and placed in a hatching container (25 cm in diameter fitted with a 100 μm sieve on the bottom). The egg chambers were suspended in a running seawater bath (20 L min-1) at 8 °C under a 14:10 h light:dark photoperiod. Sieves were checked daily for the presence of hatched nauplii. Each day, unhatched egg strings were transferred to a new sieve, which was suspended in the water bath. Sieves containing newly hatched nauplii were labelled with the date to generate cohorts of lice of the same age. Larvae were observed under a microscope to evaluate development until the copepodite stage was reached. Groups of copepodids of the same age were kept separately for further analyses.

2.2. Ionotropic receptor 25a gene expression after kairomone exposure

Groups of 150 copepodids were exposed to different concentrations of the Atlantic salmon Cath-2 peptide: 7, 70 and 700 ppb in a 2 L glass tank containing filtered seawater for 25 min at 8 °C. The Cath-2 peptide was obtained by chemical synthesis (Núñez-Acuña et al., 2016a). Exposure trials were evaluated using three biological replicates (3 pools of 150 copepodids) in each experimental group. After incubation, animals were filtered, transferred to a cryogenic tube with 1 mL of RNA Later solution (Ambion, USA) and stored at −80 °C. Total RNA was extracted from each group using Trizol (Invitrogen, USA), following the manufacturer's instructions. RNA concentrations were measured with a NanoDrop spectrometer (NanoDrop Technologies Inc., USA) and its integrity assessed by electrophoresis in a MOPS-agarose gel under denaturing conditions. cDNAs synthesis reactions were performed from 200 ng/μL of RNAs using a RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA). Quantitative PCR reactions were conducted from cDNAs in a StepOne Plus qPCR system (LifeTechnologies, USA) in 10 μL reactions including holding-stage at 95 °C for 2 min, 40 cycles of 95 °C for 15 s, 60 °C for 60 s and a melting curve from 60° to 95 °C. Master mix for *IR25a* and the endogenous control gene were performed using the PowerUp™ SYBR® Green Master Mix kit (Applied Biosystems, Thermo Fisher Scientific, USA). The endogenous control corresponded to the elongation factor-1a (EF1a) gene, which was previously validated as a housekeeping gene for the study species (Frost and Nilsen, 2003). Quantification of gene expression was conducted by the $\Delta\Delta C_T$ method, applying a *t*-test for statistical analyses with a p-value < 0.05 considered as significant. Primers for qPCR analyses are shown in Table 1. Dynamic range analyses were used for both genes before measuring gene expression to measure reaction efficiency. This range consisted of five serial dilutions of cDNAs from 200 ng as a starting level.

2.3. IR25a gene silencing using double-strand RNA (dsRNA) treatment

A specific 794 base pair (bp) region of the salmon lice *IR25a* gene was amplified using the following primers: Fwd- GCTGCCTATTTTGC AACCAG and Rev- CAGCGTTCCTTGAGAGTCTC (Table 1). T7 promoter sequence was added to the oligonucleotides (TAATACGACTCACTATA GGGAGA). *In vitro* dsRNA synthesis was conducted applying the MEGAscript™ T7 Transcription Kit (Ambion, Thermo Fisher Scientific). Obtained dsRNA was measured with a NanoDrop spectrometer. Gene silencing treatments were performed according to Eichner et al. (2014). Groups of 150 parasites at the Nauplius I stage were subdivided into 25 per experimental group and exposed to 2 μg of dsRNA using 1-mL chambers at a concentration of 20 ng/μL of seawater. Animals were incubated with dsRNA for 16 h. Two control groups were used: negative control (without any dsRNA) and animals exposed to a non-related dsRNA, corresponding to the green fluorescent protein (GFP) gene (primers at Table 1). To confirm dsRNA treatment efficacy, qPCR reactions to measure gene expression levels of *IR25a* were conducted on all of the experimental groups using the same methodology as described above.

