Isolation of several anti-stress genes from a mangrove plant *Avicennia marina*

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**Summary**

In order to isolate anti-stress genes from mangrove plants, a cDNA library of *Avicennia marina* was constructed and screened for anti-stress genes by a functional expression screening with *Escherichia coli* cells. Several stress-related gene homologues, such as chaperonin-60, clpP protease of the clp/Hsp100 family of chaperones, ubiquitin, eEF1A, drought-induced AtDi19 gene of *Arabidopsis thaliana*, and secretory peroxidase, were successfully isolated.

**Introduction**

Higher plants are generally sensitive to environmental stresses such as high salinity, drought, high temperature, high light intensity, and chilling. These environmental stresses are the main limiting factors for plant growth and distribution, and breeding of stress-tolerant trees and crops is therefore important: (i) to prevent deforestation, which causes elevation of atmospheric CO₂ concentration and global warming, and (ii) to expand the cultivation area of crops for the increasing food demand resulting from the rapid growth in world population.

The isolation of genes with the capability of improving stress-tolerance (anti-stress genes) is an important factor for the breeding of stress-tolerant plants, and mangrove plants are attractive resources for anti-salt-stress genes, since they grow under a condition of high salinity. In this study, to isolate the anti-stress genes from mangrove plants, we applied a simple screening method based on the functional expression of the mangrove genes in *E. coli* cells and on host cell acquisition of salt-stress-tolerance, and successfully isolated several stress-related genes.

**Materials and methods**

**RNA extraction and cDNA library construction**

Total RNA was extracted from the young leaves of *Avicennia marina*, grown in the green house, according to the procedure described by Chomczynski & Sacchi (1987). The poly (A)⁺ RNA was purified by oligo-dT chromatography (Maniatis *et al.* 1982). The cDNA was synthesized from 5 μg of poly(A)⁺ RNA using the λZAPII-cDNA synthesis kit (Stratagene, La Jolla CA, USA), and size-fractionated by agarose gel electrophoresis (12 cm × 14 cm 0.8% agarose gel, 15 V for 16 h). The selected fraction (above 0.7 kb) was directionally cloned into the *Eco*RI-*Xho*I sites of the vector. Recombinant DNA was packaged *in vivo* with ‘Gigapack gold’ extracts (Stratagene). Approximately 2 × 10⁶ p.f.u. of primary recombinant phages was obtained; the library was then amplified once to a titer of 1 × 10¹⁰ p.f.u./ml. When a sample of the amplified library was plated with the top agar containing 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 300 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) onto the NZY agar plates, the percentage of the blue plaque was less than 0.8%.

**Screening for anti-stress genes**

The screening of the *A. marina* cDNA library for anti-stress genes by the functional expression screening method with *E. coli* cells was carried out as described previously (Miyasaka *et al.* 2000a). The cDNA library was mass excised into phagemid DNA according to the procedure provided by the manufacturer (Stratagene). The yield of mass excision was 15–20%. For anti-stress
gene screening, approximately $8 \times 10^7$ of *E. coli* cells (SOLR strain, Stratagene) in 1.2 ml of LB medium were infected with 1 to $3 \times 10^8$ of mass excised phagemids by incubating at 37 °C for 15 min, and were plated onto a Luria-Bertani (LB)-carbenicillin (Cb; 50 μg/ml) plate with 5–6% NaCl. The plates were incubated at 37 °C for 2–3 days and the salt-stress-tolerant bacterial colonies were isolated.

**DNA sequencing and computer analysis**

Double-stranded plasmid DNA templates were prepared according to Zimmermann et al. (1990), and sequencing reactions were carried out using an ABI PRISM DNA sequencing kit (Perkin-Elmer, Foster City CA, USA). Sequencing gels were run on the ‘ABI PRISM 377 automated DNA sequencer’ (Perkin-Elmer). The databases used for the homology search were DNA Data Bank of Japan (DDBJ; release 38), Swiss-Prot (Sp) protein database (release 38), and Protein Identification Resource (PIR) protein database (release 62). The homology search was done by the FASTA program (Pearson & Lipman 1988). The isolated clones were analysed by: (a) partial sequence of cDNA by only single-run sequencing reaction, and (b) searching for possible homologies either in the DNA database or in the protein database, after translating DNA sequences into protein sequences. The nucleotide sequence data of the present study appear in the DDBJ database under the accession numbers AB049589, AB049590, and AU108463 through AU108536.

