

Changes in Gene Expression in the Brown Alga *Undaria pinnatifida* (Harvey) Suringar (Laminariales, Pheophyceae) between Natural Populations

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Accepted 4 April 2011 DOI 10.1007/s13530-011-0083-4
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Abstract

Genes involved in defense mechanisms can be used as efficient biomarkers of physiological changes in organisms caused by both endogenous and exogenous stress. Thus, the expression levels of such genes serve as a critical 'early warning system' for the environmental assessment and health of biological organisms. In this study, the transcription levels of *Hsp70* and *vBPO* in *Undaria pinnatifida* sporophytes were quantitatively compared between two distinct natural populations collected from uncontaminated Mijo (Namhae, Korea) and industrially-polluted Myodo Is. (Yeosu, Korea) in order to verify their potential as biomarker genes and the applicability of this macroalga for assessing the health status of a local marine ecosystem. The results found that the two tested genes were highly expressed in the Myodo population. The results suggest that *U. pinnatifida* itself and the selected two genes could be applicable to monitoring of marine environments in coastal regions.

Keywords: *Undaria pinnatifida*, Brown alga, Differential gene expression, *UpiHsp70*, *UpivBPO*, Real-time quantitative PCR

Introduction

Coastal areas play a number of important environmental roles, including transfer of matter, energy, and

living organisms between terrestrial and marine ecosystems. These regions contain critical habitats rich in biodiversity with high biological productivity. Such benefits promote increases in the coastal population, however, which produces sewage and industrial and/or agricultural effluents that worsen the coastal ecosystem itself. Anthropogenic pollutants act as stressors to biological organisms and induce metabolic changes, which are regulated by molecular signals related to self-defense mechanisms, such as immune, antioxidant, and detoxification systems. The molecular information involved in such mechanisms can be used as molecular biomarkers, which are defined as the change in a biological process in response to toxic exposure or to toxic effects caused by environmental changes^{1,2}.

Marine macroalgae, commonly known as seaweeds, are very important components of marine ecosystems and are valuable sources of food, biochemicals, and pharmaceuticals. They assist in supplying oxygen and are one the primary producers in the marine food web. They also assist in the structuring of aquatic ecosystems by offering a fertile habitat to many aquatic organisms. Seaweeds are probably one of the sentinel species in coastal environments since most of them are sessile, directly contact water mass, and inhabit coastal regions where land-based pollutants finally accumulate.

Undaria pinnatifida (Harvey) Suringar (Figure 1) (Miyok in Korean) shows global distribution, including in North-east Asia, Europe, North to South America, Australia, and New Zealand (Algaebase <http://www.algaebase.org>), and it inhabits intertidal to sublittoral zones in rocky shores. For these reasons, this species is worthy of investigation as a sentinel species for environmental changes. This species is particularly important as a food source in Korea, Japan, and China. Moreover, fucoidan, a sulfated polysaccharide isolated from this species shows various biological activities such as inhibition of *Herpes* virus reactivation³, anti-tumor effects⁴, and defensive effects against virus infection⁵. Another biochemical substance, polyunsaturated fatty acids, isolated from this species also shows anti-inflammatory activities⁶. Recently, we isolated *U. pinnatifida* homologues genes related to natural and anthropogenic stress responses and successfully

identified the partial nucleotide sequences of two possible candidates, Heat shock protein 70 (Hsp70) and Vanadium-dependent bromoperoxidase (vBPO). The Hsp70 chaperones are found in various cellular

compartments and are induced by a variety of biological stresses⁷. Meanwhile, Vanadium-dependent bromoperoxidase (vBPO), a haloperoxidase, plays a role in synthesizing halogenated organic metabolites related to defense and pigmentation in seaweeds⁸.

The objectives of this study were to verify the usefulness of these two potential biomarker genes isolated from the brown alga *U. pinnatifida* as well as the applicability of this macroalga for assessing the health status of a local marine ecosystem using its natural population.

Results and Discussion

Cloning of *UpiHsp70* and *UpivBPO* cDNA

The partial nucleotide sequences of two stress-related and β -actin genes in *U. pinnatifida* [Heat shock protein 70 (*UpiHsp70*), Vanadium-dependent bromoperoxidase (*UpivBPO*) and β -actin (*Upi β -actin*)], were obtained by reverse transcriptase (RT-) PCR using primers for each gene (Table 1). The 812-bp, 499-bp, and 636-bp cDNA fragments were amplified, which revealed significant matches to the *Hsp70*, *vBPO*, and β -actin genes of same or other organisms (data not shown).

