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Peroxiredoxin 6 gene: A new physiological and genetic indicator of multiple environmental stress response in Pacific oyster *Crassostrea gigas*

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Abstract

Peroxiredoxin 6 is a 1-cysteine peroxiredoxin involved in antioxidant processes. We characterised the full-length cDNA and genomic sequence of the gene encoding peroxiredoxin 6 (*Cg*Prx6) in the Pacific oyster, *Crassostrea gigas*. A phylogenetic analysis of Prx6 sequences showed that the *Cg*Prx6 segregates between vertebrate and invertebrate groups. We analysed the expression of mRNA coding for *Cg*Prx6 using RT-PCR in gills and digestive gland of oysters sampled in different contaminated and reference estuaries of the Atlantic French coast. We also studied *Cg*Prx6 exon 6 polymorphism by PCR-SSCP in the same populations. Our results showed that *Cg*Prx6 gene expression was highly regulated in the estuaries showing differential contamination levels, as expression increased with pollution level. Polymorphism analysis revealed no significant allelic frequency variations between populations. However, heterozygote deficiency seems to occur in the most contaminated estuaries, suggesting a potential selective effect of environmental stress on heterozygote frequency. Finally, the use of *Cg*Prx6 as a possible marker to monitor stress exposure in disturbed ecosystems is discussed.

Keywords: *Crassostrea gigas*; Peroxiredoxin 6; Gene expression; Polymorphism; Environmental stress

1. Introduction

The peroxiredoxin (Prx) family is composed of antioxidant proteins ubiquitously found in prokaryotic and eukaryotic (yeast, plant and animal) species (Chae et al., 1994; Leyens et al., 2003; Wood et al., 2003). Peroxiredoxins exhibit thiol-dependent peroxidase activity and they function as peroxidases only when coupled to sulfhydryl-reducing system such as thioredoxin or glutathione (Radyuk et al., 2001). Peroxiredoxins play a protective antioxidant role in cells, reducing and detoxifying hydrogen peroxide, peroxinitrite and a wide range of organic hydroperoxides (ROOH), through their peroxidase activity ($ROOH + 2e^- \rightarrow ROH + H_2O$) (Wood et al., 2003). Prx use redox-active cysteine residues to reduce peroxides. Depending on the Prx isoforms, one or two cysteine residues are directly involved in catalysis (Chae et al., 1994; Hofmann et al., 2002). Prx isoforms have been divided into three sub-groups: the 2-Cys Prx proteins which contain two-cysteine residues (N- and C-terminal conserved cysteine residues, respectively), the atypical 2-Cys proteins which contain only the N-terminal but require a supplementary non-conserved cysteine residue, and the 1-Cys Prx proteins which contain only the N-terminal residue that is sufficient for their catalytic activity (Rhee et al., 2005). In mammals, six isoforms (Prx-I to Prx-VI) have been described to date (Hofmann et al., 2002): Prx-I–IV belong to the 2-Cys subgroup, Prx-V contains two additional cysteine residues to the conserved one and Prx-VI is a 1-Cys Prx. The N-terminal Cys-SH of Prx-VI is oxidised upon exposure to hydrogen peroxide (Rhee et al., 2005) and the resulting Cys-SOH of oxidised Prx has been suggested to be reduced by glutathione as physiological electron donor (Fisher et al., 1999). Prx-VI has then been also described as non-selenium glutathione peroxidase (Munz et al., 1997; Fisher et al., 1999; Leyens et al., 2003; Manevich and Fisher, 2005), although some authors did not detect any glutathione-supported peroxidase activity (Kang et al., 1998; Radyuk et al., 2001). Several 1-cysteine peroxiredoxins, similar

Abbreviations: Prx6, peroxiredoxin 6; SSCP, single strand conformation polymorphism; UTR, untranslated region

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to Prx-VI, have been sequenced in vertebrate and invertebrate species and particularly in insects (Radyuk et al., 2001; Tsuji et al., 2001; Kim et al., 2005). However, to our knowledge, there is no study dealing with Prx in molluscs. In addition, to date, very few studies have been carried out on genetic polymorphism of peroxiredoxin, and these are geared towards humans (Kim et al., 2006).

As they belong to antioxidant enzymes, peroxiredoxins are supposed to be involved in xenobiotic stress response. Most of anthropogenic contaminants are known to induce antioxidant mechanisms as a protective response of subjected organisms (Regoli et al., 2002). Estuarian ecosystems are more and more exposed to xenobiotic inputs. Expression and polymorphism studies of candidate genes involved in such a stress response as peroxiredoxin genes may be useful to appreciate the physiological and genetic adaptation of organisms living in contaminated areas. The increasing anthropogenic pollution (hydrocarbons, pesticides, nutrient inputs) and other environmental stressors are known to induce modifications in the genetic structure of populations as an effect of selection. Indeed, it has been observed that individuals within a population may be more or less vulnerable than others to environmental stressors due to their specific phenotypes (Ma et al., 2000). Selection could be linked to varying survival capacity of some genotypes inducing differential mortality (Gillespie and Guttman, 1993; Moraga et al., 2002; Tanguy et al., 2002). Studies of genetic polymorphism in specific candidate genes could allow the characterisation of genetic biomarkers of sensitivity or resistance to environmental stressors in marine populations. These biomarkers must reflect genetic variations of the stress response, linked to variations of sensitivity (Depledge, 1996; Forbes and Depledge, 1996).

