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Screening of Differentially Expressed Genes in *Heterosigma akashiwo*, a Red-Tide Causing Organism, Induced by Exposure to High Light

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Heterosigma akashiwo has been reported as red-tide causing phytoplankton in the Korean coastal area during summer when they are exposed to high light. It also shows photosynthetic adaptability to strong light during culture in the laboratory. On the basis of these observations, we tried to find out some genes specifically expressed in *Heterosimga akashiwo* during exposure to high light, assuming that they might have some resistant mechanisms associated with light adaptation. For this purpose, we carried out DD-PCR to detect differentially expressed mRNAs from cells that had been illuminated under high light for 3 days. We found eight cDNA clones that had been expressed specificically for high light. When they were further screened by reverse Northern hybridization, three of them were identified to be positive cDNA clones. When these cDNA fragments were subjected to DNA sequencing and then their base sequences were compared to GenBank database, one of them showed sequence homology 86% identical to the partial sequence of 16S rRNA gene of eubacterium CRO-18.

key words: *Heterosigma akashiwo*, DD-PCR, high light, photosynthesis, photoadaptation, red tide, reverse Northern hybridization

INTRODUCTION

Phytoplanktons are primary photosynthetic organisms in the aquatic environments and their adaptation phenomena to light intensity and quality are considered ecologically important [1]. As photosynthetic activity is quite sensitive, in general, to environmental changes, proliferations of red-tide causing organisms are strongly affected by several environmental factors such as light, nutrients, temperature and salinity. In particular, light intensity, even moderate in nature, could be detrimental to the growth of aquatic algae. Thus, light intensity, together with day length and light quality, can greatly affect photosynthetic activities of oceanic red-tide causing organisms [2].

Growth of some red-tide causing organisms such as *Heterosigma* is known to be quite tolerant to high light, in contrast to *Prorocentrum* which shows high susceptibility to high light [3]. Therefore, these species occur in different seasons and occupies different vertical positions in the water body, depending on the light environments to which they are exposed.

When exposed to excessive light beyond the level required for the optimal growth, photosynthetic plants usually experience photoinhibition in which oxygen evolution or CO_2 fixation rate markedly declines [4]. For the most efficient use of light, phytoplanktons change the compositions of chloroplast membranes in response to light environments, including components of thylakoid membranes, such as photosynthetic pigments, reaction centers, electron carriers and ATP synthase. In particular, primary photoinhibitory damage occurs to D_1 protein, a component of PS II reaction center [5], leading to the inactivation of PS II.

Although it is possible that high-light condition might be responsible for rapid growth of *Heterosigma akashiwo* during warmer seasons, it is not yet understood in terms of molecular mechanisms how they could acclimate to strong light. On the basis of these observations, we tried to screen genes specifically expressed in the laboratory-cultured *Heterosigma akashiwo* induced by high light, using a differential display-PCR (DD-PCR) technique.

DD-PCR has been employed as useful tool to selectivley detect the differentially expressed genes from two different samples. It is currently used most efficiently to clone those genes that play important roles during cell differentiation and the genes that are differentially expressed according to environmental conditions. The original method developed by Liang and Pardee (1992) [6] has been modified in this study following the procedure of Sung and Denman (1997) [7] to minimize false positive clones that might possibly arise in the course of PCR.

MATERIALS AND METHODS

Cultivation of Heterosigma akashiwo

The cells of *Heterosigma akashiwo* were collected from the South coastal waters of the Korean peninsula in 1997. Isolated cells were cultured in the enriched medium of f/2 [8] for 7~10

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days at 25°C with illumination at a light intensity of 150 μmol quanta $m^{-2}s^{-1}$ and with the photoperiod of 14 L:10 D.

Measurement of photosynthetic oxygen evolution

Photosynthetic oxygen evolution and PS II activity of *H. akashiwo* were measured using Clark-type oxygen electrode, following the procedure of Moon *et al.* (1995) [9].

Exposure to high light

Two groups of cells in the phase of exponential growth, that had been cultured at 25°C and at a light intensity of 150 μmol quanta m^{-2} s^{-1}, were treated with low light (25 μmol quanta m^{-2} s^{-1}) and high light (800 μmol quanta m^{-2} s^{-1}) conditions, respectively, for 3 further days. Cells after exposure to light treatments were harvested by centrifugation at 3,000 rpm for 10 min at room temperature.

Isolation of total RNA

Total RNA was isolated from cell suspensions using Tri reagent (Molecular Research Center, USA) in combination with DNase I (Promega, USA) which destroyed residual chromosomal DNA from total RNA samples [10].

