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Transcriptional Responses in *Ecklonia cava* to Short-term Exposure to Hyperthermal Stress

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Abstract

Rapid climate change accelerates the damage to many valuable marine ecosystems, including macroalgal forests and coral reefs. To understand the biological responses of kelp species to increased seawater temperatures, we isolated the genes that responded to hyperthermal stress conditions in *Ecklonia cava*. Thalli of *E. cava* were exposed to seawater at 20 or 24°C, and the genes whose transcript levels changed in response to hyperthermal stress were identified with microarray hybridization. Eighty-nine candidate genes were identified, 49 of which were upregulated and 40 were downregulated by hyperthermal stress. Gene Ontology mapping showed that cellular oxidant detoxification, the regulation of cellular metabolic processes, and photosynthesis processes were affected by increased ocean temperatures. The functions of some isolated genes were also analyzed to predict the possible changes in the metabolism of *E. cava* under hyperthermal stress. The differentially expressed genes isolated here may serve as molecular biomarkers, allowing us to better understand the biological responses of marine organisms to environmental changes.

Keywords: Brown alga, Differentially expressed genes, Climate change

Introduction

Marine macroalgae, also called seaweeds, are ecologically important because they produce oxygen and are one of the primary producers in the marine food web. Among the brown macroalgae (Phaeophyta), several genera, including *Macrocystis*, *Laminaria*, *Saccharina*, *Undaria*, *Ecklonia*, and *Eisenia*, form kelp forests, the world's most productive and dynamic ecosystem. Kelp forests provide habitats for various kinds of organisms. However, they are now threatened by the rising seawater temperatures associated with rapid climate change and the pollution of coastal regions by xenobiotics.

Ecklonia cava Kjellman (Laminariales, Laminariaceae) is commonly distributed in Korea (southern part of the east coast, including Ulleungdo Island and Dokdo Island; south coast; and Jeju), the central to south coasts of China, and the central coasts of Japan, where the seawater temperature is around 12-25°C during the year. It forms kelp forests at depths of 5-20 m, and helps to maintain marine biodiversity by offering unique habitats to many other marine organisms. Therefore, the protection of *E. cava* is necessary for the conservation of the marine biodiversity in the coastal areas of the Northwest Pacific region. *E. cava* is also economically valuable. It is a source of natural pharmaceutical and medical products, including those with antioxidant effects[1,2], anti-aging effects[3], potential capacity to alleviate the symptoms of Alzheimer's disease[4], and positive effects on several human diseases[5-7].

Changes in transcript levels are the earliest and most sensitive biomarkers of the physiological responses to various environmental stresses. Therefore, the impact of environmental stress on kelp species can be identified and quantified using genes whose expression levels change in response to specific environmental changes. The metabolic and physiological changes induced by environmental stresses can also be predicted by considering the functions of the proteins encoded by these genes. We have previously reported changes in the transcript levels of several stress-responsive genes in natural populations of red alga[8] and brown alga[9] in response to marine environmental pollution. The gene expression changes induced by alginate oligosaccharides have also been investigated in a brown alga[10].

We isolated 2,740 cDNA clones of *E. cava* with an expressed sequence tag (EST) analysis, and developed the Ecava cDNA Microarray containing these cDNA clones to profile differentially expressed genes, to better understand the molecular responses to stress in *E. cava*.

In this study, we describe a strategy for isolating and identifying hyperthermal-stress-responsive genes in *E. cava* using microarray hybridization, to assess its biological responses to the rising seawater temperatures associated with global climate change. In brief, oxidative stress and DNA damage may be induced and photosynthetic processes, carbohydrate metabolism, and protein activation may be affected when *E. cava* is exposed to high temperatures.