The aim of this study was to characterize new nuclear genetic markers in the Pacific oyster *Crassostrea gigas*. First, isolation and characterization of the full-length cDNA and genomic sequence that encodes *C. gigas* peroxiredoxin 6 (*Cg*Prx6) is described. We have then performed a phylogenetic analysis to compare the *Cg*Prx6 sequence to Prx6 sequences in other species and to identify potential evolutionary divergences in the sequence. We compared *Cg*Prx6 expression patterns in field populations from estuaries exposed to differential contamination. We also studied the *Cg*Prx6 genetic polymorphism in these populations to find out the possible selective effect of environmental parameters on specific Prx6 allelic frequencies in *C. gigas*.

2. Materials and methods

2.1. Field collection

2.1.1. Study sites

Adult oysters were collected in three polluted French estuaries (Vilaine, Loire and Gironde) and one moderately contaminated estuary (Belon) considered as the 'reference site' (low domestic, agricultural, and industrial effluents) (Fig. 1). The Vilaine, Loire and Gironde estuaries are subjected to a strong anthropogenic influence and are chronically polluted by mixtures of chemicals, depending on the nature and the proportion of anthropogenic activities. The Loire undergoes a diffuse contamination with a complex mixture of chemicals (Marchand et al., 2003; RNO, 2001). The Gironde estuary shows high levels of heavy metals such as zinc, cadmium, and copper (Marchand et al., 2003; RNO, 2001). The Vilaine is thought to have high levels of pesticides (Atrazine, Diuron, Isoproturon) (SAGE Vilaine, 2000), as a result of intensive agriculture. The Vilaine estuary (and the Loire to a lesser extent) is also known to experience hypoxic events (Menesguen et al., 2001). The coordinates of the different sampling sites are, for the Belon estuary: 47°48′34N 3°42′58W; the Vilaine estuary: 47°29′52N 2°25′11W; the Loire estuary: 47°16′06N 2°12′45W and the Gironde estuary: 45°33′53N 1°02′30W.

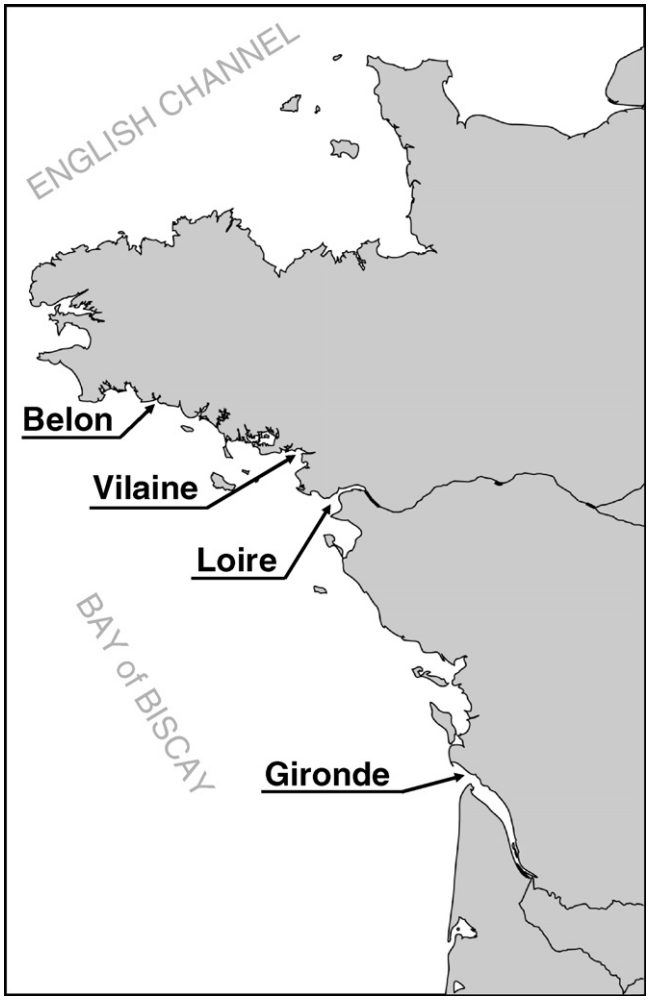

Fig. 1. Localisation of the different sampling sites.

2.1.2. Sample preparation

Adult oysters were collected from the different estuaries in January and June 2005. A total of 96 oysters were collected from each estuary (36 in January and 60 in June). Gills and digestive gland samples were collected and flash-frozen in liquid nitrogen for later RNA extraction. These samples were brought back to the laboratory and stored at −80 °C until use. Gill samples were also preserved in alcohol for DNA extraction.

Table 1
Sequences of the primers used in the *Crassostrea gigas* Prx6 cDNA and gene cloning, semi-quantitative expression and polymorphism analysis

Primer name	Sequence
Oligo dT anchor primer 1	5′-CGCTCTAGAACTAGTGGATCTTTTTTTTTTTTTT-3′
Oligo dT anchor primer 2	5′-GACCACGCGTATCGATGTCGACT$_{(16)}$V-3′
Anchor primer	5′-GACCACGCGTATCGATGTCGACT-3′
Prx Rev 1	5′-GACCACCCCTCATGACTGGGGACGTCATC-3′
Prx For 2	5′-GGCATAGAAAAGGTGTTCCCTAAGGGTGT-3′
Prx Rev 2	5′-TGGGGGATGGAGGGtAAGACCATACACTT-3′
Prx For 1	5′-GATGACGTCCCCAGTCATGAGGGGTGGTC-3′
Prx For 3	5′-TAGTATATTCCATAGCAAGCGAC-3′
Prx Rev 3	5′- GACCACCCCTCATGACTGGGGACGTCATC-3′
Prx For 4	5′- AAGATGATCGCCCTGTCCTGTGATGACGT-3′
Prx Rev 4	5′- CTTACTGCACGACAAGTAAGTGGAAGGCC-3′
Prx For 5	5′-GAAAAGGACAATGCAGGCCTTCCACT-3′
Prx Rev 5	5′- AAATCTATTTACTCTGGCTGTGGGGT-3′
Actin For	5′- CACGGCATCGTCACCAACTGGGA -3′
Actin Rev	5′- GAAGCGTACAGGGACAGTACGGC -3′
Forward primer P1	5′- GTCTCTAAATTTCAGCAAGGAGG -3′
Reverse primer P2	5′- AAATCTATTTACTCTGGCTGTGGGGT -3′