Quantitative Analysis of Gene Expression level Changes

Real-time quantitative PCR (qRT-PCR) was used to investigate the mRNA levels of *UpiHsp70* and *UpivBPO* in sporophytes between unpolluted (Mijo, Namhae) and polluted (Myodo Is., Yeosu) sites.

The normalized transcript level of *UpiHsp70* was approximately 1.68-fold higher in the brown alga collected from Myodo than from Mijo (Figure 2). Heat shock proteins (Hsps) play an important role in the cellular response to various kinds of stressful conditions and thus are important for recovery and survival of organisms⁹. The gene expression of Hsps is induced by stressful factors such as extreme temperatures, UV radiation, and xenobiotics. Hsps are designated according to their molecular weight, such as Hsp27, Hsp60,



Figure 1. Brown alga; *Undaria pinnatifida*.

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

| Gene | | Nucleotide sequence | Reference |
|------------------------------------|---------|--------------------------------|--|
| <i>UpiHsp70</i> | Forward | 5'-ACTTCCTGCAGGAGTTCAAG-3' | <i>Undaria pinnatifida</i> (FJ375361) |
| | Reverse | 5'-TGGTGATGGTGATCTTGTCT-3' | |
| <i>UpivBPO</i> | Forward | 5'-AACACGGACCTGCTCTCGC-3' | <i>Laminaria digitata</i> (AJ491787) |
| | Reverse | 5'-GCCGGGGGGATGTCGTCGCT-3' | |
| <i>Upiβ-actin</i> | Forward | 5'-TCCGTTGCCCCGAGGTGCTGTTCC-3' | <i>Saccharina japonica</i> (FJ375360) |
| | Reverse | 5'-ACCGCCACCACCACCACATCACG-3' | |

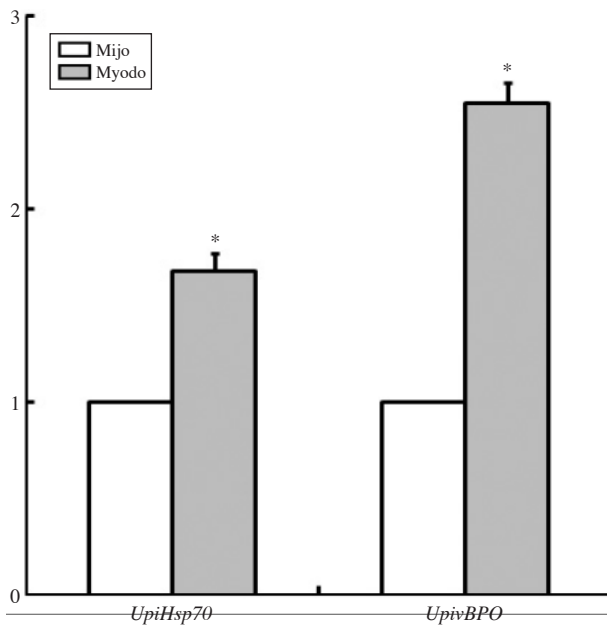


Figure 2. Differential gene expression in *U. pinnatifida* between two natural populations. Transcript levels were evaluated by real-time quantitative PCR and expressed relative to *Upiβ-actin* levels. *Significantly different from each Mijo population ($P < 0.05$).

Hsp70, and Hsp 90. Among Hsps, the 70 kDa protein (Hsp70) family is the most well studied. The Hsp70 family is a set of highly conserved proteins that assist protein folding processes, guide translocation proteins across cellular organelle membranes, disassemble oligomeric protein structures, and facilitate proteolytic degradation of unstable proteins in response to a variety of biological stresses⁷. Induction of Hsp70 and its gene expression in response to environmental stresses has been demonstrated, and its usage as a biomarker of exposure to environmental contaminants in various kinds of organisms, including seaweed, has been suggested¹⁰⁻¹⁴.

