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Isolation of cadmium-induced DNA sequence in microalga *Nannochloropsis oculata*

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Abstract: The gene induced in response to Cd exposure in microalga *Nannochloropsis oculata* was examined in this investigation. In order to isolate this gene by using nested PCR, degenerated primers were designed on the basis of highly conserved regions of the amino acid sequences of various phytochelatin synthase. The size of the nested PCR amplification product from *Nannochloropsis oculata* by using two degenerated primers was measured to be 266 bp. The 266 bp DNA fragment was sequenced, and it might be induced by Cd exposure in microalga *Nannochloropsis oculata*.

Key words: *Cadmium, DNA, Nannochloropsis oculata*
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Introduction

There has been considerable concern about the increasing levels of heavy metals like cadmium (Cd) in the environment due to the potential toxic effects on living organism (Pinto *et al*., 2003; Akinola and Ekiyoyo, 2006; Phetsombat *et al*., 2006). The photosynthetic process is very sensitive to Cd; it reduces growth, metabolism and low biomass accumulation (Payne and Price, 1999; Sanita and Gabbrielli, 1999). Cd also produces oxidative stresses in the cell possibly by generating free radicals and reactive oxygen species as we have reported previously (Lee *et al*., 2000; Lee, 2002; Lee and Shin, 2003). One protective strategy against metal excess is the expression of high-affinity binding sites to suppress uncontrolled binding of metal ions to functional groups (Ahner and Morel, 1995; Pistocchi *et al*., 2000). Phytochelatins (PC), glutathione-derived metal binding peptides, are heavy metal binding peptides that play an important role in sequestration and detoxification of heavy metals in various organisms. A few years ago the genes encoding phytochelatin synthase (PCS) were cloned from plants, fungi and nematodes. Expression of Arabidopsis PCS in Indian Mustard enhanced tolerance for Cd and Zn (Gasic and Korban, 2006). The tolerance of zebrafish to heavy metal toxicity was also enhanced by the expression of plant PCS (Konishi *et al*., 2006).

PCS was more strongly activated by Cd^{2+} than by Zn^{2+} in marine green alga, *Dunaliella tertiolecta* (Tsuji *et al*., 2003). Proline-mediated tolerance to Cd through increased phytochelatin synthesis and sequestration of Cd in transgenic microalgae was also reported (Siripornadulsil *et al*., 2002).

However, the information about cadmium-induced genes in marine microalgae is not sufficient in spite of the importance for environmental genomics. In this investigation, cadmium-induced DNA sequence was isolated and characterized from *Nannochloropsis oculata* by using nested PCR with degenerated primers for PCS.

Materials and Methods

Algal culture and cadmium treatment: A unialgal strain of Nannochloropsis oculata was obtained from the Korea Marine Microalgae Culture Collection (KMCC) and cultured in the f/2 medium of Guillard and Ryther (1962). Erlenmeyer flasks containing 100 ml medium were inoculated with 25 ml of a mother culture. All the media for microalgal growth were made with electrolyzed water. The alga was grown at room temperature with shaking at 100 rpm under a 12:12 light-dark cycle of 50 μmol photon m^{-2} s^{-1} illumination. For cadmium treatment, a 10-day old culture was further cultured for 4 days in similar medium, but with 10 μM $CdCl_2$.

Algal DNA extraction: Algal material treated with 10 μM $CdCl_2$ for 4 days was powdered in liquid nitrogen. This powdered was further homogenized in a solution of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 50 mM NaCl. Lysozyme was added and the solution was incubated at 55°C for 30 min. After incubation, proteinase K (20 mg/ml) and 10% SDS were added, and the mixture was incubated at 55°C for 30 mins. This solution was chilled on ice and then extracted with equal volume of phenol-chloroform-isoamylalcohol. The organic extraction step was repeated, and the supernatant was added to an equal volume of 4M ammonium acetate. The DNA was precipitated by adding 2 volumes of isopropanol, and collected by centrifugation for 10 min at room temperature (Sambrook and Russell, 2001).

Nested PCR with degenerated oligonucleotides: Degenerated oligonucleotides designed on the basis of the highly conserved regions of the amino acid sequences of various phytochelatin synthases were used for nested PCR. PCR was performed in a total volume of 20 μl containing 200 μM dNTPs, 2 mM $MgCl_2$, 1 unit of Taq polymerase, 1X PCR buffer, 1 μl of DNA template and 1 pmol of sp-1 primer (garccngartaytgygg) and asp-2 primer (nacccartgnggnggrga). Amplification was carried out with 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min using a PROGENE thermal cycler. The primary PCR product was used as

a template for a secondary PCR with a nested sp-2 primer (nnnnatgctngaytgytg) and asp-1 primer (nnnnggrctnggrtgncc) in a reaction mixture. A secondary amplification was also carried out with 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min using the same thermal cycler. The PCR product was subcloned and characterized by DNA sequencing (Sambrook and Russell, 2001).

