INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–0038/2019/22–6–1681–1696
DOI: 10.17957/IJAB/15.1250
http://www.fspublishers.org

Full Length Article

RNA-Seq and Validation Analysis on the Important Genes Involved in Early Responses to Salinity Stress of Malaysian Rice Seedlings (*Oryza sativa* ssp. *Indica*)

Nor Mustaiqazah Juri[1,2], Norliza Abu Bakar[2], Rabiatul Adawiah Zainal Abidin[2], Maziah Mahmood[1], Noor Baity Saidi[3], Yahya Awang[4] and Noor Azmi Shaharuddin[1,5*]

[1]*Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM Serdang, 43400 Selangor, Malaysia*
[2]*Malaysian Agricultural Research and Development Institute, Serdang, Selangor, Malaysia*
[3]*Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM Serdang, 43400 Selangor, Malaysia*
[4]*Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, UPM Serdang, 43400 Selangor, Malaysia*
[5]*Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, UPM Serdang, 43400 Selangor, Malaysia*
[*]For correspondence: noorazmi@upm.edu.my; mustaiqazah@yahoo.com

Abstract

Salinization of rice cultivation land has progressively enlarged, thus negatively impaired the world's rice bowl. Due to the polygenic nature and complexity of salinity tolerance mechanisms in plants, the development of new rice varieties with better adaptation to salinity has become a great challenge. Regarding this, transcriptomic profiling has been seen as a promising approach for a holistic understanding of salinity tolerance mechanisms in rice. Using Illumina RNA-Seq method, transcriptomes of two contrasting Malaysian rice varieties named as salt-tolerant MR211 and salt-sensitive MR220 were analysed within a short-term exposure (9 h) to salt-shock treatment (12 dS m^{-1}) at early seedling stage. Transcriptomic analysis using Tuxedo package enabled the identification of 252 differentially expressed genes (DEGs). Interestingly, 93.3% of the DEGs (n=235) were identified as higher and specifically expressed in salt-tolerant MR211 compared to the sensitive variety (MR220's FPKM ≤ 0). This group of DEGs was assigned in 33 KEGG pathways, with the highest number of transcripts accounted in purine and thiamine metabolism pathways. Meanwhile, functional annotation analyses revealed the presence of regulatory genes, annotated functional and unknown genes involved in various salt adaptation mechanisms in the salt-tolerant variety MR211. The expression accuracy and reproducibility of the 252 DEGs identified from the RNA-Seq experiment were further verified through semi-quantitative RT-PCR followed by real time PCR analysis.

Keywords: Comparative transcriptomes; Salt stress; Indica Rice; Seedling stage

Introduction

Rice (*Oryza sativa* L.) is critical crop for global food security and ranked as one of the most important crops, primarily in Asian and certain part of Latin America and African countries (Muthayya *et al*., 2014). It feeds almost half of the world's population which represented by more than 3.5 billion people, as well as important source of employment and incomes for rural farmers. Over the years, a massive growth of world's population has led to the increment of global rice demand. It has been estimated that an additional of 114 million tonnes of rice are required in order to meet the global rice demand in 2035 (Mohanty *et al*., 2013; F.A.O., 2016). Unfortunately, rice is prone to a wide range of environmental constraints. A stagnation of rice yield in many Asian countries has been recorded due to various abiotic stress factors. Among them, salt stress has been recognised as a significant threat to rice growth and development, accounting 30 to 50% or even higher losses of rice yield (Joseph and Mohanan, 2013).

Soil is categorised as saline with electrical conductivity (EC) of 4 dS m^{-1} or higher with pH less than 8 and Exchangeable Sodium Percentage (ESP) of less than 15 (Allotey *et al*., 2008). A continuous salinization process occurred through the release of soluble salts from parental rocks, application of chemical fertiliser, increased annual temperature or rise in global sea level. The coastlines of Southeast Asia made up of regions that serve as 'world rice bowl' are highly vulnerable to the effects of climate change (Yuen and Kong, 2009). In Malaysia, Indonesia and Vietnam, thousands square kilometre loss of land have been expected due to the yearly 30–50 cm increment of the sea

To cite this paper: Juri, N.M., N.A. Bakar, R.A.Z. Abidin, M. Mahmood, N.B. Saidi, Y. Awang and N.A. Shaharuddin, 2019. RNA-Seq and validation analysis on the important genes involved in early responses to salinity stress of Malaysian rice seedlings (*Oryza sativa* ssp. *Indica*). *Intl. J. Agric. Biol*., 22: 1681–1696

level (Wassmann *et al*., 2004). Granary lands especially those located on delta and coastal areas as well as the rising of sea level will lead into sea water intrusion and submersion of the fertile fields under sea, thus making it unsuitable for rice planting.

Depending on its concentration and duration, salinity stress invokes various changes in physiological and metabolic events in plants, which ultimately inhibit their production and survival. The destructive effect of soil salinity is caused by two major factors known as osmotic and ionic effects (Kosová *et al*., 2013). Osmotic effect occurs when high levels of salt ion around plant root are triggered into the decreasing of cellular osmotic potential, leading to cell dehydration. Meanwhile, ionic effect happens when the salt ion penetrates and accumulate in cell cytoplasm via plasma membrane. These effects will result in the interruption of intracellular enzyme activities, disruption of membrane structures and functions, nutrient imbalance, accumulation of reactive oxygen species (ROS), decreased photosynthetic activity, decrease in stomatal aperture as well as reduction of cell division and expansion (Rahnama *et al*., 2010).

Cereal crops specifically rice is very sensitive to salinity. However, its degree of susceptibility differs widely between varieties. Certain rice varieties possess unique ability to adapt to a toxic level of salt stress, whereas others are greatly susceptible, thus demonstrating the diversity of their genetic makeup and regulatory architecture (Ferreira *et al.,* 2015; Patishtan *et al.,* 2017). Based on standard evaluating score (SES) in rating the visual symptoms of salt toxicity (I.R.R.I., 2002), Malaysian rice variety, MR211, has been categorised as the most tolerant variety, whereas MR220 has been considered the most susceptible variety (Hakim *et al*., 2010). MR211 stably exhibits its salt-resistance trait at various growth stages including germination to seedling, vegetative to maturation as well as during callus regeneration stage (Hakim *et al*., 2014). Interestingly, MR211 has been found to be comparative to Pokkali, a widely used salt-tolerant check variety and a salt-tolerant donor in various breeding programmes.

Salinity tolerance is a quantitative trait controlled by many genes involved in different pathways. Therefore, a full understanding on the molecular responses of rice plants to varying conditions and identification of genes involved in salinity stress response is crucial to serve as a foundation in developing rice with better adaptation to salinity. In this prospect, identification of salt stress related genes is a promising approach in crop improvement programme through the development of rice varieties with higher harvestable yield during environmental stresses (Amudha and Balalubramani, 2011). Although the conventional breeding has been playing a crucial role in rice improvement, it is somewhat a slow process and time consuming. Plant biotechnology through genetic engineering and molecular breeding approaches offer much rapid development in a crop improvement programme under stressful environments. Recently, the attention on utilizing modern high-throughput genetic approach such as transcriptomics and proteomics has extensively grown. As plants vary in their response to stresses, the application of these "omics" methods in comparative studies between related plant species enables the identification of various functional genes, their transcript and protein products, including the novel ones that are responsible for stress responses and adaptation.

In this study, using Illumina RNA-Seq method, transcriptomes of two contrasting Malaysian rice varieties, salt-tolerant MR211 and salt-sensitive MR220 in responses to salt stress treatment were analysed. Transcriptomes analysis using Tuxedo package enabled the identification of significant genes differentially expressed between both varieties where further analyses had identified early responses candidate genes related to salt tolerant in rice, specifically in early seedling stage. The data generated from this study will serve as an invaluable genomic reference to further the knowledge on molecular and cellular events that specifically occur in rice seedlings during their early response to severe salinity stress. Not only constricted as the candidate genes for rice improvements through genetic engineering, the potential salt tolerant genes can also be utilised as DNA markers to fasten the selection process in conventional breeding.