**Results and discussion**

**Screening for anti-stress genes**

In the previous study (Miyasaka et al. 2000a), we choose the marine green alga *Chlamydomonas* W80, which showed a high tolerance against salt and oxidative stresses, as the genetic resource of anti-stress genes, screened the cDNA library of this alga by a functional expression screening with *E. coli* cells, and successfully isolated several anti-stress genes, such as ascorbate peroxidase, glutathione peroxidase, bcl1 (breast basic conserved), alternative oxidase, and ω-6 fatty acid desaturase (Miyasaka et al. 2000b; Takeda et al. 2000; Tanaka et al. 2001). The principle of the screening method was based on the acquisition of NaCl salt-stress-tolerance and oxidative (methyl viologen) stress-tolerance of the *E. coli* cells carrying the algal genes. In this study we choose a mangrove plant as a resource of anti-stress genes, constructed a cDNA library of *Avicennia marina*, which is one of the mangrove plants showing the highest salinity tolerance, and screened this library for anti-stress genes with this expression screening method.

We isolated 76 clones, and the DNA sequences of these clones were analyzed. Of the 76 isolated clones; 30 clones were found to be homologues of the genes previously reported; 21 clones showed significant homology to the expressed sequence tag (EST) entries in the DNA database (the functions of the coded proteins are unknown); five clones showed significant homology to the genomic DNA sequence entries (coded protein is unknown); three clones were chloroplast genomic DNA; one clone was 26S ribosomal RNA; and the other 16 clones were unknown (novel) genes (no significant homology to any entries of the database). Of the 30 homologous clones, 67% (20/30) seemed to be full-length clones (having the putative start codon).

**Heat-shock proteins and ubiquitin**

Among the 30 homologous clones, there are several stress-related genes as shown in Table 1. The acquisition of stress tolerance of the *E. coli* cells carrying these potential anti-stress genes was further confirmed by back-inoculating the phagemid DNA into the host *E. coli* cells, and by checking the acquisition of stress tolerance of the newly generated transformants. The chaperonin-60 (clone No.: Av117, DDBJ Acc. No.: AB049590) is the homologue of GroEL of *E. coli*, and is a chaperone protein found in most organisms. The main function of the GroEL protein is to help the organism deal with outside stresses (especially heat and salt) by holding proteins under those stressed conditions (Buchner 1996). ClpP (clone No.: Av129, Acc. No.: AU108486) is also a member of stress (heat, salt, and ethanol etc.)-inducible clp/Hsp100 chaperone family,

<table>
<thead>
<tr>
<th>Accession No. a</th>
<th>Identification</th>
<th>% of amino acid identity (No. of overlaps)</th>
<th>Species</th>
<th>Accession No. b</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB049589 (Av117)</td>
<td>Chloroplast chaperonin-60</td>
<td>76 (166)</td>
<td><em>Brassica napus</em></td>
<td>Pir-S38642</td>
<td>2.1E–39</td>
</tr>
<tr>
<td>AU108486 (Av129)</td>
<td>Clp protease (clpP)</td>
<td>69 (124)</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Dad-AF032123-1</td>
<td>6.4E–30</td>
</tr>
<tr>
<td>AU108479 (Av119)</td>
<td>Ubiquitin</td>
<td>97 (190)</td>
<td><em>Vicia faba</em></td>
<td>Dad-AF032123-1</td>
<td>4.8E–65</td>
</tr>
<tr>
<td>AU108477 (Av116)</td>
<td>Translation elongation factor-1 α</td>
<td>87 (183)</td>
<td><em>Zea mays</em></td>
<td>Dad-AF032123-1</td>
<td>3.9E–59</td>
</tr>
<tr>
<td>AU108475 (Av113)</td>
<td>Drought-induced 19 (AtDj19)</td>
<td>41 (95)</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Pir-SS1478</td>
<td>4.5E–13</td>
</tr>
<tr>
<td>AB049589 (Av114, 140)</td>
<td>Secretory peroxidase</td>
<td>84 (175)</td>
<td><em>Nicotiana tabacum</em></td>
<td>Dad-AF149251-1</td>
<td>1.7E–62</td>
</tr>
</tbody>
</table>

a DNA sequence data were submitted to DDBJ (DNA Data Bank of Japan) database.

b Database abbreviations: Dad = all translated sequences from DDBJ; Pir = Protein Identification Resource.