The transcription level of *UpivBPO* was 2.55-fold higher in the alga sample collected from Myodo compared to that from Mijo in the qRT-PCR analysis (Figure 2). Vanadium-dependent bromoperoxidase (vBPO) is a haloperoxidase that catalyzes the oxidation of halides, such as iodide, bromide, and chloride, by hydrogen peroxide. These halogenated organic compounds are known to be associated with defense and pigmentation in marine algal species. Thus, halogenated marine metabolites possess biological activities of great pharmacological interest, including anti-microbial activities⁸. vBPO has been isolated and characterized from all types of seaweeds, including green algae¹⁵, brown algae¹⁶, and red algae¹⁷. In previous studies,

vBPO was identified as a stress-related gene in *Laminaria digitata*¹⁸; both its protein level and gene expression level were upregulated after exposure to copper in *Ectocarpus siliculosus*¹⁴. Thus, this gene potentially has warning functions that reflect the environmental conditions in which the organism inhabits.

Algae are, without doubt, important contributors to global productivity, biological cycling, and are critical components of many habitats, especially marine ecosystems. They are also important economically, since they serve as a food source and natural product with various kinds of bioactivities. Furthermore, macroalgae in marine habitats are very good candidates for elucidating the environmental health conditions in estuarine and coastal regions. Thus, seaweeds can be potentially used for the biomonitoring and bioremediation of anthropogenic pollutants¹⁹. However, the molecular biology and genomics of these organisms are poorly understood compared to that of animals and terrestrial plants. Nevertheless, ever since the pioneering expressed sequence tags (ESTs) analysis of *Gracilaria gracilis*²⁰, a considerable number of genes from several seaweed species have accumulated in public databases²¹⁻²³. Identification and profiling of stress-related genes have been successfully achieved^{18,24}. Recently, toxicogenomic approaches were initiated in red alga, *Chodrus crispus*^{24,25}, and subsequently in brown alga, *Ectocarpus siliculosus*²⁶, both of which show extensive differential gene expression after exposure to stressors. A proteomic approach to identify proteins that are regulated by copper exposure was reported more recently¹⁴. Our research group also demonstrated in red alga, *Gracilaria textorii*, that the expression levels of genes involved in cellular defense mechanism changed in response to anthropogenic contaminants, and this was also found in natural populations²⁷. Subsequently, we tried to isolate the candidate genes related to environmental stress responses in representative brown algae in North-east Asia. As a consequence, we obtained the partial sequences of two stress-related genes (*UpiHsp70* and *UpivBPO*) in brown alga, *U. pinnatifida*, which has ecological and economical importance, and investigated the potential of these two genes as biomarkers of pollution exposure in a natural population. The transcription levels of the two genes were thus compared between two populations: a polluted site, Myodo (Yeosu, Korea) and a possibly unpolluted site, Mijo (Namhae, Korea). For the two genes tested in this study, the mRNA expression levels were higher in the Myodo than the Mijo population (*UpiHsp70*: 1.68-fold; *UpivBPO*: 2.55-fold). In regard to the functions of the tested genes, the Myodo site seemed more polluted than the Mijo site.

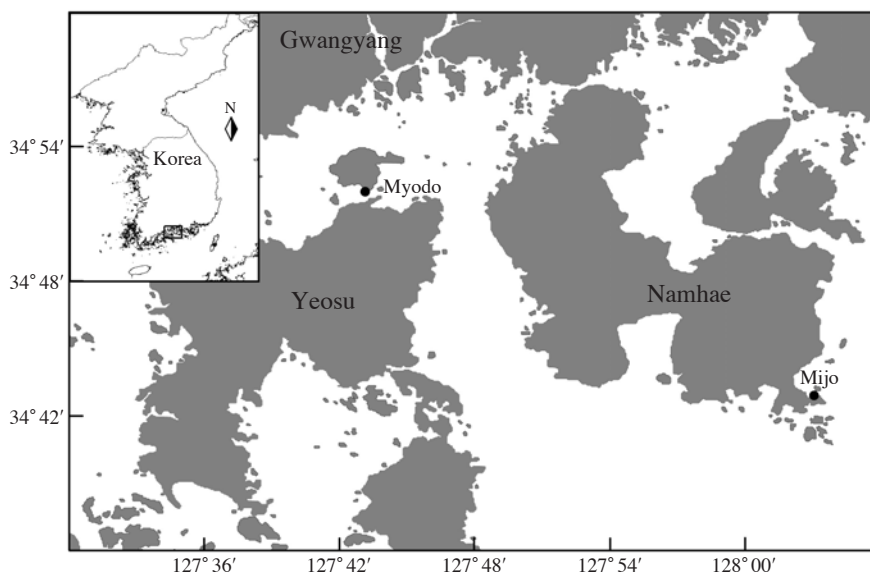


Figure 3. Map showing the two sampling locations of *U. pinnatifida*.