DD-PCR

Random hexamers (Takara, Japan) were used as primers to synthesize the 1st strand cDNA from total RNA (Yoshida *et al.* 1994). Following the procedure of Sung and Denman (1997), two types of reverse transcriptase (RTase), i.e., AMV RTase (Takara, Japan) and Superscript II (GIBCO BRL) were employed for the synthesis of the 1st strand cDNA to minimize false positive cDNA clones that might arise in PCR. To amplify cDNA clones that had been differentially expressed upon exposure to different light intensity, RAPD primer (10 mer) (Operon, USA) were used in PCR [10].

Cloning of cDNA clones

4.5% denaturing polyacrylamide gel (containing 7 M urea) was prepared to separate PCR products, by the procedure of Sambrook *et al.* (1989) [11]. Electrophoresis was carried out at 160V for about 4h, and then the gel was stained with GelStar Nucleic Acid Stain (FMC, USA) for 30 min. Differential bands that had been simultaneously amplified by both enzymes were cut from the gel and then subjected to the second PCR to get sufficient amount of expressed cDNAs. The PCR products were recovered from the gel using GeneClean II Kit (BIO 101) and ligated with pGEM-T vector (Promega, USA). Ligated recombinant DNAs were provided to transform the competent bacterial cells that had been prepared by the modified method of Hanahan (1983) [12], and the resultant transformants were identified by colony PCR [13].

Reverse Northern hybridization

Reverse Northern hybridization was carried out to identify the authentic cDNA clones that had been exclusively expressed in *Heterosigma akashiwo* cells by exposure to high light [14]. In order to synthesize labeled cDNAs, digoxygenin-11-dUTP (Boehringer Mannheim) was incorporated into the reaction mixture of the 1st strand cDNA synthesis [10]. cDNA clones, that had been selected to be specific for high light, were obtained as products of colony PCR, and then they were transferred to nylon membranes (Hybond N^+, Amersham) using Bio-Dot microfiltration system (Biorad). cDNA clones on the membranes were subjected to hybridization with DIG-labelled cDNA probes, using DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim Cat. No. 1585614) and the resultant reaction products were detected on X-ray film (Hyperfilm, Amersham) by chemiluminescent method.

Determination of DNA sequences

The authentic cDNA clones that had been identified to be specific for high light from the results of reverse Northern hybridization, were subjected to DNA sequencing analysis using LI-COR 4200 autosequencer (LI-COR, USA), and the determined sequences of the cDNA clones were examined by BLAST search on the GenBank database to find homologous genes.

RESULTS AND DISCUSSION

Effects of light intensity on the oxygen evolution from H. akashiwo

To examine any short-term responses of photosynthetic machinery of *H. akashiwo* to changing light environments, we measured oxygen evolutions from *H. akashiwo* at 25°C with illumination of varying light intensities (Figure 1). The results showed that photosynthesis was almost light-saturated around at the intensity of 1,000 μmol quanta m^{-2} s^{-1} and, moreover, photosynthetic activity was remained rather constant at a quite high level, without showing any photoinhibition up

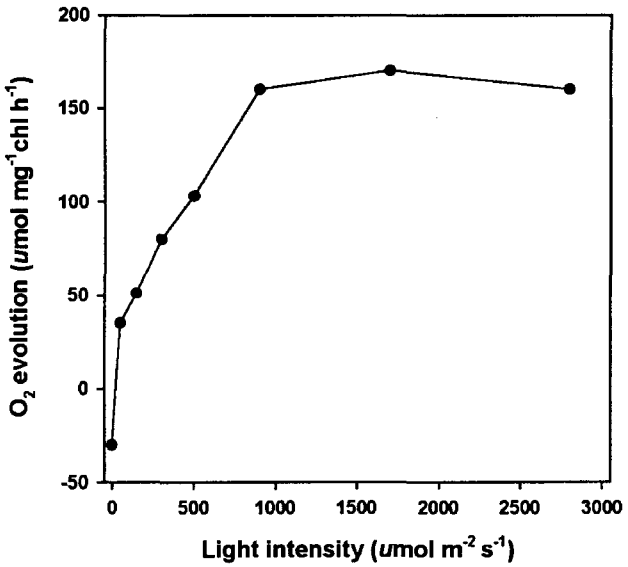

Figure 1. Effect of light intensity on the photosynthetic O_2 evolution from *Heterosigma akashiwo*.

to the light intensity of 2,800 μmol quanta $m^{-2}s^{-1}$. These observations suggest that *H. akashiwo* could be quite tolerant to high-light environments and seem to be consistent with the previous reports that *H. akashiwo* usually blooms at the surface of sea waters during bright and warmer sunny days and that it actually shows higher capacity to tolerate high light [3].

