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Using an improved Tol2 transposon system to produce transgenic zebrafish with epinecidin-1 which enhanced resistance to bacterial infection

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ABSTRACT

In order to advance the application of antimicrobial peptides in aquaculture, transgenic zebrafish expressing the antimicrobial peptide, epinecidin-1, were developed and are reported on here. First, we cloned the zebrafish mylz2 promoter for this purpose. To characterize the activity of the mylz2 promoter, various fragments of it were analyzed using a firefly luciferase transient expression assay, in which maximum promoter activity was found with a 2.5-kb fragment. In addition, the 2.5-kb fragment also expressed considerable red fluorescent proteins in skeletal muscles of transgenic zebrafish. Second, in order to improve the translation efficiency of the Tol2 transposase, we constructed untranslated regions (UTRs) of zebrafish ba1 globin flanked by a transposase. A transient embryonic excision assay (TEEA) and in vivo fluorescent observations showed high transposition efficiency during embryonic development. After optimization of the promoter and transgene efficiencies, transgenic zebrafish with the Epi-1/DsRed plasmid (pTLR-m2.5 K-K.Epinecidin-1/DsRed vector) were developed, and expressions of Epi-1/DsRed in muscles and blood were demonstrated by immunohistochemical staining techniques. Moreover, we also found that the Epi-1/DsRed gene was efficiently and significantly expressed in vivo against *Vibrio vulnificus* and *Streptococcus agalactiae* after injecting the bacteria and determining bacterial counts. A gene expression study using real-time RT-PCR revealed that Epi-1/DsRed itself induced endogenous MyD88 expression in vivo. After Epi-1/DsRed transgenic zebrafish were infected with *V. vulnificus* 204, interleukin (IL)-10, IL-22, IL-26, lysozyme, toll-like receptor (TLR)1, TLR3, TLR4a, MyD88, and nuclear factor (NF)-κB activating protein-like were upregulated, but IL-1β and tumor necrosis factor-α were downregulated at 12 h post-infection; IL-21, complement component c3b, and NF-κB activating protein-like were down-regulated, but MyD88 was upregulated at 24 h post-infection. These results suggest that using epinecidin-1 as a transgene in zebrafish can effectively inhibit bacterial growth for up to 24 h after infection.

1. Introduction

Epinecidin-1 is one of the fish antimicrobial peptides (AMPs) that were discovered in grouper (*Epinephelus coioides*) following a molecular cloning method [1]. It has secondary structures that closely resemble those of pleurocidin found in the winter flounder (*Pleuronectes americanus*) [2]. Epinecidin-1 is readily incorporated into cellular membranes of bacteria, fungi, and tumor cells which results in the formation of pores in membranes leading to pathogen and tumor cell death [3,4]. We previously characterized the lytic activities of synthetic epinecidin-1 against several gram-positive and -negative bacteria, and electroporated the plasmid containing the epinecidin-1 complementary (c)DNA sequence under a cytomegalovirus (CMV) promoter into zebrafish muscle to protect fish against *Vibrio vulnificus* infection [5].

Fish diseases are the greatest problem facing aquaculture and damaging its profitability. Although fish diseases can be prevented and controlled by vaccinations [6], there is still no truly effective vaccine or miracle drug for a variety of fish diseases. Genetic engineering to produce disease-resistant transgenic fish may provide a solution to this challenging problem [7–9]. In recent years, the Tol2 transposable element was developed as a potential and powerful genetic tool in transgenic animal studies for transgenesis, insertional mutagenesis, gene trapping, and enhancer trapping [10–12]. Tol2 belongs to the hobo/Activator/Tam3 (hAT) family of elements that feature an element size as long as that of the mariner/Tc1 family [13]. Tol2 is expected to contain a large cassette of transfer vectors when delivering large DNA fragments into chromosomes [14]. Meanwhile,

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germline transgenesis of zebrafish using the Tol1 transposon system was obtained at a high transmission rate comparable to that of Sleeping Beauty [15]. Nevertheless, the transposition efficiency of the transposase form of the Tol2 element needs to be improved, because when the Tol2 element carrying an enhanced green fluorescent protein (EGFP) reporter gene with Tol2 transposase mRNA was co-injected into fertilized eggs, only a few embryos showed EGFP [16]. Those results indicated that the composition of transposase messenger (m)RNA from the transcription to the translation step may need to be modified to enhance its ability to catalyze excision of the Tol2 element.