Results and Discussion

Identification of Differentially Expressed Genes in a Hyperthermal-Stress-Exposed *E. cava* Using a Microarray Analysis

To identify the genes associated with hyperthermal-stress-induced metabolic changes, a gene expression profile of the thalli of *E. cava* incubated in 24°C was generated using the Ecava cDNA Microarray, which contains 2,740 *E. cava* genes. We reliably identified 89 genes whose transcript levels changed by >2.0-fold during hyperthermal incubation ($P<0.05$), 49 of which were upregulated (Table 1) and 40 downregulated (Table 2).

Gene Ontology (GO) analysis was performed to interpret the functions of these 89 genes, and the results are shown in Table 3. We summarized the biological processes at level 4, the molecular functions at level 3, and the cellular components at level 4. Among the GO terms identified, cellular oxidant detoxification, regulation of cellular metabolic process, and photosynthesis from the biological processes category, and oxidoreductase activity from the molecular functions category warrant further analysis. Various metabolic processes seemed to be affected, and in particular, oxidative stress may be induced by hyperthermal stress.

Functional Consideration on Differentially Expressed Genes

The transcript levels of two genes, encoding glutathione S-transferase (GST) and peroxyredoxin (PRDX), which are associated with the detoxification of potentially toxic reactive oxygen species (ROS), were increased by hyperthermal stress. GST plays a role in detoxification and is a crucial factor in determining the sensitivity of cells to various toxic chemicals, including environmental pollutants and the products of oxidative stress[11]. The expression of the *gst* gene increased by 2.04-fold after hyperthermal stress (Table 1). PRDXs are antioxidant proteins that protect cells against elevated concentrations of ROS[12,13]. In this study, the expression of *prdx* was increased by 3.07-fold in the experimental group (Table 1). Quinone oxidoreductase (NQO1) is a flavoprotein that catalyzes the two-electron reduction and detoxification of quinones and their derivatives, such as quinone imines, and nitro compounds. It plays very important roles in protecting cells against redox cycling, and oxidative stress, for example, by scavenging cellular superoxide[14,15]. The expression of *nqo1* was decreased by 2.20-fold in *E. cava* at 24°C compared with its expression in the control group incubated at 20°C (Table 2). These results suggest that oxidative stress is induced by hyperthermal stress in *E. cava*.

A 12 kDa protein is an extrinsic protein that is only present in algal-type photosystem II (PSII). It is assumed to play a role in the evolution of photosynthetic oxygen, such as in stabilizing the structure of the photosystem II oxygen-evolving complex (OEC), in the ion environment of oxygen evolution, and in protecting the OEC from heat-induced inactivation[16]. The transcript levels of *E. cava* photosystem II 12-kDa extrinsic protein homolog were increased by 4.05-fold by hyperthermal stress (Table 1).

Small heat shock proteins (Hsps) function in the inhibition of apoptosis, the organization of the cytoskeleton, and as chaperones[17]. In contrast, chaperonins are the chaperone proteins present in bacteria and eukaryotic subcellular organelles, such as mitochondria and chloroplasts[18]. In this study, the expression of the Hsp10 chaperonin gene (*hsp10*) was increased by 2.36-fold by hyperthermal stress (Table 1), suggesting that hyperthermal stress negatively affected the structures of cellular proteins. ABC excinuclease functions in DNA repair, acting on a wide spectrum of DNA adducts[19]. The expression of *E. cava* homologous to excinuclease ABC subunit B (*uvrB*) was increased by 2.03-fold by hyperthermal stress (Table 1), suggesting that the elevated temperature induced DNA damage.

Xylans are the major hemicellulose components of the plant cell wall. In our microarray experiment, the transcript levels of a xylanase, endo-1,4-beta-xylanase (*xyn2*), increased 3.85-fold compared with that in the control group (Table 1). The characteristics and significance of xylanases for industrial use have been described previously[20,21]. This result suggests that the cells in *E. cava* thalli are lysed when exposed to unexpectedly high temperatures. However, it is not yet clear whether this cell lysis process is induced by programmed cell death.

