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Isolation of pollutant (pine needle ash)-responding genes from tissues of the seaweed *Ulva pertusa*

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Abstract

Genetic responses of the seaweed *Ulva pertusa* to pine needle ash have been compared using differential display technique. The tissue viability was assessed to evaluate the stress level with triphenyltetrazolium chloride. Total RNA, from tissues treated in seawater containing ash, was reverse transcribed and amplified by PCR with arbitrary primers. The genetic fragments responding to the stress were selectively isolated from agarose gel and sequenced with a DNA auto sequencer. According to sequence analysis, an ash-inducible gene (342 bp) and an ash-suppressed gene (1690 bp) were identified as hypothetical proteins.

Introduction

During the winter and spring, forest fires often occur in Korea (www.foa.go.kr). In the forest area, the pine tree *Pinus densiflora* is the most dominant tree. The ash derived from the fire may be one of the natural hazards that cause damage to the seaweeds near a river mouth. Especially through the short rivers, the ash reaches the coast without self-purification, and affects marine organisms, including useful seaweeds. *Ulva pertusa* Kjellman (sea lettuce) is one of the most widely distributed species in the coastal area near river mouths, and also commonly found occupying a range of shores and habitats, polluted and unpolluted. *U. pertusa* has thick, stiff foliose, perforated blades with two cell layers. It occurs throughout the year, propagating mostly in the winter and spring periods. It is simple in structure and very easy to handle in culture and seems to be consistent in its behavior when collected from different habitat sources (Burrows, 1971). The growth of tissue was accelerated in seawater containing humic-like substances (Asahina et al., 1999). Moreover, *Ulva* has been well investigated with respect to cellular developmental properties (Reddy et al., 1992; Nakanishi et al., 1996), physiology (Floreto et al., 1993), biochemical constituents (Okano & Aratani, 1979), and molecular genetics (Lim et al., 1983). Thus, it is ideally suited as a marine bioassay test organism. With the exception of obvious lethal damage associated with extreme environmental conditions, it is difficult to evaluate the occurrence and severity of stress in natural populations of seaweed. There is a need to develop molecular and biochemical markers specific for an individual stress or group of stresses to allow the prompt, unambiguous and direct determination of stress. We measured the viability against pine needle ash as a pollutant stress source and screened the differential display of gene expression for detection of stress markers at the RNA expression level.

Materials and methods

Thallus

Fresh thalli of the green alga *Ulva pertusa* Kjellman were collected from the Chongsapo area in Busan, Korea. Tissues were cleaned by brushing thoroughly

and sonicating (47 kHz) twice for 1 min in autoclaved seawater, and immersed in 1% Betadine for 2 min to eliminate epiphytes (Jin et al., 1997). They were then rehabilitated at 18 °C in PES (Provasoli, 1968) for a day before use.

Pollutant treatment

Ash from pine (*Pinus densiflora*) needles was prepared by burning in an oven at 200 °C for 1 h. After this preparation, 1 g of fresh needles resulted in 0.5 g dry ash weight, 25 mg water-soluble extract (1d, RT), and an absorbance of 0.61 at 230 nm for the 10-fold diluted extract. To measure the stress level induced by the ash, 0.1 g of the *U. pertusa* tissue was incubated under 50 μmol photons $m^{-2}\,s^{-1}$ at 18 °C for 1 d in 100 ml PES containing various concentrations of ash. Viability of the tissue was measured by the TTC method that is based on the enzymatic reduction of colorless 2,3,5-triphenyltetrazolium chloride to a red-colored triphenylformazan (Nam et al., 1998). The relative viability (%) for each ash concentration was expressed on a dose-response curve. From the curves, we determined the MNLC (maximum non-lethal concentration), LC_{50} (lethal concentration 50), and MLC (minimum lethal concentration).

RNA extraction

For the RNA extraction, pollutant-treated and control tissues were processed by the LiCl-guanidinium method (Hong et al., 1995). Briefly, 0.6 g of wet tissues was powdered in liquid nitrogen and heated in 4 ml of RNA extraction solution at 55 °C for 10 min. Total nucleic acid was precipitated after addition of the same volume of 4 M LiCl at 4 °C for 1 h. To remove DNA, a 100 μl aliquot was incubated with 4 μL of RNase-free DNase I (1 unit μL^{-1}). The total RNA was adjusted to 0.5 $\mu g\ \mu L^{-1}$ with RNasin (0.5 unit μL^{-1}) in DEPC water for the cDNA synthesis.

cDNA synthesis

Five μL of total RNA (2.5 μg) was used as a template in 20 μL of reaction mixture, according to the Invitrogen cDNA synthesis protocol. To prime total RNA, 1 μL of random hexamers (1 $\mu g\ \mu L^{-1}$) was added. cDNA synthesis was carried out at 42 °C for 2 h by avian virus reverse transcriptase (10 unit μL^{-1}).

Differential display

PCR amplification was carried out using a DNA thermal cycler (Perkin-Elmer, Norwalk, CT). Arbitrary primers of 10-base oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, CA). A 25 μL PCR reaction mixture contained 1 μL of the cDNA, 5 pM of arbitrary primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 0.5% Tween 20, 0.1 mM of each dNTP, and 1 unit of *Taq* DNA polymerase. The cycling parameters included an initial incubation at 94 °C for 5 min followed by 45 cycles of 5 sec denaturation at 94 °C, 1 min annealing at 36 °C, and 2-min extension at 72 °C (Yu & Pauls, 1992). A 10 μL sample of PCR product was loaded on a 3% agarose gel containing 0.5 $\mu g\ mL^{-1}$ ethidium bromide and run for 1.5 h at 5 V cm^{-1} in 0.5 × TAE buffer (20 mM Tris-acetate, 1 mM EDTA, pH 8.0). Amplified cDNA fragments induced by the stress were selectively isolated from the gel.

Sequence analysis

The cDNA fragments were cloned into pCR2.1 using the TA cloning kit (Invitrogen). Both strands of the cloned cDNA fragments were sequenced with a DNA auto sequencer (Perkin-Elmer ABI Prism 377). Sequences were analyzed for open reading frames (ORFs) using the ORF Finder option from NCBI (www.ncbi.nlm.nih.gov), and also aligned using the BLASTX option to detect GenBank proteins that are most likely to be related. The G + C content and molecular weight were determined using the DNAsis and ExPASY Molecular Biology programs (www.expasy.org), respectively.

Results

Ulva pertusa tissues were treated with various concentrations of ash to identify the concentration at which a stress response was induced. From the dose-response curve, the MNLC, LC_{50} and MLC induced by the ash were determined to be 60, 350 and 550 mg mL^{-1}, respectively (Figure 1). For the differential display screening of ash stress responsive genes, the tissues were treated in PES at MNLC for 24 h, LC_{50} for 1 h, LC_{50} for 6 h, and LC_{50} for 24 h. Total RNA was extracted from each stressed tissue using the LiCl-guanidinium method. The RNA yield after removing DNA was approximately 169 μg from 1 g of wet tissue.