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Research Article

Betaine Aldehyde Dehydrogenase (BADH) gene and free amino acid analysis in *Rhizophora mucronata* Lam. from Thalassery region of Kerala, India.

Anusha Sreeshan, Meera S. P., Anu Augustine*
Department of Biotechnology and Microbiology, Kannur University Thalassery Campus, Palayad P.O., Kannur-670661, Kerala, India.

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Abstract: Mangroves are salt tolerant plants inhabiting saline environment. Multiple factors contribute to their salt tolerance and we need multifaceted approach to reveal the mechanisms of salt tolerance in the plant. In the present study, leaves of the mangrove, *Rhizophora mucronata* grown in the presence and absence of salt were used, free amino acids and the expression of selected genes were analyzed. Chromatographic technique showed the accumulation of free amino acids like proline, glycine, aspartic acid, valine, leucine and glutamic acid in the presence of salt. RNA was isolated from the leaf sample and cDNA was synthesized. Gene specific primers were designed and standardized. Among the genes studied (P5CS, BADH, NHX1), Betaine Aldehyde dehydrogenase (BADH) gene was found to be expressed.

Keywords: Amino acid, Mangrove, Salinity, Salt tolerance, Stress.

Introduction

Plants serve as reservoir of various amino acids and phytochemicals.In plants like mangroves these constituents help them to survive in saline environment. In the present study we attempt to find the presence of free amino acids and the expression pattern of few salt tolerant genes in *Rhizophora mucronata,* a true mangrove species. The species inhabit saline environments and their high adaptability to salinity can be partially explained by morphological, physiological and biochemical studies (Liang *et al.,* 2008). Mangroves are always important model for studying salt tolerance. They acquire the tolerance during their development and it is contributed by several factors (Sreeshan *et al.,* 2014). Parida and Jha (2010) reviewed the different mechanisms of salt tolerance in mangroves like the morphological and anatomical features, physiological and biochemical mechanisms (salt secretion, salt exclusion and salt accumulation, accumulation of compatible solutes and osmolytes, induction of antioxidative enzymes) and other molecular mechanisms of salt tolerance in mangroves.

Nasir *et al.,* (2010) showed that halophytes are rich sources of free amino acids and the content increases under salt stress. Halophyte defense mechanisms to salt include changes in ion homeostasis, formation of osmo protectants, activation of cross - talk genes, induction of antioxidants, and development of salt glands or bladders. Much information is not available on well-defined molecular defense mechanism of halophytes against salt stress (Mishra and Tanna *et al.,* 2017). Salt stress induced genes mainly includes those which are involved in osmolyte accumulation, transporters and channel proteins, detoxification, ion homeostasis and stress hormone production, ROS generation etc. (Park *et al.,* 2016, Meng *et al.,* 2018).

Salinity tolerance is complex and involves the action of many genes, progress has been made in studying their mechanism (Negrao *et al.,* 2017). We analyzed some of the genes involved in salt tolerance, among those analyzed Betaine aldehyde dehydrogenase was found to be expressed, one which involved in glycine betaine synthesis. BADH was studied long back in plants and their role in abiotic stress was reported (Fitzgerald *et al.,* 2009). Betaine aldehyde dehydrogenase was first shown in plant salt stress response in spinach leaves (Weigel *et al.,* 1986). These are thought to protect the plant by acting as an osmolyte by maintaining the water balance between the plant cell and the environment, and by stabilizing macromolecules, enzyme activities, and membranes under stress conditions (Zhang *et al.,* 2011). Nowadays mangrove genes are widely used in transgenic research to enhance the capacity of salt tolerance in model plants (Meera *et al.,* 2013). A proper understanding of the mechanisms of salt tolerance will give us an idea about how mangrove survive in saline environment and thus pave-way to develop salt tolerant crops. In the present study we made an attempt to identify the free amino acids

*Corresponding Author:
Dr. Anu Augustine,
Department of Biotechnology and Microbiology,
Kannur University, Thalassery Campus,
Palayad P.O., Kannur- 670661, Kerala, India.
E-mail: anua@kannuruniv.ac.in

and some of the genes which were reported previously in many salt tolerant varieties contributing to their tolerance.

Materials and Methods

Rhizophora mucronata leaves were collected from Melur estuarine area of Thalassery region in Kannur district, Kerala. Plant propagules were grown in pots and watered for several months using tap water. This leaves were used as control for the experiment and the leaves of *Rhizophora mucronata* collected from estuarine region growing in salt water (260 mM concentration, at the time of collection) were used as salt treated sample. Leaves were ground in liquid nitrogen. For the free amino acid analysis 2 gram of powdered leaf was homogenized in acetone and the extract was centrifuged at 3000g for 30 minutes. The supernatant was subjected to paper chromatography using a mixture of 4:1:5 n- butanol: acetic acid: distilled water as running solution.

