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Isolation and characterization of salt-induced genes from *Rhizophora apiculata* Blume, a true mangrove by suppression subtractive hybridization

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Mangroves are plants that can tolerate salinity equivalent to that present in seawater. In the present study, we have attempted to isolate the genes upregulated at the early stage of salt stress response in *Rhizophora apiculata* Blume, a true mangrove, through suppression subtractive hybridization technique. Several genes were isolated, which were then classified to nine functional categories, viz. metabolism, protein degradation and folding, secondary metabolism, cell rescue and defence, transport facilitation, signal transduction, transcription and translation, photosynthesis and unclassified genes of unknown function. The expression patterns of 12 of the genes thus obtained were studied at 6, 12 and 24 h time points of salinity stress using real-time PCR. Most genes were found to be upregulated under salt stress and showed maximum upregulation at the 6 h time point. Two of the genes studied were downregulated after 6 h, implying that the pattern of gene expression varies with time of application of stress. The genes thus isolated may be used to confer the trait of salt tolerance to non-tolerant genotypes, which can ultimately prove beneficial for crop improvement programmes.

Keywords: Gene expression, *Rhizophora apiculata*, salt stress, suppression subtractive hybridization.

SALT stress is one of the most serious factors limiting plant growth and productivity[1]. Several studies have focused upon the better utilization of saline soil in order to improve crop production. Mangrove plants may have acquired specific genes essential for salt tolerance during the course of their evolution. Identification and characterization of such genes can contribute towards better agricultural productivity in the future. *Rhizophora apiculata* Blume belongs to the category of Indo-West Pacific stilt mangroves which show tolerance to a wide range of salinities and is the dominant population in the southern coast of India[2]. It belongs to the category of non-secretor, true mangroves which accumulate the excess salt that enters through the transpiration stream within their leaves. This species exhibits several unique biological properties like antimicrobial, insecticidal, etc.

The tolerance of mangroves to a high saline environment is also tightly linked to the regulation of gene expression. The effect of stress upon a plant is best studied at the time of its application rather than at later stages after which the plant adapts to it. Some proteins like vacuolar Na^+/H^+ ATPase, that are upregulated at the initial point of stress, which usually lasts from 30 min to a few hours[3], are switched off once the adaptive stage is reached and their expression levels become comparable to those before stress treatment. Miyama and Tada[4] have reported that in Burma mangrove (*Bruguiera gymnorhiza*), which is also a non-secretor, true mangrove, the expression level of genes changed at 6 h after salt stress treatment, but recovered at 24 h and that the expression profile under salt stress was distinctly different from that under osmotic stress.

Understanding the mechanisms of salt tolerance in mangroves and identification of salt-tolerant genes from mangroves will lead to an effective means to breed or genetically engineer salt-tolerant crops. So the present study focuses on the identification and characterization of the genes involved in short-term response to salinity, especially the salt-tolerant genes in the mangrove, *R. apiculata* by means of suppression subtractive hybridization (SSH) technique, thus providing a new avenue for crop improvement programmes.

Seeds of *R. apiculata* Blume were collected from the Mangrove Research Station at Ayiramthengu, Kollam district, Kerala. The seeds were germinated and grown in potted soil in the greenhouse under freshwater conditions

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for a period of three months. Healthy plants were randomly selected for salt stress treatment for each time point and 500 mM sodium chloride solution was used to impart salinity stress. The seedlings were carefully uprooted from the soil and the stress was imposed at the root zone. For each stress treatment given, a healthy plant treated with the same amount of double-distilled water was used as control. Stress treatment was imposed in accordance with the previous reports[4]. The leaf succulence increased in the sample but not in the control and the leaves did not show any symptoms of wilting.

