
M Parani, M N Jithesh, M Lakshmi and A Parida*

M S Swaminathan Research Foundation, III Cross Street, Taramani Institutional Area, Chennai 600 113, India

Received 20 April 2001; revised 7 February 2002

Covalent attachment of ubiquitin has been implicated in mediating proteolysis of the cellular proteins by Ubiquitin-proteasome pathway. Ubiquitin activating enzyme (EI), ubiquitin conjugating enzyme (E2), and ubiquitin protein ligase (E3) are the three enzymes involved in this process. This paper reports the isolation of a gene that codes for the ubiquitin conjugating enzyme in *Avicennia marina* (AmUBC2), and regulation of its expression at RNA level under salt stress. Deduced amino acid sequence of AmUBC2 showed 96% identity with UBC2 of *Arabidopsis thaliana* and also 73-78% identity with RAD6 DNA repair protein of *Homo sapiens*, *Rattus norvegicus*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. Multiple alignment analysis showed that the amino acid residues in the core region of UBC2 were highly conserved across different taxa in the evolutionary hierarchy. While some ubiquitin conjugating enzymes were induced under salt, heat and heavy metal stress in different tissues in plants, Northern analysis in the present study has clearly shown that the expression of UBC2 is not induced by salt stress either in root or in leaf tissues in *A. marina*. Southern hybridization of genomic DNA with gene-specific probe showed that AmUBC2 is a single copy gene.

Keywords: ubiquitin conjugating enzyme, *Avicennia marina*, proteolysis, mangroves

Introduction

The state of proteins in living organisms is dynamic being continuously synthesized and degraded. Degradation of proteins could be non-specific like hydrolysis in lysosomes (Knop et al, 1993) or selective like degradation by ubiquitin-proteasome pathway (Hershko & Ciechanover, 1992). Three enzymes are involved in conjugating a particular protein to ubiquitin before being presented to 26S proteasomes for selective degradation. The C-terminus of ubiquitin is adenylated by the ubiquitin activating enzyme (ubi or E1). The resultant ubiquitin-AMP intermediate of the ternary complex is then attacked by a sulfhydryl group of the enzyme, yielding an E1-ubiquitin thiolester. Then, E1 is replaced by ubiquitin conjugating enzyme (ube or E2) by transthiolation yielding E2-ubiquitin thiolester. Mostly, E2-ubiquitin binds to the target protein in conjunction with ubiquitin-protein ligase (ubr or E3) or rarely on its own. Subsequently, consuming ATP, the 26S proteasomes degrade the protein and release the ubiquitin (Finley & Chau, 1991; Ciechanover, 1994). Apart from protein degradation, UBC is also involved in DNA repair, cell cycle control and removal of incorrectly folded proteins under normal and stress conditions (Hochstrasser, 1996). In plants, some UBC's are also induced under stress conditions such as heat shock, heavy metal treatment (Feussner et al, 1997) and high salt (Emilie et al, 2000), and have been implicated in selective degradation of incorrectly folded proteins caused by stress (Seufert & Jentsch, 1990). Several ubiquitin genes are present in plants for the proteasome-ubiquitin pathway and hence the diversity in functional characteristics, expression and regulation of plant UBC's (Ingvardsen & Veierskov, 2001). A multigene family encodes the ubiquitin-conjugating enzyme. For example, there are 18 structurally related genes belonging to the UBC super family in *Arabidopsis thaliana*. Super family can be divided into five gene families based on the protein structure and the physiological functions. AtUBC1 gene family includes AtUBC1, 2 and 3; AtUBC4 family with AtUBC4, 5 and 6; AtUBC7 family includes AtUBC7, 13 and 14; AtUBC8 family with AtUBC8, 9, 10, 11 and 12; AtUBC15 family with AtUBC15, 16, 17, 18 (Sullivan et al, 1994; Nocker et al, 1996). There are two types of ubiquitin genes, polyubiquitin genes and ubiquitin extension genes. Polyubiquitin genes are...
arranged tandem head-to-tail repeats, and post-translational processing results in monomeric units of the protein. Ubiquitin extension genes are single monoubiquitins linked via its C-terminus to an unrelated protein of either 52 or 76 to 81 amino acids (Nishi et al, 1993).

This paper reports the isolation and characterisation of a full-length cDNA clone from a mangrove species, *Avicennia marina* that codes for the ubiquitin-conjugating enzyme. Mangroves inhabit the estuaries and backwaters of the tropical and sub-tropical coasts, and *A. marina* is one of the true mangrove tree species capable of withstanding salinity that is even higher than that of normal sea-water.