Materials and Methods

Plant Materials and Salinity Shock Treatments

Two contrasting Malaysian rice varieties, salt-tolerant MR211 and salt-sensitive MR220 supplied by Malaysian Agricultural Research and Development Institute (MARDI) were used in this study. The seeds were surface-sterilized by soaking them in 70% of sodium hypochlorite solution (Clorox®) for 15 min followed by 5 times of rinsing cycle with distilled water. The sterilized seeds then were sown on wetted tissue paper and were placed in the dark incubator at 28ºC for three days. Then, the germinated seeds with approximately 1 cm length of radicle and plumule were carefully transferred into Yoshida's nutrient solution (Yoshida *et al*., 1976) and were let grow in control condition (28°C, 12 h light/12 h dark cycle). The salt-shock treatment of seedlings started at the first day of their 4th weeks of growth in the hydroponic system or specifically in 22-day-old seedlings. Single-step application of salt (Shavrukov, 2012) was conducted by dissolving the NaCl powder in the freshly prepared full-strength working nutrient solution until a desired EC 12 dS m^{-1}. The solution's EC was measured by EzDo 6061 Waterproof EC Meter (Instrument Choice, Australia). The pH of the solutions was kept at 5.0. The untreated seedlings were set as control plant. The experimental design adopted randomized complete design (RCD) with three replicates for each sample. Shoots of seedlings were collected at the 9th h after treatment and immediately stored at -80°C.

Total RNA Extraction, cDNA Library Construction and Reads Sequencing

The triplicates samples of salt-treated plants of MR211 (S1-211, S2-211 and S3-211) and MR220 (S1-220, S2-220 and S3-220) were pooled together and ground into a fine powder in a cooled mortar and pestle under liquid nitrogen. Approximately 100 mg of the finely powdered samples were filled into pre-chilled 2.5 mL micro-centrifuge tubes. Extraction of total RNA was conducted by using RNeasy Plant Mini Kit (Qiagen, USA), followed by quantity and integrity check by using Agilent Technologies 2100 Bioanalyzer. RNA samples with RIN ≥ 6 were outsourced to Macrogen (Korea) for cDNA library construction and sequenced using Illumina Hiseq TM 2000 platform (Illumina, USA). cDNA libraries of both samples were prepared by using Illumina® TruSeq™ RNA Sample Preparation Kit, by following manufacturer's instructions. Poly-A containing mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads and fragmented using divalent cations under elevated temperature. First strand cDNA was synthesized using reverse transcriptase and random primers followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA fragments then go through an end repair process, the addition of a single 'A' base, followed by adapters ligation. The products were then purified and enriched with PCR before loaded into Illumina HiSeq2000 instrument for paired-end sequencing. The sequence files of S211 and S220 were generated in FASTQ format.

Reads Mapping and Transcripts Assembly

Raw sequenced reads generated from the S211 and S220 cDNA library sequencing were trimmed using Trimmo matic (0.32) (Bolger *et al*., 2014) to remove any low quality bases (Q ≤ 30), adapters and reads below 36 bases long. High-quality reads of S211 and S220 libraries were individually aligned to *Oryza sativa* Japonica IRGSP-1.0 genome assembly (http://rapdb.dna.affrc.go.jp/) using TopHat (http://TopHat.cbcb.umd.edu/), which incorporates the Bowtie (http://bowtie-bio.sourceforge.net/index.shtml) algorithm to perform the alignment. The assembling of the mapped reads into their respective transcripts was conducted by Cufflinks (http://cufflinks.cbcb.umd.edu/). Cufflinks utilizes the normalized RNA-Seq fragment counts to quantify the transcripts relative abundances. Fragments per Kilobase of exon per Million fragments mapped (FPKM) was used as the unit of measurement.

Identification of Differentially Expressed Genes (DEGs)

Cufflinks, which were packaged with CuffCompare, CuffMerge and CuffDiff programs, was used to sort out the significant genes that were differentially expressed between the assembled transcripts of S211 and S220. CuffMerge was used to assemble and construct consensus transcripts between S211 and S220. CuffDiff was used to determine significant genes which expressed differentially in both samples. FPKM was used as a unit for the quantification, whereas FDR cut- off = 0.05 and absolute $\log_2$ ratio (Log_2FC) ≥ 2 was set as the threshold to trace the significant genes. From the CuffDiff output, CummeRbund (http://compbio.mit.edu/cummeRbund/) package was used to generate a volcano plot and heatmap was used to display the expression of the DEGs in a graphical form.

Functional Annotation of DEGs

Functional annotation of DEGs was determined by referring on the Rice Annotation Project Database (RAP-DB) (Sakai *et al*., 2013). DEGs that were not annotated in RAP-DB were classified as unknown/novel and their possible functions were predicted by subjecting their sequences for homology search in NCBI non-redundant (Nr) database through BLASTX search *via* Blast2GO program (https://www.blast2go.com/). The identification of Gene Ontology (GO) term, KEGG pathway and conserved domains of DEGs were also performed via Blast2GO program.

Semi-quantitative RT-PCR and Real-time PCR Analysis

The reproducibility of the DEGs identified from the RNA-Seq experiment was further validated by semi-quantitative RT-PCR and real-time PCR. Nine representative DEGs were selected. The selection was made based on previous reports on their involvement in salinity stress responses, as well as representing each expression category of the DEGs from the comparative express ion in S211 and S220 samples. The specific primers for each gene were designed using Primer-BLAST in NCBI (http://www.ncbi.nlm.nih.gov/tools/primerblast). For housekeeping genes (HNR and EP), the primer sequence was taken from Maksup *et al*. (2013). The details on the primers used for semi-quantitative RT-PCR and real-time PCR were listed in Table 1.

Preparation of triplicate samples of untreated and salt-treated MR211 and MR220 seedling samples used were repeated by following the exact steps and parameters that were used in the preparation of plant samples for previous transcriptomic analysis. The triplicate samples of control and salt-treated seedlings, S211 and S220 were extracted by using RNeasy Plant Mini Kit (Qiagen, USA) following the manufacturer's protocol. The qualities of extracted RNA were analyzed in 1% agarose through gel electrophoresis and were quantified with NanoDrop ND 1000 spectrophotometer (Thermo Scientific, USA). cDNA were synthesized by using iScript cDNA Synthesis Kit (Bio-Rad, USA) following to manufacturer's protocol.

Semi-quantitative RT-PCR was conducted using TECHNE-TC 521 thermal cycler (TECHNE, U.K.). The standard PCR program used was initial denaturation at 94^{o}C for 5 min, 94°C for 15 sec (denaturation), 60°C for 1 min (annealing) and 72°C for 7 min. A final volume of 25 μL of