RNA was isolated from the leaf tissue, following the protocol by Rubio-Pina and Zapata-Perez (2011). Leaf samples were ground to a fine powder using liquid nitrogen and transferred into 2 ml tubes. 900 µl of extraction buffer (Extraction buffer: 2% (w/v) CTAB; 0.1 M Tris-HCl (pH 8); 1.4 M NaCl; 20 mM EDTA (pH 8); 2% (w/v) PVPP) and 100 µl of β-mercaptoethanol were added. The mixture was shaken for 30 sec and then incubated at 65ºC for 10 min, inverting the tube 3-4 times during incubation. To the mixture 800 µl of chloroform was added. The mixture was shaken for 30 sec and centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was transferred to a new tube, 800µl of phenol/chloroform (1:1) was added and shaken for 30 seconds. The mixture was centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was transferred to a new tube and an equal volume of chloroform/ isoamyl alcohol (24:1) was added. Samples were shaken for 30 sec and centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was transferred to new tube containing 1/3 volume of LiCl (8 M) and kept at -20ºC for 4 hrs. The samples were centrifuged at 10,000 rpm for 20 min at 4ºC. The pellets were washed with 100% and 70% ethanol. The pellets were dried and dissolved in 50 µl of molecular biology grade water.

RNA was quanified and cDNA was synthesized from approximately 1µg of total RNA was mixed with 2µl of 50 µM Oligo (dT) primer and 2µl dNTP mix (10mM) and incubated at 65°C for 5 minutes. The reaction mix was immediately chilled on ice and cDNA synthesis was done by adding 8µl of 5x first strand buffer, 1µl of RNasin (40U/µl), and 2µl reverse transcriptase (200U/µl) (Thermo Scientific). The reaction was adjusted to a total volume of 20µl with DEPC water and incubated at 42°C for 60 minutes, the reaction was inactivated at 72°C for 10 minutes.

The RT-qPCR analysis of reference and target gene transcripts in cDNA samples were conducted in a 7900 HT Fast Real Time PCR System (Applied Biosystems). Gene specific primers were designed from the gene sequence from related species using Primer3 Plus software and was synthesized from Sigma Aldrich (100 µM concentration).The primer was diluted to 50 µM concentration and amplified at different annealing temperatures within the range of primer melting temperature. Amplified products were checked on 1% agarose gel. Annealing temperature was selected based on absence of primer dimers and nonspecific amplification. The PCR reaction contained 4 µl of above synthesized 1µg cDNA as template, 12.5 µl of 2X SYBR green master mix (Thermo Scientific), forward and reverse primers (concentration 50mM) and the total volume was made upto 25µl with sterile water. All the samples were assayed under the following conditions: 95˚C for 10 min followed by 40 cycles of 95ºC for 15s and 60ºC for 1 min. The PCR products were then subjected to melt curve analysis. The melt curve analysis was done from 60-95ºC to ensure that the resulting fluorescence originated from a single PCR product and did not represent primer dimers formed during the PCR or a non-specific product. No-template controls were also included for each gene to detect any spurious signals arising from amplification of any DNA contamination or primer dimer formed during the reaction. RT-qPCR results were analyzed with the sequence detection software SDS version 2.4 (Applied Biosystems). The SYBR green fluorescent signal was standardized to a passive reference dye (ROX) included in the PCR master mix. Direct detection of the PCR product was measured by monitoring the increase in fluorescence caused by the binding of SYBR green dye to double stranded DNA. Actin was used as internal control and the experiment was done in triplicates. The primer was diluted and amplified at different annealing temperature within the range of primer melting temperature. Amplified products were checked on 1% agarose gel. Annealing temperature was selected at which there is absence of primer dimers and nonspecific amplification.

Results and Discussion

The plant was taxonomically identified as *Rhizophora mucronata*, they are average sized trees with stilt arch roots and prop roots. Their leaves have a mucronate tip and are oval shaped and wide and sometimes have brown spots on the underside surface (Fig.1).

Figure 1. *Rhizophora mucronata* leaves

Chromatographic analysis of the leaf extract showed the presence of proline, glycine, aspartic acid, valine, leucine, glutamic acid in the plants grown in saline condition whereas those from control plants showed threonine, glutamic acid, leucine, serine, aspartic acid, and tyrosine. Rf values were analyzed using Wincat software (Fig.2). Betaine and proline are compatible solutes that accumulate in response to osmotic stress, and the accumulations of these osmolytes promote osmotic balance at cellular level (Moghaieb *et al.,* 2004, Liang *et al.,* 2017). The present study showed the presence of these osmolytes in *R. mucronata* under salt stress. Free amino acid contents vary widely in leaf cells, depending on the species and the metabolic conditions. Joshi *et al.,* (1962) reported that salinity enhances amino acid biosynthesis and inhibits organic acid synthesis, these soluble nitrogenous compounds accumulated under salt stress is regarded as a components of salt tolerance mechanism (Stewart and Larher 1980). In built levels of amino acids in higher amounts were reported in many salt tolerant plants. Stewart and Larher (1980) showed plants when grown without NaCl have low levels of such solutes but show increased accumulation under salinization. Our study is in concurrence with these findings.

