The polymorphisms in the promoter of HSP90 gene and their association with heat tolerance of bay scallop

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Abstract The heat shock protein 90 (HSP90) is a highly abundant and ubiquitous molecular chaperone which plays essential roles in many cellular processes. In the present study, the messenger RNA (mRNA) expressions of HSP90 after acute heat stress were investigated in two bay scallop populations (*Argopecten irradians irradians* and *Argopecten irradians concentricus*). The heat-resistant scallop *A. i. concentricus*, which is distributed in Zhanjiang, China, exhibited significantly higher induction of HSP90 compared with that of the heat-sensitive scallop *A. i. irradians*, which is distributed in Qinhuangdao, China. The promoter sequence of HSP90 gene from bay scallop (AiHSP90) was cloned, and the polymorphisms within this region were investigated by sequencing to analyze their association with heat tolerance. A total of six single nucleotide polymorphisms (SNPs), including –1167 T-C, –1023 A-C, –799 C-T, –774 A-G, –686 C-T, and –682 A-C, were identified in the amplified promoter region, and most of them affected the putative transcription factor binding sites except for locus –1167. All the six SNP sites were found to be associated with heat tolerance after Hardy-Weinberg equilibrium (HWE) and association analysis. Moreover, haplotypes CACACC and TCTATC were also found to be associated with heat tolerance based on the result of linkage disequilibrium and association analysis. The results provided insights into the molecular mechanisms underlying the thermal adaptation of different congener endemic bay scallops, which suggested that the increased heat tolerance of *A. i. concentricus* (compared with *A. i. irradians*) was associated with the higher expression of AiHSP90. Meanwhile, the six genotypes (–1167 TT, –1023

CC, –799 TT, –774 GG, –686 CC, and –682 AA) and two haplotypes (CACACC and TCTATC) could be used as potential markers for scallop selection breeding with higher heat tolerance.

Keywords *Argopecten irradians* · Heat shock protein 90 · Promoter · Single nucleotide polymorphism · Heat tolerance

Introduction

The adaptability of a population to new environments partly depends on the presence of genetic variation in relevant genes, such as heat shock protein (HSP) genes (Sgrò et al. 2008). The HSPs are a subset of molecular chaperones firstly discovered in *Drosophila* larvae in 1962 by induction under heat stress condition (Ritossa 1962). They protect organisms against various environmental stresses and are usually classified into different families on the basis of molecular mass (Feder and Hofmann 1999). The 90-kDa HSP90 is a highly abundant and ubiquitous molecular chaperone which plays essential roles in many cellular processes (Jackson 2013). Since it was proposed that HSP90 represents an evolved mechanism that regulates the expression of genetic and phenotypic variability (Rutherford and Lindquist 1998), there was a growing interest in the association between the impairment of HSP90 function and phenotypic variability in a range of model organisms, including *Arabidopsis*, *Drosophila*, and zebrafish (Mittelman and Wilson 2010; Rutherford and Lindquist 1998; Sangster et al. 2008; Yeyati et al. 2007). Lots of research studies have indicated that HSP90 played important roles in regulating heat tolerance directly except for its potential role as an evolved buffering mechanism (Boher et al. 2012; Heads et al. 1995; Morgan and Mackay 2006; Sgrò et al. 2008; Yonehara et al. 1996).

As one of the best candidate genes for adaptation to thermal change, DNA sequence variation in HSP90 genes may be under selection and control thermal responses (Sgrò et al.

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2008). Compared to the most studies performed under laboratory conditions, it is necessary to investigate related species or geographical populations of the same species inhabiting thermally contrasting habitats to elucidate the ecological role of HSP90 in nature (Feder and Hofmann 1999; Nollen and Morimoto 2002). By far, genetic variations in HSP90 genes of several organisms have been reported to be associated with heat tolerance traits, such as cattle (Charoensook et al. 2012; Shergojry et al. 2012), *Drosophila* (Blackman and Meselson 1986; Boher et al. 2012; Hasson and Eanes 1996; Sgrò et al. 2008), snail (Arad et al. 2010), Stratiomyidae (Astakhova et al. 2013), and yeast (Jarosz and Lindquist 2010). Especially, the studies focused on the association between genetic variations in HSP90 genes of *Drosophila* species and their ecological roles are arising. The nucleotide diversity in the HSP90 gene in natural populations of *Drosophila melanogaster* from Australia revealed their involvement in the heat tolerance as well as the expression of genetic and phenotypic variability (Sgrò et al. 2008). Biogeographic origin and thermal acclimation interact to determine survival and HSP90 gene expression in *Drosophila* species submitted to heat stress (Boher et al. 2012). Although the relationship between the genetic variations in HSP90 genes and the heat tolerance traits of various organisms have been well characterized, the detailed molecular mechanisms still remain elusive.

Compared with the remarkable progress in terrestrial animals, the study on HSP90s in aquatic animals is still at the beginning, which mainly focuses on the discovery of new family members and their expression in response to changes in environmental conditions. By far, aquatic HSP90s have been identified from fish (Manchado et al. 2008; Pan et al. 2000; Wu et al. 2012), shrimp (Li et al. 2012), crab (Zhang et al. 2009), and shellfish (Fu et al. 2011; Gao et al. 2008; Pantzartzi et al. 2009), and most of them were shown to be responsive to heat stress (Manchado et al. 2008; Pan et al. 2000; Wu et al. 2012; Zhang et al. 2009). For example, the expression of HSP90AA in the flatfish increased rapidly after heat stress, which reached a peak within the first hour and followed up by a decline over the next 24 h (Manchado et al. 2008). However, there was no study in aquatic animals to identify the molecular mechanisms underlying these differences in expression levels.