Table - 1: Degenerated primers used in the nested PCR

Primer	Sequence (5'→3')	Product length (bp)
PCS sp-1	garccngartaytgygg	423
PCS asp-2	nacccartgnggnggrga	
PCS sp-2	nnnnatgctngaytgytg	237
PCS asp-1	nnnnggrctnggrtgncc	

Results and Discussion

Both essential and non-essential transition metal ions can be toxic to cells. The physiological range for essential metals between deficiency and toxicity is therefore extremely narrow and a tightly controlled metal homeostasis network to adjust to fluctuations in micronutrient availability is necessary for all organisms (Clemens, 2006). One protective strategy against metal excess is the production of high affinity binding sites to inhibit uncontrolled binding of metal ions to physiologically important functional groups. Phytochelatins are heavy metal-binding peptides that play important roles in the detoxification of toxic heavy metals. Phytochelatins also regulate intracellular concentrations of essential metals in eukaryotes, including higher plants, fungi, and microalgae. Recently, PC synthase (PCS) genes in higher plants and fission yeast have been identified and characterized (Clemens, 2006), enabling molecular biological investigation to clarify the mechanisms underlying PC synthesis. Moreover, recent routine database searches have unexpectedly identified genes that are similar to plant PCS genes in the genomes of worms and some prokaryotes (Hirata *et al*., 2005). In response to heavy metal exposure, the constitutively expressed phytochelatin synthase enzyme (PCS) is activated leading to synthesis of PCs in the cytosol (Picault *et al*., 2006). Moreover, recent attempts to enhance metal accumulation and tolerance in plant reported that

Fig. 1: Amino acid sequence alignment of putative phytochelatin synthase. Degenerate primers underlined were designed based on the highly conserved regions

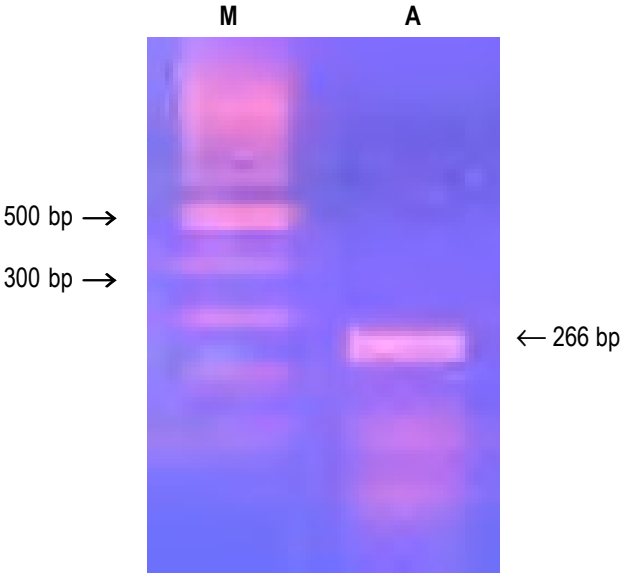

Fig. 2: PCR amplification of a PCs gene from *Nannochloropsis oculata* using degenerated primers. Lane M: 100 bp DNA ladder, Lane A: Nested PCR products from *Nannochloropsis oculata*

PCS over-expression in transgenic plants paradoxically induced cadmium hypersensitivity (Picault *et al*., 2006).

In this study, our goal is to isolate the gene induced in response to Cd exposure in marine microalga *Nannochloropsis oculata*. Cd-induced oxidative stress and the involvement of antioxidant enzymes in *Nannochloropsis oculata* following 10 μM Cd treatment were reported in our previous investigation (Lee and Shin, 2003). Increasing concentrations of Cd produced growth inhibition. The reduction was about 15% with 10 μM Cd and 50% growth with 20 μM Cd. The markedly elevated levels of various antioxidant enzymes, named ascorbate peroxidase, guaiacol peroxidase and catalase following 10 μM Cd treatment indicated the protective role of the enzymes against Cd induced oxidative stress. Moreover, the oxidative stress might result in the activations of various stress-related signals at gene levels. Therefore, in order to isolate Cd stress-related gene, degenerated primers were designed on the basis of highly conserved regions of the amino acid sequences from various phytochelatin synthases. Amino acid sequence alignment of various putative phytochelatin synthases was shown in Fig. 1. Table. 1 indicates the degenerated oligonucleotide primers designed on the basis of the highly conserved regions of the amino acid sequences from various phytochelatin synthases. The size of the nested-PCR amplification product from *Nannochloropsis oculata* using two sets of degenerated primers was measured to be 266 bp as shown in Fig. 2.