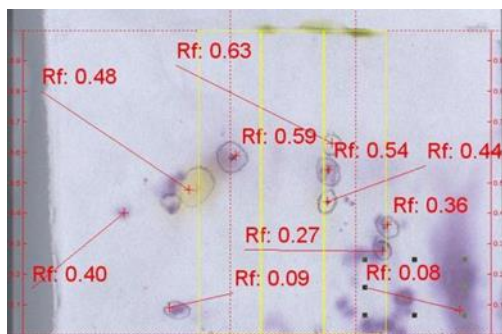

Figure 2. Amino acid spots on paper chromatogram

Clear RNA bands were observed as shown in the figure. cDNA was synthesized using oligo (dT) primers and reverse transcriptase (Fig.3). RNA samples were DNase treated.

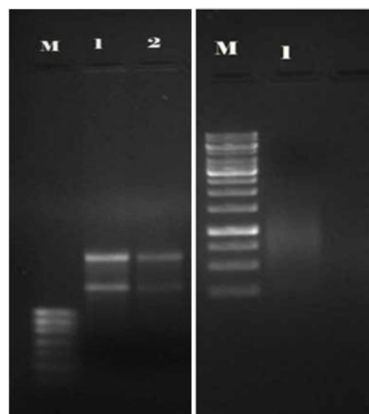

Figure 3. RNA and cDNA on 1% agarose gel

Some of the genes involved in salt tolerance were selected .Genes specific primers were designed and synthesized (Fig.4). The expression analysis of BADH, NHX1 and P5CS gene in *R. mucronata* were done by amplification of cDNA by real time PCR. The amplification of the genes was determined from the amplification plot and specificity of the product was checked by melt curve analysis. The expression analysis showed that among the genes analyzed, BADH showed a higher fold change.

Genes	5'<-----Sequence----->3'
BADH	GAAGCGATCGTCGGTGTATT
	TCAACATTGGGATGAGATGC
P5CS	TCCAAGAGGAGTCCATCTGC
	GCAGGAAGCTGGGTTAAGAA
NHX1	CAGTTCACCAACAGGTGCAG
	ACCTCCATAGCACGCCTCT

Figure 4. Gene specific primer sequences

Amplified PCR product was sequenced by Sanger Chain termination method (Amnion Bioscience, Bangalore). A partial sequence of 516 base pair was obtained. Sequence similarity was checked using BLAST tool (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) and the partial sequence was deposited in GenBank. BLAST analysis of BADH sequence showed a maximum of 82% similarity with *Jatropha curcas* BADH mRNA and 80% similarity with *Beta vulgaris* BADH mRNA. GenBank accession number for the nucleotide sequence deposited is: KP067199.

Real-time PCR analysis showed the increased expression of BADH gene in *Rhizophora mucronata.* Hibino *et al.,* (2001) first identified and cloned the BADH gene that is involved in betaine synthesis in *Avicennia marina*. BADH was up-regulated and lead to the accumulation of betaine in *A. marina*. Glycine betaine is known to be effective in protecting the plant against abiotic stresses such as salt, water deficit, heat and chilling (Le Rudulier *et al.,* 1984). It is an important osmoprotectant in bacteria, plants, and animals, but little information is available on the synthesis of glycine betaine in tree plants.

BADH gene was first reported in Barley (Arakawa *et al.,* 1990) and expressed in response to osmotic stress (Ishitani *et al.,* 1995), further, expression in Spinach (Liang *et al.,* 1997), Rice (Nakamura *et al.,* 1997), Tomato (Jia *et al.,* 2002) and *Suaeda liaotungensis* K. (Zhang *et al.,* 2008) were also reported. Betaine aldehyde dehydrogenase (BADH) plays a dual role in cereals, apart from osmoprotection, it also influences the fragrance in rice, hence of both agronomic and breeding value. The gene was isolated and expressed in wheat (Shrestha *et al.,* 2011). Plant betaine aldehyde dehydrogenases (BADHs) have been targeted in research, since last twenty years and the present study will add to its role in salt tolerance.

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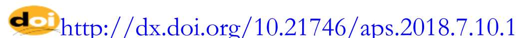http://dx.doi.org/10.21746/aps.2018.7.10.1

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