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Mutation in promoter region of a serine protease inhibitor confers *Perkinsus marinus* resistance in the eastern oyster (*Crassostrea virginica*)

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ABSTRACT

Protease inhibitors from the host may inhibit proteases from invading pathogens and confer resistance. We have previously shown that a single-nucleotide polymorphism (SNP198C) in a serine protease inhibitor gene (*cvSI-1*) is associated with *Perkinsus marinus* resistance in the eastern oyster. As SNP198 is synonymous, we studied whether its linkage to polymorphism at the promoter region could explain the resistance. A 631 bp fragment of the promoter region was cloned by genome-walking and resequenced, revealing 22 SNPs and 3 insertion/deletions (indels). A 25 bp indel at position −404 was genotyped along with SNP198 for association analysis using before- and after-mortality samples. After mortalities that were primarily caused by *P. marinus*, the frequency of deletion allele at −404indel increased by 15.6% ($p = 0.0437$), while that of SNP198C increased by only 3.4% ($p = 0.5756$). The resistance alleles at the two loci were coupled in 79.6% of the oysters. Oysters with the deletion allele at −404indel showed significant ($p = 0.0189$) up-regulation of *cvSI-1* expression under *P. marinus* challenge. Our results suggest that mutation at the promoter region causes increased transcription of *cvSI-1*, which in turn confers *P. marinus* resistance in the eastern oyster likely through inhibiting pathogenic proteases from the parasite.

1. Introduction

Proteases are proteolytic enzymes that degrade proteins by breaking specific peptide bonds. Proteases are essential in protein metabolism, cellular homeostasis, development and disease processes. Protease activities are tightly controlled as unwanted proteolysis causes cellular damage. One mechanism of controlling protease activity is through the action of protease inhibitor proteins. Thus, protease inhibitors are found in all organisms and play important roles in all pathways that involve proteases.

Protease inhibitors can be grouped based on the proteases that they inhibit into major classes such as aspartic protease inhibitors, cysteine protease inhibitors, metalloprotease inhibitors, and serine protease inhibitors [1]. They can also be classified based on sequence homology into 38 clans and 67 families [2]. Some protease inhibitors may play a role in host defense by regulating the Toll pathway [3] and apoptosis [4,5] or by directly inhibiting proteases from invading pathogens that are often important virulence or pathogenic factors [6,7].

In bivalve molluscs, protease inhibitors have been discovered and implicated in host defense in several species. In the eastern (*Crassostrea virginica*) and Pacific (*Crassostrea gigas*) oysters, plasma proteins have shown strong inhibitory effects against a variety of proteases including those from oyster pathogens *Perkinsus marinus* and *Vibrio vulnificus* [8]. Protease inhibitor activities have also been demonstrated for plasma of softshell clam (*Mya arenaria*), which are suppressed by disseminated sarcoma [9]. Kazal-type serine protease inhibitors have been identified in the bay (*Argopecten irradians*) and zhikong (*Chlamys farreri*) scallops, showing up-regulation after injection of *Vibrio anguillarum* [10,11]. Two novel serine protease inhibitors, cvSI-1 and cvSI-2, have been identified and characterized in the eastern oyster showing strong affinity and inhibition of perkinsin, the main protease of the oyster parasite *P. marinus* [12–14]. cvSI-1 also inhibits the proliferation of *P. marinus* in vitro, and its transcription is up-regulated in *P. marinus* resistant oysters [12].

P. marinus is a protist belonging to phylum Apicomplexa. It is an important pathogen of the eastern oyster and the etiological agent of Dermo disease that has devastated oyster populations along the mid-Atlantic coast of the United States [15]. *P. marinus* can cause heavy mortalities (up to 80–90%) in naïve oysters, which usually occur in late summer and fall. Some oysters may be resistant to Dermo although the genetic mechanism of resistance is not well

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understood [16,17]. In a previous study, we found an association between genetic variants of *cvSI-1* and *P. marinus* resistance in the eastern oyster [18]. The C allele of SNP198 showed consistent frequency increase in two families after disease-caused mortalities and was enriched in the disease-resistant strain. However, SNP198 is a synonymous mutation that does not result in amino acid change. We speculate that SNP198's association with disease resistance may be due to its linkage to a polymorphism at the promoter region that regulates the expression of *cvSI-1*. To test this hypothesis, we sequenced the promoter region of *cvSI-1* and obtained polymorphism for association analysis in this study. Our results show that a deletion in the promoter region is correlated with up-regulation of *cvSI-1* and has stronger association to *P. marinus* resistance than the synonymous SNP198 in the coding region.

2. Materials and methods

2.1. Cloning and sequencing of cvSI-1 and its promoter region

Genomic DNA was isolated from adductor muscles of 10 wild eastern oysters collected from Delaware Bay, New Jersey, in June 2009 using the Omega Biotek E.Z.N.A. Mollusc DNA Kit. DNA was quantified on a NanoVue plus spectrophotometer (GE Healthcare) and verified for product integrity on agarose gels. Primers SPI-2F and SPI-2R (Table 1), which were designed to amplify the full coding sequence, were used to obtain the genomic sequence of *cvSI-1*. PCR was carried out in 10 μl volume containing 20 ng of DNA, 1 × PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 200 nM of each primer and 0.2 U of GoTaq polymerase (all from Promega) using the following profile: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. PCR products were purified with MinElute PCR purification kit (Qiagen) and directly sequenced in both directions on an ABI 3130*xl* sequencer. Alignment for genomic DNA and cDNA sequences of *cvSI-1* was conducted by ClustalW and the intron/exon boundaries were identified by manual inspection.