Identification of differentially expressed genes during exposure to high light

Total RNA was isolated from the respective cells that had been exposed for three days to low-light (25 μmol quanta $m^{-2}s^{-1}$) and high-light (800 μmol quanta $m^{-2}s^{-1}$). Total RNA samples free from contaminated chromosomal DNAs were subjected to the 1st strand cDNA synthesis by using two types of reverse transcriptase, namely, AMV RTase and Superscript II, to minimize possible amplification of any false positive cDNAs in carrying out DD-PCR [7].

DD-PCR products were electrophoresed on the denatured polyacrylamide gels, and the seperated electrophoretic profiles were examined to find any differentially expressed bands between low and high light. Furthermore, the bands that had been selectively amplified by both AMV RTase and Superscript II were regarded as differentially expressed cDNA clones affected by different light regimes.

Many electrophoretic bands were recognized to be specific for high light, including three for the RAPD primer #1, one for each of the primers #10 (Figure 2), #8, #18 and two for the

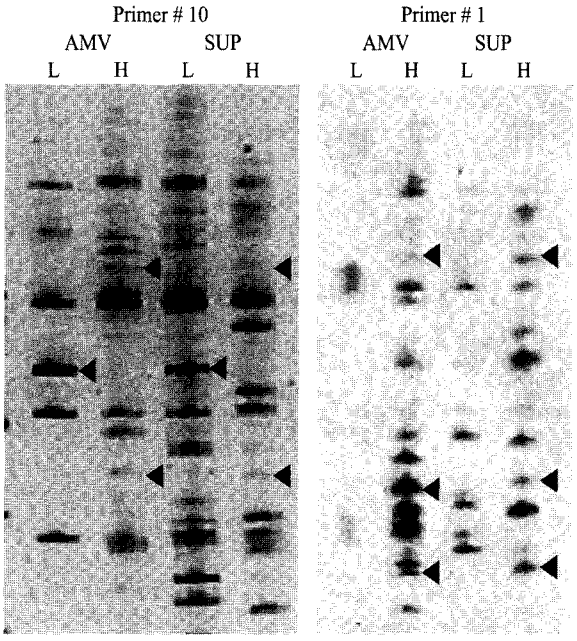

Figure 2. Differential display of the cDNA products from *Heterosigma akashiwo* that had been exposed to low-light (25 μmol quanta $m^{-2}s^{-1}$) (L) and high-light (800 μmol quanta $m^{-2}s^{-1}$) (H) for three days. Messenger RNAs were isolated from the light-treated cells, respectively. cDNAs were synthesized from the respective messenger RNAs using two reverse transcriptases, namely, AMV reverse transciptase(AMV) and Superscript II(SUP). After then, PCR was performed with the resultant cDNAs and the PCR products were displayed on 4.5% denaturing polyacrylamide gel. cDNA fragments of interest were indicated by arrows.

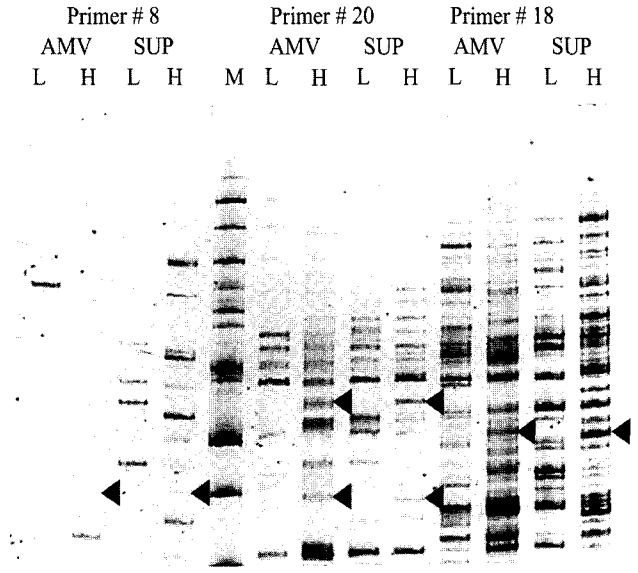

Figure 3. Differential display of the cDNA products from *Heterosigma akashiwo* that had been exposed to low-light (25 μmol quanta $m^{-2}s^{-1}$) (L) and high-light (800 μmol quanta $m^{-2}s^{-1}$) (H) for three days. cDNAs were synthesized from the respective total RNAs using two reverse transcriptases, namely, AMV reverse transcriptase (AMV) and Superscript II (SUP). After then, PCR was performed with the resultant cDNAs and the PCR products were displayed on 4.5% denaturing polyacrylamide gel. cDNA fragments of interest were indicated by arrows.

