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

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**A B S T R A C T**

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.

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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 zhihong (*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 understood [16].
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 stored at −20 °C until RNA extraction.

Two gene-specific primers (SPI1GW1 and SPI1GW2, 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 MgCl2, 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 3130xl 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 3130xl sequencer.

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.

Sequence alignment and mutation discovery were conducted by VECTOR NTI Advance 10.1.1 (Invitrogen).

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 Chocawhatchee 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 3130xl 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.

### Table 1

A summary of primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5’–3’)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPI1GW1</td>
<td>AGGGCATACAAACGAGGATCACGTC</td>
<td>Primary PCR of genome walking</td>
</tr>
<tr>
<td>SPI1GW2</td>
<td>CTGCCCGCAGTCACGATACACACCAC</td>
<td>Secondary PCR of genome walking</td>
</tr>
<tr>
<td>SPI1PR</td>
<td>AACTCTTTCTTCCGGCCATTTC</td>
<td>Promoter re-sequencing</td>
</tr>
<tr>
<td>SPI1PF</td>
<td>CCGAATCCTGAGATCTGTCA</td>
<td>Promoter re-sequencing</td>
</tr>
<tr>
<td>SPI1R</td>
<td>CGATCTTATGCTCCTCA</td>
<td>Indel detection</td>
</tr>
<tr>
<td>SPI1R</td>
<td>CGATCTTATGCTCCTCA</td>
<td>Real-time RT PCR</td>
</tr>
<tr>
<td>SPI1R</td>
<td>CTGGCGAAGGCAGCACGAC</td>
<td>Real-time RT PCR</td>
</tr>
<tr>
<td>SPI1R</td>
<td>CTGGCGAAGGCAGCACGAC</td>
<td>Real-time RT PCR</td>
</tr>
<tr>
<td>SPI1R</td>
<td>CTGGCGAAGGCAGCACGAC</td>
<td>Real-time RT PCR</td>
</tr>
<tr>
<td>cv28SR</td>
<td>TAGATGACACGGGCTTGGCTA</td>
<td>Real-time RT PCR</td>
</tr>
<tr>
<td>cv28SR</td>
<td>TAGATGACACGGGCTTGGCTA</td>
<td>Real-time RT PCR</td>
</tr>
</tbody>
</table>
RNA was extracted from each tissue using RNeasy Mini Kit (Qiagen) and treated with DNase 1 (Qiagen) to prevent DNA contamination. First strand cDNA was synthesized from 1 μg of total RNA of each sample using oligo(dT)18 and MMLV reverse transcriptase (Promega) in a volume of 25 μl. Real-time quantitative RT-PCR (qRT-PCR) was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) to quantitate the expression of cvSI-1. The 28S major rRNA gene was chosen as the reference gene for internal standard. The qRT-PCR amplification was carried out in triplicate, each in a volume of 20 μl containing 10 μl of 2 × Power SYBR Green PCR master mix (Applied Biosystems), 1 μl of the cDNA, 0.8 μl each of 5 μM SPI1RTF and SPI1RTR primer (or cv28SF and cv28SR for the 28S standard, Table 1) and 7.4 μl of deionized water. The amplification was programmed as 2 min at 50 °C, 2 min at 95 °C, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min with the fluorescent signal collection. Relative expression of cvSI-1 was measured against the expression of 28S gene with delta–delta Ct-value method and indicated as means ± S.E. Difference in expression between control and challenged samples as well as between different genotypes was examined with a student t-test using arcsine-transformed data.

3. Results

3.1. Gene structure and polymorphism in the promoter region

Direct genomic sequencing and 5’ genome walking produced a fragment of 1925 bp. The fragment includes a 1199 bp of cvSI-1 genic region and a 726 bp of the promoter region. The cvSI-1 gene is organized into three exons and two introns. The three exons from start to stop codons are 40, 120 and 113 bp in length, and separated by two introns of 663 and 189 bp (Fig. 1). All intron–exon boundaries conform to GT-AG structure [20]. Both introns are of type 1 as they interrupt the codon between the first and the second nucleotide.

The promoter region was re-sequenced in 30 oysters collected from three wild populations. Analysis of the 631 bp promoter region shared by the 30 sequences revealed 22 SNPs and 3 indels. The three indels included two of 1 bp and one of 25 bp, positioned at −200, −410 (−434 for insertion type) and −404, respectively. We selected the 25 bp indel at position −404 for further interrogation because it is a major indel that is more likely affecting transcription.

3.2. Association with disease resistance

The 25 bp length polymorphism at −404indel was easily genotyped with length polymorphism on agarose gel electrophoresis and confirmed by direct sequencing (Fig. 2A and B). To determine possible association between the locus and disease resistance, we genotyped −404indel in samples collected before and after disease-caused mortalities. Before mortalities, oysters consisted of 14.5% insertion homozygote (II), 44.9% insertion/deletion heterozygote (ID) and 40.6% deletion homozygote (DD) (Table 2). After mortalities, the frequency of II genotypes decreased from 14.5% to 3.8%, and that of DD increased from 40.6% to 51.5% (Table 3), and the shift in genotype frequency was significant (p = 0.0171). The frequency of D allele increased by 15.6%, from 63.0% to 72.8%, and the increase was significant with a p-value of 0.0437.

