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The polymorphism in the promoter region of *metallothionein 1* is associated with heat tolerance of scallop *Argopecten irradians*

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ABSTRACT

Metallothioneins (MTs), a superfamily of cysteine-rich proteins, perform multiple functions, such as maintaining homeostasis of essential metals, detoxification of toxic metals and scavenging of oxyradicals. In this study, the promoter region of a metallothionein (MT) gene from Bay scallop *Argopecten irradians* (designed as AiMT1) was cloned by the technique of genomic DNA walking, and the polymorphisms in this region were screened to find their association with susceptibility or tolerance to high temperature stress. One insert–deletion (ins–del) polymorphism and sixteen single nucleotide polymorphisms (SNPs) were identified in the amplified promoter region. Two SNPs, −375 T–C and −337 A–C, were selected to analyze their distribution in the two Bay scallop populations collected from southern and northern China coast, which were identified as heat resistant and heat susceptible stocks, respectively. There were three genotypes, T/T, T/C and C/C, at locus −375, and their frequencies were 25%, 61.1% and 13.9% in the heat susceptible stock, while 34.2%, 42.1% and 23.7% in the resistant stock, respectively. There was no significant difference in the frequency distribution of different genotypes between the two stocks ($P > 0.05$). In contrast, at locus −337, three genotypes A/A, A/C and C/C were revealed with the frequencies of 11.6%, 34.9% and 53.5% in the heat susceptible stock, while 45.7%, 32.6% and 21.7% in the heat resistant stock, respectively. The frequency of C/C genotype in the heat susceptible stock was significantly higher ($P < 0.01$) than that in the heat resistant stock, while the frequency of A/A in the heat resistant stock was significantly higher ($P < 0.01$) than that in the heat susceptible stock. Furthermore, the expression of AiMT1 mRNA in scallops with C/C genotype was significantly higher than that with A/A genotype ($P < 0.05$) after an acute heat treatment at 28 °C for 120 min. These results implied that the polymorphism at locus −337 of *AiMT1* was associated with the susceptibility/tolerance of scallops to heat stress, and the −337 A/A genotype could be a potential marker available in future selection of Bay scallop with heat tolerance.

1. Introduction

Bay scallop (*Argopecten irradians*), introduced from America in 1982, is one of the most important economic bivalve species cultivated in China. However, in the past decades, the industry of scallop aquaculture had been suffering summer mortalities seriously, and the high temperature was suspected to be one of the main environmental inducers (Zhang and Yang, 1999). High temperature stress can cause a series of damage to organisms, such as oxidative damage which is possibly the result from disturbing the balance between producing and scavenging active oxygen species (ROS) (Bowler et al., 1992). Therefore, it is necessary to understand the antioxidant mechanism of scallops under high temperature stress, which will provide clues to develop strategy for the control of summer mass mortalities.

Organisms have developed numerous antioxidant systems to prevent the tissue damage caused by ROS, such as the non-enzymatic antioxidant molecule metallothioneins (MTs) (Lazo et al., 1995; Thornalley and Vasak, 1985; Vergani et al., 2005). MTs are a superfamily of cysteine-rich proteins with multiple functions, such as homeostasis of essential metals (Roesijadi, 1996; Vašák and Meloni, 2011; Viarengo and Nott, 1993), detoxification of toxic metals (Mason and Jenkins, 1995) and scavenging of oxyradicals (Anderson et al., 1999; Coyle et al., 2002; Vašák and Meloni, 2011). The activity of MT to scavenge hydroxyl radicals ($HO^{\bullet}$) and peroxyl radicals was approximately 50 times and 100 times greater than reduced glutathione (GSH) on a molar basis (Miura et al., 1997). Recently,

Abbreviations: ARE, antioxidant response element; Bi-PASA, bidirectional PCR amplification of specific alleles; bp, base pair(s); DEPC, diethylpyrocarbonate; GRE, glucocorticoid responsive element; GSH, reduced glutathione; HSE, heat shock element; HSF, heat shock factor; HSP, heat shock protein; HWE, Hardy–Weinberg equilibrium; $HO^{\bullet}$, hydroxyl radicals; ins–del, insert–deletion; L, liter; min, minute; MT, metallothionein; MRE, metal response element; PAGE, PA-gel electrophoresis; PCR, polymerase chain reaction; RFLP, restriction-fragment length polymorphism; ROS, active oxygen species; RT-PCR, real-time PCR; SNP, single nucleotide polymorphism; TSS, transcription start site.

