Agricultural Communication

Biosci. Biotech. Res. Comm. 9(3): 445-456 (2016)

BBRC Bioscience Biotechnology Research Communications

Expression analysis of salt stress related expressed sequence tags (ESTs) from *Aeluropus littoralis* by quantitative real-time PCR

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ABSTRACT

Aeluropus littoralis is a monocot halophyte grass and provide valuable genetic resources for understanding the molecular mechanisms of stress-responsive genes, and improving tolerance to abiotic stresses in economically important crops. In an attempt to identify salt stressed responsive genes, 154 isolated expressed sequence tags (EST) from *A. littoralis* were bioinformatically analyzed and functionally annotated. Of the 129 assembled unique transcripts, 111 (86%) and 18 (14%) comprised of singletons and contigs, respectively. Among them, 58.9% could be assigned a putative identity, 20.9% with hypothetical or unknown functions and 20.9% showed no match with existing sequences. Expression pattern of 41 selected ESTs were estimated by quantitative real-time polymerase chain reaction (qPCR) in two different tissues. Expression profiling were undertaken in control and three time point of salt stress (6hrs, 24hrs and one week) followed by three time point of recovery condition (6hrs, 24hrs and one week). In the root, the genes of *SAMDC, ISB1* (6hrs), *PP2C* and *SelO, HsfA1a, TFC D, Katanin, F-box* were significantly up-regulated relative to control while *LecRLKs, ARP, HP3, PICKLE, Utp20, SYP81, CIPK20, HAK18, VDAC3, SND1, NAP1, ISB1* (6hrs and 24hrs), *NUC2, MUT, HP1* and *PIP1;3* showed down-regulation in given conditions. In the case of leaf tissue, the genes of *PP2C, SelO, Utp20, SND1, PITP, LecRLKs, STPK, KCNK12, HsfA1a, HAK18, NUC2, ARP, HP3* and *ARP* were significantly up- or down-regulated. Differential regulation of these genes were observed in root and tissue which confirm their role in salt stress tolerance. This functionally annotated EST and gene expression profiling provide initial insights into the transcriptome of *A. littoralis*.

KEY WORDS: *AELUROPUS LITTORALIS*, GENE EXPRESSION, SALT STRESS, RECOVERY CONDITION

ARTICLE INFORMATION:

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Received 1st Aug, 2016
Accepted after revision 5th Sep, 2016
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007
Thomson Reuters ISI ESC and Crossref Indexed Journal
NAAS Journal Score 2015: 3.48 Cosmos IF : 4.006

Online Contents Available at: http//www.bbrc.in/

INTRODUCTION

The global climatic changes, such as prolonged drought, temperature change and increasing salinity, cause to a serious challenge for agricultural production worldwide, affecting plant growth and yield. Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050. Therefore it is important to secure food production for a growing world population by increasing the yield of crop plants while resources become more restricted (Yamaguchi and Blumwald, 2005, Pitman and Läuchli, 2002 and Mittler and Blumwald, 2010).

The discovery of novel stress-responsive genes, identification of new cis-and trans- acting elements that are involved in stress adaptation provide an opportunity for generating stress tolerance crops (Cushman and Bohnert, 2000, Patnaik and Khurana, 2001, Ben-Saad et al., 2012 and Hashemi et al., 2016).

The use of wild plant species or halophytic relatives has been considered in plant breeding programs for developing salt and drought tolerant crops Utilising such approach, *Aeluropus littoralis* can serve as a halophyte model for identification and isolation of the novel adaptation genes. *Aeluropus littoralis* is a perennial monocot grass with the small haploid genome of 349 Mb, using the C_4 mechanism for carbon fixation (Wang, 2004). *Aeluropus littoralis* grows in dry salty areas or marshes (Saad et al., 2011) and can survive where the water salinity is periodically high (Mesléard et al., 1993) and tolerate up to 1100 mM sodium chloride (Barhoumi et al., 2007). Therefore, *A. littoralis* serves as valuable genetic resource for understanding the molecular mechanisms of stress-responses in monocots, and can potentially be used for improving tolerance to abiotic stresses in economically important crops (Saad et al., 2010).

The process of identifying new genes and characterizing their functions generally is done at three molecular biology levels viz: genomics, transcriptomics and proteomics. *Transcriptome*-based gene *discovery* in response to environmental stress offers insights into the roles of the transcriptome in the regulation of physiological and biological responses (Gracey, 2007). Because these methods strictly clarify changes in transcript level, a complex multi-component process, such as salt and drought stress, can be broken into their basic element (Umezawa et al., 2002). Partial cDNA isolation often known as expressed sequence tags (ESTs) is the rapid and cost-effective *Transcriptome*-based gene *discovery method that* has become an efficient approach for identifying *of coding* regions in a wide spectrum of organisms.

Various techniques such as differential display PCR (DDPCR) (Hubank and Schatz, 1994), cDNA-amplified fragment length polymorphism (AFLP) (Bachem et al., 1996), suppression subtractive hybridization (SSH) (Diatchenko et al., 1996), serial analysis of gene expression (SAGE) (Velculescu et al., 1995), massively parallel signature sequencing (MPSS) and recently whole transcriptome profiling (RNA-Seq) (Brenner et al., 2000) have been used for EST isolation. By use of these techniques a large number of genes expressed during different developmental, differentiation and growth stages or in response to a variety of biotic and abiotic stresses has already identified in plants (Priya et al., 2012). It is clear now, the most biological processes, growth and developmental programming are regulated by the precise control of genetic expression (Agarwal et al., 2008). Genome-wide analyses of mRNA level showed that the expression level of genes may be changed (up or down-regulation) in response to different condition (Rabbani et al., 2003), in some case differential regulation of specific genes and pathways can lead to adaptation of crop genotypes to different abiotic stress (Aglawe et al., 2012).