2.2. Cloning and sequencing of 5′ and 3′ flanking regions of Prx6 cDNA

The procedures for the generation of cDNA of the 5′ and 3′ untranslated regions (UTRs) for Prx6 were carried out according to the commercial protocol for the rapid amplification of 3′/5′ cDNA ends (5′/3′ RACE Kit, Roche, Mannheim, Germany) using specific primers. The primers were designed from the partial sequence of *C. gigas* non-selenium glutathione peroxidase (or Prx6) gene previously identified (David et al., 2005) and are shown in Table 1. Total RNA was extracted from the gills, mantle and digestive gland of oysters according to the method based on extraction in guanidium isothiocyanate (Strohman et al., 1977). Briefly, cDNA was synthesized using the oligo dT anchor primer 1 (Table 1) and M-MLV reverse transcriptase (Promega, Madison, WI, USA), and was then purified using the Wizard® DNA Clean-Up System (Promega). Amplification of the 5′ and 3′ UTRs was carried out according to the following procedure: 200 ng of reverse transcription product was treated with terminal deoxynucleotidyl transferase (Promega, Madison, WI, USA) and dATP to generate a polyA tail at the 5′ end. This product, 10 pmol each of oligo dT anchor primer 2 (Table 1) and 5′ (Prx Rev2) or 3′ (Prx For1) specific primer, was submitted to denaturation at 94 °C for 2 min, followed by 10 cycles at 94 °C for 15 s, 60 °C for 30 s, 72 °C for 40 s, then 25 cycles at 94 °C for 15 s, 60 °C for 30 s, 72 °C for 40 s, with an increase of 20 s per cycle for the elongation time, and a final step at 72 °C for 10 min. The amplification products, 10 pmol each of anchor primer and 5′ (Prx Rev1) or 3′ (Prx For2) specific primer (Table 1), were then submitted to denaturation at 94 °C for 2 min, followed by 15 cycles at 94 °C for 15 s, 57 °C for 30 s with an increase of 0.2 °C per cycle, 72 °C for 2 min, then 25 cycles at 94 °C for 15 s, 60 °C for 30 s, 72 °C for 2 min, and a final step at 72 °C for 10 min. The resulting 400- and 300-base pair cDNA fragments generated by these procedures, corresponding to 5′ and 3′ UTRs, respectively, were inserted into pGEM-T vector (Promega), cloned, and then sequenced using a LiCOR IR^2 (Sciencetech, Lincoln, NE, USA) and Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare Europe, Freiburg, Germany). Sequences were subjected to a homology search through the BLASTX program (NCBI, Bethesda, MD, USA).

2.3. Cloning and sequencing of the Prx6 gene

Total genomic DNA was isolated from oyster gills using phenol/chloroform/isoamyl alcohol (25:24:1). The *Cg*Prx6 gene sequence was amplified using 3 primers combinations designed from the cDNA sequence (Prx For 3 and Prx Rev 3; Prx For 4 and Prx Rev 4; Prx For 5 and Prx Rev 5; Table 1) using Uptitherm DNA polymerase (Interchim, Montluçon, France). Two hundred nanograms of genomic DNA was submitted to amplification, using denaturation at 94 °C for 5 min, 59 °C, 64 °C or 62 °C for 2 min depending on the different primers combinations, respectively, 72 °C for 90 s, then 40 cycles at 94 °C for 30 s, 59 °C, 64 °C or 62 °C for 40 s depending on the different primers combinations, respectively, 72 °C for 90 s, and a final step at 72 °C for 10 min with 2 mM $MgCl_2$, and 10 pmol of each primer. Cloning and sequencing were performed according to the procedures described above.

2.4. Prx6 expression study by RT-PCR

Total RNA was extracted from the digestive gland and the gills of adults of *C. gigas* sampled in different contaminated estuaries, using a method based on extraction in guanidium isothiocyanate (Strohman et al., 1977). For each sample, 20 μg of RNA was submitted to reverse transcription using oligo dT anchor primer 2 (Table 1) and M-MLV reverse transcriptase (Promega). The amplification of Prx6 mRNA was performed in 2 mM $MgCl_2$ and 10 pmol of primers Prx For1 and Prx Rev2 (Table 1). Actin mRNA was used as a PCR internal control under the same conditions with primers Actin For and Actin Rev (Table 1). After different adjusting tests, the chosen number of PCR cycles was 35 for Prx6 and 30 for actin

expression to avoid band intensity saturation for optical determination. Primers hybridization temperature used was 59 °C for Prx6 gene and 63 °C for actin gene. The resulting PCR products were electrophoresed in a 0.5× TBE/1.5% agarose gel, and visualised with UV light after staining with ethidium bromide. Band intensities were quantified using the GENE PROFILER software (Version 4.03, Scanalytics, Inc., Lincoln, NE, USA).