**Materials and Methods**

**c-DNA Library Construction**

One-year-old wild plant of *Avicennia marina* (Forsk.) Vierh. was collected and treated with 0.5 M NaCl for 48 hrs. Total RNA from the leaf tissue was isolated following the GITC method (Chomzynski & Sacchi, 1987) with minor modifications (Parani et al., 1999). Poly (A)+ RNA was purified over oligo-(dT) cellulose column and used as template for cDNA synthesis. The SuperScript™ Lambda System for cDNA Synthesis and λ Cloning (Life Technologies, USA) was used for cDNA synthesis. First strand cDNA synthesis was primed with NotI-primer adapter, and the double stranded cDNA was directionally cloned in plasmid vector (pSPORT 1) using the SalI adapter ligated at the 5' end. The SalI adapter ligated cDNAs were size fractionated over SizeSep™-400 Sepharose CL-4B spin column (Pharmacia Biotech, USA) before cloning in the plasmid vector. The ligated cDNAs were transformed in to the DH5α strain of *Escherichia coli*.

**Sequencing of Expressed Sequence Tags (ESTs)**

Several clones from *A. marina* cDNA library were randomly selected, and the insert size in each of the clones was determined by PCR using the universal M13 forward and reverse primers. The Plasmid DNA from the cDNA clones having cDNA of above 600 bp size were isolated by alkaline lysis method (Birnboim & Doly, 1979). The 5' end of the cDNAs were subjected to single-read sequencing using M13 reverse primer and Big-Dye™ Terminators in an automated sequencing machine (ABI310, Applied Biosystems, USA). The DNA sequences were clipped for removing vector and adapter sequences and manually edited for sequencing errors. The edited DNA sequences were used for searching nucleotide and protein homology to the existing genes in the databases at www.ncbi.nlm.nih.gov using BLASTN and BLASTX algorithms, respectively.

While the clones identified to be partial or having homology with unknown proteins were reserved for future studies, the clones having potentially full-length genes were completely sequenced from both the strands and further characterized. This study reports the characterization of a full-length cDNA that codes for ubiquitin conjugating enzyme in *A. marina* (Genbank Accession No. AF262934). Multiple alignment analysis of *A. marina* gene coding for the ubiquitin conjugation enzyme with other homologues was performed using a CLUSTALW Program (Thompson et al, 1994).

**Northern Analysis**

The seeds of *A. marina* were collected from the mangrove forests in Pichavaram, Tamil Nadu, and seedlings were raised in pots, and irrigated with half-strength MS solution. Full-grown plants (two months after planting) were kept either in half-strength MS solution (control) or half-strength MS solution supplemented with 500 mM NaCl (treated). Total RNA from leaf and root tissues from control and treated plants were isolated at 12 hrs interval up to 48 hrs after imposing the stress, and up to 24 hrs after withdrawing the stress according to the procedure described previously. Total RNA (15 μg) was separated in 1.2% formaldehyde gel and transferred to nylon membranes (Sambrook et al, 1989). A 200 bp long 3' untranslated region (UTR) from AmUBC2 was amplified by PCR using forward (5'-TCCCTTACTAGACGTTGG) and reverse (5'-AGTGACGCGTTCCCTACA) primers, and eluted from agarose gel. This fragment was radioactively labeled with 32P and used as gene specific probe for Northern analysis. Following hybridizations (Sambrook et al, 1989), the blot was finally washed at 1X SSC and 0.1% SDS stringency conditions and exposed to X-ray films for 2 days at 70°C. Estimation of RNA sizes was based on the mobility of RNA Ladder 0.24-9.5 Kb of GIBCO BRL and computing the relative size of AmUBC2 using a Fragment Sizer Program of SEQAID II (tm) version 2.01.
Southern Analysis

Total genomic DNA from A. marina leaves was isolated using the CTAB method with minor modifications (Parani et al., 1997). 10 µg of genomic DNA was digested with four different restriction endonucleases, EcoRI, EcoRV, HindIII and BamHI, fractionated in a 0.8% agarose gel and then transferred on to a nylon membrane (Hybond N*, Amersham) by Southern blotting (Southern, 1975). The blot was hybridized with a radioactive labeled 200 bp fragment in the 3' untranslated region (UTR) of AmUBC2 as explained in the previous section. After hybridizations (Sambrook et al., 1989), the blot was finally washed at high stringency (0.1X SSC and 0.1% SDS) and exposed for 3 days at 70°C.