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accumulating evidence demonstrated that MTs acted as potent antioxidants against various oxidative damages (Chiaverini and Ley, 2010; Lim et al., 2013; Qu et al., 2013), and many factors that mediated oxidative stress could induce MT synthesis (Sato and Bremner, 1993). For example, the mRNA level of mice MT increased 20 to 30-fold in response to ROS (Bauman et al., 1991), and the mRNA or protein level of oyster MTs increased at the recovery period against heat stress (Farcy et al., 2009; Piano et al., 2004). MTs have also been approved to scavenge radicals in vitro (Chiaverini and Ley, 2010; Lazo et al., 1995; Thornalley and Vasak, 1985; Vergani et al., 2005) and protect the organisms against cellular injury and oxidative DNA damage (Chiaverini and Ley, 2010; Qu et al., 2013). Tat-MT fusion protein could protect H9c2 cells of rat against hypoxia- and oxidative stress-induced apoptosis (Lim et al., 2013). The radical scavenging activity of MTs may consist in both a direct interception of free radicals and/or in the chelation of redox active transition metal ions responsible for radical generation via the Fenton reaction (Buico et al., 2008; Ercal et al., 2001).

In the past few years, the investigations about the sequence polymorphisms and antioxidant activity of MTs have revealed the association of MT polymorphisms with some phenotypes. For example, the SNPs −5 A/G and −209 A/G in the promoter region of the human MT2A gene were associated with the susceptibility to the toxic effects of cadmium (Giacconi et al., 2005; Kita et al., 2006) and marked zinc deficiency (Giacconi et al., 2005). The SNPs +647 A/C and rs8052394 (C/G) in the coding region of human MT1A gene were associated with longevity in Italian population (Cipriano et al., 2006) and type-2 diabetes mellitus in Chinese population (Giacconi et al., 2008; Yang et al., 2008). However, except for the above reports, there are few studies on the association of MT polymorphisms with other traits related to its antioxidant activity. Since high temperature can induce oxidative stress and MTs usually serve as antioxidants, it will be interesting to find out the association of MT polymorphisms with high temperature tolerance.

Compared with those in vertebrates, the knowledge about bivalve MTs, especially scallop MTs is very limited. Though two MT genes have been identified from scallop *A. irradians* (*AiMT1/2*) (Song et al., 2006; Wang et al., 2009), there is no information about *MT* promoter sequence, and the association between the polymorphisms of MT and related traits. In the present study, the promoter region of *AiMT1* was cloned and the sequence polymorphisms in this region were screened. The association between the polymorphisms with susceptibility/tolerance to high temperature stress was investigated to provide more evidence for the possible mechanism of heat tolerance, and potential markers for selective breeding of scallop.

2. Materials and methods

2.1. Scallop

Two hundred Bay scallops, averaging approximately 45 mm in shell length, were collected from two scallop farms of Qinhuangdao and Zhanjiang (China) in November 2010, respectively. The scallops from Qinhuangdao were Northern Bay scallop (*Argopecten irradians irradians*) which were introduced from Massachusetts of America in 1998, and the scallops from Zhanjiang were Southern Bay scallop (*Argopecten irradians concentricus*) which were introduced from Florida of America in 1991 (Liu et al., 2006). The Southern Bay scallops are more heat-resistant than the Northern Bay scallops (Liu et al., 2006), and consequently the scallops from Zhanjiang were employed as heat resistant stock and the scallops from Qinhuangdao were employed as heat susceptible stock.