To gain insight into these processes, it is necessary to study patterns of gene expression. Quantitative real-time polymerase chain reaction (qPCR) analysis is one of the most currently used approaches for measuring gene expression level (Gutierrez et al., 2008). The sensitivity, specificity and simplicity of this technique is incomparable with other methods such as Northern and *in situ* hybridization, RNase protection assays and semi-quantitative reverse transcription- polymerase chain reaction (RT-PCR) (Bustin, 2000).

In our pervious study (Fatemi et al 2016.), 154 ESTs (relative to salt and drought stresses) have been isolated from *A. littoralis* by cDNA-AFLP and their sequences were deposited in dbEST database (NCBI; www.ncbi.nlm.nih.gov/dbEST). The primary goal of this investigation was to annotate and assign putative functions of 154 isolated ESTs. Also, expression pattern of selected ESTs was analyzed in two different tissues of *A. littoralis* at salt stress and recovery condition.

MATERIAL AND METHODS

Aeluropus littoralis seeds were collected from Isfahan province (Roddasht region) in Iran and the sterilized seeds plated on full strength MS medium (Murashige and Skoog, 1962) with vitamins, 3% sucrose and 0.7% agar (pH 5.8). The cultures were incubated in germinator at 25 ± 2 C with 16 h light/8 h dark photoperiod at 100 µmol m^{-2} s^{-1} photon flux density using cool-white fluorescent light. Two weeks after germination, the seedlings were transferred to hydroponic culture containing Hoagland's solution (Hoagland and Arnon, 1950). The 30 day-old seedlings were stressed in 600 mM of sodium

chloride at six passages (received 100 mM sodium chloride per 48 hrs up to 600 mM). At the end of the sixth passage, salt stress samples were collected at 6hrs (S1), 24hrs (S2) and one week (S3) time point. In order to plant recovery, the remained plants were transferred to a sodium chloride-free Hoagland's solution, and then were collected after 6hrs (R1), 24hrs (R2) and one week (R3). Leaf and root were sampled in parallel. Control samples were taken from unstressed plants at the start of the experiment. All samples were immediately frozen in liquid nitrogen and stored at -70°C for RNA extraction.

The EST sequences of *Aeluropus littoralis* were retrieved from EST database at NCBI and were analyzed using the BLASTN, tBLASTX and BLASTX algorithms (Zhang et al., 2000). The database of gene ontology (http.//www.geneontology.org) was used to investigate the molecular function of each EST and its role in biological processes as well as its location in the cell. After selection of candidate reference genes, the gene-specific primers were designed using the Primer 3 software (Rozen and Skaletsky, 1999), and were synthesized by Metabion GmbH (Martinsried, Germany). All designed primers had 18-24 length, GC content ranging from 42% to 61% and similar melting temperatures (55-64°C). The amplicon length ranged from 60 to 282 bp. The primer sequences and GenBank accession numbers of related genes are presented in Table 1. The primer specificity was evaluated by melt curve analysis, and size of the amplicons was tested by end-point PCR on 3% agarose gels.

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted ribonucleic acid was checked by measuring absorbance at 260/280 nm using a NanoDrop spectrophotometer (Biochrom WPA Biowave II, UK). Further, the purity and integrity of RNA was tested by running on 1.2% agarose gel electrophoresis. Residual gDNA contaminating RNA extracts was removed by DNase treatment (*DNase* I RNase-free, Thermo Scientific, USA). The qPCR with three rDNA-based primers has recently been applied for DNA contamination assay by using RNA as template, (Hashemi et al., 2016).

The cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. In brief, 1 µL (200 ng) of treated RNA, 1 µl of RT primer mix (blend of oligo-dT and random primers), 1 µl Quantiscript Reverse Transcriptase (contains RNase inhibitor), 4 µl Quantiscript RT Buffer 5X (includes Mg^{2+} and dNTPs) and 13 µl of RNase-free water were added and incubated at 65°C for 20 min and then followed by incubation at 95°C for 3 min for inactivation of reverse transcriptase. The final cDNA reactions were diluted 1:10, and stored at -20°C. Targets were amplified by the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) with two-step cycling in CFX96 real-time PCR instrument (Bio-Rad, USA) according to the company's suggestions. The reaction master mix prepared by adding the following components: 1µL of cDNA (50 ng), 5 µl of 2X SYBR Green Master Mix and 0.3 µl of 10 µM of each primers and 3.4 µl of RNase-free water. Thermal cycling were performed using two-step cycling protocol according to the company's procedures as follow: 10 min initial activation step at 95° C followed by 40 cycles of 95° C for 15 sec and 60° C for 1 min.

Data acquisition were performed during the annealing/extension step. After amplification, all PCR reactions were subjected to a thermal melt with continuous fluorescence measurement from 55°C to 95°C for dissociation curve analysis. Curves were analyzed by CFX Manager (Bio-Rad) with single threshold cycle and subtracted curve fit method. At least one non-template control (NTC) was used for each primer pair master mix. The threshold cycles (Ct) were automatically calculated for all reactions in the plate using the CFX manager software (Bio-Rad). All assays were carried out in three replications. The mean values for each assay were obtained, and used for further analysis. The livak ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001) were used for calculation of relative gene expression ratio. RT2 Profiler PCR Array Data Analysis software (SABiosystems) was used to construct vocalno plot and clusterogram.