Results and Discussion

The mangrove ecosystem harbours several interesting plants, animals and microbes (Rao, 1987). The plant species of the mangrove forest that grow luxuriously under saline seawater is a rich source for genes conferring salinity tolerance. Therefore, authors have constructed a cDNA library from one of the highly salt tolerant mangrove species, A. marina, in order to isolate the genes involved in salt stress or osmotic stress. Amino acid residues in the N-terminal region of the proteins are conserved across the species, and therefore, by sequencing a few hundred nucleotides in the 5' end of the cDNA, it is possible to predict the gene function without complete sequencing of the corresponding gene. That is, the 5' nucleotide sequence can serve as a tag for the intact expressed gene, and hence, called as Expressed Sequence Tags (ESTs). Large numbers of ESTs are sequenced in several species for rapid gene discovery (Rounsley et al., 1996; Covitz et al., 1998). The approach was used for gene isolation from the A. marina cDNA library. One of the ESTs (Am494) showed significant homology with ubiquitin conjugating enzyme. This clone was approximately 900bp as determined by PCR and the open reading frame (ORF) was intact at the 5' end from 60 bp. As the ORF of the reported UBCs were about 500 bp, this could be a full-length clone. Since some of the ubiquitin conjugating enzymes were reported to be upregulated under salt stress, Am494 was completely sequenced and further characterized.

Complete sequencing of Am494 revealed that it is 850bp long with a longest ORF of 459bp (60bp-518bp) coding for 152 amino acids. The 5' untranslated region (UTR) is 59bp and the 3'UTR is 332bp with a 22-residue Poly (A) tail from 829bp. The 3'UTR did not contain a typical poly-adenylation signal but a putative signal ATTAAA is located at position 763-768bp.

Homology search for the deduced amino acid sequence of the longest ORF of Am494 was carried out with the global database using BLASLX algorithm. It showed strong homology with UBC2 of A. thaliana (AtUBC2). AtUBC2 is a member of a UBC gene family AtUBC1 that includes AtUBC1, 2 and 3. Am494 clone showed 95, 96 and 87% identical amino acids with AtUBC1 (Accession No. L19351), AtUBC2 (Accession No. L19353) and AtUBC3 (Accession No. L19352), respectively. It also showed 96% identical amino acids with UBC2 from Medicago sativa and Triticum aestivum. However, it showed only 35-45% identical amino acids with UBCs outside this family like UBC4, 7, 8, 15 of A. thaliana (Accession No. L19354, U33757, Z14989, AF028338) (Table 1). Percentage of identical amino acids was only 45-46% with UBC7 of maize (Accession No. AF034946), and UBC4 of tomato and pea (Accession No. L23762, L29077). Similarity percentage between the nucleotide sequences of AmUBC and AtUBC2 was 85%. But it showed no significant similarity with any of the UBC outside the AtUBC1 gene family (Table 1). This clearly showed Am494 to be a homologue of AtUBC2 of the AtUBC1 gene family, and hence named AmUBC2. In addition, AmUBC2 also showed 73-78% identical amino acids with RAD6 DNA repair protein of Homo sapiens, Rattus norvegicus, Caenorhabditis elegans, Drosophila melanogaster, Arabidopsis thaliana and Saccharomyces cerevisiae (Accession Nos P49459, M62388, U08139, M63729, L19353, P06104 respectively). Multiple alignment analysis of AmUBC2 and AtUBC2 from plants and the RAD6 DNA repair protein homologues in yeast and animals presented in Fig. 1, reveals that the UBC enzymes across different taxa carries a highly conserved cysteine residue. It is hypothesized that ubiquitinylation takes place via a thiol-ester at this cysteine residue (Sullivan & Vierstra, 1991). Mutation of the highly conserved cysteine-88 residue in the yeast Saccharomyces cerevisiae RAD6 protein has been experimentally shown to destroy the protein’s vital ubiquitin-conjugating activity (Sung et al., 1990).

