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Identification and characterization of pufflectin from the grass pufferfish *Takifugu niphobles* and comparison of its expression with that of *Takifugu rubripes*

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ABSTRACT

Pufflectin found in *Takifugu rubripes* (Tr pufflectin) is the first animal lectin reported to show sequence similarity to monocotyledonous plant lectins. In the present study, we identified and characterized an orthologous lectin from *Takifugu niphobles* (Tn pufflectin), a species closely related to *T. rubripes*. Tn pufflectin exhibits 86% identity to Tr pufflectin with two conserved mannose-binding domains. Tn pufflectin was mainly expressed in the skin, gills, brain, and muscles; however, it was expressed at a lower level in the other examined tissues. Recombinant Tn pufflectin, expressed by *Escherichia coli*, exhibited binding activity specific for D-mannose. The expression of pufflectin in the gills was much lower in *T. niphobles* than in *T. rubripes*; notably, the former and latter are resistant and susceptible, respectively, to the monogenean parasite *Heterobothrium okamotoi*, which parasitizes gills. This suggests that pufflectin might be utilized by the parasite for host recognition.

1. Introduction

Lectins comprise a group of proteins or glycoproteins, other than antibodies and enzymes, that bind carbohydrates (Barondes, 1988) and exert a wide variety of biological roles, both inside and at the surface of the cell (Vasta and Ahmed, 2008). Pufflectin, which we detected in skin mucus from *Takifugu rubripes* (Tr pufflectin; Tsutsui et al., 2003), has been suggested to correlate with host defense, particularly against the monogenean gill-targeting parasite *Heterobothrium okamotoi* because it could bind the parasite. However, a defensive role for Tr pufflectin has not been completely verified. Interestingly, *Takifugu niphobles*, a species closely related to *T. rubripes*, features a marked difference in susceptibility to *H. okamotoi*; although this organism parasitizes *T. rubripes*, it cannot colonize *T. niphobles* (Ogawa, 2002; Ohhashi et al., 2007). Therefore, we anticipated that a comparison of biochemical characteristics and expression patterns in these two pufferfish species might reveal the biological roles of pufflectin.

Many fish express lectins not only inside the body (e.g., peripheral blood and liver) but also in the mucus covering the body surface, an important first line of defense against infection. The skin mucus from many types of fish has long been known to exhibit hemagglutination activity, suggesting the presence of lectins in the mucus. To date, sequence analysis studies have revealed marked diversity among fish mucus lectins; previously, seven families of lectins were identified from the skin mucus of eight species of fish (de Santana Evangelista et al., 2009; Suzuki et al., 2003; Muramoto and Kamiya, 1992; Muramoto et al., 1999; Okamoto et al., 2005; Tasumi et al., 2002, 2004; Tsutsui et al., 2003, 2005, 2007, 2009, 2011a, 2011b). Among these, pufflectin is a novel animal lectin for which the amino acid sequence exhibits homology with mannose-binding lectins from monocotyledonous plants (Tsutsui et al.,

Abbreviations: GNA, *Galanthus nivalis* agglutinin; PBS, phosphate buffered saline; RACE, rapid amplification of cDNA ends; UTR, untranslated region; SDW, sterile distilled water; CRD, carbohydrate recognition domain; RT-PCR, Reverse transcription-PCR; PBS-Tw, 0.05% Tween 20 in PBS.

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2003), and accordingly, we proposed the designation of this lectin as the "Lily-type" lectin (Suzuki et al., 2003). In our previous study, we also found that pufflectin could bind to the body surface of adult *H. okamotoi,* which localizes in the gills of *T. rubripes* where lectin is expressed (Tsutsui et al., 2003).

In the present study, we identified and characterized pufflectin in *T. niphobles* (Tn pufflectin). In this report, we will additionally discuss the relationship between pufflectin and parasitism of the monogenean parasite *H. okamotoi*.