RESULTS

BIOINFORMATIC ANALYSIS AND SELECTING CANDIDATE ESTS

In our pervious study, 154 *A. littoralis* ESTs from four different library were isolated by cDNA-AFLP method, and used in this study. EST sequences were retrieved through Entrez Gene -EST database- at the National Centre for Biotechnology Information (NCBI). The four libraries names were LIBEST_028119 (69 ESTs with GenBank accession number of JK191110.1-JK191042.1), LIBEST_027583 (25 ESTs with GenBank accession number of JK671243.1-JK671267.1), LIBEST_027584 (34 ESTs with GenBank accession number of JK671209.1-JK671242.1), and LIBEST_027576 (26 ESTs with GenBank accession number of JK671176.1-JK671201.1).

All ESTs were evaluated by the CAP3 DNA sequence assembly program. Assembling of the 154 ESTs produced a total of 18 assembled contigs and 110 singletons. Of the 129 assembled unique transcripts, 111 (86%) and 18 (14%) comprised of singletons and contigs, respectively. A total of 129 non-redundant dataset were compared to the GenBank non-redundant database using BLASTX

to assign putative function. Of all aligned sequences, 58.9% could be assigned a putative identity, 20.9% with hypothetical or unknown functions and 20.9% showed no match with existing sequences. The size of most ESTs (79%) ranged from 100 to 400 bp. Sequence similarity searches using BLASTX were performed to compare the selected ESTs to subset of non-redundant protein sequences (nr) of green plant (taxid: 33090), *Oryza sativa, Zea mays, Arabidopsis thaliana, Brachypodium distachyon, Setaria italica* and *Sorghum bicolor* with several blast e-value cutoffs. We found that the *A. littoralis* transcript set (129 ESTs) showed a greater number of sequence similarity matches with *Setaria italica* and *Zea mays* transcripts. Based on functional annotation, 41 ESTs were selected for relative expression analysis in root and leaf tissues at different time-point of salt stress and recovery condition. The primer features are listed in Table 1.

DNA CONTAMINATION ASSAY

For DNA contamination assay, the RNA samples have been tested by qPCR with. In our pervious study, a new procedure for testing DNA contamination was explained (Hashemi et al., 2016). To monitor residual gDNA contamination in RNA sample, the total RNA samples were examined by three rDNA-based primer pairs in qPCR. Generally, observation of any band on the agarose gel or melting curve peak in qPCR analysis were considered as gDNA contamination. In this study, all RNA samples were tested by this procedure, and *DNase*-free RNA was used for cDNA synthesis.

PRIMER VALIDATION

The cDNA synthetized from control and treatment samples was also tested by qPCR. Pooled cDNA tissue samples containing equal amounts of the cDNA from control and treatment conditions were used to determine the primer pairs annealing temperature and their specificity. Primers annealing temperature were adjusted to 55-60°C, and their specificity was checked by melt curve analysis and electrophoresis in 3% agarose gels. Single sharp peak with no primer-dimer was used for relative expression analysis. From 41 ESTs tested in the melt curve analysis, three of the genes (*C-NAD-MDH2*, *TTL*, *SAP* and *PI/PC-TP*) were not amplified and excluded from further analysis. The sharp peak of *5PTASE11* was only observed in root samples while no peak was detectable in leaf samples. The C_t value of *CHR11* in root time point were higher than 35 and therefore excluded from analysis. For *RBPL39* and *HP2* genes, sharp peak were amplified only in leaf time points while unambiguous *TFC D* peak was only observed in root tissue. The trend of regulated genes in root and leaf tissues are presented in Figure 1.

ROOT TIME POINT ANALYSIS

Distribution of C_t values amplified from root samples showed that in control and salt stressed samples, C_t value of most genes were lower than 25. Percent distribution of C_t values in C_t range of <25 in different time-points including control. Salt Stress: S1, S2, S3, Recovery condition: R1, R2 and R3 were 54.63%, 59.26%, 50.00%, 53.70%, 6.48%, 5.56% and 59.26%, respectively. These values indicated that the mRNA level of most genes in control and salt-stressed samples were higher than in the mRNA level of recovered samples (except R3). For normalization of expression levels in *Aeluropus littoralis,* different set of reference genes as well as their optimal number were recommended for root and leaf samples (Hashemi et al., 2016).

The three genes namely, *RPS3*, *EF1A* and *UBQ* were used as normalizer in root samples. For identification of genes with statistically significant gene expression changes, a volcano plot were used. Volcano plots are used to look at fold change and statistical significance, simultaneously (Allison et al., 2006).

In this study, expression values of 31 genes across six time point of root samples were compared to control samples by volcano plots (Figure 2). Values above the blue line and outside of the vertical lines were determined to statistically significant fold changes with 95% confidence (α=0.05). Genes with fold change higher than 2 or lower than -2 and p-value < 0.05 are indicated in blue in Figure 2.

Based on volcano plot analysis, seven genes including *ZF30*, *URM12*, *CAND1*, *SPIKE1*, *TBC1*, *HP1*, *MTL1* and *5PTASE* had not significant difference relative to control group. At time point S1, the expression level of *SAMDC* (3.1) and *ISB1* (3.3) were higher while *LecRLKs* (-12.1), *ARP* (-18.2) and *HP3* (-4.8) were downregulated (p-value < 0.05). Despite of the expression level of *PP2C* (4. 2) and *GlyI* (-7) were higher and lower than 2 and -2, but their fold changes were not significant (value inside parentheses is fold change). In S2 time point, two gene of *PP2C* (5.7) and *SelO* (4.8) were significantly upregulated while *PICKLE* (-4.5), *Utp20* (-3.8), *SYP81* (-3.6), *CIPK20* (-4.1), *HAK18* (-3.8) and *LecRLKs* (-18.9) were significantly downregulated (p-value < 0.05). *PP2C* (13.4) and *SelO* (11.9) and *SAMDC* (4.1) were upregulated in S3 time point while *LecRLKs* (-6.2) were significantly downregulated.