2.4. Swimming behaviour analyses of dsIR25a copepodids

Swimming behaviour was evaluated using silhouette video

photography (SVP) (Browman et al., 2003; Fields et al., 2018; Núñez-Acuña et al., 2018). All of the experimental groups (dsIR25a, dsGFP control, and non-treated control) were incubated in a 2 L glass tank with water containing Cath-2 as described above. A fourth group consisted of copepodids that were not incubated with the Cath-2 peptide. Three replicates of each experimental group were evaluated (150 parasites per group). A flashing light from a 1000 W Xenon arc lamp (Oriel Instrument, USA) was projected from above to induce a swimming response (Fields et al., 2018). Each replicate received 16 ON:OFF (13 s:47 s) cycles of the lamp. Video sequences were recorded using StreamPix software (v 5.0, Norpix Inc., Canada) and swimming response data was extracted from the first 10s after the beginning of the light stimulus (ON) in each of the 16 cycles. SVP image sequences were analysed to determine % activity (% of animals responding to the signal) and swimming speed in response to the light stimulus (Fields et al., 2018). The behavioural responses of the lice were characterised by frame-by-frame analysis of the video recordings (MANTRACK software, JASCO Scientific, Canada). Statistical differences were evaluated by a one-way ANOVA followed by Tukey's post-hoc test.

3. Results

3.1. Gene transcription analyses

The efficiency of qPCR reactions was 102.3 and 99.87% for *IR25a* and *EF1a* genes, respectively. A significant increase in the expression of *IR25a* gene was observed in copepodids exposed to all the concentrations of the Atlantic salmon Cath-2 peptide ($p < 0.05$) in a dose-dependent pattern (Fig. 1). The most significant increase with respect to the control group was observed at the highest concentration of Cath-2 (700 ppb), which had 27.8% more expression than control ($p < 0.01$).

After dsRNA treatment, qPCR analyses confirmed that the silencing assays were successful (Fig. 2). Expression of *IR25a* was significantly lower in the dsIR25a group after exposure to Cath-2 compared to the negative control ($p < 0.001$), reducing the basal expression levels of the target gene in 89.7% (Fig. 2). The dsGFP group did not exhibit significant differences in the expression of this gene with respect to the negative control group and, therefore, were significantly different from the dsIR25a group.

3.2. Swimming behaviour of the dsIR25a treated group

Gene silencing treatment was associated with a reduction in the swimming activity of salmon lice after being exposed to attractant signals (Fig. 3). The number of copepodids that responded to the signal decreased by 17% in treated animals with respect to the control ($p < 0.05$). Treated animals decreased their activity level with respect to controls as animals that were not incubated with Cath-2. There were no significant differences between the dsGFP and negative control groups (Fig. 3a). Furthermore, there was a significant reduction in swimming speed of dsIR25a group with respect to controls (18 mm/s slower, $p < 0.05$). The group without a chemical signal in the water was 31 mm/s slower than control ($p < 0.001$). There were no differences with the dsGFP group with respect to the negative control (Fig. 3b).

4. Discussion

The small peptide Cath-2, found in the mucus of salmon, activates a chemosensory transduction pathway (including the chemosensory receptor IR25a) in sea lice (Núñez-Acuña et al., 2016a). The expression profile of IR genes in the salmon lice is strongly correlated with chemical signals in seawater (Núñez-Acuña et al., 2018). In addition, expression patterns of IR genes were associated with the anterior portion of the animal's body which is where chemosensory structures are located (Komisarczuk et al., 2017). These observations are consistent with the dose-dependent expression patterns obtained in this study (Fig. 1) and provide strong evidence that *IR25a* is a critical element of the louse chemosensory system.

Furthermore, in salmon lice species, Cath-2 acts as a chemical activation cue, triggering physiological and behavioural responses in the parasites (Núñez-Acuña et al., 2018). Salmon lice showed a concentration-dependent increase in swimming speed in the presence of Cath-2. Silencing the *IR25a* gene altered host-seeking behaviour in salmon lice, resulting in a pronounced reduction in the number of animals responding to the light stimulus and a decrease in their average swimming speed. These results support the hypothesis that the *IR25a* gene is involved in host-recognition through the Cath-2 peptide released from the salmon skin. Besides, the percentage of copepodids responding to the light source in the dsIR25a treated group was reduced to a level similar to that displayed by the negative control that had no chemical cue added to the water (NO-Cath-2) (Fig. 3). The swimming speed of the dsIR25a group was also lower than the positive control (Cath-2), indicating that silencing of the *IR25a* gene decreased the swimming activity of parasites that had been activated by Cath-2.

