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Differential Gene Expression in a Red Alga *Gracilaria textorii* (Suringar) Hariot (Gracilariales, Florideophyceae) between Natural Populations

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Abstract

The bio-molecules involved in defense mechanisms can be used as efficient biomarkers for physiological changes in organisms caused by both of internal and external stress. Thus, the expression level of genes which encoding such molecules serve as critical 'early warning system' for environmental assessment as well as health diagnosis of biological organisms. In this study, *Cytochrome P450*, *Heat shock protein 90*, *Ubiquitin* and *β-actin* gene were isolated for the first time from a red alga *Gracilaria textorii*. The quantitative differential gene expression analyses of three genes, *GteCYP1A*, *GteHsp90* and *GteUB*, were carried out in *G. textorii* sporophytes collected from two different localities, polluted Sujeong (Masan, Korea) and potentially unpolluted Danggeum (Daemaemuldo Is., Korea). The transcripts of all three tested genes were highly expressed in the Sujeong population. The results suggest: 1) the Sujeong site was more polluted than the Danggeum site; 2) *G. textorii* could be applicable to marine environment monitoring in coastal regions.

Keywords: *Gracilaria textorii*, Red alga, Differential gene expression, *GteCYP1A*, *GteHsp90*, *GteUB*, Real-time quantitative PCR

Living organisms are affected by various environmental factors originating from chemical, physical or biological sources. Defense mechanisms, such as immune, antioxidant and detoxification systems, have evolved in every biological organism for their survival against those factors. The biological substances involved in such mechanisms can be used as biomarkers, which can be defined as changes in a biological response to toxic exposure or to the toxic effects due to environmental changes, ranging from molecular to cellular, and from physiological responses to behavioral changes, and community structure, even to the function and structure of ecosystems[1,2]. Molecular biomarkers are now recognized as being particularly important in establishing ecological inferences to observed biochemical and/or physiological responses as an 'early warning' of environmental changes.

Marine macroalgae, also called as seaweeds, have ecological importance as they assist in supplying oxygen and are one of the primary producers in the marine food web. Most are sessile; thus, macroalgal species can reflect the environmental state in which they are distributed. They are mainly distributed in coastal and estuarine regions, where land-based pollutants finally accumulate. Thus, macroalgae may offer important advantages for defining the organism-environment interface and response to natural or anthropogenic stressors.

Gracilaria textorii (Suringar) Hariot (Figure 1) are globally distributed, including North-east Asia, South Asia, Indian Ocean Is., Pacific Ocean Is., Australia and New Zealand (Algaebase http://www.algaebase.org), and range in habitat from intertidal to sublittoral zones. *G. textorii* are distributed in all the Korean waters, including Jeju Island. The sporophytes can be observed from February to August in the intertidal zone, with gametogenesis starting from about July through to August. The sporophytes are disappear after

discharging their gametes. In the sublittoral zone; however, sporophytes can be found throughout the year, with the population size increasing during July to August (personal observation of YSO). Thus, this species is worth noting as a candidate for environmental risk assessment in coastal regions. For this purpose, we have challenged to isolate antioxidants and stress-responsive genes and the partial nucleotide of *cytochrome P450* (*CYP1A*), *heat shock protein 90* (*Hsp90*), and *Ubiquitin* (*UB*) homologues of *G. textorii* were obtained by reverse transcriptase polymerase chain reaction (RT-PCR). The CYP1A subfamily is responsive to environmental contaminants, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides and petroleum products, as well as an array of drugs. The induction of this enzyme is significantly related to the contaminant levels in the environment[3]. Heat shock protein 90 (Hsp90) is a cellular chaperone protein, with pivotal roles in folding, activation and assembly of client proteins involved in signal transduction, cell cycle control and transcriptional regulation[4]. The misfolded and unassembled proteins, in other words, after the function of hsp-90-chaperoned protein has been disrupted, undergo rapid destruction via an ubiquitin-dependent proteasome pathway[5]. Ubiquitin is a small, 76-amino acid protein that can be covalently attached to target proteins destined for removal from the cell. Ubiquitination serves as a signal for the degradation of short-lived or unnecessary proteins by proteasomes in the cell[6,7].