Under recovery conditions at time point R1, the expression level of *HsfA1a* (3.2), *SelO* (18.9), *TFC D* (81.1), *SAMDC* (11.1), *Katanin* (9.5), *F-box* (4.3) were increased relative to control while, the genes of *Utp20*

Table 1: showing the primer features

Gene symbol	Accession number	Name	Function	E-value	Sequence
PIP3;1	JZ191087	Plasma membrane intrinsic protein	water channel activity	3e-63	TGTCATGGGCGTCTCCAAGT GCAGTAGACGAGCGCGAAGA
VDAC3	JZ191051	Voltage dependent anion channel 3	regulation of anion transmembrane transport	1e-13	TCCAGACCCAGCTGAAGCAC GCCTGGTACACCAAGATCCTCA
SYP81	JZ191048	Syntaxin of plants 81	Vesicle trafficking protein that functions in the secretory pathway.	2e-32	CAGCATGGCGTGGCTCTTAT AGCATCTTGAAAGCGCATGG
NAP1	JZ191072	Nucleosome assembly protein1	modulate chromatin structure by regulation of nucleosome assembly/disassembly	0.29	CAGGGCTCCACAAATCCAAC ACGACCTGCTGAGTGCAAGC
CAND1	JZ191057	Cullin-associated and neddylation-dissociated	promotes the exchange of the substrate-recognition F-box subunit in SCF complexes	2e-06	TGGCAGTGACTACAGCATACGG ACTGCGCACAGAGCGGTACT
SAMDC	JZ191058	S-adenosylmethionine decarboxylase	Essential for polyamine homeostasis, and normal plant embryogenesis, growth and development.	5e-14	CCATCCATGGTCCTGCTTTC GGGTTGAAGCCCATGACCTC
Katanin	JZ191064	Katanin p80 WD40	microtubule severing	1e-61	TGATCCCTCCCTTCCCAGTT CCTGAGCGAATGCGTAAACC
F-box	JZ191080	F-box protein	Unknown	1e-60	TGCCCATGAACCATTGTACG GCCCTGCAGATCAGGTCAAC
SND1	JZ191081	Staphylococcal nuclease domain-containing protein 1-like	posttranscriptional gene silencing by RNA, response to salt stress	1e-18	GCGGATCTGGCAGTATGGAG ACCGCTGCCTGAACAGACTT
GTF3C5	JZ191082	General transcription factor 3C polypeptide 5-like	Involved in RNA polymerase III-mediated transcription	2e-37	TTCCAAGTGGCCATCAGGTT AAAGGGCTTCCTGCCTCTTG
ISB1	JZ191092	Importin subunit beta-1	protein transporter activity	1e-42	GCTCCAGCCAAATGTCAAGC GGTCTTGGTCAACAGCTTCAGG
NUC2	JZ191093	Nucleolin 2-like	Involved in pre-rRNA processing and ribosome assembly	7e-06	AAGTCCAGTGTTGCGGTTGC CCGCATTTCTCTTCCCCTTC
GlyI	JZ191094	Glyoxalase I	carbohydrate metabolic process	0.079	GTGGCATGGACTTGCTACGG CCGTGGCATCACAGAGGATT
CIPK20	JZ191099	CBL-interacting protein kinase 20	protein serine/threonine kinase activity	2e-09	CAGGAGATGAGGCCAGCACT CTGTTGCTGTTGCTGCTTGG
HAK18	JZ191100	High-affinity potassium transporter	potassium ion transmembrane transporter activity	7e-37	GGCCAGACATTTCAGACCACA AGCCCTGATGACCGTGTTTC
ZF30	JZ191101	Zinc finger CCCH domain-containing protein 30	regulation of transcription	3e-08	GCTCTTGTTGGCTCCCCTCT TCACCATTTACGCCCCAATC
URM12	JZ191103	Ubiquitin-related modifier 12	involved in tRNA modification	4e-17	ACTGCGATTGGGAGCTGTGT CGTGGAGATGAAGACCACCA
5PTASE11	Jk671224	Inositol polyphosphate 5-phosphatase	response to abscisic acid, response to auxin, response to jasmonic acid	6e-11	CACATGGAACATGAATGGCAAG TGAACTCCTTGCTCCGAAAAGA
PITP	Jk671260	Sec14p-like phosphatidylinositol transfer family protein	transporter activity	3e-55	GAAAGTAAAGATTGCGGAGAC GGGTGCGAACTCTGAAAC
SPIKE1	Jk671264	DOCK family guanine nucleotide exchange factor	vesicle-mediated transport	1e-24	TAAACAACACGGTGGCAGGTA GCTCCCCATCAAATGTCCATA
TBC1	Jk671226	TBC1 domain family member 5 homolog B	act as a GTPase-activating protein for Rab family protein(s)	3e-18	CGGGATGGGAGCAACAAC CACGGATAAGGGCACTGGT