2.2. Construction of genomic DNA walking library and cloning of AiMT1 promoter

The adductor muscle of scallops from the two stocks was collected and their genomic DNAs were extracted by using the proteinase K and phenol method (Yang et al., 2011). The integrity of genomic DNA was routinely checked with 0.8% agarose gel electrophoresis, and the purity of genomic DNA was detected by colorimetric assay. The genomic DNA walking library was constructed using the GenomeWalker™ Universal Kit protocol (Clontech). Following digestion with restrictive enzyme *Ssp* I, *Dra* I and *Pvu* II (NEB), the genomic DNA was purified by phenol/chloroform, and then ligated to the GenomeWalker™ adaptor.

Two gene specific primers, P3 and P4 (Table 1), were designed based on the genomic DNA sequence of *AiMT1* to clone the sequence of promoter by genome walking approach. The first round PCR amplification was performed by using adaptor primer P1 and gene specific primer P3 (Table 1). With 1 μL of 1:50 dilution of the first round PCR product as nested PCR template, the nested PCR reaction was performed by using the adaptor primer P2 and gene specific primer P4 (Table 1). All PCR amplifications were performed in a PCR Thermal Cycle (TaKaRa, GRADIENT PCR). The PCR products were gel-purified and cloned into the pMD18-T simple vector (TaKaRa, Japan). After being transformed into the competent cells of *Escherichia coli* Top10, the positive recombinants were identified through an anti-Amp selection and PCR screening with a sense vector primer P9 and an antisense vector primer P8 (Table 1). Three of the positive clones were sequenced on an ABI 3730 XL Automated Sequencer (Applied Biosystems).

2.3. Sequence analysis

The promoter sequence of *AiMT1* was analyzed using the Transcription Element Search System (TESS) (http://www.cbil.upenn.edu/cgi-bin/tess/tess) and the Patch System (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi?). The possible transcription start site was predicted using the Neural Network Promoter Prediction (NNPP) (http://www.fruitfly.org/seq_tools/promoter.html).

2.4. Identification of polymorphic sites in the promoter region of AiMT1

A pair of gene specific primers, P5 and P6 (Table 1), was designed based on the genomic DNA sequence of *AiMT1* to amplify a 910 bp fragment including the promoter region (519 bp). PCR reaction was performed as previous description (Yang et al., 2011) and the temperature profile was as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; a further 10 min elongation at 72 °C.

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
Genomic DNA walking	
P1 (AP1)	GTAATACGACTCACTATAGGGC
P2 (AP2)	ACTATAGGGCACGCGTGGT
P3 (GSP1)	AACTTTATTGCCACCACTCTTGTCACA
P4 (GSP2)	AGACGCCATCAGGGCAGGTAGAAGACT
Clone primers	
P5 (promoter-F)	ATGTAAGGGCACTTATGCTTGATCTTTAGT
P6 (promoter-R)	AGCAGTCAGGACCAGCACAGCCA
P7 (oligo (dT)-adaptor)	GGCCACGCGTCGACTAGTACT17
Sequencing primers	
P8 (M13-47)	CGCCAGGGTTTTCCCAGTCACGAC
P9 (RV-M)	GAGCGGATAACAATTTCACACAGG
Bi-PASA PCR	
P10 (−337 P)	TTCAGGTTGGAAAGTTCATGTAAGGGCACT
P11 (−337 Q)	CAACACACCTTCGGAAGACATCACAGC
P12 (−337 A)	GGGGGGGGGGCATATTTTGAACAGTTC
P13 (−337 B)	GGGGGGGGGGCTCAGCTAGACATGAT
RT primers	
P14 (AiMT1-RTF)	GCAAATGTGGCTGTGCTGGTC
P15 (AiMT1-RTR)	CAGACTGGCAATCATCGGAGC
β-Actin primers	
P16 (β-actin-RTF)	CAAACAGCAGCCTCCTCGTCAT
P17 (β-actin-RTR)	CTGGGCACCTGAACCTTTCGTT