Recent reports suggested that zebrafish possess well-developed immune systems and are ideal hosts for understanding fish responses to bacterial infections [17,18]. In addition, several papers reported the function of innate parameters related to disease resistance, prophylactic treatment, and genetic traits [19]. The precise outcome of an infection depends on complex bacterial-host cell interactions which are still poorly understood. at present As protecting fish against infectious diseases is a major challenge for aquaculture worldwide, transgenic fish with transgenes of AMP genes can provide a new strategy and can be used to develop disease-resistant fish strains. Previous studies used non-fish AMP genes as the transgene. Under the concept of an all-fish model, studying fish AMPs as transgenes for producing transgenic fish to resist bacterial infections may play an important role in fish aquaculture in the future. We introduced the epinecidin-1 gene into zebrafish and observed the enhanced resistance against bacterial infection in epinecidin-1-transgenic zebrafish. We also examined the immune-related gene expression by real-time reverse-transcriptase polymerase chain reaction (RT-PCR), immunohistochemistry, and fluorescence microscopy. Expression of epinecidin-1 by the transgenic fish was significantly correlated with decreased bacterial numbers and changes in immune-related gene expression. These data demonstrate that epinecidin-1 may be a candidate gene for producing commercial transgenic fish such as *Archocentrus nigrofasciatus* var., *Arc. nigrofasciatus*, *Pterophyllum scalare*, and *Astronotus ocellatus* with resistance to bacterial pathogens in the future.

2. Materials and methods

2.1. Promoter isolation, sequence analysis, construction, microinjection, and luciferase assay

Zebrafish and medaka genomic DNA was extracted using the PureLinkTM Genomic DNA mini kit (Invitrogen, Madison, WI, USA) according to the manufacturer's protocol. The PCR was performed using 200 ng genomic DNA, a final concentration of 1 μM of primers (primers for the mylz promoter that contain the XhoI and NheI sites; Table 1), 1.25 units of TaKaRa Ex Taq (Takara Bio, Tokyo, Japan), 1× Ex Taq buffer (Takara Bio), and dNTPS (0.2 mM each, Takara Bio) in a total volume of 50 uL in a model 2700 thermocycler (Applied Biosystems, Foster, CA, USA). The PCR condition was 30 cycles of 20 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C. The 2.5-kb amplicon was gel-purified and ligated to the TOPO TA vector (Invitrogen). Several clones were sequenced by Genomics (Taipei, Taiwan) and searched against the genome database using the BlastGen program (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=7955). Primers used for the promoter activity assay are shown in Table 1 and were synthesized by Quality Systems (Taipei, Taiwan). The PCR product was digested with SacI and XhoI restriction endonucleases (REs), and ligated into the SacI and XhoI sites of the pGL-3-basic vector (Promega). For the transgenic fish study, we excised the 2.5-kb mylz promoter regions using EcoRI and ligated them into the pDsRed2-1 vector (Clontech Laboratories, Inc., Mountain View, CA, USA).