TTL	Jk671266	tubulin-tyrosine ligase	cellular protein modification process	1e-05	AAGAGGCAGTATCCTAATCAC AAACTCATTCTGCCAATCTA
KCNK12	Jk671259	potassium channel subfamily K, member 12	potassium ion transmembrane transport	1.5	TCGGAATCTGCCCTGAATCT TATGTATCCCGGTCCACCACT
LecRLKs	JK671176	G-type lectin S-receptor-like serine/threonine-protein kinase	Protein kinase activity	5e-16	CGGCCGACAATGGGTGAAG GGGCATGCCAACCTCCTGTAG
SAP	JK671180	Putative senescence-associated protein	-	2e-11	TGACACACCCCACACATACAA GGTTTAGACCGTCGTGAGACAG
ARP	JK671182	Auxin-repressed protein	-	6e-08	GGAAGTTTTGGGCTGTCTTTA ATTTCGATGTTGCCTACTCTCTA
HP1	JK671187	Hypothetical protein1	Similar to F-box family protein	1.5	CCAACAACTCAGCTCCAA GATGTGAAAATAAGCACGCTA
MTL1	JK671192	Mitochondrial translation factor 1	Mitochondrial protein translation and group II intron splicing	6e-50	ATTTCGGCAAAAGGAATGGAG GAAGCTTGATGAGGCGACAGA
HP2	JK671195	Hypothetical protein2	-	0.006	GTTTGGGCATTGGGTCCTCAAGT CGAGCAACAGCAGCAAGAGCAC
MUT	JK671196	Mutator-like transposase	Transposable element gene	3e-18	GATGCCCATCTTGACAATAC GCAGTGGGGAAGTTGATTT
HP3	JK671200	Hypothetical protein3	-	0.003	GGAAGTTTTGGGCTGTCTTTAC AGATTTCGATGTTGCCTACTCTC
HsfA1a	Jk671211	heat shock factor A1a	transcription factor activity	4e-10	GCAGTGCCCAGTTGTCTT TTGGGCCTGGTGTCATA
PP2C	Jk671236	Protein phosphatase 2C	protein serine/threonine phosphatase activity	6e-34	TAATATGCAGGGGAGGAAA CAGCGAGTACACCACCAA
C-NAD-MDH2	Jk671223	Malate dehydrogenase	Cytosolic-NAD-dependent malate dehydrogenase 2	4e-20	AAAACGTCGTTCAAAGAG GCCATAAGATCCGTCAG
5PTASE11	Jk671224	Inositol polyphosphate 5-phosphatase	response to abscisic acid, response to auxin, response to jasmonic acid	6e-11	CACATGGAACATGAATGGCAAG TGAACTCCTTGCTCCGAAAAGA
HsfA1a	Jk671211	heat shock factor A1a	transcription factor activity	4e-10	GCAGTGCCCAGTTGTCTT TTGGGCCTGGTGTCATA
PP2C	Jk671236	Protein phosphatase 2C	protein serine/threonine phosphatase activity	6e-34	TAATATGCAGGGGAGGAAA CAGCGAGTACACCACCAA
C-NAD-MDH2	Jk671223	Malate dehydrogenase	Cytosolic-NAD-dependent malate dehydrogenase 2	4e-20	AAAACGTCGTTCAAAGAG GCCATAAGATCCGTCAG
PI/PC-TP	Jk671213	Putative phosphatidylinositol/ phosphatidylcholine transfer protein SFH8-like	phosphatidylinositol transporter activity, transporter activity	6e-13	TTGGCACATGCTTCCACATC AGGACTGCCCCATCCATCAT
PICKLE	Jk671232	CHD3-type chromatin-remodeling factor	DNA helicase activity	1e-37	AGGGGTATGCTGAACTTGT CACCTTCGCCTCAATAA
RBPL39	Jk671237	RNA-binding protein 39-like	mRNA processing	3e-05	GGTGCCACTGGTCTGA AAAGGGGAAGCTACAGGAG
SelO	Jk671243	Selenoprotein O-like	transferase activity	3e-13	TCAAGGGTAGCGGAAAGAC GGATGCTGCTGCGTAGAAC
TFC D	Jk671246	tubulin folding cofactor D	GTPase activator activity	4e-25	TAAAAGATGCCGCAACATA GAAGGTGGGGAGCAAG
CHR11	Jk671250	chromatin-remodeling protein 11	ATP-dependent chromatin remodeling, nucleosome binding	7e-119	CGCTGTTTTCTCTTTGATT CGCTTTTGCCCTATTCTA
Utp20	Jk671251	small subunit processome component 20 homolog	rRNA processing, associates with U3 snoRNA.	2e-60	-CTTTCAGTTGCGTTTAGATGT CGCTTTCAGAAGTGATAAGG
STPK	Jk671258	Serine/threonine-protein kinase ULK4-like	protein kinase activity	1e-32	CATTTTCTGCCACTGTATCCT ACTTTTACACAACCATGCTCC