Table 1: List of designated primers for candidate and housekeeping genes

Primer name	Sequence (5'-->3')	T_m	GC%	Amplicon size (bp)
PSII_F	AAGGGTAGCCAAGGGAAAGG	59.3	55	163
PSII_R	TCCAGCAAGAGTGATTGCCC	60.32	55	
MT_F	CAGAAAGGAGCCAGCATGGA	60.03	55	82
MT_R	TCCAGGATCGAGGTAAGCCA	60.03	55	
HOX16_F	CTCCGCCACAGCAATCTGTA	60.11	55	138
HOX16_R	GCGGATCAAGTTCTCAACGC	59.9	55	
SAPK6_F	GTATGACGGCAAGATGGCAG	59.06	55	70
SAPK6_R	GTAAGCACCGACGAGCATCA	60.46	55	
FER2_F	CCATCGTCACACCCTTGACA	59.97	55	75
FER2_R	CCAAGGCCAACTCCATAGCA	60.03	55	
PAO_F	TCCAGGACTACGTCCATGCT	59.11	55	84
PAO_R	AGCTAAGGAATTCCCAGCAACA	60.89	45	
Thaumatin_F	TCATGGTGCATGGCATTGTTG	60.07	48	145
Thaumatin_R	CCCAAACCAACCGCTAAAACT	59.32	48	
UBC_F	TCAAGACCAAGGTGTACCATCC	59.7	50	193
UBC_R	GCCTCGTACTCGTGCTTGTA	59.83	55	
VI_F	AAGATCGCCATCGTCTGCAC	60.81	55	152
VI_R	ACATGCGTAGCAATCGAACG	59.35	50	
HNR_F	GGCAGGTTCTGCAGTGGTAT	58.61	55	95
HNR_R	TAAGGTCGGTATCGCCAATC	58.45	50	
EP_F	TGAGCAAAATGGTGGAAAGC	59.64	45	97
EP_R	CAGTTGCAACCCCTGTATGA	58.00	50	

Table 2: Quality assessment of reads, mapping and assembly of the sequenced data

Sample	Data	Read Count	Q30 (%)
S211	Raw reads	79,900,758	86.35
	High quality reads (% of cut-off)	76,456,236 (4.3%)	
	Total mapped reads (%)	68,764,731 (89.94%)	
	Total gene	20,858	
S220	Raw reads	67,200,972	82.89
	High quality reads (% of cut-off)	57,323,996 (14.7%)	
	Total mapped reads (%)	51,815,160 (90.39%)	
	Total gene	19,315	

PCR reaction mix were prepared, containing 1 μg of cDNA template, 0.6 μM of each forward and reverse primer, 0.2 Mm of each dNTP mixture (Promega, USA), 2.5 units of Taq DNA Polymerase (Invitrogen, USA), 1× PCR buffer minus Mg (Invitrogen, USA), 1.5 m*M* MgCl2 and autoclaved distilled water. Three μL of the PCR products were run on 2% agarose gel electrophoresis and the size of band were determined according to VC 100 bp DNA Ladder (Vivantis, USA). The image of ethidium bromide-stained PCR products in agarose gels were quantified using Image J software (Antiabong *et al*., 2016). Analysis on the quantified band intensity of each candidate gene in different cDNA samples was performed according to Luke Miller's method at lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j.

Real-time PCR was performed using 96-well plates BIO-RAD CFX96 Real-time PCR System thermocycler (Bio-Rad, Berkeley, CA, USA). The PCR reaction per well contained 1 μg of the first strand cDNA, 0.4 μM forward and reverse primers and 1× master mix of SensiFAST SYBR No-Ro x Kit (Bioline, U.K.). For negative control (NTC), water was added to a master mix instead of cDNA. Ten μL of PCR reaction components were transferred to the wells in triplicate. The PCR condition was set to 95°C for 2 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. Melting curve analysis were performed after the last cycle finished at 65°C to 95°C to verify the specificity of the amplification. The analyses were carried out with auto baseline and manual threshold chosen from the exponential phase of the PCR amplification. The Cq values of each candidate genes between different cDNA samples were performed according to relative quantification $\Delta\Delta$Cq ($2^{-\Delta\Delta Cq}$) method (Livak and Schmittgen, 2001) using multiple reference genes for normalization.

Results

RNA-Seq, Reads Mapping and Transcripts Assembly

The sequencing of S211 and S220 cDNA libraries resulted in the generation of 79,900, 758 and 67,200,972 raw paired-end reads, respectively. Quality assessments were carried out to minimize errors that might have occurred during the sequencing process. The quality assessments of the sequencing data are shown in Table 2. Through FastQC program, the base calling accuracy of the raw sequencing reads was measured using Phred quality score (Q score) $\geq$ 30. It represents 99.9% of base call accuracy, which equivalent to the probability of an incorrect base call 1 in 1000 times (Illumina, 2011). Result in Table 2 shows that 86.35 and 82.89% of S211 and S220 raw reads were having base calling accuracy of Q $\geq$ 30. After trimming off the adapters, low quality sequence reads of Q $\leq$ 30 and reads below 36 bases long, total high-quality reads of S211 and S220 were reduced to 76, 935,356 and 57,323,996, respectively. This small percentage (4.3% in S211 and 14.7% in S220) of raw reads cut-off indicated that a high quality of paired-end sequenced reads was successfully generated by Illumina Hiseq 2000 sequencing platform. With the combination of TopHat and Bowtie as program aligner, 89.94% (68,764,738) and 90.39% (51,815,160) of the total S211 and S220 quality reads were successfully aligned to the rice reference genome; *Oryza sativa* Japonica (IRGSP1.0). Next, *via* Cufflinks program the mapped reads of S211 and S220 were assembled into their respective transcripts. Assembly of S211 resulted in more genes as compared to S220, in which 20,853 and 19,315 genes were identified in S211 and S220, respectively. This result indicates that the two rice varieties (MR211 and MR220) might have different regulation of gene expressions at the transcriptional level, in response to salt stress even though they share a similar genetic heritage.

Global Transcripts Annotation

The availability of Rice Annotation Project Database (RAP-DB) provides a highly reliable and up to date set of rice gene

annotations. This enabled the distinguishing of the annotated (known transcripts) and un-annotated (unknown transcripts) transcripts. It reveals that 59.06% (12,315 transcripts) of S211 total transcripts were annotated in RAP-DB that was categorized as known transcripts, whereas 40.94% (8,543 transcripts) were un-annotated and identified as unknown. For S220, 61.09% (11,799 transcripts) were identified as known transcripts, and 38.91% (7,516 transcripts) were identified as unknown (Fig. 1).

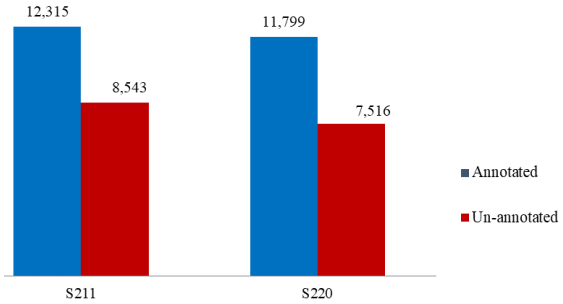

Fig. 1: Bar graph of the division of annotated (known) and un-annotated (unknown) global transcripts of S221 and S220. Blue bar represents genes that are annotated in RAP-DB and red bar represents genes with no hit (un-annotated) in the RAP-DB database

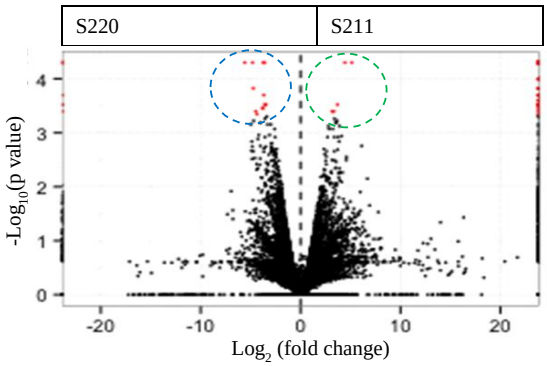

Fig. 2: Volcano plots representing estimated fold changes (log2, x-axis) and statistically significant differences ($-\log_{10}$, p-value, y-axis) of genes in S211 and S220. Each dot represents a gene. Fold changes >0 indicated genes with higher expression in S211, whereas fold changes <0 indicated genes with lower expression in S211 compared to S220. Red dots: significant differentially expressed genes. Black dots: non-significant expressed genes. DEGs with FPKM=0 were not shown it this figure

Identification of Differentially Expressed Genes (DEGs)

RNA-Seq permits a high sensitivity measurement of differential gene expression in a single assay. At FDR=0.05 and $\log_2FC \geq 2$, 252 expressed genes were identified as DEGs. The visualization of the DEGs over the insignificant ones and their mode of expression were shown in Fig. 2. Here, Log_2 Fold Change (FC) of S211 FPKM over S220 FPKM formula was used to determine the DEGs mode of express ion (higher or lower expressed) in S211 as compared to S220. The positive (+) Log_2FC value indicated the genes were higher expressed in S211, whereas the negative (–) Log_2FC value indicated the genes were lower expressed in S211 as compared to S220. Based on the dots (genes) distribution in Fig. 2, higher number of DEGs (n=11, red dots in green circle) were distributed in negative side of fold change-axis in S211 as compared to the positive side of axis (n=6, red dots in blue circle). DEGs with $\log_2FC$ values equal to +infinity (+INF) or -INF were not presented in the volcano plot, but were presented in next cluster analyses.