The RING finger domain is defined as a series of

Table 1. Gene expression in *E. cava* after hyperthermal stress, as determined by microarray analysis. Numbers indicate significant increases (fold changes) in expression relative to the expression in the control group ($P < 0.05$).

Gene description	Accession No.	Fold change
photosystem II 12-kDa extrinsic protein (*Ectocarpus siliculosus*)	CBJ28280	4.05
pyruvate dehydrogenase (*Rhodanobacter* sp. 115)	EIL98005	2.52
glycyl-tRNA synthetase (*Gemella morbillorum* M424)	ZP_07953692	4.83
LigA protein (*Streptomyces hygroscopicus* ATCC 53653)	ZP_07293342	5.98
pyrophosphatase, MutT/nudix family protein (*Burkholderia pseudomallei* NCTC 13177)	ZP_02490244	2.64
vacuolar membrane PQ loop repeat protein (*Trichophyton equinum* CBS 127.97)	EGE04466	3.57
glutathione S-transferase (*Ectocarpus siliculosus*)	CBJ48330	2.04
elongation factor 1 alpha gene (*Phaeostigma* sp. SS-2008)	EU414713	4.96
Gram-positive anchor (*Staphylococcus pettenkoferi* VCU012)	EHM68064	2.04
excinuclease ABC subunit B (*Agrobacterium* sp. H13-3)	YP_004279234	2.03
dehydrogenase E1 component (*Parvimonas* sp. oral taxon 393 str. F0440)	ZP_08757219	2.27
GDP/GTP exchange factor Sec2p (*Trichophyton equinum* CBS 127.97)	EGE09564	2.17
pmbA protein (*Neorickettsia sennetsu* str. Miyayama)	YP_506432	2.18
BAHD family acyltransferase, clade V (*Selaginella moellendorffii*)	XP_002992205	3.48
replication factor C protein (*Trichophyton tonsurans* CBS 112818)	EGD93764	2.03
hydroxyproline-rich glycoprotein DZ-HRGP-related (*Oryza sativa* Japonica Group)	NP_001065817	5.78
acyl-CoA thioesterase II (*Providencia rettgeri* DSM 1131)	ZP_06126244	2.06
G2 glycoprotein (Hantavirus Z37)	AAD56175	2.15
splicing factor, arginine/serine-rich 1/9 (*Nannochloropsis gaditana* CCMP526)	AFJ69494	2.19
amidase, putative (*Cordyceps militaris* CM01)	EGX94310	2.19
peroxiredoxin (*Laminaria digitata*)	CAQ52405	3.07
RING-finger-domain-containing protein (*Cordyceps militaris* CM01)	EGX87820	2.40
ribonuclease (*Paracoccus* sp. TRP)	ZP_08666042	2.03
ABC transporter (*Thermosediminibacter oceani* DSM 16646)	YP_003824754	2.67
YSIRK type signal peptide (*Streptococcus* sp. C150)	ZP_08047422	2.17
myb-domain-containing protein (*Polysphondylium pallidum* PN500)	EFA86488	4.09
serine acetyltransferase (*Methylacidiphilum fumariolicum* SolV)	ZP_10017420	2.00
NAD-dependent epimerase/dehydratase family (*Salinibacter ruber* M8)	YP_003571095	2.01
glyceraldehyde 3-phosphate dehydrogenase (*Ectocarpus siliculosus*)	CBJ31240	2.36
DNA mismatch repair protein (*Polysphondylium pallidum* PN500)	EFA76223	2.32
DNA-directed RNA polymerase sigma subunit RpoD (*Rothia dentocariosa* ATCC 17931)	YP_003982893	2.75
tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase (uncultured gamma proteobacterium)	BAL55389	3.06
DnaB-domain-containing protein (*Thiorhodovibrio* sp. 970)	ZP_09812059	2.86
FMN oxidoreductase (*Pusillimonas* sp. T7-7)	YP_004417796	2.02
light-harvesting complex protein (*Ectocarpus siliculosus*)	CBJ33312	2.94
cycloartenol synthase (*Medicago truncatula*)	CAA75588	2.52
histidine kinase (*Vibrio ichthyoenteri* ATCC 700023)	ZP_08744004	2.62
copper resistance protein D (*Kineococcus radiotolerans* SRS30216] SRS30216)	YP_001468149	2.57
inner-membrane translocator (*Roseobacter* sp. AzwK-3b)	ZP_01904755	2.00
ubiquitin fusion degradation protein (*Grosmannia clavigera* kw1407)	EFX06648	2.13
endo-1,4-beta-xylanase (*Verticillium dahliae* VdLs.17)	EGY23605	3.85
general secretory pathway protein L (*Xylella fastidiosa* 9a5c)	NP_298813	2.49
zinc-binding protein (*Vibrio vulnificus* MO6-24/O)	YP_004189937	2.90
10-kDa chaperonin (*Phytophthora infestans* T30-4)	XP_002998938	2.36
Populus EST from mildly drought-stressed leaves	CU231114	2.17
cationic amino acid transporter, putative (*Ricinus communis*)	XP_002515785	2.00
vacuolar membrane PQ loop repeat protein (*Trichophyton equinum* CBS 127.97)	EGE04466	2.55
ABC transporter (*Nakamurella multipartita* DSM 44233)	YP_003200206	3.95
ribulokinase (*Salmonella enterica* subsp. *Arizonae*)	YP_001571890	2.09

