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Thermal and Organic Chemical Stress Responsive Genes in Soft Coral, *Scleronephthya gracillimum*

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Abstract

The extensive isolation of genes responsive to stressful conditions from a soft coral *Scleronephthya gracillimum* was described. Soft coral colonies were exposed to thermal and chemical stressors to induce the expression of stress related genes. Differentially expressed genes by natural or anthropogenic stressors were identified by construction of standard and stress exposed-paired subtractive cDNA library. Thirty-two and thirty-seven kinds of candidate genes were identified from thermal or benzo[a]pyrene stress exposed group, respectively, which are associated with cell cycle, cell signaling, transcription, translation, protein metabolism, and other cellular functions. The expected function of each gene was described. The isolated and identified differentially expressed genes have a great potential to identify environmental stressors in global environmental changes and could act as molecular biomarkers for biological responses against environmental changes. Finally, it may open a new paradigm on soft coral health assessment.

Keywords: Thermal stress, Benzo[a]pyrene, Differentially expressed genes, Subtractive cDNA library, Soft coral, *Scleronephthya gracillimum*

The coral reefs, including soft coral communities, are known to be World's most valuable ecosystems in terms of ecological, economic and cultural capital but are in serious decline mainly due to the human-associated activities. Although the importance of the species has been continuously emphasized and management efforts have been successful locally, the worldwide decline of coral populations due to pollution[1], disease[2], and climate change[3] is reaching a crisis. Over the last 30 years, coral reef assessment has provided an extensive description of certain responses at population and community levels in terms of coral cover, diversity and population dynamics of other reef species. However, with only these descriptive approaches for assessment are incapable of identifying the causes of deterioration of coral reef ecosystems. More specifically, physiological responses such as changes in respiration[4,5], photosynthetic efficiency[6], growth rate[7], and bleaching[8] have commonly employed as measures of coral health. However, most of physiological measurements do not identify the stressors or the underlying molecular mechanisms controlling a response. Changes in gene expression and protein production are key elements of the stress response and usually occur before physiological damage is evident. Thus, diagnosis and quantification of the impact of stressors on corals can be possible by using the genes whose expression would turn on or off under a specific type of environmental change.

The soft coral, *Scleronephthya gracillimum* (Alcyonacea, Octocorallia, Anthozoa, Cnidaria), is found predominantly at depths of 15-40 m, in the subtropical ocean regions surrounding Jeju Island, Korea (Fig. 1). This species contribute to the species diversity of this area and offer a wide variety of habitats for other benthic marine animals, and facilitate the survival and maintenance of this unique and highly diverse biological community.

The representative environmental stressors in marine ecosystem are anthropogenic contamination such as sewage including persistent organic pollutants and a variety of toxic chemicals from land runoff. Benzo [a]pyrene (BaP) is one of the polycyclic aromatic hydrocarbons (PAHs) and a representative marine ecotoxicant. It has been well reported its bioaccumulative potential in many organisms[9] resulting in DNA damage, endocrine disruption, and reproductive disturbance. In this study, we described the strategy on extensive isolating and identifying both physical and chemical responsive genes by subtractive cDNA library construction (thermal and BaP) in *S. gracillimum*, and their potential usage as biomarkers to assess the health status of local marine ecosystem.