2.5. PCR-SSCP (single strand conformation polymorphism) analysis

Among the six exons we characterized in *Cg*Prx6 gene, we chose exon 6 for SSCP analysis because among the others, two were too short for this method, one did not present any polymorphism, and the two others did not allow robust and reproducible polymorphism analysis and interpretation. Exon 6 of the Prx6 gene was amplified using the forward primer P1 and the reverse primer P2 (Table 1). All PCR amplifications were performed in a volume of 25 μL containing 1× Taq polymerase buffer, 2 mM $MgCl_2$, 40 μM deoxynucleotides (dNTPs), 10 pmol of each primer, 0.5 units of Taq Uptitherm DNA polymerase (Interchim, Montluçon, France) and about 100 ng of total genomic DNA. After an initial pre-cycle (denaturation 5 min at 94 °C, primer hybridisation 2 min at 58 °C, elongation 1 min 30 s at 72 °C), 40 amplification cycles were performed as follows: 30 s at 94 °C, 40 s at 58 °C, 1 min 30 s at 72 °C, with a final elongation 10 min at 72 °C. The PCR products were then combined with 20 μL of loading buffer (bromophenol blue, xylen cyanol, saccharose), heated for 5 min at 95 °C, then rapidly chilled on ice to melt and retain single strand DNA. After loading on a neutral 10% polyacrylamide gel (37.5:1, acrylamide: bisacrylamide), the samples were electrophoresized at a constant voltage (120 V) in a 0.6× TBE buffer, for 20 h at 10 °C. After electrophoresis, the gels were stained with ethidium bromide and visualised under UV light. Single strand DNA bands from the PCR products visualized on the gel as different conformation types obtained were gel-purified by diffusion into water by freezing at −20 °C and thawing. The same PCR-amplifications as for SSCP analysis were done on this recovered DNA. PCR products were then purified using Qiaex II gel extraction kit (Qiagen, Courtaboeuf, France), cloned and sequenced as described above.

2.6. Molecular phylogeny analysis

Phylogenetic analysis was performed on the amino acid sequences from full-length Prx6 cDNA from various organisms, including invertebrates and vertebrates. Amino acid sequences were aligned using ClustalW Software (Thompson et al., 1994). Molecular phylogenetic trees were constructed using the phylogenetic component of the MEGA3.1 software (Kumar et al., 2004). Amino acid differences were corrected for multiple substitutions using a gamma correction. In this correction, α, the shape parameter of the gamma distribution, was set to 2. With $\alpha = 2$, the distance between any two amino sequence, d_{ij}, is approximately equal to Dayshoff's PAM distance per site. Support for the major nodes within trees was evaluated by bootstrapping the data; 1000 bootstrap replicates of the whole data set were generated.

2.7. Statistical and genetic analysis

Comparisons of expression level between estuaries and between winter and summer have been realised with non-parametric Kruskal and Wallis tests using the STATISTICA Software (Statsoft, Maisons-Alfort, France).

The population genetic parameters (allelic frequencies, observed heterozygosity (H_o), heterozygosity based on Hardy–Weinberg expected values (H_e)) were calculated per population with the GENETIX 3.0 software (Belkhir et al., 1996). Within each population, deviation from Hardy–Weinberg equilibrium was determined by computing the inbreeding coefficient (Fis) as follows: $Fis = (H_e - H_o)/H_e$. The significance of Fis was assessed by a Fisher's exact test using the GENEPOP 3.2 software (Raymond and Rousset, 1995). Allelic frequencies were analysed to detect possible heterogeneity between the different estuaries. A population differentiation genetic coefficient (Fst) was computed using the GENETIX 3.0 software (Belkhir et al., 1996). Fst significance was assessed by permutated data sets (1000 permutations). The standard Bonferroni technique (Lessios, 1992) was used to adjust the significance levels of multiple tests: the predetermined significance level, α, was divided by the number of tests, k, to obtain α' the corrected significance level ($\alpha' = \alpha/k$ where $\alpha = 0.05$, k = number of tests carried out).

3. Results

3.1. Molecular characterization of Prx6 gene

The cDNA sequence of Prx6 from *C. gigas* showed a 663-bp coding region corresponding to 221 amino acids with a calculated molecular weight of 24.3 kDa and an isoelectric point of 6.59 (MWCALC software, Infobiogen, France). The 5′ and 3′ untranslated (UTR) sequences comprise 82 and 169 bp, respectively. Several ATTTA(G) motifs are present in the UTRs of the Prx cDNA sequence, one ATTTA motif is located in the 5′UTR and two ATTTG motifs in the 3′UTR. The size of the Prx6 gene is 3697 bp from the 5′UTR to the polyA site (Fig. 2). The sequence contains 6 exons of 28, 53, 48, 24, 25 and 43 amino acids, respectively, separated by five introns of 794, 1016, 717, 164 and 89 bp, respectively. All the introns of the *Cg*Prx6 gene start and end with the consensus GT and AG splicing signals.

3.2. Molecular phylogeny

A range of 20 vertebrate and invertebrate Prx6 amino acid sequences was analysed to build a phylogenetic tree. The tree placed *Cg*Prx6 sequence in the invertebrate group but closer to vertebrates than other invertebrates (Fig. 3). Moreover, *Xenopus tropicalis* sequence was found in the same sub-group than *C. gigas*.