Cluster or heat map analysis of the generated DEGs (n=252) was conducted to visualize the differences in their level of expression between S211 and S220, which was represented by the differences of color intensity. Column with lighter orange indicates lower expression, while darker orange indicates higher expression of gene. Fig. 3 showed the largest cluster of DEGs (93.3%, n=235) were dominated by specific variety-induced genes, in which 88% (n=221) of them were specifically expressed in salt-tolerant S211 and 6% (n=14) were specifically expressed in salt-sensitive S220.

DEGs Annotation

Based on RAP-DB gene annotation, out of 252 DEGs, 197 (78%) of the DEGs were categorized as known genes (annotated), whereas the other 55 (22%) as unknown genes (un-annotated) (Fig. 4). Within the 197 DEGs that were annotated in RAP-DB database, several have a multiple number of genes short names. As a total, the annotated DEGs were represented by 224 genes, where 199 of the genes were encoded for proteins, while the other 25 were described as non-coding RNA (ncRNA) (Table 3). Within the 199 of protein encoded genes, 13.6% (27) of them has a multiple transcript with a maximum number of 6 transcripts per gene, resulting in a total number of 237 transcripts. The function of the transcripts was identified and categorized based on the transcripts description provided by RAP-DB database. More than half (108; 51%) of the transcripts were categorized as having a well-annotated protein features in RAP-DB database such as peroxidase (Os07t0677100-01), protein with similar function in protein databases such as Alpha-galactosidase (Os07t0452100-0) and some are just provided with their domain or family name. The other half of the transcripts were made up of proteins of unknown function in DUF family protein (9; 4%), hypothetical gene (8; 3%), hypothetical conserved gene (31; 13%), hypothetical protein (14; 26%), conserved hypothetical protein (61; 26%) and non-protein coding transcript (6; 2%) (Fig. 5).

Analyses of Un-annotated DEGs

In order to predict the function of 55 un-annotated genes

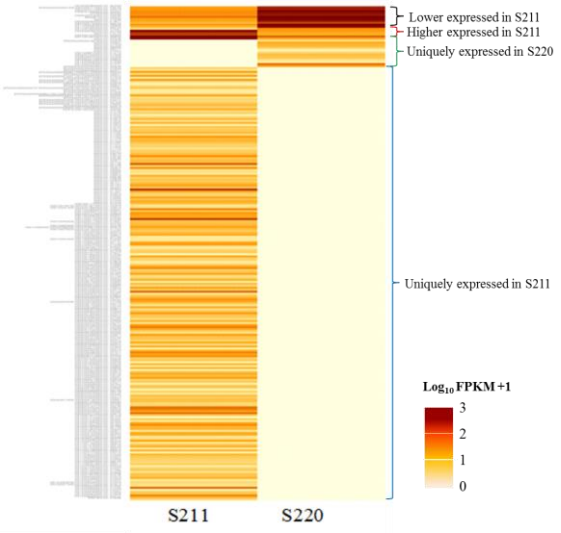

Fig. 3: Clustered heat map of the statistically significant expressed genes identified between S211 and S220. Left and right column represent S211 and S220, respectively. Each horizontal line refers to one gene, which made up of one or multiple number of transcripts. The color bar at the right side of the figure represent the expression level of the genes expression in FPKM values; those in the lightest orange have lower expression relative to the geometrical means, while dark orange indicates genes with higher expression

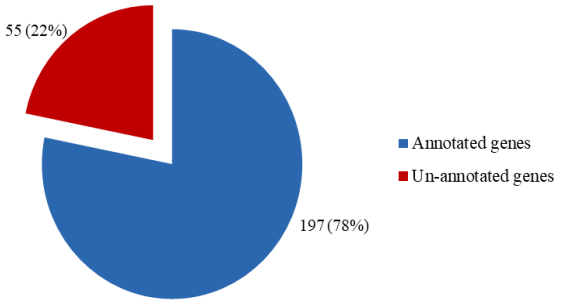

Fig. 4: The categorization of 252 DEGs into known (197, blue) and unknown genes (55, red). The categorization was made based by referring to gene annotation from RAP-DB database

that represented by 101 transcripts, their transcript sequences were subjected to BLASTX and InterProScan search of Blast2Go program. As expected, most of the transcripts which previously found as un-annotated in RAP-DB showed the highest similarity to *Oryza sativa* species itself, followed by other monocot plant species such as *Zea mays*, *Setaria italica* and *Oryza branchyta* (Fig. 6). The sequence similarity search through BLASTX showed that 62% (n=63) out of the 101 un-annotated transcripts were significantly hit (E values ≤ 10−5) to the proteins deposited in the NCBI non-redundant (Nr) database (Fig. 7A), with 59% (37), 38% (24) and 3% (2) of the transcripts were identified as well-annotated, hypothetical protein and unknown protein in Nr database, respectively (Fig. 7B). The other 38% (38) of RAP-DB un-annotated transcripts did not

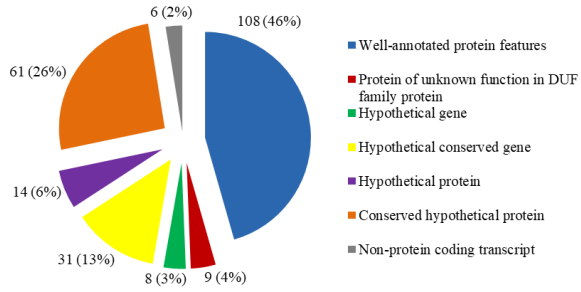

Fig. 5: The pie chart represents the classification of known DEGs transcripts into their respective function based on RAP-DB database

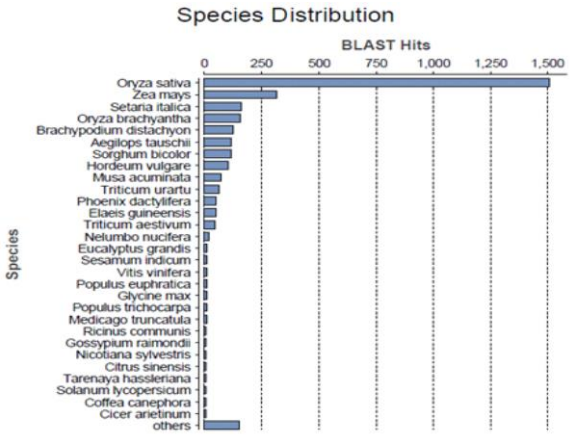

Fig. 6: Species distribution of the top BLAST hits for un-annotated DEGs transcripts. Hits shown for 101 transcripts. The most matches are to *Oryza sativa*, followed by other monocot plant species