Bay scallop (*Argopecten irradians*), first introduced from America in 1982, has become 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. In order to prevent the tissue damage caused by heat stress, scallops have developed various self-protective strategies, such as the inducing synthesis of HSP90s. So far, one HSP90 gene has been identified from bay scallop (AiHSP90) (Gao et al. 2008), but there is no available information about

the polymorphisms in its promoter and their associations with heat tolerance. In the present study, two closely related populations of bay scallop inhabiting at different latitudes were employed to investigate the molecular characters of HSP90 and their relationship with heat tolerance. The messenger RNA (mRNA) expression level of HSP90 genes were determined in two scallop populations, and the polymorphisms in the promoter region of HSP90 and their association with the heat tolerance were analyzed, aiming at finding a correlation between the population-specific molecular characteristics of the HSP90 gene promoter and the different adaptations of the examined populations to heat stress.

Materials and methods

Scallop and acute heat stress treatment

Two hundred bay scallops, averaging approximately 45 mm in shell length, were collected from two scallop farms in Qinhuangdao (119.57° E, 39.95° N) and Zhanjiang (110.38° E, 21.19° N) (China), respectively. The population distributed in Qinhuangdao (Northern Bay scallop, *A. i. irradians*) is a heat-sensitive species, which were introduced from Massachusetts in America in 1998, while the other population distributed in Zhanjiang (Southern Bay scallop, *Argopecten irradians concentricus*) is a more heat-resistant species, which were introduced from Florida in America in 1991. The scallops from Zhanjiang are more heat-resistant than the scallops from Qinhuangdao (Liu et al. 2006). The adductor muscle of each scallop from these two populations was removed and kept at −80 °C until DNA isolation.

The acute heat stress treatment experiments at different temperatures were employed with another 60 scallops from the above two scallop farms. As 16 °C is the suitable temperature to culture bay scallop, the scallops were first cultivated in 24-L tanks containing aerated seawater at 16 °C for 1 week, and then 30 individuals of each population were averagely divided into five groups. The scallops in four groups were cultivated in 24-L tanks containing aerated seawater at 23, 27, 31, and 35 °C for 2 h, respectively. And, other six scallops were still kept in 24-L tanks containing aerated seawater at 16 °C as the blank group. Gill from the scallops were collected and stored at −80 °C with addition of 1-mL TRIzol reagent (Invitrogen) for subsequent RNA extraction.

The acute heat stress treatment experiments at the same temperature for different time were employed with another 72 scallops from the above two scallop farms. After cultivated in 24-L tanks containing aerated seawater at 16 °C for 1 week, 30 individuals of each population were cultivated in 24-L tanks containing aerated seawater at 28 °C and 6 individuals were randomly sampled at 1, 2, 4, 8, and 12 h after treatment, respectively. The other six scallops were still kept in 24-L

tanks containing aerated seawater at 16 °C as the blank group. Gill were collected from those scallops and stored at −80 °C with addition of 1-mL TRIzol reagent (Invitrogen) for subsequent RNA extraction.

RNA isolation and cDNA synthesis

Total RNA was isolated from the gill of scallops using TRIzol reagent (Invitrogen). The first strand complementary DNA (cDNA) synthesis was carried out based on Promega M-MLV RT Usage information using the DNase I (Promega)-treated total RNA as template and oligo(dT)-adaptor primer P1 (Table 1). The reaction mixtures were incubated at 42 °C for 1 h and then terminated by heating at 95 °C for 5 min. The cDNA mix was diluted to 1:100 and stored at −80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR (RT-PCR).

DNA extraction and construction of genomic DNA walking library

The genomic DNA was extracted from the muscle of scallops using proteinase K and phenol/chloroform method (Yang et al. 2013). The genomic DNA walking library was constructed using the GenomeWalker™ Universal Kit protocol (Clontech). Following digestion with restrictive enzymes *Ssp* I, *Dra* I, and *Pvu* II (NEB), the genomic DNA was purified by phenol/chloroform and then ligated to GenomeWalker™ adaptor (Yang et al. 2013).

Real-time PCR analysis of AiHSP90 mRNA expression in two bay scallop populations

For SYBR Green fluorescent RT-PCR, two AiHSP90-specific primers, sense primer P2 and reverse primer P3 (Table 1), were used to amplify the corresponding products. The scallop β -actin, amplified with primers P4 and P5 (Table 1), was chosen as reference gene for internal standardization. DEPC water for the replacement of cDNA template was used as negative control.

The SYBR Green RT-PCR assay was carried out and analyzed as previously described (Yang et al. 2012). The comparative average cycle threshold method was used to analyze the mRNA expression level of AiHSP90, and the value stood for an n -fold difference relative to the calibrator (Zhang et al. 2010). All data were given in terms of relative mRNA expressed as mean $\pm$ S.E. ($N=5$). Differences were considered significant at $P<0.05$.