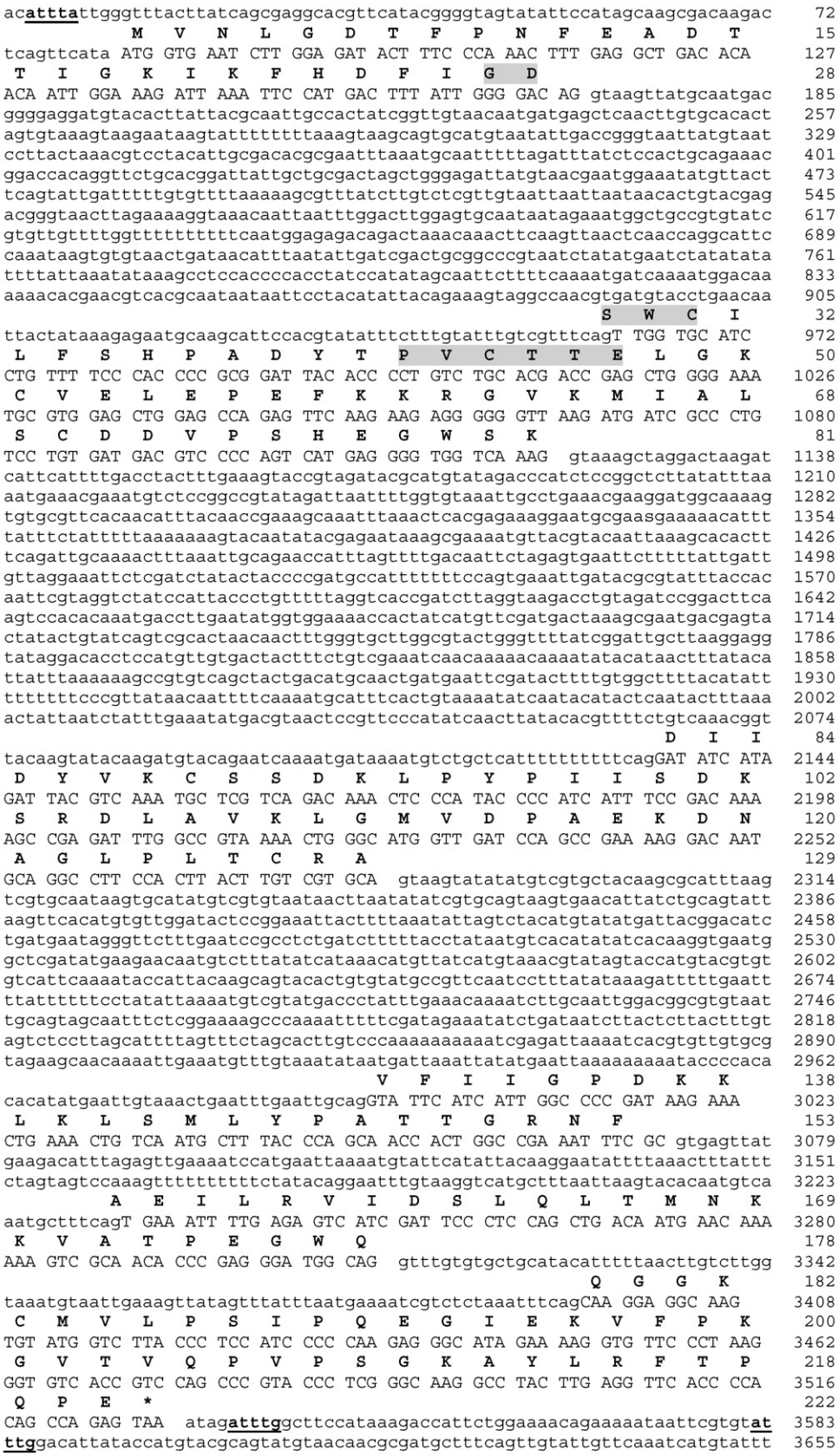

Fig. 2. The nucleotide sequence and predicted amino acid sequences of *Crassostrea gigas* peroxiredoxin 6 gene (Genebank accession number AM265552). Bold characters indicate the predicted amino acid sequence. The polyadenylation sequence is underlined and messenger stability motifs ATTTA and ATTTG are in bold characters and underlined. Catalytic centers for peroxidase activity (PVCTTE) and phospholipase A2 activity (GDSWC) are highlighted.