As a comparison, SNP198 was also genotyped in the same samples. Before mortalities, the oysters consisted of 5.8% AA, 43.5% AC and 50.7% CC genotypes (Table 2). No significant shifts were observed after disease-caused mortalities (p = 0.6892). The C allele, which was shown to be associated with P. marinus resistance in two families and selected strains [18], did not show significant (p = 0.5756) increase in frequency after disease-caused mortalities in the wild population. The increase was only 3.4% compared with 15.6% for D allele at −404indel.

Analysis of bi-locus genotypes indicated that the D allele at position −404 was most often coupled with C allele at SNP198, while I allele at position −404 was often linked to A allele at SNP198. The dominant genotypes were DD/ACSNP198 (40.3%) and ID/ACSNP198 (35.1%), suggesting that D/ACSNP198 and I/ACSNP198 are likely the two ancestral haplotypes (Table 3). Of the 137 oysters genotyped, 79.6% of oysters had bi-locus genotypes that conserved the ancestral haplotypes. The other 20.4% oysters had bi-locus genotypes indicative of historical crossover between the two loci.

3.3. cvSI-1 gene expression

Expression of cvSI-1 was determined in different organs of 10 oysters challenged with P. marinus and 10 control oysters. In control oysters, the mean relative cvSI-1 gene expression in different organs ranged from 2.88 × 10^{-4} to 3.59 × 10^{-2} (Fig. 3A). cvSI-1 was preferentially expressed in digestive gland. The expression level in digestive gland was over 100 times higher than the average expression in hemocyte, gill and mantle. The same organ distribution of cvSI-1 expression was found in oysters challenged by P. marinus. However, the relative expression in challenged oysters was higher than in control oysters in all organs, and the up-regulation was 2.48 fold in hemocyte, 2.88 fold in gill, 1.73 fold in mantle and 5.45 fold in digestive gland. The up-regulation of cvSI-1 by P. marinus was only statistically significant (p = 0.0189) in digestive gland. These results show that cvSI-1 is up-regulated by P. marinus challenge in all organs, but only significantly in digestive gland.

To determine possible effects of promoter mutation on gene expression, −404indel was genotyped in the oysters used in the expression study. Among the 10 control and 10 challenged oysters, 12 oysters had II (homozygote for insertion) and eight oysters had ID (heterozygote) genotypes at −404indel. Compared with II oysters, ID oysters had higher mean expression of cvSI-1 in all organs in both control and challenged groups, although the difference was only significant in digestive gland of the challenged oysters (Fig. 3B). In control oysters, the mean relative cvSI-1 expression in digestive gland was 0.035 in II oysters (N = 6) and 0.051 in ID oyster (N = 4). In challenged oysters, cvSI-1 expression in digestive gland was 0.087 ± 0.023 in II oysters (N = 6) and 0.47 ± 0.15 in ID oysters (N = 4), and the difference was significant (p = 0.0026). cvSI-1 expression in challenged ID oysters was also significantly (p = 0.0063) higher than ID oysters in the control group. These results suggest that the D allele at −404indel is associated with up-regulation of cvSI-1 in all oysters and organs, although significant up-regulation only occurs in digestive gland of challenged oysters.

4. Discussion

Pathogens use proteases to degrade host proteins during invasion and pathogenesis [21]. Hosts may, on the other hand, produce protease inhibitors to inactivate proteases from pathogens for defense, although protease inhibitors have broad functions in normal metabolism and development. The idea that oysters may use protease inhibitors to defend against pathogens was first suggested by Faisal et al. [8], who demonstrated strong inhibitory effects of oyster plasma on proteases from P. marinus and V. vulnificus. A serine protease inhibitor (cvSI-1) was later identified in the eastern oyster that inhibits protease from P. marinus and the proliferation of the parasite itself in vitro [12,14]. In a previous study, we found that the C allele at SNP198 of cvSI-1 was associated
Fig. 1. Genomic and deduced amino acid sequences of cvSI-1 gene. Coding sequences are shown in capital letters and non-coding sequences are in lowercase letters. The start codon is boxed and the stop codon is marked with an asterisk. Polymorphic sites in the promoter region are underlined and variants are indicated. Three indels are in bold, and the deletion variants are indicated by "-".

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with *P. marinus*-resistance [18]. However, SNP198 is a synonymous mutation, and we speculate that its linkage to a polymorphism in the promoter region that regulates cvSI-1 expression may explain the resistance.