conserved cysteine and histidine residues[22]. The *Arabidopsis HOS1* gene encodes a protein containing a RING finger motif that regulates gene transcription and organismal development in response to cold environments[23]. In our microarray experiment, the hyperthermal treatment induced (by 2.40-fold) the expression of a gene encoding a RING-finger-domain-containing protein (Table 1).

The DEAD/DEAH box helicases are involved in various aspects of RNA metabolic processes[24]. The tran-

Table 2. Gene expression in *E. cava* after hyperthermal stress, as determined by microarray analysis. Numbers indicate significant decreases (fold changes) in expression relative to the expression in the control group ($P<0.05$).

Gene description	Accession No.	Fold change
cell death suppressor protein (*Ecklonia cava*)	–	−2.54
chromosome partitioning ATPase (*Mycobacterium* sp. KMS)	YP_935769	−2.06
glycosyl transferase (*Photorhabdus asymbiotica* subsp. *asymbiotica* ATCC 43949)	YP_003043163	−3.00
glycosyl transferase family protein (*Roseiflexus castenholzii* DSM 13941)	YP_001432834	−2.36
glycosyl transferase (*Bacteroides* sp. 3_1_19)	ZP_06986244	−3.55
glycosyl transferase family 2 (*Achromobacter arsenitoxydans* SY8)	ZP_09298426	−3.91
membrane protein, PAP2 family (*Burkholderia thailandensis* MSMB43)	ZP_02464598	−3.21
DEAD/DEAH box helicase domain protein (*Burkholderia ubonensis* Bu)	ZP_02379212	−6.03
serine/threonine protein kinase (*Naegleria gruberi*)	XP_002671568	−4.46
serine/threonine protein kinase (*Thermobispora bispora* DSM 43833)	YP_003653441	−2.12
protein kinase B (*Pseudomonas aeruginosa* PAK)	AAD22550	−2.68
protein kinase C-like protein (*Aspergillus oryzae* RIB40)	XM_001823989	−2.04
protein kinase domain-containing protein (*Arabidopsis thaliana*)	NP_187156	−3.25
alpha-1,3-glucan synthase (*Aspergillus fumigatus* Af293)	XM_747819	−2.49
disease resistance protein At3g14460 (*Vitis vinifera*)	XP_002272291	−2.39
DegT/DnrJ/EryC1/StrS aminotransferase (*Candidatus Solibacter usitatus* Ellin6076)	YP_826104	−2.17
UvrD/REP helicase (*Mycobacterium tusciae* JS617)	ZP_09685033	−3.05
transcription-repair coupling factor (*Aminomonas paucivorans* DSM 12260)	ZP_07739822	−2.16
polysulphide reductase NrfD (*Bradyrhizobium* sp. ORS 375)	ZP_09418269	−2.35
heme peroxidase-related protein (*Ectocarpus siliculosus*)	CBJ27919	−2.50
eukaryotic initiation factor 3b (*Ectocarpus siliculosus*)	CBJ29502	−2.84
tRNA uridine 5-carboxymethylaminomethyl modification enzyme gidA (*Schizosaccharomyces japonicus* yFS275)	XP_002175477	−4.06