SSH was performed only for 6 h salinity stress, since the maximum variation in gene expression has been reported at that time point. Leaves were collected from both stress-treated and untreated plants and RNA was isolated by modified CTAB method[5]. Leaves were the organs of choice for this study, since non-secretor species accumulate the salt ions in their leaves. Poly(A)$^+$ RNA was isolated from the total RNA of both the samples using Nucleotrap mRNA isolation kit (Macharey Nagel, Duren, Germany), which was further used for cDNA construction. The subtractive hybridization was performed according to the PCR Select^{TM} Subtraction kit (Clontech, USA). The cDNA of the treated samples (tester cDNA) and the untreated samples (driver cDNA) were equalized in their concentration before proceeding with the subtraction procedure. Poly(A)$^+$ RNA (from human skeletal muscle) provided with the kit was used as a control.

Tester and driver cDNA were digested separately with *Rsa*I to generate short, blunt-ended cDNA fragments, following which specific adaptors were ligated to tester cDNA, but not the driver cDNA. This was followed by two rounds of hybridization reactions to enrich for differentially expressed sequences and two subsequent PCR amplifications to selectively amplify differentially expressed cDNA. The primary PCR was performed for 27 cycles at 94°C, 10 sec; 66°C, 30 sec; 72°C, 90 sec and the secondary PCR for 11 cycles at 94°C, 10 sec; 68°C, 30 sec; 72°C, 90 sec. The secondary PCR product was purified and 0.005 cm^3 volume was then cloned into T/A cloning vector (pGEM-T Easy Vector System, Promega Corporation, USA) and transformed into competent cells of *Escherichia coli* strain JM109. Positive transformants were selected by blue/white screening, from which approximately 200 clones were randomly selected for plasmid isolation using alkaline lysis method[6]. The plasmids were sequenced with T7 5′-ATTATGCTGAGTGATATCCC-3′ and SP6 5′-ATTTAGGTGACACTATAGAA-3′ primers using ABI PRISM 3100 DNA sequencer (Applied Biosystems, USA) with Bigdye R Terminator v3.1 cycle sequencing kit (Applied Biosystems).

Sequences of poor quality (abundant N) were eliminated and the vector sequences were removed manually. The functional assignment of expressed sequence tags (ESTs) was based on a homology search of the insert sequences against the non-redundant (nr) peptide database of NCBI (http://www.ncbi.nlm.nih.gov/BLAST) using BLASTX. The degree of sequence identity between cDNA clone and a known sequence was checked using the BLASTX probability *E* value. The ESTs that matched the database with an *E* value of 10^{-4} were considered to have significant similarity.

Degenerate primers were designed based on previous studies[7] to amplify the core region of the β-actin gene (~390 bp), which was cloned into pGEM-T easy vector (Promega Corporation) and propagated in *E. coli* strain JM109. From this region new gene-specific primers ActF 5′-TCCATAATGAAGTGTGATGT-3′ and Act R 5′-GGACCTGACTCGTCATACTC-3′ of ~100 bp amplicon were designed. These primers were used in real-time PCR analysis for amplification of β-actin gene selected as the endogenous control.

Real-time PCR was carried out at 6, 12 and 24 h time points to confirm the differential expression of the genes identified through SSH. Total RNA was isolated from three-month-old *R. apiculata* seedlings which were given salt stress for 6, 12 and 24 h respectively. Similar to the previous treatments, the leaf succulence increased in the stress-treated plants but not in the control. The leaves showed no symptoms of wilting throughout the entire period of experimentation.

First strand cDNA was synthesized using 2 µg of isolated RNA, 0.5 µg oligo(dT)15 primer, 1 mM dNTP mix, 40 U RNasin ribonuclease inhibitor, 1× MMLV reverse transcriptase reaction buffer and 200 U of MMLV reverse transcriptase (Promega, USA). The forward and reverse primers for real-time PCR were designed using The Primer Express™ v 3.0 software (Applied Biosystems). The primer sequences are given in Table 1. The PCR reactions were carried out in triplicate in 0.01 cm^3 volume containing 0.005 cm^3 Power SYBR® Green PCR Master Mix (Applied Biosystems), 300 nM of each primer and 0.002 cm^3 of template cDNA. Real-time RT-PCR was carried out at 95°C for 10 min (denaturation and enzyme activation) followed by 35 cycles of 95°C at 15 sec and 60°C at 1 min) in 7900 Fast Pro Thermal Cycler (Applied Biosystems) and visualized in 2% agarose gel. β-actin was used as internal control to normalize the PCR efficiency.