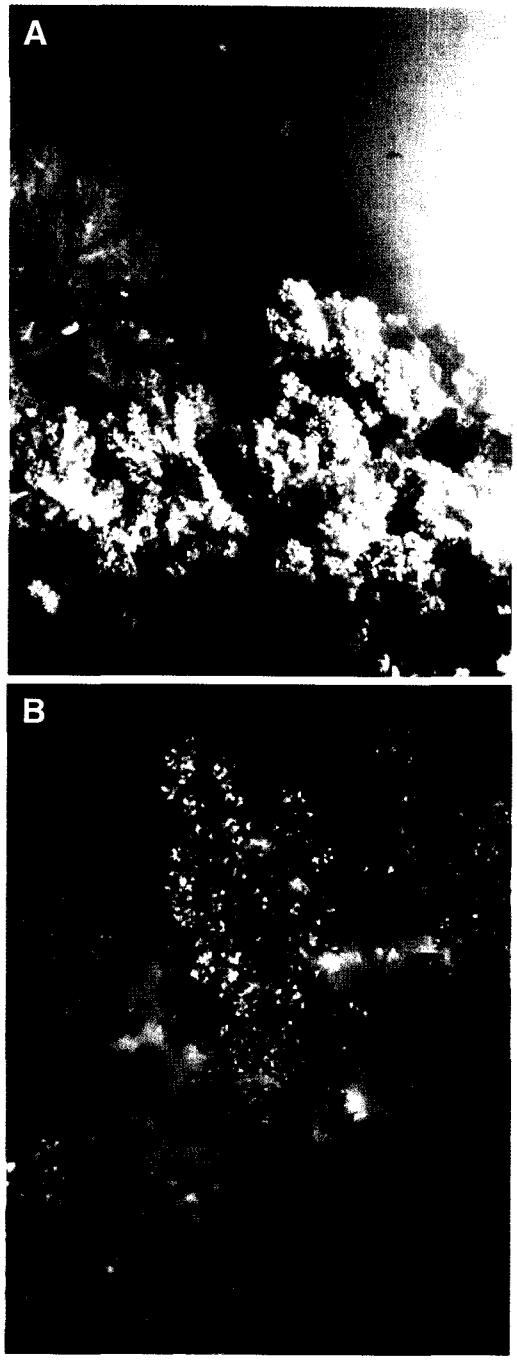

Fig. 1. A soft coral *Scleronephthya gracillimum*. A, the scenery of soft coral community at a depth of 20 m, in Munsom I., off Seogwipo, Jeju; B, detailed view of a colony of *S. gracillimum*.

Among the 500 clones which were randomly sequenced in subtractive cDNA library constructed from thermal stress exposed colonies, we obtained 32 authentic cDNA clones whose expressions were up-regulated by higher temperature. Potential functions of the genes were listed in Table 1. We also obtained 37 partial cDNA clones whose expressions were increased after the BaP exposure among the randomly sequenced 700 clones in subtractive cDNA library that was specifically constructed with organic pollutant exposed colonies. Presumptive functions of the genes were arranged in Table 2. The GenBank/EMBL/DDBJ accession number of each gene showing highest homology to isolated cDNA clones after using Blastx algorithm of the NCBI server was indicated.

Table 1. Up-regulated genes by thermal stress in *S. gracillimum*.

Gene function	GenBank/ EMBL/DDBJ Accession No.
Cell cycle	
G1 to S phase transition factor	NP_942101
Cell signaling	
GTP binding protein	NP_998640
Activated protein kinase C receptor	AAP20196
Serine/threonine protein kinase	BAC99099
Zinc finger protein 403	AY633742
EGF-like domain	NM_133930
Transcription	
Histone H2A	AAP94647
Histone H2A variant	P08991
Histone H3.2	AAB36495
Translation	
Ribosomal protein S15	AAK92184
40S ribosomal protein	XP_235014
Ribosomal protein S2	AAM33437
60S ribosomal protein L44	P90702
Ribosomal protein L5	AAM33437
Elongation factor 2	AAG13312
Polyadenylate binding protein	AAB88449
Protein folding and degradation	
Heat shock protein 70	AA038780
Ubiquitin-S27a	BAC56381
Mitochondria	
ADP, ATP carrier protein precursor	LAC27140
ABC transporter	AAS09272
Metabolism	
Fructose 1,6-bisphosphatase	AAT01078
Adenosylhomocysteinase	AAQ96656
Tyrosine 3-monooxygenase	NP_997770
cAMP responsive element modulator	NO_038526
Induced cAMP early repressor	CAC34846
Miscellaneous	
Gnb2l1-prov protein	AAH41541
Hemagglutinin/amebocyte aggregation factor	Q01528
Ferritin GF1	AAP83793
Sarcoglycan delta	AAQ97851
Actin, cytoskeletal 1	P53473
Profilin	P18320
Tubulin alpha-1 chain	P18258

Table 2. Up-regulated genes by organic chemical stress in *S. gracillimum*.