probable UGA2-succinate semialdehyde dehydrogenase (*Sporisorium reilianum* SRZ2)	CBQ70025	−3.30
pentatricopeptide repeat-containing protein (*Medicago truncatula*)	XM_003607640	−2.11
ABC-type multidrug transport system protein (*Lactobacillus paracasei* subsp. *paracasei* 8700:2)	ZP_04674707	−2.24
transcriptional regulator, AraC family protein (*Plesiocystis pacifica* SIR-1)	ZP_01906447	−2.02
Mg^{2+}/Co^{2+} transporter (*Burkholderiales bacterium* JOSHI_001)	ZP_09750163	−2.04
quinone oxidoreductase (*Devosia geojensis*)	ZP_07283096	−2.30
GPI-anchored cell surface glycoprotein (flocculin) (*Candida dubliniensis* CD36)	XP_002419999	−2.15
membrane protease subunit stomatin/prohibitin-like protein (*Streptomyces* sp. SA3_actG)	ZP_07980836	−2.13
endonuclease III/similar to 8-oxoguanine DNA glycosylase isoform 1b (*Ectocarpus siliculosus*)	CBJ27158	−2.40
class III chitinase (*Aspergillus oryzae* RIB40)	XP_001824581	−2.14
thiazole synthase (*Desulfovibrio vulgaris* str.)	YP_002435224	−2.22
phospholipid:diacylglycerol acyltransferase (PDAT1) gene (*Ricinus communis*)	HQ875348	−2.87
2-octaprenyl-6-methoxyphenyl hydroxylase (*Fulvimarina pelagi* HTCC2506)	ZP_01439917	−3.12
polyprotein (*Agaricus bisporus* var. *bisporus*)	ACZ04926	−2.77
hemolysin activator protein precursor (*Erwinia amylovora* ATCC BAA-2158)	CBX79525	−2.89
ATPase central domain-containing protein (*Verminephrobacter eiseniae* EF01-2)	YP_998469	−3.30
tyrosine recombinase xerC (*Sphingobium chlorophenolicum* L-1)	YP_004553517	−2.47
vacuolar protein sorting-associated protein, putative (*Phytophthora infestans* T30-4)	XP_002904386	−2.29

script levels of a protein containing a DEAD/DEAH box helicase domain in *E. cava* was decreased by 6.03-fold by hyperthermal stress (Table 2). This result suggests that elevated seawater temperatures influence RNA metabolism in *E. cava*.

The expression levels of genes encoding glycosyl transferase, an enzyme that establishes natural glycosidic linkages[25], and its related proteins were decreased by 2.36-3.55-fold by hyperthermal stress (Table 2). These results suggest that carbohydrate metabolism is affected by exposure to abnormally high temperatures.

Serine/threonine-specific protein kinases (STKs) play roles in many cellular processes, including glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration[26]. The transcript levels of an *E. cava* STK homolog were decreased by 4.4-fold by hyperthermal stress in this study. Protein kinase B (PKB) is another serine/threonine-specific protein kinase, and the expression of the *pkb* gene was decreased by 2.68-fold by hyperthemal stress.