The Ubiquitin dependent proteolysis pathway plays a vital role in the selective protein degradation in cellular organisms. UBC2-proteasome complex in yeast
could efficiently target H2B for ubiquitylation (Prasad et al., 2000). It is also hypothesized that the interaction between UBC and the proteasome may be regulated by DNA repair and could be induced upon physiological stress. One report shows that mcUBC1 coding for ubiquitin conjugating enzyme in the halophytic ice plant, Mesembryanthemum crystallinum, is upregulated under salt stress. In ice plant, mRNA level of UBC4 (but named as mcUBC1) was induced under 200 mM and 400 mM NaCl stress only in root but not in leaf tissues. However, UBC9 (but named as mcUBC2) was not induced either in root or leaf tissue (Yen et al., 1999; Emilie et al., 2000). A UBC gene was induced under heat as well as heavy metal stress (Feussner et al., 1997). However, it could not be properly annotated and assigned to a particular UBC gene family, because its sequence homology with other plant UBCs covered only smaller sequence parts. Therefore, it would be interesting to study the spatial and temporal regulation of AmUBC2 in A. marina. Northern hybridization of total RNA from A. marina roots and leaves with gene specific probe for AmUBC2 was used to study regulation of this gene at RNA level. A single mRNA species (approx 830 bp) was observed with this probe indicating the expression of RNA transcripts for AmUBC2.
Table I — Percentage of identical amino acids and nucleotides (given in parenthesis) between the UBC from *Avicennia marina* (annotated as UBC2) and other UBCs from *Arabidopsis thaliana*. One gene from each gene family of *A. thaliana* UBC super family was taken for comparison.

<table>
<thead>
<tr>
<th></th>
<th>AmUBC2 (AF262934)</th>
<th>AtUBC2 (L19353)</th>
<th>AtUBC4 (L19354)</th>
<th>AtUBC7 (U33757)</th>
<th>AtUBC8 (Z14989)</th>
<th>AtUBC15 (AF028338)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amubc2</td>
<td>100 (100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atubc2</td>
<td>96 (85)</td>
<td>100 (100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atubc4</td>
<td>32 (*)</td>
<td>32 (*)</td>
<td>100 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atubc7</td>
<td>37 (*)</td>
<td>39 (*)</td>
<td>26 (*)</td>
<td>100 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atubc8</td>
<td>45 (*)</td>
<td>46 (*)</td>
<td>34 (*)</td>
<td>34 (*)</td>
<td>100 (100)</td>
<td></td>
</tr>
<tr>
<td>AtUBCI5</td>
<td>35 (*)</td>
<td>35 (*)</td>
<td>27 (*)</td>
<td>31 (*)</td>
<td>37 (*)</td>
<td>100 (100)</td>
</tr>
</tbody>
</table>

(*) No significant similarity between the nucleotide sequences.

---

Fig. 2 — Northern blot analysis of AmUBC2 RNA transcripts in Leaf tissues (a) and Root tissues (b) of *Avicennia marina* under salt stress. Total RNA was isolated from leaves (a) and roots (b) of plants that had not received NaCl treatment, Control (Lane 1) and plants that were salt stressed (0.5M) for 12 hours (Lane 2); 24 hours (Lane 3); 48 hours (Lane 4); 12 hours after withdrawal from the salt stress (Lane 5); 24 hours after withdrawal from the salt stress (Lane 6). 15 µg of total RNA was loaded and probed with 32P labeled AmUBC2 3' UTR cDNA probe. To confirm equal loading, blot (a) was stripped and reprobed for 18S r-RNA (c). The same was done for root also (data not shown). For size estimation, GIBCO BRL 0.24-9.5 Kb size ladder was used.

---

Fig. 3 — Southern blot analysis of *Avicennia marina* genomic DNA with the AmUBC2 3’ UTR specific cDNA probe. 10 µg of genomic DNA was digested with restriction enzymes EcoRI, EcoRV, HindIII and BamHI. The blot was hybridized with 32P labeled AmUBC2 3’ UTR cDNA probe. Expression of UBC2 is not induced under salt stress either in the root or in the leaves even after 48 hours of salt treatment (Fig. 2). This result establishes that up regulation of UBC mRNA is specific to only certain members of the gene family. However, the steady
state level of different UBCs in different tissues and reason for up regulation of only specific UBCs under stress remains to be studied. In this context, it has to be noted that UBC2 is not only involved in proteolysis but also in DNA repair, which is an essential constitutive function for any organism.

Southern hybridization studies using AmUBC2 cDNA was done to find out the gene copy number. In hybridizations of 3' UTR AmUBC2 specific probes to the Avicennia marina total genomic DNA, at high stringency washing conditions, all the four enzymes (EcoRI, EcoRV, HindIII and BamHI) used to digest total genomic DNA, showed one major hybridizing fragment (Fig. 3). Thus, it is concluded that AmUBC2 gene is present as a single copy gene in the genome of Avicennia marina.

Thus, the present communication would help in understanding and elucidating the functions of this crucial enzyme of protein turnover and DNA repair in different organisms.

Acknowledgement
The authors gratefully acknowledge the Department of Biotechnology and the Department of Atomic Energy, Government of India, New Delhi, for financial support.

References