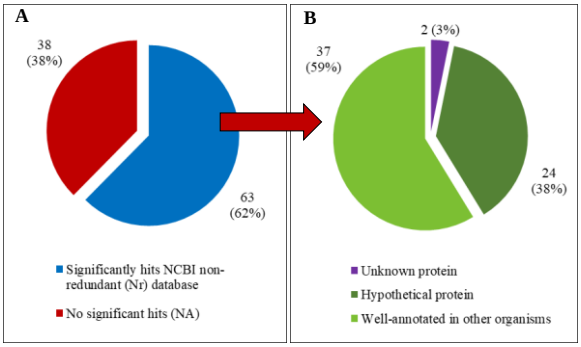

Fig. 7: The pie chart represents the classification of novel (un-annotated in RAP-DB) DEGs transcripts into their respective categories based on NCBI non-redundant (Nr) database. (**A**) Through BLASTX analyses of 101 DEG that are un-annotated in RAP-DB, 62% of them showed a significant homology with sequences in NCBI Nr database (blue), whereas no any significant hit showed for other 38% (red). (**B**) The 62% of DEGs were made up of unknown protein, hypothetical protein and well-annotated in other organism

show any significant hit to the Nr databases. Finally, the unknown 101 transcripts were subjected to InterProScan

Table 3: List of non-coding RNA (ncRNA) that differentially expressed in S211 and S220 transcriptome

Gene ID	Gene short name	Nt-long	FPKM		Log_2 FC
			S220	S211	
XLOC_076474	EPlOSAG00000010041	28	0	0.865774	+INF
XLOC_039918	EPlOSAG00000016097	43	0	0.575149	+INF
XLOC_021678	EPlOSAG00000011469	54	0	23.8607	+INF
XLOC_052636	EPlOSAG00000005246	60	0	3.67958	+INF
XLOC_033848	EPlOSAG00000023865	61	0	1.68755	+INF
XLOC_032277	EPlOSAG00000029899	61	0	2.58104	+INF
XLOC_075383	EPlOSAG00000020747	66	0	1.59626	+INF
XLOC_048158	EPlOSAG00000007308	71	258.286	19.2929	-3.74283
XLOC_000382	EPlOSAG00000026081	78	0	2.22391	+INF
XLOC_049777	EPlOSAG00000020839	85	0	0.943189	+INF
XLOC_033849	EPlOSAG00000004226	87	0	1.42672	+INF
XLOC_009677	EPlOSAG00000009250	89	0	18.3569	+INF
XLOC_023271	EPlOSAG00000008592	111	0	18.8066	+INF
XLOC_008302	EPlOSAG00000029781	112	0	2.93336	+INF
XLOC_045889	EPlOSAG00000034812	124	0	7.70292	+INF
XLOC_069651	EPlOSAG00000016607	125	0	5.55763	+INF
XLOC_064675	EPlOSAG00000016604	126	0	1.93191	+INF
XLOC_020919	EPlOSAG00000035844	133	0	3.39477	+INF
XLOC_027438	EPlOSAG00000034296	157	0	4.96465	+INF
XLOC_054293	EPlOSAG00000008962	159	0	2.30738	+INF
XLOC_003898	EPlOSAG00000025663	161	0	15.3638	+INF
XLOC_066531	EPlOSAG00000011994	172	0	2.9652	+INF
XLOC_075383	EPlOSAG00000035695	180	0	1.59626	+INF
XLOC_003433	EPlOSAG00000046849	188	0	11.3563	+INF
XLOC_040538	EPlOSAG00000018916	143	13.8461	0	-INF

(IPS, http://www.ebi.ac.uk/InterProScan) program for their functional predictions. IPS is embedded in 16 secondary protein databases that allow the scanning of novel sequences (protein and nucleic acid) to determine their domains, families and functional sites. Only 29 out of 101 transcripts were matched to the IPS program. The other 72 transcripts did not show any IPS match. The IPS search found 2 of the transcripts (XLOC_032754_1 and XLOC_016841_1) hits on COILS database (http://embnet.vital- it.ch/software/COILS_form.html), which denote regions named as coiled coil domain (Hartmann *et al*., 2016). This coil structure is a domain for Ubiquitin-conjugating enzyme E2 that catalyzes the covalent attachment of ubiquitin to target proteins. Through cross-referencing to Phobius (http://phobius.sbc.su.se/), TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) databases, 19 transcripts were identified as a part of transmembrane protein and were spanning a signal peptide at their N-terminus.

Gene Ontology (GO) and Pathway-based Analysis of DEGs

To overview putative function of the DEGs in response to salt stress, all 252 DEGs represented by 338 transcripts sequences (237 known transcripts and 101 novel transcripts) were blasted into the GO and KEGG databases via Blast2GO program (version 2.3.5) (http://www.blast2go.org/). Based on their expression profiling (Fig. 3), the transcript sequences were divided into two groups and blasted to the Blast2GO program separately. Group I composed of transcripts (n=303) categorized as specifically and higher expressed in salt-tolerant S211, while Group II composed of transcripts (n=35) categorized as specifically and higher expressed in salt-sensitive S220. GO at hierarchy level 2, comprising the highest node for each category in combined graph, was chosen to summaries the GO output (Fig. 8). The output of Blast2GO showed that Group I DEGs were enriched into 21 functional terms that belong to the biological process (BP; 9), molecular function (MF; 6) and cellular component (CC; 6). Biological process (BP) term was dominated by "metabolic process", "cellular process", "single-organism process" and "response to stimulus". Molecular function (MF) term was dominated by "catalytic activity" and "binding", whereas "cell" and "organelle" were most dominant for cellular component (CC) term. Group II DEGs were enriched into 15 functional terms that belong to the biological process (BP; 7), molecular function (MF; 4) and cellular component (CC; 4). To identify their involvement in metabolic and signal transduction pathways, pathway enrichment analyses were conducted to all the DEGs. Out of 303 total transcripts of Group I DEGs, 61 (20%) transcripts were assigned to 33 KEGG pathways. This high number of metabolic pathways indicated the diverse survival strategies of MR211 to cope with salinity stress, with the highest number of transcripts accounted in "purine metabolism" (19, 23%) and "thiamine metabolism" (11, 13%) pathways. Others prominent pathways were including "drug metabolism-other enzymes" (5, 6%) and "starch and sucrose metabolism" (5, 6%) (Fig. 9). For

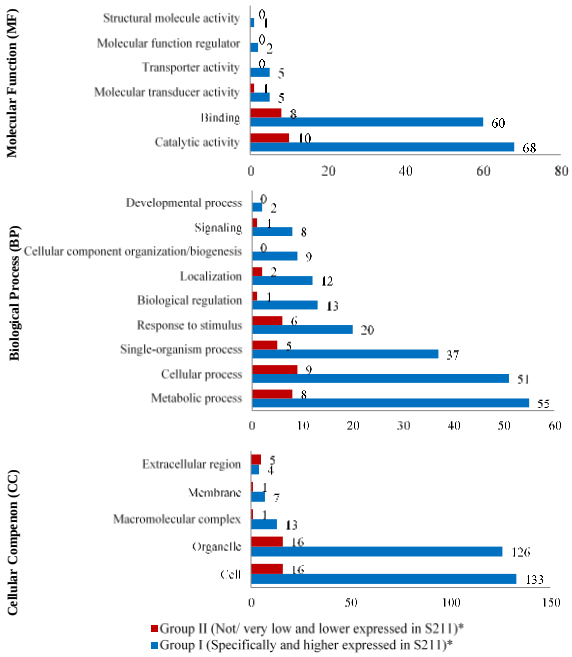

Fig. 8: The Gene Ontology (GO) terms demonstrated by DEGs transcripts. The GO terms were categorized into three main categories (Biological process: BP, molecular function: MF and cellular component: CC) which composed of 28 subcategories with GO classifications at level 2. Top 10 most presented categories and the number of transcripts belong to each category are shown. Asterisk (*) indicated the genes expression in S211 as compared to S220

Group II DEGs (DEGs that "not/very low" and "lower expressed" in S211 compared to S220), 28.2% (11) out of its 39 total transcripts were assigned to 9 KEGG pathways with phenylpropanoid biosynthesis was denoted as the most enriched pathway (4, 37%) (Fig. 9).