Cloning and sequence analysis of AiHSP90 promoter

Two gene-specific primers, P6 and P8 (Table 1), were designed based on the genomic DNA sequence of AiHSP90 to

clone the promoter by genome walking approach (Yang et al. 2013). The first-round PCR amplification was performed by using gene-specific primer P6 and adaptor primer P7 (Table 1). The nested PCR reaction was performed by using 1 μ L of the first-round PCR product (1:50 dilution) as template and gene-specific primer P8 and adaptor primer P9 (Table 1). All PCR amplification was performed in a PCR Thermal Cycle (TaKaRa, GRADIENT PCR). The PCR products were gel-purified and cloned into pMD18-T simple vector (TaKaRa, Japan). After being transformed into the competent cells of *Escherichia coli* Top10, the positive recombinants were identified through anti-Amp selection and PCR screening with sense vector primer P11 and antisense vector primer P12 (Table 1). Three of the positive clones were sequenced on an ABI 3730XL Automated Sequencer (Applied Biosystems).

The promoter sequence of AiHSP90 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).

Identification and analysis of polymorphisms in the promoter region of AiHSP90

One gene-specific primer, P10 (Table 1), was designed based on the promoter sequence of AiHSP90 and used to amplify a 705-bp fragment together with gene-specific primer P8 (Table 1). The PCR products from six scallops of two populations were separated by electrophoresis on 1 % agarose gels, respectively, and the fragments were excised and purified. The objective fragments were then cloned into pMD18-T vector (TaKaRa), transformed into *E. coli* Top10, and at least two clones were sequenced for each fragment using an ABI 3730 Automated Sequencer (Applied Biosystem). The alignments of nucleotide sequence of the AiHSP90 promoter region were performed using Vector NTI Suite 9, and the polymorphisms in promoter region were identified from the sequence alignments of different individuals.

Based on the sites of polymorphisms identified by Vector NTI Suite 9, the amplified promoter region of AiHSP90 was analyzed by TFSEARCH program (<http://www.cbrc.jp/research/db/TFSEARCH.html>) to predict the putative transcription factor binding sites.

Screening of polymorphisms in two scallop populations with differential heat tolerance

The six single nucleotide polymorphisms (SNPs) identified in the promoter region were screened by sequencing to examine their association with the heat tolerance of bay scallops. The genomic DNA (100 ng μ L^{−1}) of 30 individuals from each

Table 1 Primers used in this study

Primer name	Sequence (5'-3')	PCR objective
P1 (oligo(dT)-adaptor)	5'-GGCCACGCGTCGACTAGTACT17-3'	Clone primers
P2 (Ai90-RTF)	5'-AGGAAAAGGCAACCGCAGACA-3'	RT-PCR
P3 (Ai90-RTR)	5'-ATGTGTTCCAGGCTCCTCCAATG-3'	RT-PCR
P4 (AiActinF)	5'-CAAACAGCAGCCTCCTCGTCAT-3'	RT-PCR
P5 (AiActinR)	5'-CTGGGCACCTGAACCTTTCGTT-3'	RT-PCR
P6 (GSP1)	5'-CGGATTTTGTCAAGAGCGTCGGAGC-3'	Genomic DNA walking
P7 (AP1)	5'-GTAATACGACTCACTATAGGGC-3'	Genomic DNA walking
P8 (GSP2)	5'-TGGTGTTCGCGTGTAGTTTCTGCCTG-3'	Genomic DNA walking
P9 (AP2)	5'-ACTATAGGGCAGCGTGGT-3'	Genomic DNA Walking
P10 (proF)	5'-TCTATGTTATACGTGTTGGCATCTCCAGTC-3'	Promoter cloning
P11 (RV-M)	5'-GAGCGGATAACAATTCACACAGG-3'	Vector primer
P12 (M13-47)	5'-CGCCAGGGTTTCCAGTCACGAC-3'	Vector primer

population was used as template to amplify the promoter region with gene-specific primers P8 and P10 (Table 1). The PCR products were gel-purified and sequenced with gene-specific primers P8 and P10 (Table 1).

The genotype data at the six tested SNP sites was analyzed to test Hardy-Weinberg equilibrium (HWE). Moreover, linkage disequilibrium (LD) test and haplotype analysis were also conducted according to the genotyping results. All the data were analyzed by using SHEsis software (<http://analysis.bio-x.cn>) (Yong et al. 2005). A *P* value less than 0.05 was accepted as significant.

Results

Heat-induced temporal expression of AiHSP90 mRNA in two bay scallop populations

The acute heat stress treatment experiments were performed after the scallops were cultivated in 24-L tanks containing aerated seawater at 16 °C for 1 week, and the basal HSP90 expression level in *A. i. concentricus* is slightly higher than that in *A. i. irradians* without no significant difference ($P>0.05$).