PCR amplification of the cDNA obtained after the subtraction process gave discrete bands of differentially expressed transcripts as shown in Figure 1 *a*. Further, PCR analysis with actin primers gave bands in the unsubtracted samples which were absent in the subtracted sample, indicating that the housekeeping gene has been efficiently subtracted out as shown in Figure 1 *b*. About 200 white colonies of transformants were randomly selected for plasmid isolation and sequencing. Presence of insert in these colonies was checked by colony PCR and plasmids were isolated from those having an insert size greater than 200 bp. Of the approximately 100 clones

Table 1. Primers used for real-time PCR analysis

Target gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Annealing temperature (°C)
APK protein kinase	GAAAGCTCCCGGAAATACCAA	CCGCAGCAGAACTGAAACTTC	58
Cytochrome P 450/(S)-N-methylcoclaurine 3′-hydroxylase	GATTTTAGCCTCTTTGCTGTATCTCTTT	TCCTTTTGTAAGGTAATGCCAAACT	57
Methylene tetrahydrofolate reductase	GGGCCTTAATGATGTCAATGC	TGCCTCATCCTTCCATGCA	58
Hevamine A precursor	GAATCAATGGACCACTTCCCTAA	TGAGCACATTTGGTGGAATATAGC	58
Serine threonine kinase	TGTATGTGGACTTGTGTTAGGATGTTC	TGTCTTGACAAACCACCATGGT	59
Aquaporin	TGCCATGCCAAGGATTACCT	CAATCAGAGCCCTGTAAAACGA	58
Tau class glutathione transferase	GCTCCCCTGCTTCCTTCAG	TTGTCAATGTAATCAGCCCAGAA	58
Voltage-dependent anion selective channel	GAGCTAGTGCTCTCATCCAGCAT	TCCTTGTGTCTACCTCTCCTGAGAT	59
AFR protein	GGACCCAAGCTATCGCACTCT	TTATCACAGAAGTCTGCCACTCTGA	60
Geranylgeranyl reductase	TGAAACAAAAGAACGAGGGAAAA	AAATTCATTTCCGTCGAGTAGCA	59
Splicing factor	GAAAATGTGGAAGCAGCAGGTA	AATGCGAGCAAACCATCTCAT	58
Vegetative storage protein	AACTTGGGTTCGCCGTGTAG	AACCCTTGAGGTCGAGTTAGCA	58