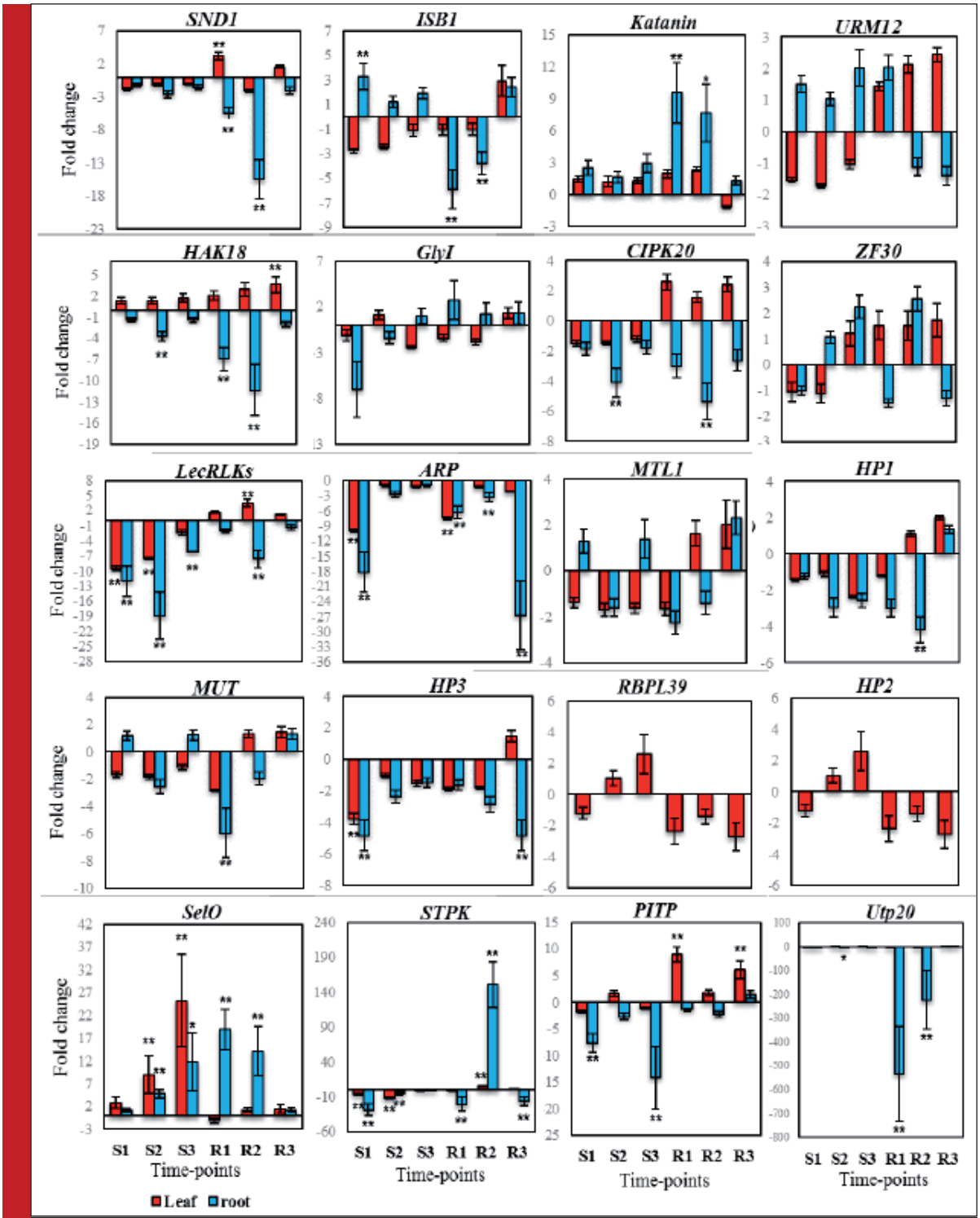

FIGURE 1: Trend of regulated genes during different time point of salt stress and recovery condition.

(-533.5), *VDAC3* (-4.7), *SND1* (-5.5), *NAP1* (-10.5), *ISB1* (-5.9), *NUC2* (-11.7), *HAK18* (-6.9), *ARP* (-6.3), *MUT* (-5.9) showed downregulation in mRNA level. The genes found to be upregulated at time point R2 were similar to that of time point R1. *HsfA1a* (3.4), *SelO* (14.1), *TFC D* (49.6), *SAMDC* (5.5), *Katanin* (7.6), *F-box* (3) showed upregulation in R2 time point. A number of eleven genes including *Utp20* (-223.1), SYP81 (-5.7), *SND1* (-15.4),

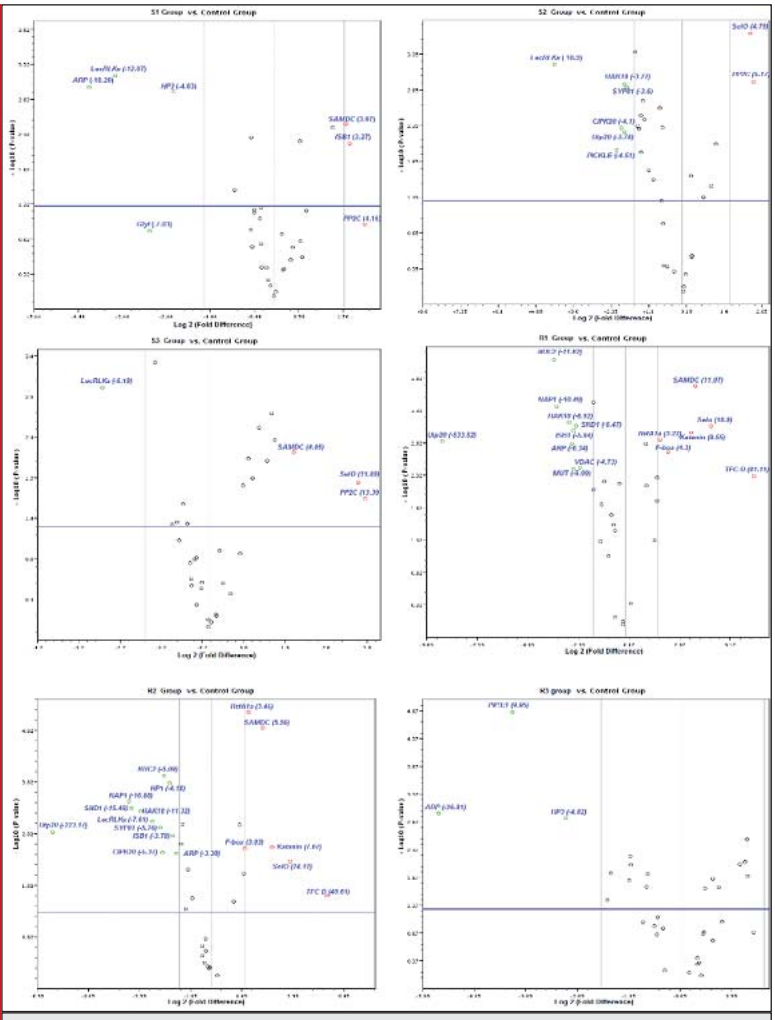

FIGURE 2: Volcano plots of the fold changes and p-values distribution. A total number of 31 genes were tested in the root salt-stressed and recovered samples. The central vertical line represents a fold change of 1 (no change) and two another vertical lines represent 2 fold upregulation or downregulation cutoffs. The horizontal blue line represents the p-value cutoff for identification of genes that are statistically significant. Value inside parentheses is fold change.