DEGs Expression Profiling Via Semi-quantitative RT-PCR and Real-time PCR

To validate the accuracy and reproducibility of the in silico RNA-Seq expression of the representative DEGs in S211 and S220, the expression of DEGs were further verified through semi-quantitative RT-PCR followed by real-time PCR analysis. Semi-quantitative RT-PCR result showed that the expression of four candidate genes, namely *FER2*, *Thaumatin*, *VI* and *UBC* were in line with data generated from the RNA-Seq data (Fig. 10). The expression of *FER2*, *Thaumatin* and *VI* were significantly higher in S211 and for *UBC*, it was found as very lowly expressed in S211 as compared to S220. *VI* gene generated a significant different of expression with approximately 2.5-fold higher of express ion in salt treated salt-tolerant MR211 (S211) compared to salt treated salt-tolerant MR220 (S220). Meanwhile, two DEGs, namely *MT* (*NRAMP1*) and *HOX16* showed a contradict trend of express

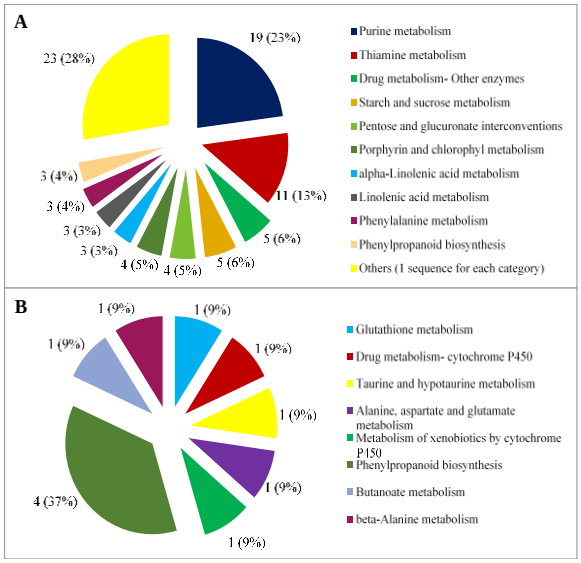

Fig. 9: The KEGG classification demonstrated by the DEGs transcripts of (**A**) DEGs that were expressed specifically and higher in S211 (Group I) and (**B**) DEGs that were no/ very low and lower expressed in S211 (Group II) as compared to S220

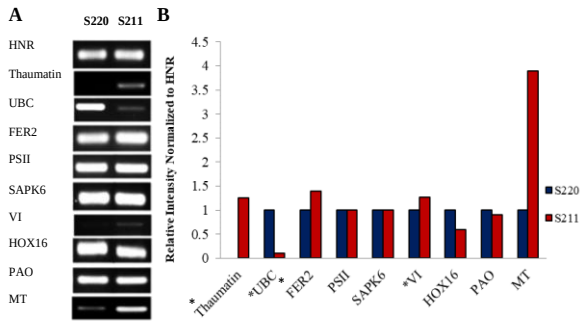

Fig. 10: Validation of the expression of nine representative DEGs in S211 and S220 cDNA via semi-quantitative RT-PCR. (A) The PCR products were run in 2.0% gel electrophoresis. (B) Analysis of quantified band intensity. Data are expressed relative to the S220 levels of expression and was normalized to *HNR* (housekeeping gene). Blue bar represents the expression of target gene in S220 cDNA sample, whereas the red bar represent the expression of target gene in S211 cDNA. Asterisks (*) denote genes with consistent expression with the RNA-seq data

ion as compared to the RNA-Seq data. *MT* (*NRAMP1*) showed a significant higher expression in S211 (3.5-fold), whereas *HOX16* showed a lower expression in S211, as compared to S220 (Fig. 10). In the RNA-Seq data, *MT* (*NRAMP1*) was found to have a lower express ion in salt treated salt-tolerant MR211 (S211) as compared to salt treated salt-sensitive MR220 (S220), whereas *HOX16* was found as specifically expressed in S211. The other three candidate genes; *PSII*, *SAPK6* and *PAO*, they exhibited a similar (no difference) level of expressions in S211 and S220. Based on their RNA-Seq data, *PSII* and *PAO* showed a higher express ion in salt treated salt-tolerant

MR211 (S211), while SAPK6 was found specifically expressed only in S211 as compared to salt treated salt-sensitive MR220 (S220).

Further with real-time PCR analysis, only *UBC*'s expression were in line with previous RNA-Seq data in which *UBC* was found as very lowly expressed in S211 as compared to S220 (Table 4). On the other hand, a less pronounced-lower expression of *PAO* in S211 as compared to S220 was observed during this PCR analysis (Fig. 11). This was contradicted with previous RNA-Seq data where *PAO* was expressed higher in S211 as compare to S220. The comparison made on the resulted genes express ion via semi-quantitative RT-PCR and the real-time PCR had showed consistent patterns of genes expression between these two-profiling approaches. Via semi-quantitative RT-PCR and real-time PCR analysis, *PAO* showed a lower trend of express ion in salt treated salt-tolerant MR211 (S211) compared to salt treated salt-sensitive MR220 (S220), whereas *PAO* exhibited only slight or no different level of expression in S211 and S220. No significant differences in expression in *PAO* and *UBC* were noticed for both genes in S211 and S220 since the expression differences in S211 and S220 samples was less than 2-fold (Fig. 11).

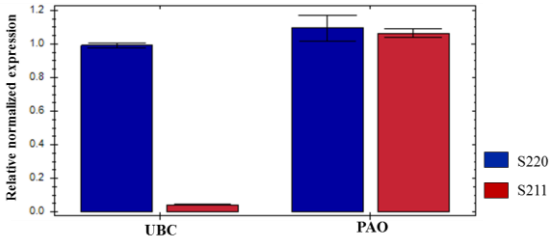

Fig. 11: Quantitative real-time PCR analyses of *PAO* and *UBC* genes in S211 and S220. *HNR* and *EP* genes were used as reference genes, whereas S220 was used as the control sample. Blue bar represents the expression of target gene in S220 whereas the red bar represents the expression of target gene in S211

Table 4: List of candidate genes to be validated via semi-quantitative RT-PCR and real-time PCR analysis

Gene	Transcript ID	FPKM		Log_2FC	Expression categorization
		S220	S211		
Pheophorbide a oxygenase (*PAO*)	Os03t0805600-00	13.41	173.77	3.70	Expressed higher in S211
Ferritin 2 (*FER2*)	Os12t0106000-01	22.61	494.57	4.45	
Photosystem II (*PSII*)	Os07t0147500-01	63.01	637.37	3.34	
Homeobox-leucine zipper protein (*HOX16*)	Os02t0729700-01	0	2.48	+INF	Specifically expressed in S211
Serine/threonine-protein kinase (*SAPK6*)	Os02t0551100-01	0	6.43	+INF	
Invertase inhibitor (*VI*)	XLOC_017425_1	0	6.26	+INF	
Thaumatin	Os06t0691200-01	0	3.67	+INF	
Metal transporter (*MT*)	Os07t0258400-01	493.792	18.64	-4.73	Expressed higher in S220
Ubiquitin-conjugating enzyme e2 11 (*UBC*)	XLOC_016841_1	8.96352	0	-INF	Specifically expressed in S220

Note: +INF and -INF are respectively referred to genes with positive and negative infinity of log_2FC value

Discussion

The application of RNA-sequencing method in comparative differential transcriptomic expression between two Malaysian rice varieties with a contrast level of tolerance to salt stress successfully generated an invaluable source of transcripts database. Hundreds of salt-shock responsive genes (DEGs) were identified thus contribute for a better understanding on the polygenic salt-shock adaptation of the tolerance variety, MR211. The DEGs that were identified as higher and specifically expressed in salt tolerant MR211 compared to the sensitive MR220 had become the attention for the downstream analysis. Generally, functional annotation of these groups of DEGs had revealed the presence of regulatory genes such as transcription factors (TFs), protein kinases and protein phosphatases, as well as functional genes that involves in various adaptation mechanisms such as mechanical support, ROS-scavenging system, ion exclusion and intracellular compartmentalization.