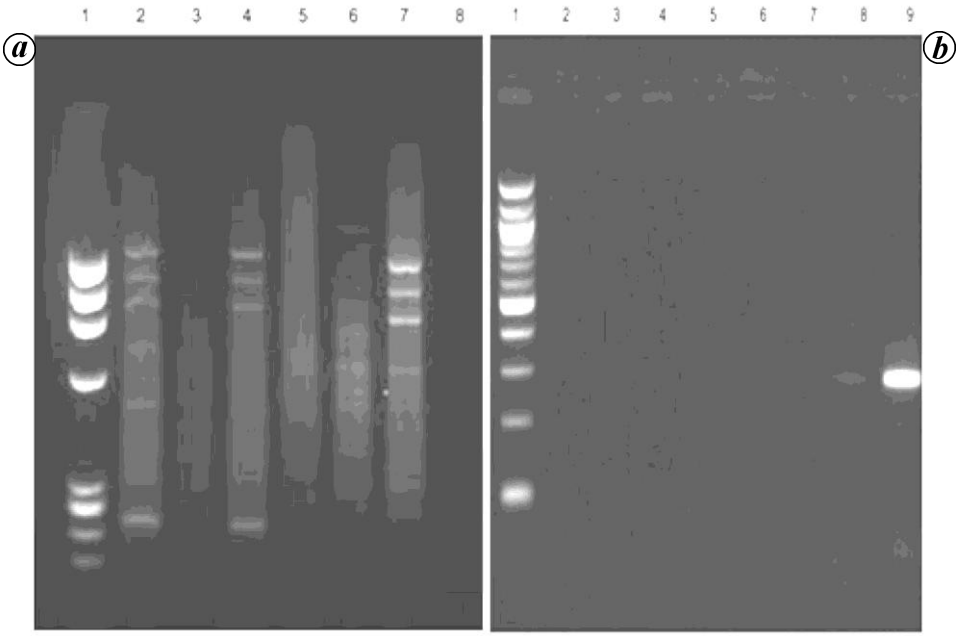

Figure 1. Amplification of differentially expressed cDNAs by primary and secondary PCR. ***a***, Lane 1, φX174 DNA/*Hae*III digest size marker; lanes 2–4, Primary PCR products of subtracted skeletal muscle tester cDNA sample (positive control; provided with PCR Select^{TM} subtraction kit (Clontech, USA)), treated sample (tester) and unsubtracted skeletal muscle tester cDNA (negative control) respectively; lanes 5–7; Secondary PCR products of subtracted skeletal muscle tester cDNA (positive control), subtracted cDNA from *Rhizophora apiculata* (tester) and unsubtracted skeletal muscle tester cDNA (negative control respectively); lane 8, Non-template control. Distinct bands are visible in lane 6 in contrast to lane 3. ***b***, PCR analysis of subtraction efficiency using actin primer. Lane 1, Marker; lanes 2–5, PCR product after 18, 23, 28 and 35 cycles from subtracted cDNA respectively; lane 6–9, PCR product after 18, 23, 28 and 35 cycles from unsubtracted cDNA respectively. Actin gene was found to be less abundant in subtracted cDNA.

that were sequenced, 66 unique clones were putatively identified through BLASTX.

The assignment of genes into groups according to their function was done based on several previous reports[7,8]. Only those genes corresponding to an *E* value greater than 10^{-4} in the BLASTX analysis were selected for functional classification (see Table S1, Supplementary Information online). The functions of 19 of these sequences are unknown and form the largest category of upregulated sequences. Among those genes whose function could be