NAP1 (-16.8), *ISB1* (-3.7), *NUC2* (-5), *CIPK20* (-5.3), *HAK18* (-11.3), *LecRLKs* (-7.6), *ARP* (-3.3), *HP1* (-4.1) showed downregulation in R2 time point. In the R3 time point, three genes of *PIP1;3* (-9.9), *ARP* (-26.8) and *HP3* (-4.8) only showed downregulation in mRNA level.

Visualization of gene expression differences among different time point was done by RT2 Profiler PCR Array Data Analysis program (Figure 3 A). The result of the cluster analysis is depicted in a clustergram that shows all 31 genes analyzed and the magnitude each is expressed in control and 6 different root time points including group 1 (S1), group 2 (S2), group 3 (S3), group 4 (R1), group 5 (R2), group 6 (R3). Four major clusters of genes were observed: cluster I and II contained upregulated genes and cluster III and IV contained downregulated genes.

LEAF TIME POINTS ANALYSIS

Distribution of Ct values in leaf samples showed that the most of genes had Ct range of 25-30. Percent of Ct values in range of 25-30 in different time-points including control, S1, S2, S3, R1, R2 and R3 time-points were 57.41%, 61.11%, 42.59%, 48.15%, 50.00%, 45.37% and 54.63%, respectively. Comparing of Ct values in root and leaf samples showed that the mRNA level in root samples were higher than leaf samples. The geometric mean of *U2SURP* and *GTF* Ct values were chosen as normalizer in leaf samples.

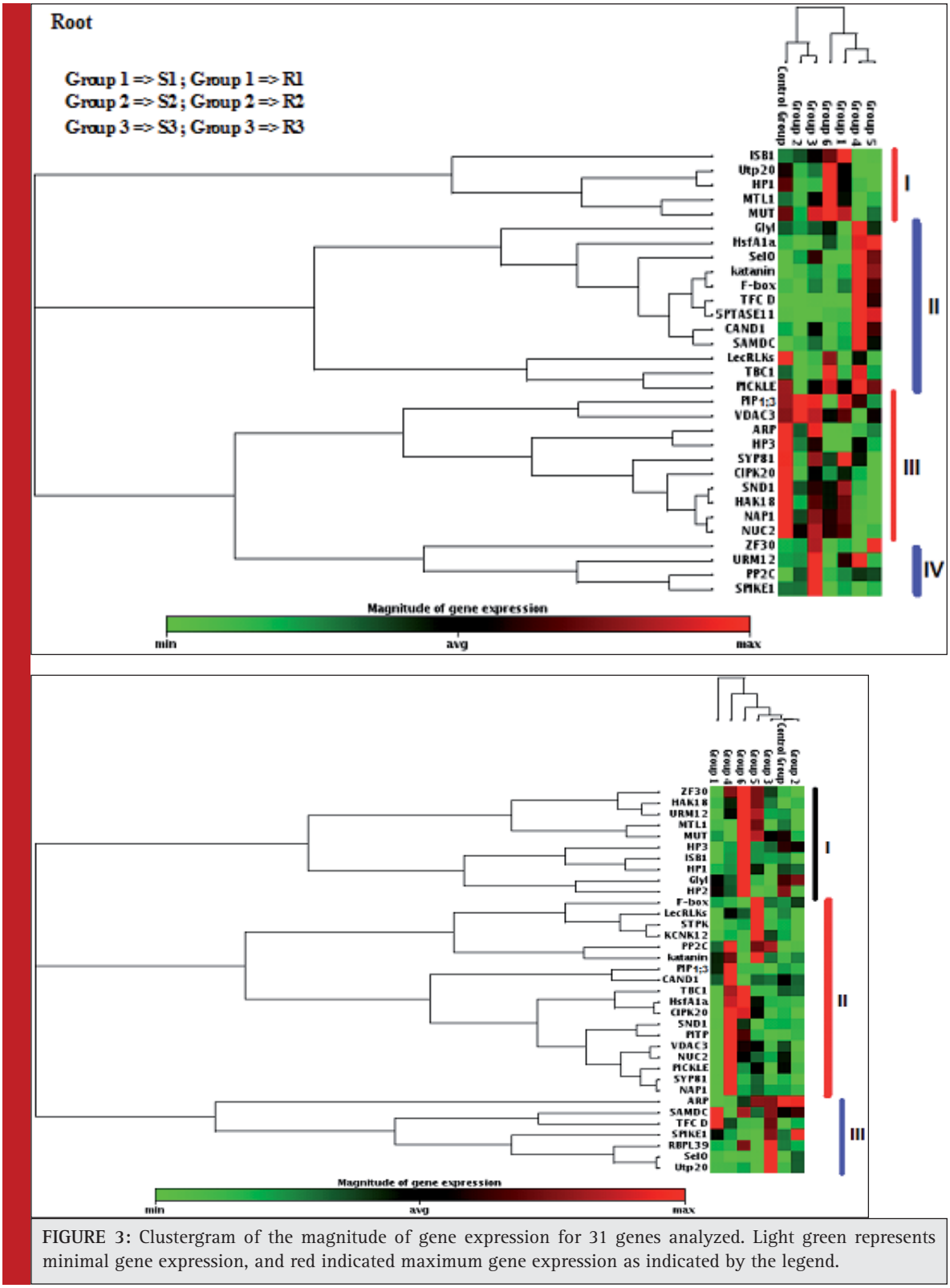

FIGURE 3: Clustergram of the magnitude of gene expression for 31 genes analyzed. Light green represents minimal gene expression, and red indicated maximum gene expression as indicated by the legend.