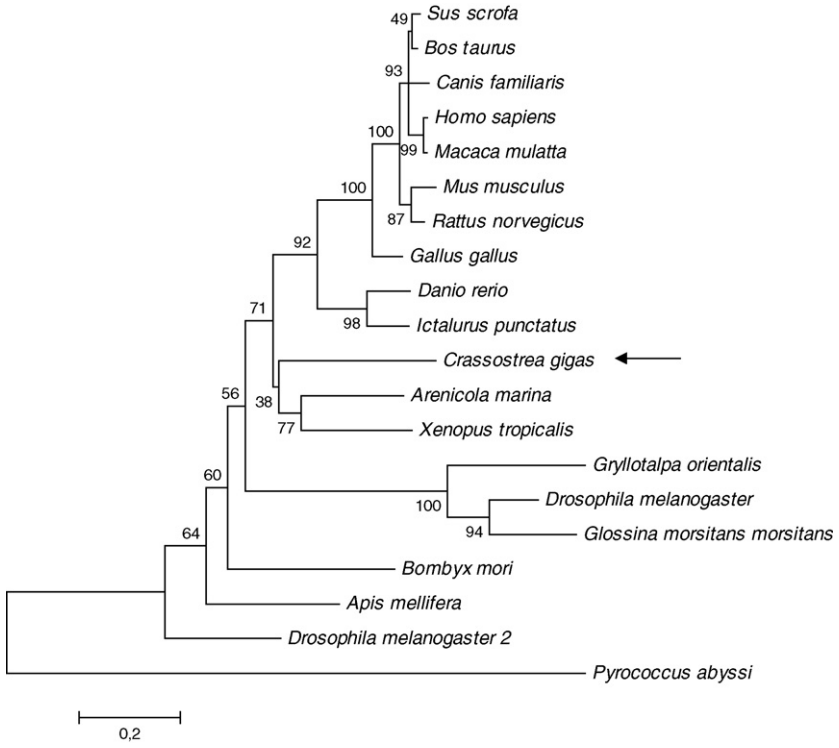

Fig. 3. An unrooted phylogeny showing the most likely relationship between representative Prx6 amino acid sequences. Branch lengths are proportional to estimates of evolutionary change. The number associated with each internal branch is the local bootstrap probability, which is an indicator of confidence. The sequences are *Homo sapiens* (NP_004896), *Mus musculus* (NP_031479), *Rattus norvegicus* (NP_446028), *Macaca mulatta* (XP_001101473), *Danio rerio* (NP_957099), *Arenicola marina* (AAY96294), *Gallus gallus* (NP_001034418), *Ictalurus punctatus* (ABG77029), *Xenopus tropicalis* (NP_001011325), *Sus scrofa* (NP_999573), *Bos taurus* (AAX08984), *Aedes aegypti* (CH477238.1), *Bombyx mori* (DG311144.1), *Pyrococcus abyssi* (AJ248286.2), *Gryllotalpa orientalis* (AY771361.1), *Canis familiaris* (XM_537190), *Apis mellifera* (XM_395319), *Drosophila melanogaster* (AAG47823 and (2) AAG47822), *Glossina morsitans morsitans* (AY625505), *Crassostrea gigas* (AM265552).

3.3. Expression analysis

Expression analysis of *Cg*Prx6 gene by RT-PCR revealed significant differences of expression between the different estuaries (Fig. 4). During winter, expression was significantly higher in the Loire and Vilaine estuaries for both tissues, and in the Gironde estuary in digestive gland, compared to the Belon estuary ($H = 8.7$; $p = 0.03$ and $H = 43.7$; $p < 0.001$ in gills and digestive gland, respectively). During summer, expression was significantly higher in the Vilaine and the Loire estuaries than in the three others for gill tissue ($H = 42.2$; $p < 0.001$), and in the Vilaine estuary for digestive gland tissue ($H = 55.6$; $p < 0.001$). In the Belon estuary, no detectable expression was observed during the summer.

Significant differences were also observed between winter and summer (Fig. 4): expression was significantly lower in summer in the Gironde estuary ($H = 38.0$; $p < 0.001$ and $H = 35.9$; $p < 0.001$ in gills and digestive gland, respectively), in the Loire estuary ($H = 23.6$; $p < 0.001$ and $H = 37.2$; $p < 0.001$ in gills and digestive gland, respectively), in the Vilaine estuary ($H = 32.5$; $p < 0.001$ and $H = 4.2$; $p = 0.04$ in gills and digestive gland, respectively) and in the Belon estuary in gills ($H = 39.3$; $p < 0.001$). In the Belon estuary, no difference of expression level was observed between winter and summer in digestive gland ($H = 1.2$; $p = 0.3$).

3.4. Polymorphism analysis

PCR-SSCP performed on exon 6 of the *C. gigas* peroxiredoxin 6 gene allowed us to characterize 10 different alleles that were named A, B, C, D, E, F, G, H, I and J. The allele frequency distribution in the field populations is presented in Table 2. The sequences of the different alleles revealed that five of them (B, D, H, I and J) contain a fragment with a polymorphism resulting in a modification of the corresponding amino acid, the sequence of allele A being used as a reference: lysine 211 is changed to asparagine. All other mutations that differentiate these alleles are synonymous, except for allele I where isoleucine 189 is changed to valine. The other alleles (C, E, F and G) showed a modification in the third base of the codon that does not change the corresponding amino acid.

Heterozygote deficit was seen in the three populations from the highly contaminated estuaries. It was significant in the Loire population (Fis = 0.092; $p = 0.02$) although after Bonferroni's correction the deficit is no longer significant. However, this value will be considered cautiously but not rejected. The deficit was not significant in the Gironde and Vilaine populations (Fis = 0.055; $p = 0.19$ and Fis = 0.083; $p = 0.07$ respectively).

In order to verify that oyster individuals sampled in each estuary in January and June belong to the same populations, we used

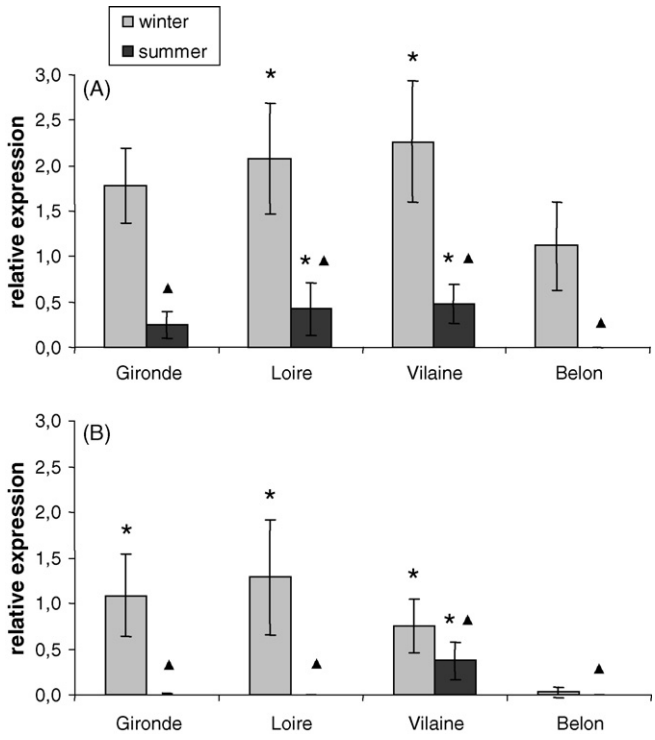

Fig. 4. Expression of the *Cg*Prx6 gene in *C. gigas* from different estuaries of Atlantic French coast (A) in the gills, and (B) in the digestive gland ($n = 36$). Expression is presented as the calculated ratio DO_{Prx6}/DO_{actin} after RT-PCR. (*) Means significant difference with $p < 0.05$ compared to Belon estuary and (▲) means significant difference between summer and winter.