Gene function	GenBank/ EMBL/DDBJ Accession No.
Cell cycle	
G1/S-specific cyclin E1	P49707
Cell signaling	
GDP-dissociation inhibitor	CAB46230
Notch protein homologue	S18188
NK-4 homeobox protein	AAP88432
Hypoxia-inducible factor 1 alpha subunit	AAR19225
Cathepsin B precursor	AAQ83887
Cathepsin Z precursor	AA064476
Focal adhesion kinase	ANN15022
F-box protein	NP_056606
Ras-related protein YPTC6	Q39572
Transcription	
Histone H1	AAP94647
Histone H2A variant	P08991
Translation	
Poly A binding protein	NP_957176
Protein folding and degradation	
Heat shock protein 90	CAC38753
Mitochondria	
NADH dehydrogenase	NP_062096
ATP synthase beta subunit	AAT06141
Cytochrome P450	CAB62060
Metabolism	
Alpha-glucosidase	YP_004082
Phosphoenolpyruvate carboxykinase	AAO32202
Glucosidase	NP_999069
Proteophosphoglycan	CAB46679
Na^+ glucose cotransporter type 1	BAA22950
Annexin VII isoform 1	NP_001147
Protein disulfide isomerase	AAK71636
Immune response related	
Macrophage expressed protein	AAR82935
Uromodulin	NP_003352
Miscellaneous	
Kin-1-prov protein	AAH46697
rBAT protein	CAA64747
UV excision repair protein RAD23	P54726
Epidermis specific serine protease	BAA84941
Gnb2l1-prov protein	AAH41541
Actin, cytoskeletal IB	P53473
Arsenite transporter	NP_004308
Pyruvate dehydrogenase	JC5089
Gstm2-prov protein	AAH54171
Myophilin	Q24799
Galaxin	BAC41519

Discussion

As the initial stage of environmental stress responsive gene isolation in *S. gracillimum*, 32 and 37 reliable candidate genes from thermal and organic pollutant exposed colonies were obtained. Each candidate genes could represent potential biomarkers for assessing the health condition of a soft coral, *S. gracillimum* after evaluating their usefulness through the gene expression analyses using real-time PCR (data not shown). The health condition of soft corals may reflect the health condition of entire soft coral community. We describe the expected functions for most of gene candidates in the following sections.

Thermal Stress Specific Gene Candidates

G1 to S phase transition factor and GTP binding protein are involved in regulation of cell growth. Two kinds of protein kinase related genes, activated protein kinase C receptor and serine/threonine protein kinase, were isolated. Eukaryotic protein kinases[10] are enzymes belong to a very extensive protein family which share a conserved catalytic core common with both serine/threonine and tyrosine protein kinases. Zinc finger protein 403 (ZFP 403) homologues were known as xenobiotic stimulus responsive genes. The partial cDNA containing EGF-like domain was identified. The EGF-like domain is composed of 30-40 amino acids containing 6 cysteines and found originally in epidermal growth factor and also in a range of proteins involved in cell signaling.

Three kinds of histone homologues, Histone H2A, Histone H2A variant, and Histone H3.2, were found in subtractive cDNA library. Histones are the chief proteins of chromatin. They act as spools around which DNA winds and they play a role in gene regulation.

We also obtained five kinds of ribosomal proteins. Ribosomes are the particles that catalyze mRNA-directed protein synthesis in all organisms. Many of ribosomal proteins, particularly those of the large subunit, are composed of a globular, surfaced-exposed domain with long finger-like projections that extend into the rRNA core to stabilize its structure. Most of the proteins interact with multiple RNA elements, often from different domains and the proteins serve to organize and stabilize the tertiary structure of rRNA. Polyadenylate binding protein (PABP) recognizes the 3′ mRNA poly (A) tail and plays an essential role in eukaryotic translation initiation and mRNA stabilization/degradation.

The HSP70 family is a set of highly conserved proteins that are induced by a variety of biological stresses, including heat stress. Ubiquitin is a small, 76-amino acid protein which 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[11-13]. Recently, its usefulness as a biomarker for environmental stressors was demonstrated[14].

ADP, ATP carrier protein precursor catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane. ATP-binding cassette transporter genes (ABC-transporter genes) are a superfamily of genes which encode the ABC-transporter proteins. These are transmembrane proteins function in the transport of a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids and sterols, and drugs[15].

Fructose 1, 6-bisphosphatase has catalytic activity in carbohydrate metabolism. Adenosylhomocysteinase is a competitive inhibitor of S-adenosyl-L-methionine-dependent methyl transferase reactions. Therefore adenosylhomocysteinase may play a key role in the control of methylations via regulation of the intracellular concentration of adenosylhomocysteine. Tyrosine 3-monooxygenase is an adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathway. It binds to a large number of partners, usually by recognition of a phosphoserine or phosphothreonine motif. cAMP responsive element modulator (CREM) is one of the nuclear factors involved in the regulation of gene expression by cAMP and has an important role in spermatogenesis. Induced cAMP early repressor has been proposed to function as a tumor or cell proliferation suppressor[16].