Deletion fragments containing various lengths of the mylz2 promoter region ligated into the pGL-3 vector were analyzed. Plasmids with various lengths of the mylz2 promoter region were called 2.5 k, 2.3 k, 2.1 k, and 1.9 k after the actual promoter region length. Each pGL-3-mylz2 (Fig. 1a) series of fragments with the pRL-TK vector (the pRL-TK vector was used as an internal control reporter) in a 1:1 ratio was microinjected into the one-cell-stage of zebrafish eggs; after injection, eggs were incubated at 28 °C for 72 h for the luciferase assay. Ten eggs placed in an Eppendorf tube for each fragment of the promoter were ground with 50 μl of passive lysis buffer (Promega, Madison, WI, USA) on ice. This was centrifuged at 13,000 rpm for 10 min; 10 μl of the supernatant was mixed with 50 μl of luciferase assay reagent II (Promega), and the emission was measured in a Fluoroskan Ascent FL luminometer (Thermo Labsystems, Ramsey, MN, USA). The promoter analytical values are shown as the mean ± SEM. Statistical significance was set at $p < 0.05$ or <0.01 and was tested using the SAS statistical program (SAS Institute, Cary, NC, USA). All data were analyzed by one-way analysis of variance (ANOVA).

2.2. Transposon constructs

After obtaining medaka genomic DNA, the genomic DNA, primers (5′Tol2L200KpnI, 3′Tol2L200XhoI, 5′Tol2R150SacII, and 3′Tol2R150SacI; Table 1), Taq DNA polymerase, buffer, dNTPs, and double-distilled water (ddH_2O) were subjected to a PCR (Applied Biosystems 2700; Applied Biosystems, Foster City, CA, USA). The PCR conditions were 30 cycles of 20 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. After amplification of the PCR products from the L200 and R150 primers, the DNA fragment was digested using different REs, gel-eluted, and then ligated into a pBluescript SK(−) vector (Fig. 2a). Construction of plasmid DNA of Epi-1/DsRed (pTLR-m2.5 K-K.Epinecidin-1/DsRed; Fig. 2c; Supplementary Fig. 4) was by PCR amplification with epinecidin-1 cDNA from our previous publication [1] (with primers 5′ Epi BamHI, 3′ Epi AgeI, 5′ K.Epi BamHI, and 3′ SV40pA SacII; Table 1), and it was ligated into the pTLR vector.

2.3. Transposase constructs

RNA was extracted from ten medaka eggs using Trizol as previously described [5]. cDNA was produced using specific primers (5′Tol2NheI and 3′Tol2BamHI; Table 1) and 10 mM dNTP's at 65 °C for 300 s, then put on ice. After adding the first-strand buffer, 0.1 M DTT and SuperScript III RT in a 20-μl volume were incubated at 55 °C for 60 min, and then the reaction was stopped at 70 °C after 15 min. The amplicons containing the transposase were generated by the following PCR conditions: 30 cycles of 20 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C. The PCR products were ligated with the pCRII-TOPO vector (TOPO TA Cloning; Invitrogen). Insertion of the plasmids was confirmed by DNA sequencing. The next step was to amplify the bovine growth hormone polyadenylation signal (BGH pA) by PCR (with primers T7P and 3′BGHpA BamHI) from pcDNA3.1 (−) (Invitrogen), subject them to gel elution, and digest them with BamHI. After digestion, they were ligated into the pCRII/Tol2 vector (pCRII/Tol2-BGH pA). For the in vitro transcription experiment, we subcloned the pCRII/Tol2-BGH pA fragment (NsiI RE cut), ligated it into pBluescript SK(−) (PstI RE cut), and named the plasmid the pBS/Tol2 (BGHpA) vector. The second step was to clone the zebrafish ba1 globin 5′ and 3′ untranslated regions (UTRs; GenBank: AL929176) by PCR amplification with zebrafish genomic DNA (with primers F′T7-ba15′UTR, R′ba15′UTR, F′ba13′UTR, and R′ba13′UTR; Table 1). The pCS-TP vector (a gift from Dr. Koichi Kawakami, National Institute of Genetics, Shizuoka, Japan) was digested using HindIII after gel elution. It was next ligated with the ba15′UTR and ba13′UTR by HindIII and BamHI RE digestion, and the three fragments were put into one tube at the same time for ligation