The relative expression levels of HSP90 mRNA were significantly upregulated in both heat-resistant *A. i. concentricus* (AiHSP90R) and heat-sensitive *A. i. irradians* (AiHSP90S) after acute heat stress treatments at different temperatures ($P<0.05$) except those of *A. i. irradians* at 23 °C ($P>0.05$), and both of them showed temperature-dependent patterns (Fig. 1a). The transcripts went up with the increase of the treated temperature and reached the maximum levels at 2 h after the treatment of 35 °C. After 2 h of acute heat stress treatment at 23, 27, 31, and 35 °C, the mRNA levels of HSP90 in *A. i. concentricus* were 4.21-fold ($P<0.05$), 12.46-fold ($P<0.01$), 26.50-fold ($P<0.01$), and 28.88-fold ($P<0.01$) of that in the

blank group, respectively, while those in *A. i. irradians* were 1.85-fold ($P>0.05$), 6.07-fold ($P<0.01$), 12.88-fold ($P<0.01$), and 14.62-fold ($P<0.01$) of that in the blank group, respectively. There were significant differences in the mRNA levels of HSP90 between *A. i. concentricus* and *A. i. irradians* after the heat stress treatments at 23, 27, 31, and 35 °C ($P<0.05$).

In addition, the relative expression levels of HSP90 mRNA showed a time-dependent pattern ($P<0.05$) (Fig. 1b). They increased with the prolongation of the treated time and reached the maximum levels at 8 and 12 h for *A. i. irradians* and *A. i. concentricus*, respectively. After acute heat stress treatment at 28 °C for 1, 2, 4, 8, and 12 h, the mRNA levels of HSP90 in *A. i. concentricus* were 9.03-fold ($P<0.05$), 17.26-fold ($P<0.01$), 16.02-fold ($P<0.01$), 21.11 ($P<0.01$), and 25.53-fold ($P<0.01$) of that in the blank group, respectively, while those in *A. i. irradians* were 9.03-fold ($P<0.05$), 6.93-fold ($P<0.01$), 7.01-fold ($P<0.01$), 13.35-fold ($P<0.01$), and 11.65-fold ($P<0.01$) of that in the blank group, respectively. The differences between the mRNA levels of HSP90 in *A. i. concentricus* and *A. i. irradians* after the heat stress treatments at 28 °C for 2, 4, 8, and 12 h were all significant ($P<0.05$).

The sequence features of AiHSP90 promoter

The promoter region of AiHSP90 (793 bp) as well as an intron (587 bp) was amplified by genomic DNA walking approach, and the sequence presented in Fig. 2 was from *A. i. irradians*. The A, T, G, and C base contents of the AiHSP90 promoter region were 33.92, 31.28, 16.52, and 18.28 %, respectively, indicating that the AiHSP90 promoter region was an AT-rich region (65.20 %). Four possible transcription start sites (TSS1–TSS4) located at –261, –436, –1,141, and –1,276 bp upstream of the AiHSP90 ATG codon were identified based on the NNPP database (Fig. 2).

There were several core promoter elements in the amplified promoter and intron regions of AiHSP90, including four

Fig. 1 Temporal expression of AiHSP90 mRNA in bay scallop *A. i. irradians* and *A. i. concentricus* relative to β -actin in gills after acute heat stress treatment at different temperatures (a) and for different time (b) as measured by RT-PCR. Vertical bars represent the mean $\pm$ S.E. ($N=5$). * $P<0.05$

TATA boxes located at -322 , -534 , -591 , and $-1,301$ bp upstream of the ATG codon, and one CAAT box located at -568 bp upstream of the ATG codon (Fig. 2). With the help of TESS and Patch databases, several putative cis-acting elements were found in the AiHSP90 promoter, such as six classical heat shock elements (HSE1–HSE6) with the core consensus sequence $5'$ -nGAAn- $3'$. The nearest HSE1 (tTTCttTTAttTTCt) and the farthest HSE6 (aGAAtcTTCaGAAt) located at -475 and -961 bp upstream of the ATG codon, respectively (Fig. 2). The other four HSEs, including HSE2 (tTTCtcGATatTTCg), HSE3 (gGAAatATCg), HSE4 (agTTCcGAAGAAataTTCt), and HSE5 (ctgTTCtaGAAaaTTTc) located between -711 and -930 bp upstream of the ATG codon (Fig. 2). These HSEs might be involved in the regulation of heat shock response by interacting with heat shock factors (HSFs).

The polymorphisms in the promoter region of AiHSP90

A 705-bp fragment in the AiHSP90 promoter region was amplified from heat-resistant and heat-sensitive scallops, and 18 positive clones from six scallops of both populations were

sequenced. Six SNPs, including -1167 T-C, -1023 A-C, -799 C-T, -774 A-G, -686 C-T, and -682 A-C, were found in the amplified promoter region (Fig. 3), and the sequence presented in Fig. 3 was from *A. i. irradians*.

The putative transcription factor binding sites were screened at these six sites by TFSEARCH analysis, and their difference between two alleles was marked in Fig. 4. There was no difference between the two alleles at locus -1167 . At two loci (-774 and -682), there were some more putative transcription factor binding sites in one allele than their corresponding allele. There was one more putative binding site for Pbx-1 in A allele than G allele at locus -774 , and there was one more putative binding site for CdxA in A allele than C allele at locus -682 . At the other three loci (-1023 , -799 , and -686), the polymorphisms changed the respective transcription factor binding sites in each allele. For example, at locus -1023 , one more putative HSF-2 binding site was found in C allele instead of one more putative binding site for Hb and HNF-3b in A allele. At locus -799 , two more putative Oct-1 binding sites were found in C allele instead of one more putative binding site for NIT2 and CF2-II in T allele. At locus -686 , one more putative CdxA binding site was found in C

Fig. 2 Sequence analysis of AiHSP90 promoter. The *negative numbers on the right* indicate upstream sequence relative to the translation start codon, and the putative promoter elements are marked. The putative transcription start sites (TSS) are shown in *bigger letters* and *curved arrow*. The TATA box and CAAT box are shadowed by gray. The HSEs are indicated with *underline*, and the core sequences (GAA/TTC) are *boxed*. The intron region is indicated by the *dotted line*

allele instead of one more putative binding site for Nkx-2 in T allele. The nucleotides are numbered on the left. The polymorphism sites are underlined, and the variants are described below (Fig. 4).