The IR25a gene is a member of the class of IR proteins involved in chemoreception (Croset et al., 2010). In invertebrates, IRs bind to chemical signals in the environment using specific binding domains (Benton et al., 2009; Benton, 2015). These structural features correspond to three transmembrane regions and two specific binding domains (S1 and S2), which were also found in the olfactory organs of the spiny lobster (Corey et al., 2013) and in the sea lice *C. rogercresseyi*, indicating some degree of conservation in these domains among Arthropoda (Núñez-Acuña et al., 2014). However, the *IR25a* gene found in *L. salmonis* (Licebase accession code 0000004402) do not share the same sequencing, although binding domains are present within its

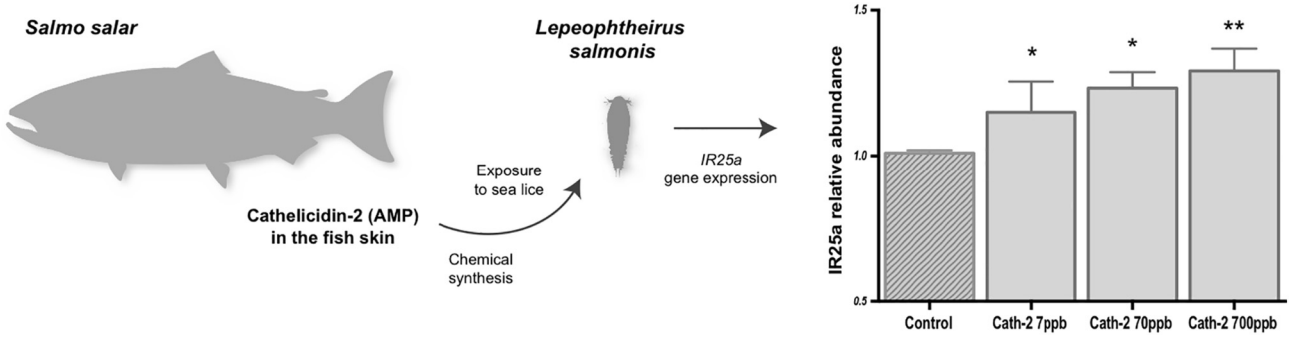

Fig. 1. Gene expression levels of *IR25a* in copepodids exposed to the cathelicidin-2 peptide. Salmon lice were exposed for 25 min to the peptide at 7, 70 and 700 ppb and gene expression levels were measured through qPCR. The control group consisted of animals incubated in seawater for 25 min but without peptide. Each group included 150 animals, and exposure trials were conducted in triplicate. *Elongation factor-1* gene was used as endogenous control. *: p–value < 0.05; **: p–value < 0.01.