Fig. 3 shows the nucleotide sequences of the nested PCR product. This sequence indicates the Cd-induced DNA fragment in marine alga *Nannochloropsis oculata.* Further molecular biological studies in *Nannochloropsis oculata* will allow testing of this DNA

1 ATCGGGGCTC GGATGGCCCC CAAGCACAGT CAGTTTGCCA GACTCACAGA TTTCAGCTAT

61 TGCCAACGGT TCCATTCTTG GGGATACATT ATTTTCTAGT ACAAATGAGA ATTTAGCAAT

121 GGTAAGTGGT TATTCAGGAT TTCCTCAGTC AATAAAGGGG GCTGCAGATC TCCATACAAG

181 CTCTATATAC GATCATCATG TGAACTCAGC TGACGGGGAT GTTGGTTGGC TTAAGACAGA

241 TAAATTCGGC AGCAATCAAG CATTCA

Fig. 3: Nucleotide sequences of the nested PCR product from *Nannochloropsis oculata*

sequence for toxic Cd sequestration and detoxification. More detailed experiments will be needed to fine out and isolate full length Cd-induced gene in *Nannochloropsis oculata*. The information on the Cd-induced DNA sequence obtained can be utilized as indicators for environmental stresses. For understanding the roles of the Cd-induced gene in the alga, experiments using transgenic alga with induction or suppression of the gene will be needed. The induction or repression of genes under the cadmium stress should be helpful in understanding the mode of action of marine organisms to defend themselves against cadmium toxicity.

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References

Ahner, B.A. and F.M.M. Morel: Phytochelatin production in marine algae: II. Induction by various metals. *Limnol. Oceanogr*., **40**, 658-665 (1995).

Akinola, M.O. and T.A. Ekiyoyo: Accumulation of lead, cadmium and chromium in some plants cultivated along the bank of river Ribila at Odo-nla area of Ikorodu, Lagos state, Nigeria. *J. Environ. Biol*., **27**, 597-600 (2006).

Clemens, S.: Evolution and function of phytochelatin synthases. *J. Plant Physiol*., **163**, 319-332 (2006).

Gasic, K. and S.S. Korban: Expression of Arabidopsis phytochelatin synthase in Indian mustard (*Brassica juncea*) plants enhances tolerance for Cd and Zn. *Planta*, **225**, 1277-1285 (2006).

Guillard, R.L. and J.H. Ryther: Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve). *Can. J. Microbiol*., **8**, 229-239 (1962).

Hirata, K., N. Tsuji and K. Miyamoto: Biosynthetic regulation of phytochelatins, heavy metal-binding peptides. *J. Biosci. Bioeng*., **100**, 593-599 (2005).

Konishi, T., S. Matsumoto, Y. Tsuruwaka, K. Shiraki, K. Hirata, Y. Tamaru and M. Takagi: Enhancing the tolerance of zebrafish (*Danio rerio*) to heavy metal toxicity by the expression of plant phytochelatin synthase. *J. Biotechnol.,* **122**, 316-325 (2006).

Lee, J.A., H.W. Shin and M.Y. Lee: Effects of various environmental stresses on the peroxidase activities from rice seedlings. *Korean J. Environ. Biol*., **18**, 331-336 (2000).

Lee, M.Y.: Effect of Na_2SO_3 on the activities of antioxidant enzymes in geranium seedlings. *Phytochemistry*, **59,** 493-499 (2002).

Lee, M.Y. and H.W. Shin: Cadmium-induced changes in antioxidant enzymes from marine alga *Nannochloropsis oculata. J. Appl. Phycol.,* **15**, 13-19 (2003).

Payne, C.D. and N.M. Price: Effects of cadmium toxicity on growth and elemental composition of marine phytoplankton. *J. Phycol*., **35**, 293-302 (1999).

Phetsombat, S., M. Kruatrachue, P. Pokethitiyook and S. Upatham: Toxicity and bioaccumulation of cadmium and lead in *Salvinia cucullata* . *J. Environ. Biol.,* **27**, 671-678 (2006).

Picault, N., A.C. Cazale, A. Beyly, S. Cuine, P. Carrier, D.T. Luu, C. Forestier and G. Peltier: Chloroplast targeting of phytochelatin synthase in Arabidopsis: Effects on heavy metal tolerance and accumulation. *Biochimie,* **88**, 1743-1750 (2006).

Pinto, E., T.C.S. Sigaud-Kutner, M.A.S. Leitao, O.K. Okamoto, D. Morse and P. Colepicolo: Heavy metal-induced oxidative stress in algae. *J. Phycol.*, **39**, 1008-1018 (2003).

Pistocchi, R., A.M. Mormile, F. Guerrini, G. Isani and L. Boni: Increased production of extra and intracellular metal-ligands in phytoplankton exposed to copper and cadmium. *J. Appl. Phycol*., **12**, 469-477 (2000).

Sambrook, J. and D. W. Russel: Molecular Cloning A Laboratory Manual. 3rd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA (2001).

Sanita, D.T.L. and R. Gabbrielli: Response to cadmium in higher plants. *Environ. Exp. Bot*., **41**, 105-130 (1999).

Siripornadulsil, S., S. Traina, D.P. Verma and R.T. Sayre: Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. *Plant Cell,* **14**, 2837-2847 (2002).

Tsuji, N., N. Hirayanagi, O. Iwabe, T. Namba, M. Tagawa, S. Miyamoto, H. Miyasaka, M. Takagi, K. Hirata and K. Miyamoto: Regulation of phytochelatin synthesis by zinc and cadmium in marine green alga, *Dunaliella tertiolecta*. *Phytochemistry,* **62**, 453-459 (2003).