The association between AiHSP90 gene polymorphisms and heat tolerance of bay scallops

The association between the sequence polymorphism and heat tolerance was investigated by examining the distributions and frequencies of all the six promoter polymorphic loci in the heat-sensitive and heat-resistant populations. By analyzing the sequencing maps, it was found that the sequencing map of

homozygous genotype had unimodal peak, while that of heterozygous genotype had overlapping peaks (Fig. 5). For example, the sequencing map of two genotypes, -1167 TT and -1167 CC, displayed their specific unimodal peak of T or C, respectively, while the sequencing map of genotype -1167 TC had overlapping peak of both T and C. Among the six polymorphic loci, four loci (-1167, -799, -774, and -686) formed three genotypes reflected by three kinds of sequencing maps, while the other two loci (-1023 and -682) formed two genotypes reflected by two kinds of sequencing maps.

The Hardy-Weinberg equilibrium for genotype frequencies was analyzed with the goodness-of-fit χ^2 test. Statistical analysis revealed that the genotype frequency of alleles at all the

Fig. 3 The polymorphisms in the promoter region of AiHSP90. The negative numbers indicate upstream sequence relative to the translation start codon and are numbered on the right. The polymorphism sites are underlined, and the variants are described below

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cgacggcccg ggctggtaaa aagtaattat aactgtaaca gttagtatat agtcataaaa -1301
tacagtagat atctatgtta tacgtgttgg catctccagt ctataattgt ttgataatttt -1241
ccatgtttta tcaaatgtac aaaatgagca ttctctcgtc tttttcccg tctattatac -1181
agtgtaatat gtatattaga ccttgggtat tcttagtcca acgttaaatt atgcactgta -1121
                                     c
taactgttac tgcctaacgt tagctaattg taatgattat aataaacact aaataattta -1061
cagaaggctg tatcaggagc tactttcatt ttacacact tcattgtaca aaacaacaa -1021
                                     a
gaattccgaa acggtccctt attgaacacc ggatatcggt gcagcagaat cttccagaat -961
agcgattact tccgaccgag gcggtccaga ctgttctaga aaatttctgt ttacgctgtc -901
aaaaataggt acattccgac ttccgcaaat agttccagaa gaaatattct attatttggg -841
ctaataatac tttttcaaag ctaatataca aaatgcataat gcatctttat attaaacaaa -781
                                     t
ttaatcgccg atcaaatatt gtggaatat cgtaaaccg tttatgataa aaagttttct -721
                                     a
cgatatattcg ggaaattgtg tagtgatata taatcgtaaa gcgtgcttcc catctgacaa -661
                                     t c
ggatagcagc cggcacgccg aaaggatcag gcagaaacta acaccgaaaa acaccatata -601
aataggaaca aaggtgagaa aatatgtttc aattgtaaga atggattatt gagtataaaa -541
agggaatac gtagactttt ttccgatcat tttatcataa tttcgatgat ttttctttta -481
ttttctctga tgaatataaa gcacgtgggt acgtacagta ggcctacgtg ctgcgacgtg -421
aacaagcaag tatgaccata gtgttgaaac cgattttgta tgaacattta ataaggaaat -361
taattcatgt agtttgatcg aaatgaatta ttatataaat tacgtataac actttttcag -301
taatgataa ataatgacc caccagtaaa gtttacgcct agaaaagcat ggttttatta -241
aatcggtctc gtgcgggtga tccaaacatt tattatgcat tctgcacttt tgcattccat -181
tttaatgat ttactagaat tatatttatg ctttcgcttt cattgtcttg tgaaaaattc -121
agtgaccaac atgaaattca aaggagaccg tccttaaata aacttgtctg ttaatagtat -61
gttaatctaa tcattgtaat caaccaaagt ttaattctgt taaattatgt atgatttcag -1
ATG                                                                                   +3

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six loci were not in HWE ($P < 0.05$) except that at locus -799 in the heat-resistant population and locus -686 in the heat-sensitive population ($P > 0.05$) (Table 2). The allele and

genotype frequencies at the six loci in the AiHSP90 promoter region were summarized in Table 2. The allele and genotype frequencies at the six loci (-1167, -1023, -799, -774, -686,

Fig. 4 Difference of putative binding sites for transcription factors between different alleles. Polymorphic loci are shadowed in gray, and the first polymorphic locus of each line is numbered on the left. The positions and directions of putative binding sites for transcription factors are labeled by the arrowhead. The TFSEARCH scores are shown on the right