Similar to root analysis, expression values of 31 genes across 6 time point of leaf samples were compared to control samples by volcano plots (Plots have not shown). Based on volcano plot analysis, expression level of 18 genes including *ZF30, URM12, TBC1, HP1, MTL1, PICKLE, PIP1;3, VDAC3, SYP81, NAP1, CAND1, SAMDC, Katanin, F-box, ISB1, GlyI, CIPK20, HP2, MUT, RBPL39, SPIKE1* were unchanged relative to control group in different leaf time point.

In S1 time point, *PP2C* (3.4) were upregulated while *NUC2* (-3.3), *LecRLKs* (-9.5), *ARP* (-9.9) and *HP3* (-3.8) and *STPK* (-6.7) were significantly downregulated (p-value < 0.05). In S2 time point, *SelO* (8.9) were significantly upregulated while *LecRLKs* (-7.5), *STPK* (-12.3) and *KCNK12* (-4.7) were significantly downregulated. In S3 time point, only upregulation of *PP2C* (3.4), *SelO* (8.9) and *Utp20* (-533.5) were observed among all analyzed genes. *PP2C* (3.4), *SND1* (3.1) and *PITP* (8.9) showed upregulation in R1 time point while *ARP* (-7.5) were significantly downregulated. In the R2 time point, the expression level of *PP2C* (6.5), *LecRLKs* (3.5), *STPK* (5.1) and *KCNK12* (4.1) were significantly were increased relative to control. Finally, *HsfA1a* (3.4), *HAK18* (-6.9), *PITP* (8.9) showed upregulation in R3 time point. The leaf clustergram were presented in Figure 3 B. Three major clusters of genes were observed. The most significantly up or down-regulated genes allocated into cluster II. This cluster represents genes reacting to recovery conditions.

DISCUSSION

The development of salt or drought-adopt crops either through the use of the crops wild relatives as genetic resources or domestication of naturally tolerant species have been proposed as a strategy to face with the environmental challenges. Halophytes as crops naturally salt-tolerant species are now being promoted in agriculture, particularly to provide forage, medicinal plants, aromatic plants (Flowers et al., 2010). Although, improving crop salt tolerance by genetic engineering is not easy, halophyte germplasm can furnish "climate-ready" genes for plant breeding program (Jaradat, 2010). Different aspects of *A. littoralis* properties such as life style, morphological, anatomical, ecological, physiological and molecular characteristics have been investigated so far (Hashemi et al., 2013, Hashemi-Petroudi et al., 2014).

In the present study, we have focused on gaining insight on differential regulation of some responsive ESTs in response to salt stress and recovery condition. Expression pattern of 41 selected ESTs were estimated by qPCR in root and leaf tissue. In the root, the genes of *SAMDC, ISB1* (6hrs), *PP2C* and *SelO*, *HsfA1a*, *TFC D*, *Katanin*, *F-box* were significantly up-regulated relative to control while *LecRLKs, ARP*, *HP3*, *PICKLE*, *Utp20*, *SYP81*, *CIPK20*, *HAK18*, *VDAC3*, *SND1*, *NAP1*, *ISB1 (*6hrs and 24hrs*)*, *NUC2*, *MUT, HP1* and *PIP1;3* showed down-regulation in given conditions. In the case of leaf tissue, the genes of *PP2C*, *SelO*, *Utp20*, *SND1*, *PITP*, *LecRLKs*, *STPK*, *KCNK12, HsfA1a, HAK18, NUC2*, *ARP*, *HP3* and *ARP* were significantly up- or down-regulated. Interestingly, expression of some genes was induced by salt stress while also significantly repressed by recovery condition. Here interestingly the transcriptional regulator *HsfA1a* could be found. *HsfA1a* was formerly described to be a main component of the heat and drought stress response (Liu et al., 2013, Wang et al., 2015).

In this study, the protein phosphatase 2C (PP2C) were activated in salt stress and recovery condition. The PP2Cs from various organisms have been implicated to act as negative modulators of protein kinase pathways involved in diverse environmental stress responses and developmental processes (Xue et al., 2008). The *SYP81* showed down regulation in given conditions. Syntaxins (with the exception of syntaxin 11) are transmembrane proteins which their functions respect to organism growth, physiology and development are not well known (Teng et al., 2001). The coexpression analysis showed that, the most significantly up or down-regulated genes allocated into cluster II (leaf tissue). Genes found in Cluster II are interesting candidates for physiological reactions related to the recovery of the plant after salt stress. This cluster represents genes reacting to recovery conditions.

CONCLUSION

In this study we bioinformatically analyzed the 154 ESTs from *A. littoralis* and the functionally annotation showed that 58.9% of ESTs had a putative function, 20.9% were hypothetical or unknown functions and 20.9% showed no match with existing sequences. The qPCR expression analysis of 41 selected ESTs showed different regulation in leaf and root tissue. The gene expression profiling has done in this study will also provide insight into the role of selected ESTs in different time points of salt stress and recovery condition. Differential regulation of these genes also point at their role in salt stress tolerance in plant. These information facilitate understanding the molecular mechanisms of stress related genes and could be used as valuable starting point for further research on these genes.

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