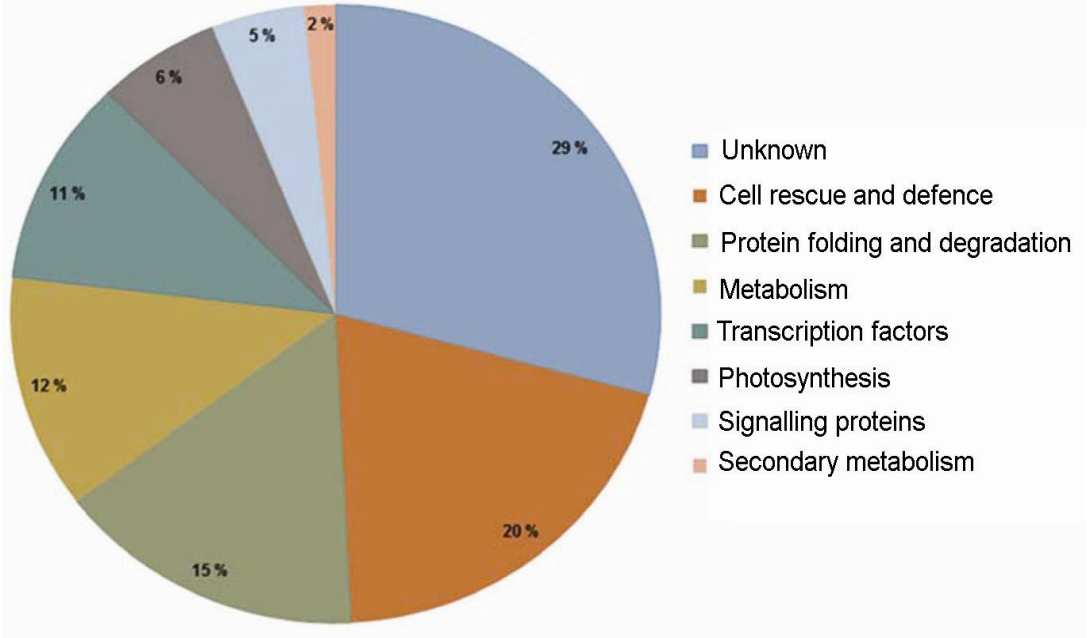

Figure 2. Functional categorization of genes identified by substractive hybridization technique. Percentage of genes showing upregulation under each category is also given.

predicted, the cell rescue and defence related proteins constituted the largest group. The 66 unique clones were functionally categorized into nine groups with the defence-related proteins contributing the largest number of transcripts (Figure 2).

Real-time PCR analysis is commonly employed for the confirmation of differential expression of the transcripts found to be upregulated by SSH[9]. Real-time PCR analyses of 12 genes, viz. cytochrome P 450/(S)-*N*-methylcoclaurine 3′-hydroxylase (*CYT*), aquaporin (*AQP*), tau class glutathione transferase (*TGT*), APK protein kinase (*APK*), methylene tetrahydrofolate reductase (*TFR*), hevamine A precursor (*HAP*), serine threonine protein kinase (*STK*), voltage-dependent anion channel (*VDC*), AFR protein (*AFR*), geranylgeranyl hydrogenase (*GGR*), splicing factor (*SF*) and vegetative storage protein (*VSP*) were done. All genes except *AFR* showed marked upregulation upon salinity stress treatment. Seven of the genes studied showed maximum upregulation at 6 h time point and their expression levels reduced at 12 and 24 h of stress. Only *TGT* showed maximum upregulation at 12 h (Figure 3).

The 66 unique clones were functionally categorized into nine groups. The largest group was the one comprising genes of unknown function. The genes in this category may be novel ones present only in mangroves or known genes whose sequence may be significantly different from those previously reported. Hence one can speculate that mangroves may indeed contain unique genes or gene sequences, whose relevance under salinity stress remains to be studied. The defence-related proteins contributed the largest number of transcripts with known function under stress. The transcripts upregulated in the present study were found to vary considerably from the transcript profile of the roots of *Rhizophora stylosa* under salinity stress[7], implying that distinct mechanisms operate in the roots and leaves which act synchronously to re-establish homeostasis. Several genes involved in stress response, viz. peroxidase, aldehyde dehydrogenase, serine threonine kinase, hydrolase, quinone oxidoreductase, splicing factor-like protein, aquaporin, geranylgeranyl hydrolase, etc. which were upregulated at the time of stress in *R. apiculata* were also upregulated in the Burma mangrove[4]. Hence similar mechanism may be involved in stress response in both the plants.

Twelve of the genes identified by SSH were analysed by real-time PCR. Of these, ten genes showed maximum upregulation at 6 h time point, with the exception of *TGT* which showed the maximum upregulation at 12 h, probably due to its role in the removal of toxic compounds produced due to stress. *AFR* showed downregulation upon salinity stress treatment at all time points, although downregulation may imply toxicity induced by stress and might be a false positive outcome with respect to the forward subtraction procedure. *SF* and *VSP* genes were found to be upregulated at 6 h time point, but were downregulated by 12 h, implying that these genes may have significant function in the initial phase of stress response. This might also suggest that in *R. apiculata* adaptation to salt stress might be occurring somewhere between the 6 h and 12 h time points.