-1023 C	TCATTGTACA AAACAAACAA GAATTCGAA ACGGT		
	<-----	HFH-2	score 89.7
-1023 A	TCATTGTACA AAACAAAAAA GAATTCGAA ACGGT		
	----->	Hb	score 87.5
	<-----	HNF-3b	score 86.7
-799 C	TATACAAAT GCATATGCAT CTTTATATTA AACAA		
	<-----	Oct-1	score 91.4
	----->	Oct-1	score 90.3
-799 T	TATACAAAT GCATATGTAT CTTTATATTA AACAA		
	----->	NIT2	score 95.0
	----->	CF2-II	score 86.0
-774 A	TATTAAACAA ATTAATCACC GATCAATAT TGTGG		
	----->	Pbx-1	score 85.3
-682 A	GTAGTGATAT ATAATCGTAA AGCGTGCTTC CCATC		
-686 C	GTAGTGATAT ATAATGTGAC AGCGTGCTTC CCATC		
-682 C	GTAGTGATAT ATAATGTGAC AGCGTGCTTC CCATC		
-686 T	GTAGTGATAT ATAATGTGAC AGCGTGCTTC CCATC		
	----->	CdxA	score 87.2
	----->	Nkx-2.	score 85.3

Fig. 5 Detection of the genotypes at six SNPs in the AiHSP90 promoter region. The unimodal peak indicates the homozygous genotype, and the overlapping peaks indicate the heterozygous genotype

and -682) were all significantly different between two populations ($P < 0.01$) (Table 2). The frequencies of individuals with genotypes -1167 TT, -1023 CC, -799 TT, -774 GG, -686 CC, and -682 AA in the heat-resistant population were significantly higher than those in the heat-sensitive population ($P < 0.01$), while the frequencies of individuals with genotypes -1167 CC, -1023 AA, -799 CC, -774 AA, -686 TT, and -682 CC in the heat-sensitive population were significantly higher than those in the heat-resistant population ($P < 0.05$) (Table 2). Therefore, the six genotypes (-1167 TT, -1023 CC, -799 TT, -774 GG, -686 CC, and -682 AA) of AiHSP90 were considered as gene markers associated with heat tolerance.

D' value is one of the important parameters to measure the LD between two loci. According to the D' value, the LD between two loci could be divided into three cases, including completely independent ($D' = 0$), complete LD ($D' = 1$) and a certain degree of LD ($0 < D' < 1$). The result of the pair-loci LD test revealed that the six loci in AiHSP90 gene promoter were in strong LD with a pairwise $D' > 0.85$ (Fig. 6). Considering that the LD between different polymorphic loci in one gene usually forms different haplotypes, the six loci were selected for haplotype analysis. Statistical analysis showed that these six polymorphic loci could form four haplotypes with frequency greater than 0.01, and the frequency of haplotypes CACACC (OR=8.000, 95 % confidence interval (CI) 2.694–23.755) and TCTATC in the heat-resistant population was significantly higher than that in the heat-sensitive population ($P < 0.01$), while the frequency of haplotype TCCGCA in the heat-sensitive population was significantly higher than that in the heat-resistant population (OR=15.357, 95 % CI 3.327–70.889, $P < 0.01$) (Table 3). There was no difference in the frequency of haplotype TACACC (OR=1.913, 95 % CI 0.242–15.095) between two populations ($P > 0.05$) (Table 3).

Discussion

Though the fluctuation of HSP levels is not “a silver bullet” and organisms may exhibit different strategies for adaptation to adverse environmental conditions (Bedulina et al. 2013; Mizrahi et al. 2012), stress-induced synthesis of HSPs represents a generalized molecular mechanism for almost all cells (Basiricò et al. 2011), and moreover, the expression timing and/or intensity of HSP90 were found to be different in various thermally adapted species in response to acute heat stress (Arad et al. 2010; Astakhova et al. 2013; Boher et al. 2012; Sgrò et al. 2008). By far, the induction of HSP90 synthesis by heat stress has previously been reported in various aquatic species (Manchado et al. 2008; Pan et al. 2000; Wu et al. 2012; Zhang et al. 2009); however, there were no comparative studies on the expression of HSP90 in different thermally adapted species of aquatic animals. In the present study, the relative mRNA expressions of HSP90 in two bay scallop populations were investigated after acute heat stress treatment. The heat-resistant scallop *A. i. concentricus* inhabiting approximately 21° N (the temperature of seawater could reach 31 °C in summer) displayed a stronger induction of HSP90 mRNA in a temperature-dependent and time-dependent manner, compared with the heat-sensitive scallop *A. i. irradians* from approximately 40° N (the temperature of seawater was generally below 28 °C in summer). Considering the important roles of HSP90 in modulating heat stress response (Boher et al. 2012; Heads et al. 1995; Morgan and Mackay 2006; Sgrò et al. 2008; Yonehara et al. 1996), it was speculated that the high inducing level of HSP90 might help *A. i. concentricus* to overcome the high temperature stress in summer, and the regulation of HSP90 expression was possibly one of the adaptation mechanism of scallop evolved to drastic temperature fluctuations.