Figure 1. A red alga; *Glacilaria textorii*. The photograph was taken at a depth of 5 m in Seogwipo, Jeju, Korea by Prof. Yoon Sik Oh.

The objects of this study were to verify the usefulness of three potential biomarker genes isolated from a red alga *G. textorii* and the applicability of this macroalga for assessing the health status of a local marine ecosystem using its natural population.

Cloning of *GteCYP1A*, *GteHsp90*, *GteUB*, and *Gteβ-actin* cDNA

The partial nucleotide sequence of three stress-related and β-actin genes in *G. textorii* [Cytochrome P450 (*GteCYP1A*); heat shock protein 90 (*GteHsp90*), Ubiquitin (*GteUB*) and β-actin (*Gteβ-actin*)], were obtained by reverse transcription (RT-) PCR using primers for each gene (Table 1). The 249-bp, 446-bp, 420-bp and 586-bp cDNA fragments were amplified, which revealed significant matches to the *CYP1A*, *Hsp90*, *Ubiquitin* and *β-actin* genes of other organisms (data not shown). The nucleotide sequence and deduced amino acid sequence were submitted to the DDBJ/EMBL/GenBank nucleotide sequence database as *GteCYP1A* (accession number EU095965), *GteHsp90* (accession number EU095964), *GteUB* (accession number EU095963) and *Gteβ-actin* (accession number EU095962).

Table 1. Information on the primer sets used for the reverse transcription polymerase chain reactions to amplify the target genes.

Gene	Primer	Sequence	Reference
GteCYP1A	CYP-F CYP-R	5′-gtcactcataccaccgaatttgtca-3′ 5′-gcatcccaaacttgatggcgatctc-3′	*Chondrus crispus* (CO653243)
GteHsp90	Hsp90-F Hsp90-R	5′-ggataactgcgaggaaataatccca-3′ 5′-gtattctttgagttgctgaacacag-3′	*Rhodomonas salina* (DQ228111)
GteUB	UB-F UB-R	5′-ggacaaggaaggaattcctccggat-3′ 5′-gtccacacttcttcttacggcagtt-3′	*Gracilaria lemaneiformis* (DQ019224)
Gteβ-actin	Actin-F Actin-R	5′-gacatggagaagatttggcaccaca-3′ 5′-gtccttacggatgtcgacgtcgcac-3′	*Ulva nertusa* (AB097503)

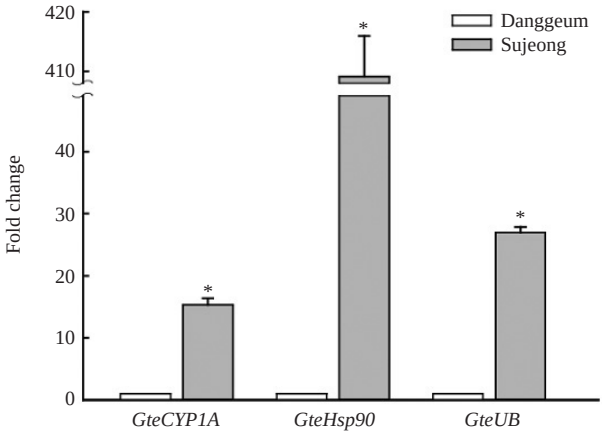

Figure 2. Differential gene expressions in *G. textorii* between two natural populations. Transcript levels were evaluated by real-time quantitative PCR, and expressed relative to the *Gteβ-actin* levels. *Significantly different from each Danggeum population ($P < 0.05$).