With the single step addition of 12 dS/m NaCl concentration on the nutrient solution, the rice seedlings especially the root parts were experiencing a remarkable effect of cellular dehydration that lead to the reduction of the cytoplasmic volume (Schneider *et al*., 2012). This condition contributes to a significant mechanical stress on plants that affects the structure and growth of cell wall. To cope with the osmotic effects, the express ion of several cell- wall related proteins were required by plants in order to protect their cell wall architecture. Two main protection mechanisms have been proposed. First through maintaining their cell wall plasticity (Park *et al*., 2003, 2004) and second, by increase cell wall thickening (Rayon *et al*., 2015). Regarding that, the finding of several cell-wall related transcripts that were specifically and highly expressed in salt treated MR211 as compared to MR220 had revealed; mechanical support through cell wall architecture is one of MR211 salt stress adaptation mechanism. The following are among those genes.

As one of skeletal component of plant cell wall, xyloglucan work as a tether between cellulose microfibrils. The content of xyloglucan will affect cell wall rigidity, either tightening or loosening of cell wall. It has been reported that the loosening activity of cellulose microfibrils in various plant species through the degradation of xyloglucan could increase cell wall elasticity which is required for cellular adaptation in various environmental changes. In poplar (Park *et al*., 2004) and *Arabidopsis* (Park *et al.*, 2003) for example, the increased expression of xyloglucanase that caused marked degradation of

xyloglucan resulted in acceleration of stem elongation and increasing of leaf cell volume, respectively. In our dataset, two xyloglucanase transcripts known as XET/XTH expressed specifically in MR211 (Os03t0108300-01 and -02; FPKM=4) in which represent the greater cell wall elasticity (CWE) of this rice variety compared to MR220. As been reported by previous studies (Diego *et al*., 2013), plant varieties with more resistant to drought showed a strong decrease in their elasticity modulus (ε) in association with a higher CWE. In contrast, the highly drought-sensitive varieties did not show any significant change in their ε and CWE. Therefore, it can be presumed that these results reflect differences in MR211 and MR220 wall structure.

Denoted as the main skeletal structure of primary cell wall, cellulose played such a crucial role in providing structural support for plants in various adverse conditions. As being reviewed by Rayon *et al*. (2015) the reduction of cellulose content in response to osmotic stress had caused the secondary cell wall becoming thinner. This makes it unable to withstand with the negative pressure produced during the course of water transport and increasing the collapse of xylem vessel. For that reason, the ability of plants to increase or maintaining their synthesis of cellulose would be an advantage for stress adaptation. Regarding that, cellulose synthesis enzyme named as cellulose synthase played it roles. As being reported by Sengupta and Majumder (2009), after being treated with 40 dS/m of NaCl, a higher expression of cellulose synthase had been detected in wild halophytic rice, *Porteresia coarctata* compared to the salt-sensitive domesticated rice. Being specifically expressed in salt treated MR211 (FPKM=1.8), it is proposed that this cellulose synthase (Os12t0477200-00) may be one of the main adaptations mechanisms of MR211 to cope with osmotic stress, which achieved by increasing the deposition of cellulose inside the primary walls, thus maintaining the secondary wall thickness.

The ROS are strong oxidizing compounds that lead to oxidative damage of lipids, protein and nucleic acid and eventually lead to plant cell death. The regulation of ROS in cells is achieved through a complex network of small-molecule antioxidants and enzymes. In the present study, we had identified abundant number of transcripts that take part as the major player in ROS scavenging network and excitingly, those genes were found to be specifically expressed in our salt-tolerant variety, MR211. They were peroxidase (EC 1.11.1.7) (Os04t0688500-01; FPKM=21.8) and peroxidase 1 (Os05g0499300-01; FPKM=2.5) which function in catalyzing the breakdown of H_2O_2. We also identified four transcripts that encoded for ankyrin-like protein (ANK) (Os01t0189700-01, -02, -03 and -04; FPKM=2.4). Those genes were suggested as key signalling molecule regulating plant antioxidant metabolism and defence responses (Seong *et al*., 2007a). ANK responses in salinity stress were progressively studied in a number of plant species such as *Capsicum annum* (*CaKR1*) (Seong *et al*., 2007b), *Arabidopsis thaliana* (*ITN1, SHG1*) (Sakamoto *et al*., 2014) and tobacco (*NEIP2*) (Yang-Rong *et al*., 2015). ANK also has been studied in rice but so far, their scope of study were mainly focus on biotic stress responses such as in *Magnaporthe oryzae* attack and several abiotic stress such as light/dark treatments (Jianyan *et al*., 2009).

Besides, three transcripts of similar to *Ferritin 2* (*Fer2*) (Os11t0106700-01, Os11t0106700-02 and Os12t0106000-01) were identified and expressed around 5-fold higher in MR211. Iron (Fe) is among crucial element in photosynthesis apparatus and in Haber-Weiss cycle. Free iron contributes to the production of excess hydroxyl radicals (Fei *et al*., 2008). As an iron-binding protein located in chloroplast, Ferritin can store iron in a complex form and release them if necessary, thus reveal its role to protect plant cells from oxidative damage in chloroplast induced by manifold stresses.

The ABC transporters are important for plant development and survival. They were localized in most of plant cell membranes such as plasma membrane, vacuole and chloroplast. Governs by active transport through ATP hydrolysis, they cover a multitude of transporter activity for various compounds as a part of plants strategy to cope with the deleterious effects of abiotic stresses. Based on our dataset, 6 ABC transporter gene family (Os10t0432200-01, Os12t0409700-00, Os12t0409900-01, XLOC_015979_1, 3 and 4) were found to be specifically expressed in salt-tolerant variety, MR211 (FPKM=1.5-5.4). Originally, ABC transporters were identified to be involved in plant detoxification from various endogenous and exogenous toxic compounds. During severe salt-stress specifically, high level of Na^+ and Cl^- in apoplast will alter plant ion homeostasis, thus resulting in hyperosmotic stress, ionic imbalance and toxicity (Oliveira *et al*., 2013). As has been found in our DEGs transcript dataset, it can be proposed that detoxification pathway was started with the oxidation of the toxic compounds which are subsequently conjugated to a hydrophilic molecule such as glucose by glutathione S transferases (EC: 2.5.1.18, Os10t0528400-01) to prevents the newly formed compounds from crossing membranes by diffusion. Finally, ABC transporters played their role in transporting the compound-conjugates into the central vacuole or excreted into the apoplast thus reduce the toxicity of the compounds. The same toxic ion compartmentalization strategy was also being used by bryophytes species to re-establish cellular ion homeostasis in salt environments (Wang *et al*., 2008). At this point, MR211 showed its efficient mechanism which enable to eliminate accumulation of salt ion in plant cells to avoid toxic level as been shown halophyte, a salt-tolerant plant species (Zhao *et al*., 2016a). Beyond detoxification, we also noticed the expression of *ABCG11* (Os12t0409700-00; FPKM=2.18134) another interesting ABC transporter family member that are responsible in production and deposition of cuticle that made up of wax and cuticle on leaf surface thus enable the plants to control non-stomatal water loss during water scarcity (Crouzet *et al*., 2013).