One or two genes belonging to each of the nine functional categories were selected for expression analysis by real-time PCR. These genes are also reported to have

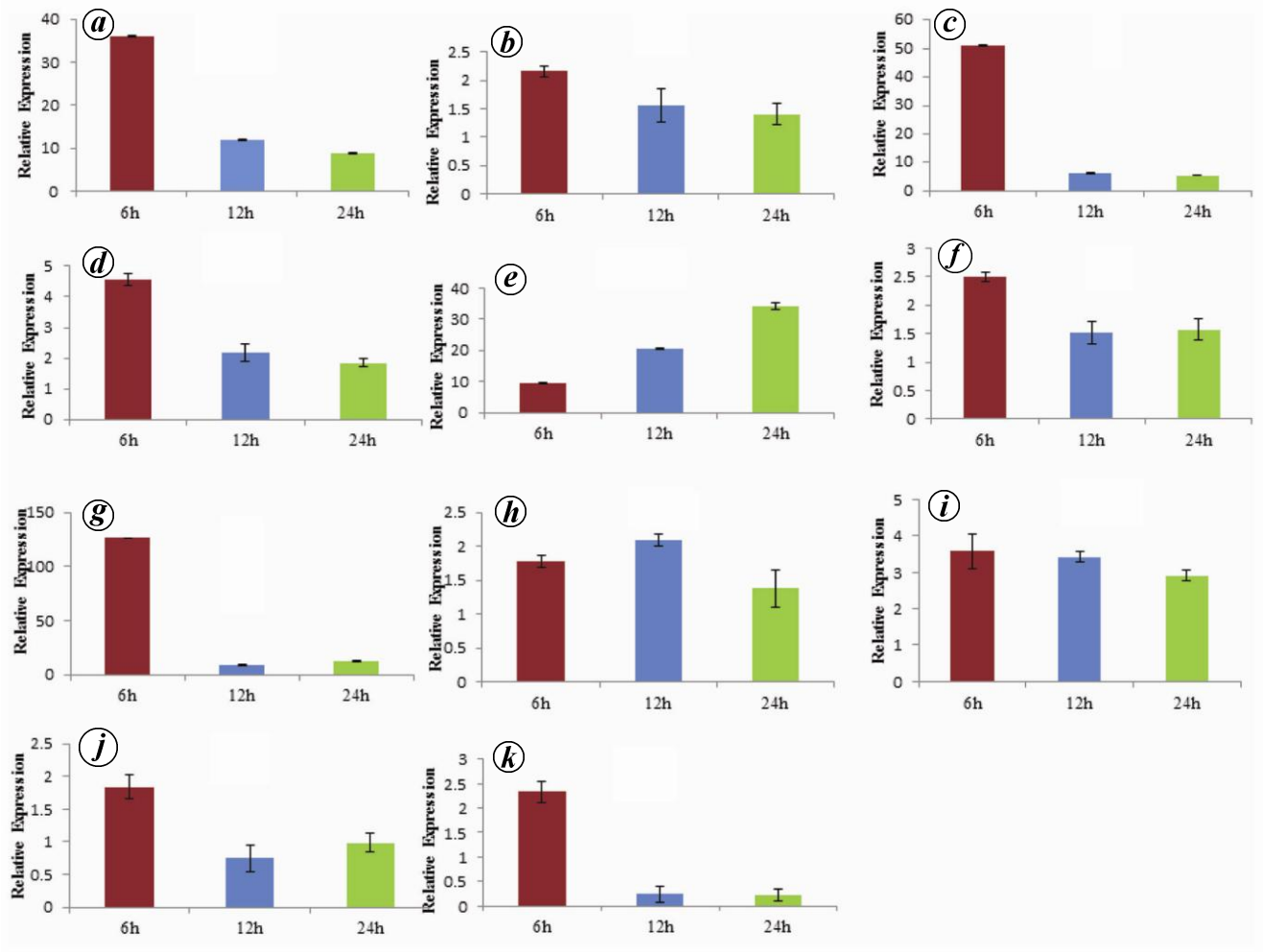

Figure 3. Quantitative real-time PCR analysis for the differential expression of genes at 6, 12 and 24 h of salt treatment. ***a***, Hevamine A precursor; ***b***, Voltage-dependent anion selective channel; ***c***, Aquaporin; ***d***, Methylene tetrahydrofolate reductase; ***e***, Tau class glutathione transferase; ***f***, APK protein kinase; ***g***, Cytochrome P 450/(S)-*N*-methylcoclaurine 3′-hydroxylase; ***h***, Serine threonine kinase; ***i***, Geranylgeranyl reductase; ***j***, Splicing factor; ***k***, Vegetative storage protein. Data are presented as mean ± SE and error bars represent SE.

specific functions under stressed conditions. TGT belonging to the category of cell rescue and defence-related proteins, are exclusively found in plants and show specificity to their substrates. Recent studies report that they are induced by various abiotic stresses and may play an important role in enhancing stress tolerance[10,11]. Another interesting protein in the same class is HAP, which is an endochitinase-like protein. Chitinase-like proteins have been shown to play an important role in controlling the influx of Na^+ ions into the plant, thereby contributing to salt stress tolerance[12].

AQP and VDC are transport proteins widely acclaimed for their role in stress response[13]. Aquaporins function in the intracellular compartmentalization of water[14,15]. Genes involved in signal transduction like *STK* and *APK* also respond to salinity stress. APK1 proteins have been shown to bind to the membranes by myristoylation and phosphorylate tyrosine, serine and threonine residues of their targets. Several transcription factor-like proteins were also upregulated on exposure to salt stress. Among them, a putative splicing factor was also found to be upregulated, which may have roles in the post-transcriptional processing of other mRNAs. AFR protein belongs to the family of F-box proteins which are involved in the regulation of various developmental processes in plants. They also play a critical role in controlled degradation of cellular proteins[16].