Guanine nucleotide-binding protein beta subunit 2-like 1 (Gnb2l1-prov protein) seems to bind protein kinase C acting as an intracellular receptor to anchor the activated PKC to the cytoskeleton. Sarcoglycan delta is a component of the sarcoglycan complex, a subcomplex of the dystrophin-glycoprotein complex which forms a link between the f-actin cytoskeleton and the extracellular matrix. Profilins are thought to play a central role in the regulation of de novo actin assembly by preventing spontaneous actin polymerization through the binding of actin monomers, and the adding of monomeric actin to the barbed actin filament ends[17].

BaP Stress Specific Gene Candidates

G1/S-specific cyclin E1 is essential for the control of the cell cycle at the G1/S transition. GDP dissociation inhibitors are proteins that regulate the GDP-GTP exchange reaction of members of the rab family. Notch protein is transcriptional regulator playing a central role in Notch signaling. The signaling pathway involved in cell-cell communications regulates a broad spectrum of cell fate determinations. NK-4 encodes a homeodomain transcription factor which is required for development of the dorsal mesoderm and its derivatives in the *Drosophila* embryo[18]. Hypoxia-inducible factor 1 (HIF1) is a transcription factor that regulates the expression of genes associated with adaptation to the reduced oxygen pressure[19].

A cathepsin is a member of protease family, which is believed to participate in intracellular degradation and turnover of proteins. It also has been implicated in tumor invasion and metastasis. Focal adhesion kinase (FAK) regulates the cancer cell adhesion and invasion into extracellular matrix (ECM). In addition, phosphorylation of FAK correlates with the increase of cell motility and invasion[20]. F-box proteins regulate diverse cellular processes including cell cycle transition, transcriptional regulation and signal transduction[21]. Ras-related protein induces morphological reversion of a transformed cell line. Ras is known to be an oncogene.

The Poly A binding protein (PABP) binds to the 3′-poly (A) tail of mRNA found on most eukaryotic mRNAs and together with the poly (A) tail has been implicated in governing the stability and the translation of mRNA[22].

Heat shock protein 90 (HSP90) is a cellular chaperone protein required for the activation of several eukaryotic protein kinases including the cyclin-dependent kinase CDK4. Cytochrome P450 is a family of powerful detox enzymes.

UV excision repair protein RAD23 encodes a protein acting in nucleotide excision repair (NER) of UV-damaged DNA[23].

Methods

Soft Coral and Environmental Stressors Exposure

The *S. gracillimum* soft coral colonies were collected at water depths of approximately 15-25 m near Seogwipo, Jeju Island, Korea, using standard scuba techniques. After transport to the aquatic facility in the laboratory, the specimens were allowed to acclimate for 7 days in 22°C filtered seawater with a salinity of 35 ppt at a light: dark cycle of 14 : 10 hr. After this acclimation period, a thermal-stressed group was assigned to 28°C water tank for 24 hr. Control group was kept in 22°C seawater. A BaP-exposed group was assigned to seawater including 100 ppb BaP (dissolved in 0.1% DMSO) (Sigma) for 24 hr and the remaining group was kept in seawater including 0.1% DMSO and used as a control. The colonies were gone into the next step of subtractive cDNA library construction.

RNA Isolation

The total RNA was extracted by following the method to be optimized for *S. gracillimum*[24]. In brief,

the soft coral polyp tissues were mortar-pulverized in liquid nitrogen. The polyp powder was then homogenized in 700 μL of lysis solution [35 mM EDTA, 0.7 M LiCl, 7% SDS, 200 mM Tris-Cl (pH 9.0)], and RNA was extracted with 700 μL of water-saturated phenol. One-third-volume of 8 M LiCl was added to the retained aqueous phase, and this was maintained at 4°C for 2 hr. The RNA was precipitated after centrifugation at 14,000 rpm for 30 min and the precipitate was resuspended in 300 μL of DEPC-treated water. The RNA was re-precipitated with a 1/10 volume of 3 M sodium acetate (pH 5.2) and the same volume of isopropanol. The precipitated RNA was rinsed with 70% ethanol (diluted in DEPC-treated water), and dissolved in an appropriate volume of DEPC-treated water (30-40 μL). RNA samples were run on formaldehyde gel in order to verify RNA integrity.

Subtractive cDNA Library Construction

We constructed two subtractive cDNA libraries to identify the differentially expressed genes responding to thermal stress and the chemical contaminant such as BaP. RNA was extracted from soft coral polyp tissues of control and experimental groups. Each subtractive cDNA library was constructed by using PCR-select cDNA subtraction kit (BD Biosciences, San Jose, CA) following the manufacturer's direction. Sequencing of positive clones was carried out with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

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