Quantitative Analysis of Gene Expression Changes by Real-time PCR

Real-time quantitative PCR was used to investigate the mRNA levels of *GteCYP1A*, *GteHsp90* and *GteUB* in the sporophytes between unpolluted (Danggeum, Daemaemuldo Is.) and polluted (Sujeong, Masan) sites. The normalized transcript level of *GteCYP1A* was approximately 15.3-fold higher in the red alga collected from Sujeong than from Danggeum (Figure 2). The transcript of the *GteHsp90* gene was also 409.1-fold higher in the alga sample collected from Sujeong in the qRT-PCR analysis (Figure 2). Finally, a higher level (27.0-fold) of the *GteUB* transcript in the alga collected from Sujeong compared to those from Danggeum was observed in the qRT-PCR analysis (Figure 2).

Discussion

Macroalgae are very good candidate organisms, which can elucidate the environmental health condition in estuarine and coastal regions. Thus, seaweeds can be potentially used for the biomonitoring and bioremediation of such pollutants[8]. However, the molecular biology on macroalgae is poorly understood compare to that of animals and terrestrial plants. Nevertheless, beginning with the pioneering work of an expressed sequence tags (ESTs) approach on *Gracilaria gracilis*[9], a considerable number of genes in several seaweed species have accumulated in public data bases[10-12]. Identification and profiling of stress-related genes have been successfully achieved[13,14]. More recently, toxicogenomic approaches were initiated in *Chodrus crispus*, which show extensive differential gene expression after exposure to stressors[14,15].

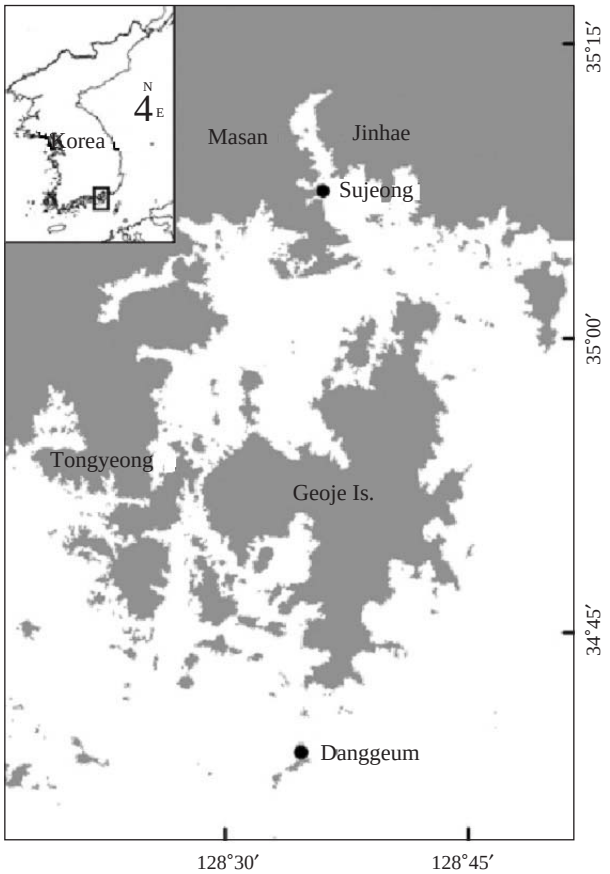

Figure 3. The map showing the two sampling locations of the *G. textorii*.

The main goal of this study was to investigate the possibility of using three potential biomarker genes to distinguish whether the organisms in a natural population have been exposed to pollution. In the initial stage of this study, isolation of the stress-related genes in *G. textorii* was planned. As a result, *G. textorii* homologues of the *CYP1A*, *Hsp90* and *Ubiquitin* genes were successfully isolated. The transcript levels of these three genes were then compared between two populations: a polluted and a possibly unpolluted site, Sujeong (Masan, Korea) and Danggeum (Daemaemuldo Is., Korea), respectively.

The cytochrome P450s (CYP1A) superfamily, play an important role in drug metabolism. The CYP1A are efficient metabolizers of organic pollutants such as polychlorinated biphenyls, polychlorinated-*p*-dibenzodioxins, and polychlorinated dibenzofurans[16]. A CYP1A-immunopositive protein was significantly higher in the mussel *Mytilus* sp. at contaminated sites

in response to organic pollutants[17]. The expression of *CYP1A* can be induced by many kinds of organic compounds, making it a robust biomarker used to investigate organismal exposure to organic pollutants.