Fst calculation to compare genetic structures of these samples. No significant difference was observed, in any estuary. Therefore, for each estuary, the samples collected in January and in June were considered as a single population for genetic analysis. The differentiation index Fst, estimated between pairs of populations, showed no significant genetic differentiation between the populations of the four estuaries. Indeed, all the calculated Fst were not significantly different from 0 ($p \geq 0.1$). However, allele A was more represented in Belon estuary than in the three others (6–8% of difference) (Table 2).

Table 2
Allelic frequencies of exon 6 of Prx6 in *C. gigas* populations from different estuaries of Atlantic French coast (n: sample size; H_o: observed heterozygosity; H_e: expected heterozygosity)

Allele	Estuary			
	Gironde	Loire	Vilaine	Belon
A	0.224	0.240	0.229	0.302
B	0.057	0.099	0.069	0.052
C	0.620	0.547	0.569	0.521
D	0.057	0.078	0.085	0.068
E	0.005	0.005	0.005	0.000
F	0.005	0.005	0.021	0.016
G	0.005	0.000	0.000	0.000
H	0.010	0.010	0.005	0.016
I	0.010	0.010	0.005	0.026
J	0.005	0.005	0.011	0.000
n	96	96	94	96
H_e	0.562	0.631	0.614	0.632
H_o	0.531	0.573	0.564	0.677

4. Discussion

In this study, we describe the first genomic sequence coding for the peroxiredoxin 6 or glutathione peroxidase seleno-independent in the Pacific oyster *C. gigas*. The Prx6 sequence shows a consensus region PVCTTE in the N-terminal which is a signature of 1-Cys type peroxiredoxins. This consensus sequence corresponds to the catalytic centre for the peroxidase activity (Wood et al., 2003; Manevich and Fisher, 2005). It contains the N-terminal Cys-SH that is oxidized upon exposure to peroxide (Rhee et al., 2005). The resulting Cys-SOH of oxidised Prx is then reduced. However, this enzyme does not use thioredoxin as the physiological reductant (Manevich and Fisher, 2005). The physiological electron donor for peroxidase activity is controversial since some authors consider glutathione as the donor and others do not (Fisher et al., 1999; Manevich and Fisher, 2005). Based on the crystal structure (Choi et al., 1998; Manevich and Fisher, 2005), a catalytic triad has been proposed for peroxidase activity: Cys47-His39-Arg132, where the Cys47 could be hydrogen bonded to His39 and electrostatically activated by Arg132. In *Cg*Prx6, this triad may exist as Cys44-His36-Arg128. Other non-conserved cysteine residues are present in the *Cg*Prx6 sequence but none corresponds to the second signature of 2-Cys type peroxiredoxin. Human and mouse Prx proteins also have additional non-conserved Cys residues (Fisher et al., 1999; Manevich and Fisher, 2005). We also observed a GDSWC motif in *Cg*Prx6. In mammals, Prx6 contains a sequence called a lipase motif (GDSWG or GXSXG) based on Ser as the catalytic centre for phospholipase A2 activity, as Prx6 appeared as bifunctional protein (Manevich and Fisher, 2005). Based on the crystal structure, a catalytic triad (Ser32-His26-Asp140) has also been proposed for phospholipase A2 activity (Choi et al., 1998; Manevich and Fisher, 2005). In *Cg*Prx6, this triad may exist as Ser29-His23-Asp136. Few genomic DNA sequences of Prx6 are present in databases. Most of them show a gene organisation with five exons and four introns and correspond to a gene length that varies between 11 and 22 kbp (Prx6 gene of *Macaca mulata* (accession number NC_007858.1), *Mus musculus* (accession number NC_000067), *Homo sapiens* (accession number NC_000001), *Pan troglodytes* (accession number NC_006468.2) and *Danio rerio* (accession number NC_007131.1)). Phylogenetic analysis showed that the *Cg*Prx6 sequence is intermediate between vertebrate and invertebrate groups, but the lack of more Prx6 sequences for molluscs and other invertebrates prevents a more complete evolutionary analysis.