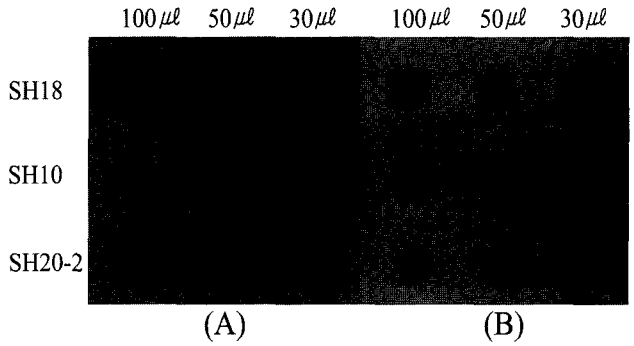

Figure 4. Reverse Northern dot blot of the colony PCR products of the cloned cDNA fragments that represent differentially expressed RNAs. Colony PCR products were dot blotted onto Hybond N^+ nylon membranes and hybridized with cDNA probes that had been labeled with dioxygenin-11-dUTP. (A) Hybridization with the cDNA probe prepared from *H. akashiwo* cells that had been exposed to low light, (B) hybridization with the cDNA probe prepared from *H. akashiwo* cells that had been exposed to high light.

primer #20 (Figures 3 & 4). Each band were named after the arbitrary number of its RAPD primer, together with its electrophoretic mobility. Thus, the respective three bands for the primer #1 were named, SH 01-1, SH 01-2 and SH 01-3 from the top, and also the band corresponding to the primer #8 was named as SH 08, and so on. Meanwhile, the band that was presumed to be specific for low light was also found when DD-PCR was carried out with the RAPD primer #10, and that was named as SL 10. Therefore, we temporarily speculated that these bands were expressed specifically in response to high light or low light in *H. akashiwo*.

Reverse Northern hybridization

To further screen the authentic cDNA clones of *H. akashiwo* specific for high light, reverse Northern hybridization was carried out. For the purpose of preparing the two kinds of probes, for the high-light and for the low-light grown cells, respectively, each cDNA was labeled by incorporating digoxygenin-11-dUTP into the 1st strand cDNA.

Then, after cloning the putative positive cDNA fragments, they were transferred to nylon membranes and hybridized with the respective cDNA probes (Figure 4A, B). Figure 4A represents hybridization signals between SH 18, SH 10, SH 20-2 cDNA clones and the probe prepared from low-light grown cells, while Figure 4B represents the signals of hybridization between the above-mentioned cDNA clones and the probe prepared from high-light grown cells. The results suggest that those cDNA clones represent the authentic genes whose expression is greatly enhanced during exposure to high light.

When the cDNA fragments were subjected to DNA sequencing (Figure 5) and the determined base sequences were analyzed for homology search using GenBank database of NCBI, only the SH 20-2 cDNA clones showed 86% homology with partial sequences of 16S rRNA of eubacterium CRO-18 (accession No. AF141567).

When plant cells are exposed to high light stress, they are known to develop various tolerance mechanisms. In particular, complex antioxidant systems are considered to play a pivotal role in the protection of cell membranes and organelles from photooxidative damages [15]. In the mechanism of light tolerance for *H. akashiwo*, active synthesis of proteins probably supports the defense mechanisms on the levels of chloroplasts as well as nuclei. Therefore, the high light-induced expression of 16S ribosomal RNA genes, that had been observed in the present study, might be explained, at least in part, by preferential synthesis of chloroplast - encoded proteins, probably needed in large quantity in response to high light in *H. akashiwo*.

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SH20-2 (391 letters):
GTTGC GATCC TGGCT TAGGA TGAAC GCNGG TAGTA TGCTT AACAC ATGCG  50
AGTCG AATGA TGGTA ACATC ATGGC GAACG GGTGA GTACA AAATA AGAAT 100
CTGCC TTTTA GTTGG GTTAA ATACC TGGNG TACAG AAAGA TCCAC GATCG 150
CTAAG AGAAG AGCTT ATTTA AGATT AGGTT GNTGG TAGGG TAAAG GCCTA 200
CCAAG CCGAC TATCT TTANC TGGTC TGAAA GGACG GTCAN CCACA CCGGG 250
ACTGA GACAA GGCCC AGGCT TCAAA CCGAA GCCAG CAGTG AGGAA TTTTG 300
GACAA TGAGC GAAAG CTTGA TCCAG CAATA CTACN TGGGG GAAGA AGGCT 350
CAATT GGTCG TAAAC CCCTT TCATT AGGGA GGATC GCAAC A  391
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Figure 5. The nucleotide sequence of a selected cDNA clone whose corresponding genes were expressed when *H. akashiwo* cells were exposed to high light. cDNA fragments identified by DD-PCR were sequenced and then homology to these cDNAs were searched by BLAST on the GenBank database of NCBI.

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