The aim of this study was to understand the responses of *E. cava* to hyperthermal stress at the transcription

Table 3. Gene Ontology (GO) classification of the genes differentially expressed in *E. cava* after hyperthermal stress.

Biological process (Level 4)	Organic substance biosynthetic process Cofactor metabolic process Heterocycle metabolic process Translational initiation Cellular nitrogen compound metabolic process Organonitrogen compound metabolic process Cellular oxidant detoxification Macromolecule metabolic process Response to toxic substance Organic cyclic compound metabolic process Cellular aromatic compound metabolic process Cellular biosynthetic process Response to radiation Regulation of cellular metabolic process Generation of precursor metabolites and energy Cellular macromolecule metabolic process Protein metabolic process Photosynthesis
Molecular function (Level 3)	Pigment binding Transferase activity Lyase activity Oxidoreductase activity Peroxidase activity Organic cyclic compound binding Heterocyclic compound binding
Cellular component (Level 3)	Ribonucleoprotein complex Intracellular part Membrane protein complex Photosynthetic membrane Protein complex Intracellular Intracellular organelle part Intracellular organelle Membrane-bound organelle Oxidoreductase complex Intrinsic component of membrane Extrinsic component of membrane

level, to allow the metabolic and physiological changes in *E. cava* during climate change to be predicted. For this purpose, we developed the Ecava cDNA Microarray, which contains 2,740 cDNA clones of *E. cava*. In this study, the cDNA microarray was used to identify the genes responsive to hyperthermal stress. We successfully identified the genes of interests and predicted the changes in cellular metabolism that occur in response to exposure to high temperatures based on the functions of those genes.

Despite the ecological importance of kelp species, the genetic information for kelp species available in public databases is extremely limited at present, although many other organisms have undergone whole-genome and transcriptome sequencing. Indeed, genomic information is only available for one kelp species, *Saccharina japonica*[27]. Therefore, the accumulation of genomic information and the functional analysis of genes of interests are required to extend our knowledge of the biological features of kelp species.

Methods

Sample Collection and Exposure to Hyperthermal Stress

Sporophytes of *E. cava* were collected at water depths of approximately 5-15 m near Seogwipo, Jeju, Korea, using standard scuba techniques. After transport to the laboratory aquatic facility of IntheSea Korea (Seogwipo, Jeju), an expert group of marine biodiversity, the thalli of the plants were punched with a sterilized stainless-steel borer (2.0 cm in diameter). The disks were incubated in ES-enriched seawater medium[28] and al-

lowed to acclimate for 1 day at 20°C in the dark in 2 L round-bottom flasks containing 1.5 L of medium. About 30 disks were incubated per flask. After acclimation, the hyperthermally exposed group was incubated in a 24°C incubator for 48 h in the dark to keep the metabolic rate low. Half of the medium was changed after 24 h. The control group was maintained at 20°C in the dark.

Preparation of the Ecava cDNA Microarray

A set of 2,740 *E. cava* cDNA clones, obtained with an EST analysis, was constructed and their open reading frames were amplified with polymerase chain reaction (PCR) using forward (5′-CTCCGAGATCTGGACGAGC-3′) and reverse (5′-TAATACGACTCACTATAGGGC-3′) primers, under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 5 min. The PCR-amplified products were purified on Sephadex G-50 columns, examined with 1.5% agarose gel electrophoresis, air-dried, and resuspended in 50% DMSO solution. The prepared DNAs were spotted onto silanized glass slides (UltraGAPS-II™, Corning, Charlotte, NC, USA) with an OmniGrid™ Microarrayer (GeneMachines, San Carlos, CA, USA). Each slide was crosslinked with 300 mJ of short-wave UV irradiation (Stratalinker; Stratagene, La Jolla, CA, USA) and stored under humid, light-controlled conditions until use.