Table 2 Distribution of different genotypes at six SNP sites of AiHSP90 in the heat-sensitive and heat-resistant populations

The polymorphisms in the promoter region of AiHSP90		Sensitive population no. (%)	Resistant population no. (%)	χ^2 (<i>P</i>)
-1167 T/C				
Allele	T	41 (89.13)	24 (50.00)	16.86 (<i>P</i> <0.01)
	C	5 (10.87)	24 (50.00)	
Genotype	T/T	20 (86.95)	10 (41.67)	10.45 (<i>P</i> <0.01)
	T/C	1 (4.35)	4 (16.66)	
	C/C	2 (8.70)	10 (41.67)	
HWE test Pearson's χ^2 (<i>P</i>)		13.84 (<i>P</i> <0.05)	10.67 (<i>P</i> <0.05)	
-1023 C/A				
Allele	C	40 (86.96)	20 (41.67)	20.87 (<i>P</i> <0.01)
	A	6 (13.04)	28 (58.33)	
Genotype	C/C	20 (86.96)	10 (41.67)	10.43 (<i>P</i> <0.01)
	A/A	3 (13.04)	14 (58.33)	
HWE test Pearson's χ^2 (<i>P</i>)		23.00 (<i>P</i> <0.05)	24.00 (<i>P</i> <0.05)	
-799 C/T				
Allele	C	11 (23.91)	46 (95.83)	50.90 (<i>P</i> <0.01)
	T	35 (76.09)	2 (4.17)	
Genotype	C/C	5 (21.74)	22 (91.67)	28.03 (<i>P</i> <0.01)
	C/T	1 (4.35)	2 (8.33)	
	T/T	17 (73.91)	0 (0.00)	
HWE test Pearson's χ^2 (<i>P</i>)		17.83 (<i>P</i> <0.05)	0.05 (<i>P</i> >0.05)	
-774 A/G				
Allele	A	43 (93.58)	28 (58.33)	15.70 (<i>P</i> <0.01)
	G	3 (6.52)	20 (41.67)	
Genotype	A/A	21 (91.30)	14 (58.33)	9.75 (<i>P</i> <0.01)
	A/G	1 (4.35)	0 (0.00)	
	G/G	1 (4.35)	10 (41.67)	
HWE test Pearson's χ^2 (<i>P</i>)		9.52 (<i>P</i> <0.05)	24 (<i>P</i> <0.05)	
-686 C/T				
Allele	C	9 (19.57)	48 (100.00)	63.67 (<i>P</i> <0.01)
	T	37 (80.43)	0 (0)	
Genotype	C/C	4 (17.39)	24 (54.17)	33.28 (<i>P</i> <0.01)
	C/T	1 (4.35)	0 (0.00)	
	T/T	18 (78.26)	0 (0.00)	
HWE test Pearson's χ^2 (<i>P</i>)		0.00 (<i>P</i> >0.05)	17 (<i>P</i> <0.05)	
-682 A/C				
Allele	A	2 (4.35)	20 (41.67)	18.25 (<i>P</i> <0.01)
	C	44 (95.65)	28 (58.33)	
Genotype	A/A	1 (4.35)	10 (41.67)	9.12 (<i>P</i> <0.01)
	C/C	22 (95.65)	14 (58.33)	
HWE test Pearson's χ^2 (<i>P</i>)		24 (<i>P</i> <0.05)	23 (<i>P</i> <0.05)	

HWE Hardy-Weinberg equilibrium

The expression of inducible HSP90 gene is mainly regulated at transcriptional level depending on the interaction between transcription factor and their putative cis-acting elements in the promoter region of HSP90 genes. Although the promoters of HSP90s and their response to environmental stress have been well studied in vertebrates (Ammirante

et al. 2007; Wilkerson et al. 2007), the corresponding information in invertebrates especially in mollusc is still limited (Pantzartzi et al. 2009). In the present study, a fragment of 1,380 bp including an intron (587 bp) in the promoter region of AiHSP90 was amplified from bay scallop, and six putative cis-acting elements HSEs were identified, implying the

Table 3 The haplotype analysis of AiHSP90 polymorphic loci -1167, -1023, -799, -774, -686, and -682 in the heat-sensitive and heat-resistant populations

Number	Haplotype	Sensitive (frequency)	Resistant (frequency)	χ^2 (P)	OR (95 % CI)
1	C A C A C C*	0.109	0.500 ↑	16.368 (5.27e-005)	8.000 (2.694–23.755)
2	T C T A T C*	0.000	0.761 ↑	59.862 (1.07e-014)	–
3	T A C A C C	0.042	0.022 ↓	0.199 (0.655)	1.913 (0.242–15.095)
4	T C C G C A*	0.417	0.043 ↓	17.818 (2.46e-005)	15.357 (3.327–70.889)

OR odds ratio, 95 % CI 95 % confidence interval, “↑” increased, “↓” decreased

*P values <0.01

structural basis of AiHSP90 for heat stress response. In mollusc, it has been reported that the promoter of HSP90 gene could be activated by heat stress (Pantzartzi et al. 2009). The heat-induced expression pattern of AiHSP90 mRNA was consistent with the structure of the AiHSP90 promoter. Moreover, it is of note that there was one intron before the initiation codon and several promoter elements, including two transcription start sites, two TATA boxes, one CAAT box, and one HSE, were located in this region. Similar results have been described in the HSP90s of human (Shen et al. 1997), Australian sheep blowfly (Concha et al. 2012), and Stratiomyidae (Astakhova et al. 2013), in which HSEs were detected in the intron of the HSP90 genes. Therefore, it is suspected that the intron found in the present study may be involved in the transcriptional regulation of AiHSP90 gene.