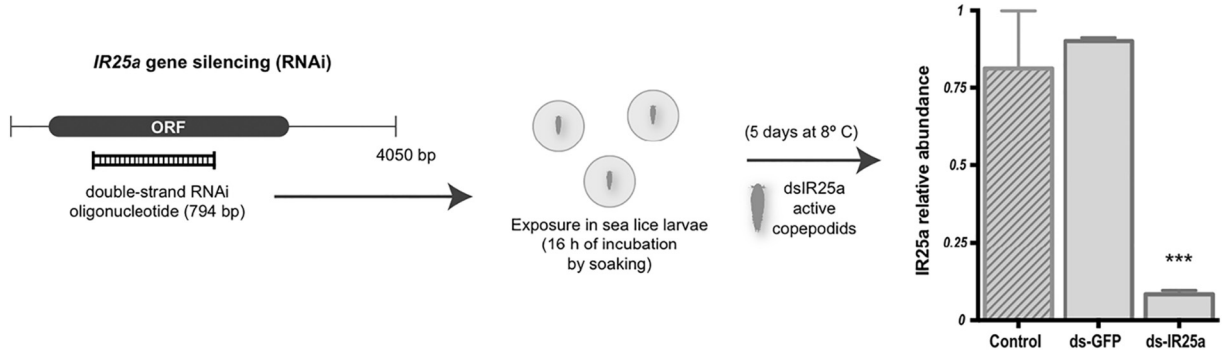

Fig. 2. Experimental design and efficacy of the gene silencing experiment. Double-stranded RNA was designed to be complementary to *IR25a* and was used for incubation in salmon lice larvae for 16 h at nauplius stage I. When the animals reached the copepodid stage, *IR25a* gene expression levels were measured by qPCR. The control group was not incubated with any dsRNA, the dsIR25a group was incubated with dsRNA silencing *IR25a*, and the dsGFP group was a second control incubated with a non-related oligonucleotide (*GFP* gene). ***: p–value < 0.001.

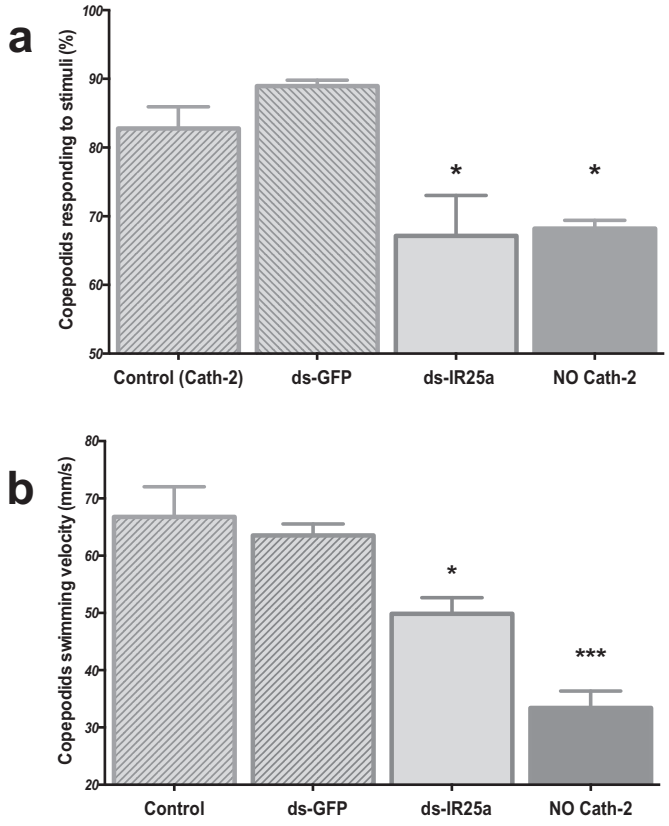

Fig. 3. Swimming behaviour of *Lepeophtheirus salmonis* copepodids after gene silencing treatment and incubation with the cathelicidin-2 peptide. Groups of 150 animals from dsRNA treatments - Control, dsGFP, and dsIR25a - were incubated with the cathelicidin-2 peptide for 25 min and their swimming behaviour was observed using silhouette video photography. A negative control group was also conducted (lice without peptide in the water). Three replicates per group were evaluated. A: Percentage of salmon lice copepodids that responded to the light signal. B: Swimming speed of copepodids in response to the light stimulus. Statistical differences relative to the control group are identified as * p–value < 0.05, *** p–value < 0.001.

sequence. Further molecular studies could explain the evolution of the different IRs structures that marine ectoparasites possess.

Acknowledgements

This study was funded by FONDAP # 15110027, CONICYT-PCHA/Doctorado Nacional/2015-21151044 awarded by Conicyt-Chile, and by the Norwegian Institute of Marine Research project “Fine-scale interactions in the plankton” (Project # 81529). The experiments reported were conducted during an internship to GNA funded by Conicyt-Chile (Grant 2015-21151044). The authors are thankful for the assistance of Reidun Bjelland and Steven Shema (IMR, Austevoll, Norway). We are grateful for the thorough and constructive comments of an anonymous reviewer which improved the completeness and clarity of the manuscript.

Conflict of interest statement

The authors declare no conflicts of interest.

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