RNA Isolation

Total RNA was extracted according to a previous report[29], with some modification. In brief, frozen tissues of the algal species were pulverized in a mortar with liquid nitrogen. Approximately 200 mg of the algal powder was homogenized in 750 μL of CTAB buffer (3% CTAB, 1.0 M NaCl, 0.7% PVP, 10 mM EDTA, 100 mM Tris-HCl, pH 9.0) and incubated at 65°C for 5 min. After centrifugation at 12,000 × *g* for 10 min at 4°C, the supernatant was added to 1/2 volume of chloroform, mixed well, and centrifuged at 12,000 × *g* for 10 min at 4°C. The aqueous phase was retained and mixed with 1/4 volume of equilibrated phenol (pH 4.3) and 1/4 volume of chloroform. After centrifugation at 12,000 × *g* for 10 min at 4°C, the supernatant was retained, mixed with 1/2 volume of 8 M LiCl, and stored at 4°C for at least for 2 h (preferably overnight). The RNA was precipitated by centrifugation at 12,000 × *g* for 30 min at 4°C and the precipitate was resuspended in 300 μL of diethylpyrocarbonate (DEPC)-treated water. The RNA was precipitated with 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), dissolved in an appropriate volume of DEPC-treated water (30-40 μL), and stored at −80°C.

Fluorescently Labeled Target Preparation and Hybridization

The pooled RNAs from each group were used for the experiments. The synthesis and hybridization of the targeted cDNA probes were performed with Agilent's Low RNA Input Linear Amplification Kit PLUS (Agilent Technologies, Palo Alto, CA, USA), according to the manufacturer's instructions. Briefly, 1 μg of each total RNA was mixed with the dT-promoter primer and MMLV reverse transcriptase and incubated for 2 h at 40°C. The reverse-transcribed samples were amplified linearly with T7 RNA polymerase for 2 h at 40°C. During amplification, the control cRNAs and test cRNAs were labeled with Cy3-CTP and Cy5-CTP, respectively. The fluorescently labeled control and test samples were combined and resuspended in 80 μL of hybridization buffer (3 × SSC and 0.3% SDS, 50% formamide). After the hybridization mixtures were boiled for 3 min at 95°C and cooled for 3 min on ice, they were directly introduced onto the Ecava cDNA Microarray and placed in a humidified hybridization chamber (Genomictree Inc., Daejeon, Korea). Competitive hybridization reactions occurred between the labeled targets and probes on the microarray during incubation for 16 h at 42°C in a hybridization chamber. The hybridized microarrays were washed with gentle agitation to eliminate nonspecific binding, as follows: 5 min at 42°C in 1 × SSC and 0.2% SDS; 5 min at room temperature in 1 × SSC and 0.2% SDS; and 2 min at room temperature in 0.1 × SSC (twice). Finally, the microarrays were spin-dried and stored in the dark until they were scanned.

Data Acquisition and the Identification and Functional Annotation of Differentially Expressed Genes

The hybridization images were analyzed with GenePix 4000B (Axon Instruments, Terumo, CA, USA) and analyzed with the GenePix Pro 6.0 program (Axon Instruments). The average fluorescence intensity of each spot was calculated, and the local background was subtracted. All data normalization and the selection of genes displaying changes in expression were performed with GeneSpring GX 7.3.1 (Silicon Genetics, Redwood City, CA, USA). The identified genes were filtered with a cut-off value based on the two-component error model after intensity-dependent normalization (LOWESS). The average normalized ratios were calculated by dividing the mean normalized signal channel intensity by the mean normalized control channel intensity. Analysis of variance and a unpaired *t* test were performed to identify those genes that were differentially

expressed in the exposed and unexposed *E. cava* tissues. A fold-change threshold of 2.0 was used to select the differentially expressed genes. A P value of <0.05 was considered significant.

Blast2GO was used to functionally annotate the differentially expressed genes in the Gene Ontology database (http://www.geneontology.org/)[30].

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