Since stress-induced synthesis of HSPs represents a generalized molecular mechanism, the differences in stress adaption

of individual animals may be partly attributed to the existence of polymorphisms in a series of related genes (Basiricò et al. 2011). It has been reported that the polymorphisms occurring naturally in the functional promoter regions of HSPs affect their expression as well as contribute to differential stress tolerance (Ane et al. 2010; Schwerin et al. 2001; Sgrò et al. 2008). In the present study, a total of six SNP sites were found in the promoter region of AiHSP90, which changed the binding sites for certain transcription factors, and thus might affect the transcriptional efficiency. This speculation could partly explain the different expression pattern of AiHSP90 mRNA under heat stress in two scallop species, and similar result has also been reported in bovine HSP70 gene, which showed one cytosine ins-del polymorphism in AP2 box region of HSP70.1 gene that affected the mRNA expression of HSP70.1 after heat stress, and finally, bovine with different genotypes displayed different thermal stress response (Deb et al. 2013).

Fig. 6 The linkage disequilibrium analysis of the six SNPs in the promoter region of AiHSP90. SHEsis software (<http://analysis2.bio-x.cn/myAnalysis.php>) was used for this analysis. The color scheme is white ($D=0$), pink ($0<D<1$), and red ($D=1$) (color figure online)

However, there were also some reports with inconsistent results. For example, in two Stratiomyidae species, though polymorphisms in the promoter region of HSP90 results in different binding activities for transcription factor GAF, which plays a crucial role in chromatin modification and provides fast switching on of HSPs genes in *Drosophila* (Omelina et al. 2011), there was no drastic differences in HSP83 mRNA accumulation in the compared species under both normal physiological and heat stress conditions (Astakhova et al. 2013).

Considering SNPs in the promoter region of AiHSP90 gene that might be the reason of the different expression pattern of AiHSP90 mRNA under heat stress in two species, association analysis between these SNPs and heat tolerance of bay scallop was performed based on case-control studies. Statistical analysis revealed that the genotype frequency of alleles at all the six loci was not in HWE ($P < 0.05$) except that at locus -799 in the heat-resistant population and locus -686 in the heat-sensitive population ($P > 0.05$). As mutational events were usually followed by environmental selection (Tanguy and Moraga 2001), which could be reflected by deviation from HWE (Schaid and Jacobsen 1999), the disequilibrium of the genotype frequency at the six loci in the AiHSP90 promoter region might imply a genetic adaptation process. Moreover, the allele and genotype frequencies at the six loci were all significantly different in two populations ($P < 0.01$), and they could be considered as the heat sensitivity/tolerance-related SNP candidates. A case-control study is an analytical study in which two existing groups differing in outcome are identified and compared on the basis of some supposed causal attribute, which has been widely used for association analysis between DNA sequence variants and various traits, and also applied to identify risk-enhancing or protective genotypes (Deb et al. 2013; Li et al. 2009; Xiong et al. 2013; Yang et al. 2013). As the -1167 TT, -1023 CC, -799 TT, -774 GG, -686 CC, and -682 AA genotypes were more prevalent in the heat-resistant population than in the heat-sensitive population, they were suggested to be significantly associated with the resistant phenotype of bay scallops to heat stress and could be potential markers in the selection of scallops with heat-resistant traits. The characterization of such mutation has important implications for understanding of the genetic adaptation processes in organisms (Tanguy and Moraga 2001).

As most of the resistance traits are of quantitative and they are always controlled by multiple genes (Zeng 1993), the contribution of a single SNP might be a small part of the related traits. In the present study, the comprehensive LD and haplotype analyses of SNPs in AiHSP90 gene promoter were performed to examine the possible interactions between the genotypes and the heat tolerance. The pair-loci LD test revealed that the six SNPs were in strong LD with a pairwise $D' > 0.85$. The LD between different polymorphic loci in one gene usually leads to the formation of different haplotypes. In the present study, haplotype analysis of the six SNPs revealed that they could form four haplotypes. The frequency of

haplotypes CACACC and TCTATC in the heat-resistant population was significantly higher than that in the heat-sensitive population ($P < 0.01$), while the frequency of haplotype TCCGCA in the heat-sensitive population was significantly higher than that in the heat-resistant population ($P < 0.01$). Therefore, the haplotypes CACACC and TCTATC were believed to confer higher heat tolerance to the scallops compared with that of other haplotypes. The population genetic studies about the balancing selection have confirmed that genetic adaptation not only maintains diversity at the selected sites but also increases diversity at closely linked neutral sites (Xiong et al. 2013). The knowledge also provides an explanation for the different numbers of heat-resistant haplotype candidates and SNP candidates in the present study.

In summary, the promoter region of AiHSP90 gene, which exhibited different mRNA expression patterns in two bay scallop populations under heat stress, was cloned, and its sequence polymorphisms were investigated. Totally, six SNPs and two haplotypes in the promoter region of AiHSP90 were associated with heat tolerance of bay scallops, and they could be applied in the future molecular-assisted selection program of bay scallop. The present results indicated that the peculiarities of HSP90 gene expression were closely coupled with the adaptation to the fluctuating thermal habitats for scallops.

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