To obtain sequence of the promoter region, genome-walking libraries were constructed with pooled DNA from the 10 oysters (1 μg each) according to the manufacturer's instructions (Clontech). Two gene-specific primers (SPI1GW1 and SPI1GW2, Table 1) were designed based on the first intron sequence of *cvSI-1* and used for nested PCR according to the protocol provided by the manufacture. Specific PCR fragment was purified by PCR clean-up kit (Promega), inserted into pGEM-T vector and then transformed into *Escherichia coli* DH5α cells (Fisher Scientific). Eight positive recombinant plasmids were sequenced in both directions on an ABI 3130*xl* sequencer.

Table 1
A summary of primers used in this study.

Primer name	Sequences (5′–3′)	Usage
SPI-2F	TATTAAGAAAACAATGGACGTAG	Genomic sequence amplification
SPI-2R	GATTTATTTCAAAACAAGAAAACC	Genomic sequence amplification
SPI1GW1	AAGGCATACACAGAGGATGAGAGTTC	Primary PCR of genome walking
SPI1GW2	ACTGCCACCGTGAACCATTACGAAAC	Secondary PCR of genome walking
SPI1PF	ATCTTGAGTTCGGGATTTC	Promoter re-sequencing
SPI1PR	GCAAATGATGTAAGTCCTGA	Promoter re-sequencing
SPI1PindF	CATGGTCGATTTTAACGCTCT	Indel detection
SPI1PindR	CGATTCTTATCGCCCTTCAA	Indel detection
SPI1RTF	GTGTATGCCTTTTCGGACTG	Real-time RT PCR
SPI1RTR	TGTTGACAACCGTGCAAGAC	Real-time RT PCR
cv28SF	GTTGACGCAATGTGATTTCTGC	Real-time RT PCR
cv28SR	TAGATGACGAGGCATTTGGCTA	Real-time RT PCR

2.2. Re-sequencing of the promoter region and mutation discovery

For re-sequencing, two primers (SPI1PF and SPI1PR, Table 1) were designed targeting a 631 bp fragment of the promoter region and used for amplification in 30 wild oysters collected from Choctawhatchee Bay (Florida), Delaware Bay (New Jersey) and Martha's Vineyard (Massachusetts), 10 from each population. PCR amplification was carried out as described above, except that the annealing temperature was 54 °C. PCR products were purified and directly sequenced in both directions on an ABI 3130*xl* sequencer. Sequence alignment and mutation discovery were conducted by VECTOR NTI Advance 10.1.1 (Invitrogen).

2.3. Genotyping and association with resistance

To test if mutation at the promoter region is associated with disease resistance, a 25-bp indel at position −404 was selected for assay design, genotyping and association studies. Two primers that flank the −404indel and are away from other polymorphic sites, SPI1indF and SPI1indR (Table 1), were designed for genotyping by length polymorphism. PCR was conducted as described above, except that the annealing temperature was 52 °C. After amplification, fragment length polymorphism at −404indel was detected with agarose gel electrophoresis, after confirmation by sequencing.

We genotyped −404indel in a wild population before and after disease-inflicted mortalities. The assumption is that frequency of resistant genotypes will increase after disease-caused mortalities, while that of susceptible genotypes will decrease if the locus is associated with disease resistance. Wild spats (2–3 cm) were collected in fall 2006 from Cape Shore, Delaware Bay, separated into single oysters and deployed in cages at the same site where *P. marinus* infection routinely causes heavy mortalities. Mortality was negligible prior to June 2007. A before-mortality sample was collected on June 26, 2007, and cumulative mortality reached 80% on September 17, 2008 at which time an after-mortality sample was collected. Most of the mortality occurred in late summer and early fall of 2007 and 2008. Pathological analysis of 25 oysters (including 6 gapping or dying oysters) on September 2, 2008 found 100% prevalence of *P. marinus* with an average infection intensity of 3.9 on a scale of 0–5, and no MSX (multinucleated sphere unknown caused by *Haplosporidium nelsoni*) was detected. For this study, we genotyped 69 oysters collected from before- and 68 oysters from after-mortality samples.

SNP198 has been shown to be associated with *P. marinus* resistance in families and selectively bred populations [18]. To determine whether −404indel has stronger association with disease resistance than SNP198, SNP198 was genotyped in the same set of samples using the HRM assay as described by Yu et al. [18]. Frequency difference between before- and after-mortality samples was examined by chi-square test.

2.4. Promoter mutation and expression

To test if mutation at the promoter region affects transcription, we took samples from a *P. marinus* challenge experiment and determined genotypic affects of −404indel on *cvSI-1* expression. The challenge experiment was conducted as previously described [19]. Oysters were collected from the control (injected with sterilized seawater) and challenged (injected with *P. marinus*) groups at day 30, 10 from each group. Gill, mantle, digestive gland and hemolymph were collected, immediately placed in RNAlater, and stored at −20 °C until RNA extraction.