In a previous study, we observed that the *Cg*Prx6 gene was expressed in several tissues: the gills, mantle and digestive gland (David et al., 2005). We followed *Cg*Prx6 expression during experimental hypoxia exposure and observed an up-regulation of transcription during hypoxic stress exposure. In the present study, we used semi-quantitative RT-PCR to assess *Cg*Prx6 gene expression in natural populations from three highly contaminated estuaries and one less contaminated estuary in the gills and

the digestive gland. We observed a significantly higher *Cg*Prx6 expression in winter in three highly contaminated estuaries, the Gironde, the Loire and the Vilaine estuaries, compared to the less contaminated Belon estuary, depending on the tissues. These results suggest an increase of *Cg*Prx6 transcription with contamination and stress exposure. Prx6 has been shown to have a peroxidase activity (Hofmann et al., 2002; Rhee et al., 2005). Such an enhanced expression of this gene in highly contaminated estuarine populations must reflect a response to oxidative stress that might occur in organisms subjected to various anthropogenic contaminations. Indeed, hydrocarbon, pesticides, heavy metals, and other chemicals are known to induce ROS production in organisms inhabiting such contaminated areas (Regoli et al., 2003; van der Oost et al., 2003). *Cg*Prx6 induction should allow the organism to control the cellular increase of ROS quantity, and thereby avoid important cellular damages. In summer, however, we observed a lower level of *Cg*Prx6 mRNA in both tissues and the four estuaries, but the decrease was weaker in the digestive gland of animals from the Vilaine estuary than in the other estuaries. The strong decrease of expression between winter and summer may be interpreted in different ways. First, the summer season is supposed to be less rainy than winter reducing contaminant inflow and then consequently reducing ROS synthesis due to lower contaminant levels. Second, the reproduction cycle may influence transcription patterns, as most energy is mobilised for gametogenesis and breeding preparation. Transcription of some genes as Prx6 may then decrease. However, we still observed a higher expression level in the Vilaine estuary than in the three others in summer, in both tissues. The Vilaine estuary is known to be submitted to temporary hypoxic events, mostly in summer (Menesguen et al., 2001). This higher level of expression may be related to this occurrence of hypoxic events. Up-regulation of Prx6 has also been observed in mammals in response to various physiological and environmental factors (Munz et al., 1997; Kubo et al., 2006). Kubo et al. (2006) suggest that Prx6 is regulated by transcriptional control rather than by translational efficiency. All these observations lead to consider Prx6 gene expression as a potential biomarker of stress exposure.

The polymorphism we observed in the coding sequence of *Cg*Prx6 gene allowed us to compare the allelic distribution in the different estuarine populations of the Pacific oyster *C. gigas*. No significant structure was observed between paired populations (Fst non significantly different from 0). However, the Fis calculation revealed a heterozygote deficit in the three highly contaminated sites (only significant in the Loire). Several hypotheses could explain such a deficit. Technical artifacts such as non-amplifying (null) alleles or bad alleles detection can be ruled out because they should be systematic for all samples. One hypothesis could be that oyster populations are not panmictic and the heterozygote deficit could result from Wahlund effect consisting in subdivision of local population into isolated and differentiated sub-populations (Castric et al., 2002). Analysis of polymorphism at other loci in the same populations could help confirm the occurrence of such effect. Moreover, inbreeding through the mating of close relatives may have caused the observed heterozygote deficiency (Lenfant, 2002). A second hypothesis suggests that heterozygote deficiency could be a consequence of selection. The higher level of chemical contamination in the three estuaries showing heterozygote deficiency for Prx6 gene may act as selective force. This interpretation does not fit the common observations of heterozygote advantage in fitness. It is commonly observed that heterozygotes use less energy for their metabolism than homozygotes, thus increasing their tolerance to environmental stressors (Holley and Foltz, 1987; Hawkins et al., 1989). In bivalves, David and Jarne (1997) associated heterozygosity at nine allozymic loci with better viability. Heterozygote advantage has also been described in relation to enhanced growth rates in clams (Gentili and Beaumont, 1988; Scott and Koehn, 1990). However, heterozygote deficiency in molluscs has also been reported to be associated to selection against heterozygotes. Juveniles of mussels resulting from pair matings revealed such a heterozygote deficit at allozymic loci (Mallet et al., 1985). The authors suggest that a genotype-dependent larval mortality constitutes the most probable cause of these observations. Environmental conditions could also select some particular alleles and also decrease heterozygosity rate for some loci in populations exposed to pollutants such as in *Idotea baltica* (De Nicola et al., 1992). Tremblay et al. (1998) observed in different stocks of mussels submitted to summer mortality that the more susceptible stock had a lower degree of multiple-locus heterozygosity. They suggested the hypothesis that high levels of heterozygosity are related with lower costs of maintenance. In oyster populations, Zouros and Foltz (1983) proposed that heterozygotes take fitness advantage as adults, primarily of their faster growth, and that the lower number of heterozygotes in populations results from some form of non-random fertilization. This non-random fertilization may be caused by differential time of spawning of individuals due to differences in body size (Zouros and Foltz, 1983). According to these authors, selection cannot be excluded.

Considering the frequencies of the different alleles we observed in this study, no significant frequency variations have been detected between estuaries. However, allele A was more represented in Belon estuary than in the three others (6–8% of difference). This non-significant decrease of allele A frequency in the most contaminated estuaries suggests that contaminants may act as selective factors on *Cg*Prx6 locus for this particular allele. Individuals carrying *Cg*Prx6-A allele may be potentially counter selected by environmental stress and then considered as less tolerant to pollution. However, differences between allele A sequence and other allele sequences do not systematically result in amino acid modification. The mutations observed in the nucleotide sequence of these alleles could be linked to mutations elsewhere in the coding sequence. Indeed, we analysed polymorphism only at exon 6 of the *Cg*Prx6 gene and allele A may be characterized by coding mutations located in other exons. On the other hand, if there is selection against allele A, it could result from a hitchhiking effect (Maynard Smith and Haigh, 1974; Barton, 2000).

The present study has provided a new physiological and genetic marker of environmental chemical stress in *C. gigas*. Expression analysis has shown that *Cg*Prx6 is regulated at the

transcriptional level by environmental factors. Polymorphism analysis revealed deficit in heterozygotes at this locus that may potentially be associated to environmental forcing. Further investigations and comparison with other exons and loci are now needed to confirm these conclusions.

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