In this study, we cloned the promoter region of cvSI-1 by genome-walking and identified a major indel at −404 bp from the start codon. We show here that the −404indel has stronger association with *P. marinus*-resistance than SNP198 as the resistant D allele at −404indel increased by 15.6% while the resistant C allele at SNP198 increased by only 3.4% after mortalities that were primarily caused by *P. marinus*. This result suggests that −404indel is more likely the causative mutation or more closely linked to it than SNP198. The frequency of allele C at SNP198 increased significantly after *P. marinus*-caused mortalities in two families [18], while the increase in wild populations used in this study was small and insignificant. This is consistent with the idea that SNP198 is not the causative mutation and its association with resistance in families is due to its linkage to the causative mutation. Linkage decay in wild populations reduces the strength of the association. Indeed, our data show that resistant alleles at −404indel and SNP198 are in the same linkage phase or haplotype in only 79.6% of the oysters. In the other 20.4% oysters, the C allele at SNP198 is not coupled with the resistant D allele at −404indel. This may explain why the C allele did not show significant frequency increase after mortalities in the wild population. Because of linkage decayed over time, we don’t expect significant association between a locus and resistance in wild populations unless the locus is the causative mutation or very closely linked to it. The finding of positive association between D allele at −404indel and *P. marinus*-resistance in a wild population suggests the −404indel could be the causative mutation.

Expression analysis provided additional support for −404indel or another mutation in the promoter region as the causative mutation affecting *P. marinus*-resistance. The observation that oysters with D allele at −404indel had significantly higher cvSI-1 expression after *P. marinus* challenge than oysters without it suggests that the D allele may confer resistance through up-regulation of cvSI-1. This is consistent with the observation that resistant oysters showed higher cvSI-1 expression in another study [12]. Although we don’t have data on post-transcription regulation, it is plausible that up-regulation of cvSI-1 transcription leads to increased production of cvSI-1 proteins which enhances inhibition of *P. marinus* proteases and proliferation. We should caution that we did not interrogate all mutations in the promoter region, and we cannot rule out the possibility that other mutations near −404indel are responsible for up-regulation and resistance, although the 25 bp indel is a major mutation in the promoter region. Nevertheless, the association and expression data suggest that mutation in the promoter region is responsible for observed up-regulation of cvSI-1 and *P. marinus* resistance. Our results add support for cvSI-1’s role in host-defense and provide a plausible mechanism linking genetic variation to disease-resistance. In humans, genetic variations in promoter sequences that alter gene expression play a prominent role in increasing susceptibility to complex diseases [22,23]. This study provides an example in oysters.

It should be noted that the expression of cvSI-1 is up-regulated after *P. marinus* challenge regardless of genotype. This finding suggest that cvSI-1 is up-regulated as a defense response against *P. marinus*, and the up-regulation is further modified by mutation at the promoter region, where oysters with D allele at −404 had higher up-regulation of cvSI-1 than oysters without.

It is also interesting to note that cvSI-1 is preferentially expressed in digestive gland and also significantly up-regulated after *P. marinus* challenge in the digestive gland. This is interesting because we expect genes related to host defense are highly expressed in hemocytes. Molluscan hemocytes were thought to play important roles in defense not only by direct phagocytosis and killing of foreign invaders, but also by producing bioactive molecules [24–26]. As cvSI-1 is involved in *P. marinus*-resistance and plasma shows inhibition of perkinsin, it should have high

![Fig. 2. (A) Length polymorphism at the 25 bp indel at cvSI-1 −404 genotyped by gel electrophoresis. The left lane is the 100 bp ladder. Lanes 1 and 2 are insertion homozygote. Lanes 3 and 4 are insertion/deletion heterozygote. Lanes 5 and 6 are deletion homozygote. (B) Sequence analysis of the indel at −404 of cvSI-1 with chromatograms showing the 25 bp insertion (upper) and deletion (lower). The insertion sequence is underlined and insertion site is indicated by an arrow. The first SNP site after indel is labeled by asterisk.](image-url)
expression level in hemocytes. However, our data show that the highest expression of cvSI-1 is observed in digestive gland, which is consistent with the results of La Peyre et al. [12]. It is possible that cvSI-1 is involved in digestion regulation in digestive gland in addition to its role in host defense in hemocytes. However, parasite challenge caused significant up-regulation of cvSI-1 in digestive gland rather than in hemocytes. We postulate that stomach and digestive tracks may play a major role in host defense in addition to its role in digestion. Consistent with this hypothesis, some other host defense genes in marine bivalves are also preferentially expressed in digestive gland. Xue et al. [27] reported that a bacterium-inhibiting lysozyme in the eastern oyster, cv-lysozyme 3, was mainly expressed in digestive gland. A lysozyme from zhikong scallop (C. farreri), CPlysG, with a role in killing bacteria, was highly expressed in hepatopancreas [28]. McLernon [29] studied lysozyme activities in several marine bivalves and found that lysozyme was primarily associated with digestive gland, and the primary role of lysozyme-like enzyme in marine bivalves was the utilization of bacteria. Previous studies on host defense response in marine bivalve have focused on hemocytes [30–32]. If our hypothesis is true, future studies should pay more attention to digestive gland as an important defense organ.

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