Heat shock proteins (Hsps) play an important role in the cell's response to a various kinds of stressful conditions and thus are important for recovery and survival of organisms[18]. The gene expression of Hsps is induced by stressful factors such as extremes of temperatures, UV radiation, and presence of xenobiotics.

Ubiquitin covalently attaches to target proteins destined for removal from the cell. Short-lived or unnecessary proteins are broken down into short peptides by this massive protein complex. Ubiquitin is also responsible for numerous important biological processes, including stress responses, DNA repair, transcriptional regulation, long-term memory, immune responses, and organelle biogenesis[19]. Under environmental stress, various enzymes are damaged and lose their function. When this happens, ubiquitin levels rise in response to the increased number of denatured enzymes, which are then removed from the cell.

In all three genes tested in this study, the mRNA expression levels were much higher in the Sujeong than the Danggeum population (*GteCYP1A*: 15.3-fold; *GteHsp90*: 409.1-fold; *GteUB*: 27.0-fold). In regard to the function of tested genes, the Sujeong site seemed more polluted than the Danggeum site. In fact, Masan Bay is a well known polluted site in Korea; thus, the status of the contaminants in this area, such as polychlorinated biphenyls (PCBs) and chlorinated pesticides[20]; polycyclic aromatic hydrocarbons (PAHs)[21]; and polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)[22], have been intensively investigated. According to these reports, the concentrations of each hazardous chemical were shown to much higher in the Bay area than the open sea. Conversely, offshore of Tongyeong and Geoje, which includes Daemaemuldo Is., have been designated as 'Blue Belt' for oyster aquaculture by the Korean Government and US FDA.

In conclusion, the differential expressions of three biomarker genes in two natural populations, which inhabit polluted and unpolluted sites, were elucidated. From the differences in the mRNA levels, the Sujeong site could be considered more polluted than Danggeum site. This interpretation coincided with chemical analyses reports conducted in Masan Bay. Thus, the use of biomarker genes in *G. textorii* of natural population seems significant enough for environmental quality assessment. This approach can be expended to various stress-related genes, such as Catalase, Glutathione S-transferase, Glutathione peroxidase and Superoxide dismutase, etc. A comparison of the biomarker gene transcript level in objective organisms from natural setting could be introduced for the early diagnosis of environmental pollution.

Materials and Methods

Plants Collection and RNA Preparation

The sporophytes of *G. textorii* were collected from two intertidal zones at different locations, polluted Sujeong, Masan (May 15, 2007) and potentially unpolluted Danggeum, Daemaemuldo Island (May 16, 2007). Thalli of the plant were quickly frozen in dry ice. After being transport to the laboratory, the samples were stored at −80°C. Total RNA was extracted by the optimized method for macroalgal species[23].

Target Gene Isolation by Reverse Transcriptase (RT-) PCR

Isolated total RNA was analyzed spectrophotometrically. The ratio of the absorbances at 260 and 280 nm ranged from 1.6 to 1.8 and that for those at 230 and 260 nm from 1.8 to 2.0. To synthesize first-strand cDNA, 2 μg of total RNA was reverse transcribed with oligo-d$(T)_{15}$ primer using a Reverse Transcription System (Promega, USA). The primers used to amplify the target genes are listed in Table 1. Nucleotide sequences from conserved regions were selected as primers, after performing multiple alignments of each gene, using known sequences for algal species: *Chondrus crispus* (*CYP1A*, CO653243), *Rhodomonas salina* (*Hsp90*, DQ228111), *Gracilaria lemaneiformis* (*Ubiquitin*, DQ019224) and *Ulva nertusa* (*β-actin*, AB097503). Amplification was carried out in an MJ Research (USA) PCT-200 thermal cycler, involving denaturation for 5 min at 95°C, followed by 30 amplification cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C, with a final extension for 7 min at 72°C. The PCR products were extracted from agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Germany), and then ligated into a pGEM-Teasy vector (Promega, USA). Plasmids containing the PCR products were isolated from *E. coli* transformant colonies selected from ampicillin and X-Gal screening. Purified DNAs were sequenced using an ABI 3100 DNA Sequencing System (Applied Biosystems Inc., USA), with T7 and SP6 primers.