As salinity inhibits photosynthesis in plants, several photosynthetic proteins were also upregulated to counteract this effect. GGR catalyses the hydrogenation of geranylgeranyl diphosphate to phytyl diphosphate. Phytyl diphosphate is the precursor for the synthesis of tocopherol which, in turn, prevents the chlorophyll-photosensitized oxidation of thylakoid components when plants are subjected to environmental stress[17]. Genes involved in several metabolic processes were also upregulated by stress. *TFR* has been found to be upregulated under conditions of abiotic stress in wheat and generation of sufficient

methyl units is reported to be crucial and a prerequisite to challenge by multiple stresses[18]. Of the categorized genes, 19 candidates showed no significant matches, and their relevance under stress conditions remains unknown. Among them, the vegetative storage protein has been previously reported to be upregulated in another mangrove, *Bruguiera gymnorrhiza*[9], but information regarding its exact function is currently unavailable.

The *CYT* gene deserves special mention as it is the only gene involved in secondary metabolism that has been upregulated in this study. In fact, *CYT* acts in the benzylisoquinoline alkaloid pathway and its overexpression has been shown to result in up to 450% increase in total morphinan alkaloids without altering product distribution. It is an important target towards improving morphine biosynthesis[19]. The actual role of this enzyme in mangroves and its activity during salinity stress are yet to be studied in detail.

Several of the genes identified in the present study have been previously shown to be upregulated under stress conditions or are suspected to be involved in stress response due to their unique functional attributes. Real-time PCR of 11 of the transcripts also confirms their differential upregulation in response to salt stress. The maximum upregulation was shown at 6 h time point, after which a downregulation in expression was observed for two of the genes. The study hence proves that adaptation to salt stress might be occurring somewhere between 6 and 12 h time points. The differentially unregulated genes can be further studied for their specific function under conditions of salinity stress and also for their contribution towards adaptation to salinity stress in mangroves.

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