Myodo Is. is located in the center of Gwangyang Bay, which is surrounded by POSCO's Gwangyang Steel Works, Yeosu Petrochemical Complex, and Yecheon Industrial Complex. Thus, the coast of the bay area is assumed to be heavily contaminated by various types of industrial pollutants. In fact, previous reports which examined the sedimentary conditions of Gwangyang Bay revealed high levels of heavy metals²⁸; organochlorine pesticides²⁹; polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (co-PCBs)³⁰. On the other hand, Mijo faces the open sea and is part of Hallyeo Maritime National Park in Korea, where seawater quality, flora, and fauna are surveyed and managed by the Korean government.

In conclusion, the transcription levels of biomarker genes in *U. pinnatifida* from a natural population served as an effective measure of the environmental conditions, since higher levels of mRNA were observed in the contaminated site group. This approach can be expended to more genes that are involved in a wide spectrum of stress-related pathways, such as hypoxia, oxidative stress, apoptosis, and so on. Therefore, transcription level comparison between objective organisms might have prognostic value for environmental pollution.

Methods

Plants Collection and RNA Preparation

Sporophytes of *U. pinnatifida* were collected from

two intertidal zones at different locations, polluted Myodo Island, Yeosu (April 12, 2009) and potentially unpolluted Mijo, Namhae (April 11, 2009) (Figure 3). 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 from the soft part of thalli by the optimized method for macroalgal species³¹.

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 while that at 230 and 260 nm ranged 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, Madison, USA). The primers used to amplify the target genes are listed in Table 1. Nucleotide sequences from conserved regions were selected as primers using known sequences for the following algal species: *Undaria pinnatifida* (*Hsp70*, FJ375361), *Laminaria digitata* (*vBPO*, AJ491787), and *Saccharina japonica* (*β -actin*, FJ375360). Amplification was carried out in an MJ Research PCT-200 thermal cycler (Waltham, USA), involving denaturation for 5 min at 95°C , followed by 30 amplification cycles of 30 s at 95°C , 30 s at 50°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), followed by ligation into pGEM-Teasy vector (Promega, Madison, USA). Plas-

Table 2. The list of the real-time quantitative PCR primers used for the target and *Upi β -actin* genes of *U. pinnatifida*.

| Gene | | Nucleotide sequence |
|------------------------------------|---------|---------------------------------|
| <i>UpiHsp70</i> | Forward | 5'-CGCTAAGATGTCTGAAGGGGCAGAT-3' |
| | Reverse | 5'-CGGGGTCACGTCGAGCAGAAG-3' |
| <i>UpivBPO</i> | Forward | 5'-ACGGCCGTTTGTCTTACTCCA-3' |
| | Reverse | 5'-TGCGATCTACCCATTACCAC-3' |
| <i>Upiβ-actin</i> | Forward | 5'-ACCGGTATTGTGCTGGACTCT-3' |
| | Reverse | 5'-GTTTCTCTTTGATATCTCTTACGA-3' |

mids containing 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., Foster City, USA), with T7 or SP6 primers.

mRNA Quantification by Real-time Quantitative PCR

The expression levels of the two biomarker genes in *U. pinnatifida* collected from both localities were quantified by real-time quantitative PCR analyses. Five individuals of each site were used for analyses. Real-time quantitative PCR was performed in triplicate in 384-well plates using an Applied Biosystems Prism 7900 Sequence Detection System (Applied Biosystems Inc., Foster City, USA); 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, Santa Clara, USA). RNAs with an rRNA ratio (large subunit/small subunit) > 1.6 were used for real-time quantitative PCR. cDNA was synthesized using a Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, 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 with 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.

Statistical Analysis

All data are presented as mean \pm standard deviation (S.D.) of triplicate experiments. Group means were compared by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. A value of $P < 0.05$ indicated statistical significance.

Acknowledgements

This work was supported by the Korea Ocean Research & Development Institute Project ("A sustainable research and development of Dokdo", PM55861), by "Marine and Extreme Genome Research Center Program" of the Ministry of Land, Transportation and Maritime Affairs, Republic of Korea (KORDI Project No. PM56202) to SY, and the Korea Research Foundation Grant funded by the Korea Government (MOEHRD, Basic Research Promotion Fund, KRF-2006-311-C00697) to JHK.

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