2. Materials and methods

2.1. Amplification and cloning of cDNA fragments of Tn pufflectin

The gills were dissected from five *T. niphobles* individuals (average body weight 11.2 g) after anesthetization with 2-phenoxyethanol and were extensively washed with phosphate buffered saline (PBS) containing 100 units of heparin to remove as much blood as possible. The tissue was soaked in 10 volumes of RNAlater® (Life Technologies, Carlsbad, CA, USA) for 1 day at 4 °C with gentle rocking and stored at −20 °C until use. Total RNA was isolated from approximately 100 mg of tissue with 1 ml of RNAiso according to the manufacturer's instructions. The total RNA quality was evaluated using a MultiNA microchip electrophoresis system (Shimadzu Corp., Kyoto, Japan). Total RNA from each individual (10 μg each, 50 μg total) was combined, and mRNA was purified using an Oligotex™-dT30 <Super> mRNA Purification Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. cDNA was synthesized from 250 ng of mRNA using a GeneRacer™ Kit (Life Technologies) according to the manufacturer's instructions. Reverse transcription was conducted using SuperScript® II (Life Technologies). Each PCR amplification described below was conducted in a total volume of 20 μl containing 0.4 units of KOD FX (Toyobo, Osaka, Japan), 1.6 mM of dNTP, and 300 nM each of forward and reverse primer, unless otherwise stated. To amplify a cDNA fragment containing the 5′-untranslated region (UTR), PCR was conducted with GeneRacer™ 5′ Primer (900 nM), Pufflectin-5′RACE primer (300 nM), and 1 μl cDNA. The reaction conditions were 2 min at 94 °C, followed by 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 90 s. The resulting PCR product was diluted 50 times with sterile distilled water (SDW) and used as a template for a nested PCR with the GeneRacer™ 5′ Nested Primer and Pufflectin-R primer. The reaction conditions were 2 min at 94 °C, followed by 25 cycles of 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 60 s. To amplify a cDNA fragment containing the 3′-UTR, PCR was conducted with Pufflectin-F primer (300 nM), GeneRacer™ 3′ Primer (900 nM), and 1 μl cDNA. The reaction conditions were 2 min at 94 °C followed by 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 90 s. The sequences of primers used in this study are summarized in Table 1. Each PCR product was inserted into the pGEM®-T Easy vector (Promega Corp., Madison, WI, USA) using T4 DNA ligase (Promega). The ligation product was used to transform One Shot® TOP10 Chemically Competent *E. coli* (Life Technologies).

2.2. cDNA sequence analysis

Plasmids from colonies were isolated by alkaline lysis. Cycle sequencing was conducted using a BigDye® Terminator v3.1 (Life Technologies), and nucleotide sequences were determined on a 3130 Genetic Analyzer (Life Technologies). Raw sequence data editing was performed with the Bioedit biological sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Tn pufflectin molecular weight and pI predictions were performed using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The deduced amino acid sequences of Tn pufflectin (accession number: LC089015), *T. rubripes* pufflectin (Tr pufflectin, AB089820), and mannose-binding lectins from monocotyledonous plants (Table 2) were aligned using CLUSTALW software (http://www.genome.jp/tools/clustalw/) in "slow/accurate" mode with the default parameter settings. For some alignments, the signal peptides were predicted by Phobius (http://phobius.sbc.su.se/) and predicted signal peptides and amino acid residues longer than Tn pufflectin were manually removed prior to alignment construction (Table 2). The Tn pufflectin carbohydrate recognition domain (CRD) structures were modeled using SWISS-MODEL (Arnold et al., 2006; Biasini et al., 2014; Guex et al., 2009; Kiefer et al., 2009). The sequences of CRD1 and CRD2 with additional amino acids pre- and post-each CRD (Lys21-Pro53 and Asp47-Thr86, respectively) were submitted to the "Automated mode." From among the models generated for each CRD, we selected the models with the highest quality scores and used the Deep View/Swiss-Pdb Viewer to manipulate the pdb files.

2.3. Reverse transcription-PCR (RT-PCR)

Total RNAs from the liver, kidney, spleen, intestine (approximate midsection), heart, gills, gonad, muscle, skin, and brain were isolated from two *T. niphobles* individuals as described above, with slight modifications. Briefly, shortly after dissection, the tissues (50 mg each) were placed in tubes containing RNAiso and were immediately homogenized. Two milliliters of RNAiso were used for skin samples; 1 ml was used for all other samples. Total RNA was treated with the TURBO DNA-free™ Kit (Life Technologies) according to the manufacturer's instruction to eliminate interference

Table 1
Primers used in this study.

Name	Sequence
GeneRacer™ 5′ Primer	5′-CGACTGGAGCACGAGGACACTGA-3′
GeneRacer™ 5′ Nested Primer	5′-GGACACTGACATGGACTGAAGGAGTA-3′
GeneRacer™ 3′ Primer	5′-GCTGTCAACGATACGCTACGTAACG-3′
Pufflectin-5′RACE	5′-ACAGGACAGGGTTGGAAACACCCAGTGA-3′
Pufflectin-F	5′-ATCCTCCTCCTTCTGCTTGCAGTCTA-3′
Pufflectin-F2	5′-TCCTCCTTCTGCTTGCAGTC-3′
Pufflectin-F4	5′-AGCTCAAGAGGGGAGACTCTGTGTTATCCAAAAAC-3′
Pufflectin-R	5′-GTGATGAGAGTTTGGCTTTC-3′
Pufflectin-R3	5′-CTTTTATTTGAACCCAGTTTACAGGACAGGGTTGG-3′
FML-SP2	5′-ATCTTCCTCCTTCTGCTTGCAGTCTA-3′
FML-AP4	5′-GTGATGAGAGTTTGGCTTTC-3′
rTnPufflec-F	5′-CCATGGCCTCCGTCACAGTCTTGAA-3′
rTnPufflec-R	5′-CTCGAGCTTCATGCCATGATCATA-3′
b-actin-F	5′-CAATGGATCCGGTATGTGC-3′
b-actin-R	5′-CGTTGTAGAAGGTGTGATGCC-3′