5′-Rapid Amplification of cDNA Ends (RACE) of *GteUB*

First-strand cDNA was synthesized using a SMART PCR cDNA synthesis kit (BD Biosciences, USA). For

Table 2. The list of the real-time quantitative PCR primers used for the target and *Gteβ-actin* genes of *G. textorii*.

Gene		Nucleotide sequence
GteCYP1A	Forward	5′-GAGCTCCAAACATGGCGCGA-3′
	Reverse	5′-GTCCCTCTGCATAGAAGGGA-3′
GteHsp90	Forward	5′-GAGAACAAGGAGGACTACAA-3′
	Reverse	5′-GGCCTTCTTCGACTCACCAG-3′
GteUB	Forward	5′-GCAAGCAGCTCGAGGATGAT-3′
	Reverse	5′-GCGGGAGTCGCGCATAGCAC-3′
Gteβ-actin	Forward	5′-TCAACCCAAAAGCCAACAGAG-3′
	Reverse	5′-GCAAAATCGAGGGCAACATAGCA-3′

5′-RACE, an oligonucleotide primer (5′-GCG GGA GTC GCG CAT AGC AC-3′), corresponding to *G. textorii Ubiquitin* (*GteUB*), and the SMART IV oligonucleotide (5′-AAG CAG TGG TAT CAA CGC AGA GT-3′; BD Biosciences, USA) were used. The PCR conditions were as follows: 25 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 60 s. The PCR products were separated on a 1% agarose gel. The amplified DNA fragments were eluted and purified using a gel extraction kit (Qiagen, Germany), and then ligated to a pGEM-T Easy vector (Promega, USA). Sequencing of positive clones was carried out on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA).

mRNA Quantification by Real-time Quantitative PCR

The expression levels of the three biomarker genes in *G. textorii* collected from both localities were quantified using real-time quantitative PCR analyses. Real-time quantitative PCR was performed in triplicate in 384-well plates using an Applied Biosystems Prism 7900 Sequence Detection System (Applied Biosystems, USA); the *β-actin* gene was used as an internal control. Briefly, total RNA was extracted, as described above, and analyzed on an Agilent 2100 bioanalyzer (Agilent Technologies, USA). RNAs with a rRNA ratio (28S/18S) >1.6 were used for the real-time quantitative PCR. cDNA was synthesized using a Superscript First-Strand Synthesis System (Invitrogen, USA). The sequences of the forward and reverse primers for each gene and *β-actin* are shown in Table 2. The nucleotide sequences of each target gene fragment amplified by real-time PCR were confirmed (data not shown). The thermal conditions for PCR were 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Each real-time quantitative PCR was conducted on serially diluted cDNA (1, 0.5, 0.25 and 0.125), which was used to generate relative standard curves for the *β-actin* and target genes. The transcript of the *β-actin* gene was used as an internal standard in all experiments.

A SYBR green DNA PCR Kit (Applied Biosystems, USA) was used for the real-time PCR analysis. The relative differences in the expressions between groups were expressed using threshold cycle (Ct) values, as follows: Ct values for *β-actin* and the target genes were derived from standard curves, using each control cDNA as templates (serially diluted 1, 0.5, 0.25 and 0.125), with the significance of the corresponding standard curves confirmed.

Statistical Analysis

All data are presented as means ± standard deviations (SD). Two-group comparisons of the control and chemical-exposed groups were made using Student's *t* test. Statistical significance was assigned at $P < 0.05$.

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