Transcription factors are a group of proteins that take part in the initiation and regulation of transcription process of DNA into RNA. Having DNA-binding domains, these proteins able to bind to a specific regulatory sequence of DNA called enhancer or promoter that located thousands of base pairs upstream or downstream from the gene being transcribed, including the stress-responsive genes (Wang *et al*., 2016). Based on our dataset, two TFs that were identified specifically expressed in salt treated MR211. They are bHLH transcription factor-like protein (Os07t0407700-01, -2, -3; FPKM=3.3) and MYB transcription factor-like protein (Os05t0206200-01; FPKM=4.4). These two types of TFs factor have been abundantly reported for their involvement in modulating the acclimation response of plants to severe environments including salt stress. More interestingly, through a comparative transcriptomic study in two contrasting rice varieties in response to salinity stress (N22 and Pokkali), these both types of TFs were also being denoted as highly enriched in Pokkali, a widely known salt-tolerant variety (Shankar *et al*., 2016).

The tolerance or susceptibility of a specific plant variety within a species to salt stress is determined by their complex defense network initiated by various series of inducible reaction. Through this reaction, plants perceive stress signals and transmit them through the cellular machinery to activate adaptive responses resulting in the activation of specific genes involved in specific mechanism. In higher plants, perception and transduction of stress signals is largely carried out by receptor-like kinases (RLKs) (Tanaka *et al*., 2012). Hundreds of *RLKs* genes have been identified in numerous plant species such as in rice and Arabidopsis. Based on their extracellular domain, *RLKs* were classified into 10 subfamilies representing their diversification in function and mode of signals perception. Taking together, the identification of *RLKs* which specifically expressed in MR211 during severe salinity stress treatment has suggested that high expressions of those *RLKs* work as the 'front line' in sensing of salinity stress signals in order to activate downstream defense pathways.

In our data, three of the *RLKs* (Os07t0145400-01, XLOC_015510_4 and Os01t0133900-00) were made up from the largest subfamily among the RLKs, known as leucine-rich repeat receptor-like kinases (LRR-RLKs), with the expression 0.9 to 2.2 fold higher in MR211 compared to MR220. As been presented in RNA-Seq analyses on *Caragana korshinskii*, a shrub with highly developed root systems and strong stress adaptation that were treated with salinity stress, 24 and 7 contigs has been predicted to encode leucine-rich repeat (LRR) receptor-like protein kinase were differentially expressed in response to drought stress and salt stress, respectively (Li *et al*., 2016). In other species, LRR-RLKs play their role in the regulation and adaptation of *Medicago truncatulata* to salt stress by causing in the accumulated fewer sodium ions than controls thus make the plants less inhibited by the presence of salt ion in the medium (Lorenzo *et al.*, 2009). By using reverse genetic approach, their data demonstrate that LRR-RLKs play a role in a wide variety of signal transduction pathways related to hormone and abiotic stress responses in *Arabidopsis* (Hove *et al*., 2011). Recently, Dievart and her coworkers had revealed 37 of new uncharacterized LRR-RLK genes in rice that putatively involved in abiotic stress responses and were targeted as the potential target genes for breeding of salt- and drought-tolerant cereals (Dievart *et al*., 2016).

Another one receptor kinase that is also play a vital role during development and in the adaptive response to various stimuli is Legume-type lectin-domain containing receptor kinases-like (*Lec*RK). In our data, this gene (Os08t0123900-00) was found to express 2.3-fold higher in MR211. Based on their conserved hydrophobic-binding site, *LecRKs* may serve in the recognition of small hydrophobic ligands, such as plant hormones or MAMP (André *et al*., 2005; Bouwmeester and Govers, 2009). Preliminary reports have demonstrated that *LecRLKs* provide assistance in alleviating salt stress, as suggested for *Arabidopsis* LecRK-V.1 (LecRK-b2; At1g70110), *Pisum sativum* LecRLK and *Glycine soja* GsSRK. Overexpression of *LecRLK* has been demonstrated to improve germination rate, pigment content, and biochemical status of the salt-stress -affected plants (Joshi *et al*., 2010; Sun *et al*., 2012). Expression-based studies also suggest putative roles of several *LecRLKs* in abiotic stresses, though experimental validation is still pending. He *et al*. (2004) have demonstrated that the *LecRK-1.3* (AtLecRK2; AT3G45410) gene was differentially up-regulated during salt stress, while Deng *et al*. (2009) showed that the *LecRK-V.1* gene was responsive to osmotic stress. The *LecRLKs* response to wounding is reported for *Arabidopsis* LecRK-V.5 and *Populus nigra PnLPK* genes (Garcia-Hernandez *et al*., 2002; Nishiguchi *et al*., 2002).

In other aspect, *LecRK* are regarded as ideal candidates for monitoring cell wall integrity and are possibly functional in adaptive responses. Even though they are implied to function in diverse biological processes such as pollen development and plant defense, their exact biological role is still not clear. In Arabidopsis, lectin receptor kinase *lecRK-a1* was shown to be induced during disruption of the plant cell wall (Riou *et al*., 2002) that caused by wounding, senescence of leaves, and in response to oligogalacturonides. Expression data of *LecRKs* in Arabidopsis show that they are activated by various biotic and abiotic stimuli and were differentially expressed in various accessions (Bouwmeester and Govers, 2009).

We also identified novel wall-associated kinases (WAKs) RLKs (XLOC_017725_1) that were expressed 3.9-fold higher in MR211. WAKs have been reported as candidates for physical linkers that provide signal between the cell wall and the cytoplasmic compartment (Kohorn, 2000; Verica *et al*., 2003). Previous studies have shown that various WAK and WAK-like kinase (WAKL) members were involved in biotic and abiotic stress responses and

were required for cell elongation and development (Lally *et al*., 2001; Wagner and Kohorn, 2001). As example, *WAK1* is an aluminium early responsive gene and its overexpression resulted in aluminium tolerance (Sivaguru *et al*., 2003). Microarray results confirmed that the highest expression of *WAKL4* was found in roots and that salt (NaCl) stress significantly induced *WAKL4* expression with 5-fold increment in salt-treated Arabidopsis seedlings. Consistent with our observations, the salt-induced expression seems to be related to elevated minerals and not to general water deprivation as various drought and osmotic stress conditions failed to induce *WAKL4* expression.

Plant-specific serine/threonine kinases snRK subfamily 2 (SnRK2) proteins was discovered to take part in abiotic stress signal transduction pathways in plants, specifically during early events of ABA signalling pathway (Kulik *et al*., 2011). The SnRK2s regulate ABA-responsive transcription factors including ABA-responsive promoter elements (ABREs)-binding transcription factors (ABFs) and activate ABA-responsive genes and ABA-responsive physiological processes (Umezawa *et al*., 2009; Vlad *et al*., 2009). In our work, s*nrk2a*-like gene (Os 02t0551100-01) was specifically expressed in salt treated MR211 with 6.4-fold higher in expression (FPKM value) compared to MR220. Consistent with our finding, similar regulation of ABA signalling has been detected in other various species such as wheat (Zhang *et al*., 2011), *Populus trichocarpa* (Song *et al*., 2016) and *Jatropha curcas* L. (Chun *et al*., 2014).

Coenzyme A (CoA) is an important cofactor in various biosynthetic, degradative and energy-yielding metabolic pathways (Begley *et al*., 2001). CoA biosynthesis is catalyzed by phosphopantetheine adenylyltransferase (PPAT), a pantothenate kinase (PANK) family protein (EC: 2.7.1.33). Overexpression of PPAT in *Arabidopsis thaliana* had led to the increasing of CoA + acetyl-CoA levels as well as enhanced vegetative and reproductive growth and salt/osmotic stress resistance (Rubio *et al*., 2008). In our finding, PANK (Os 11t0311100-01) was specifically expressed in salt treated MR211 (FPKM=8) compared to MR220.

The resulted osmotic effect and accumulation of reactive oxygen species (ROS) triggered by salt stress could lead into detrimental impact on plant photosynthesis. It leads to disruption of all photosynthesis machineries including photosystem I (PSI) and II (PSII), electron transport, carbon fixation, ATP generating system and stomatal conductance, which eventually will end up with loss of crop yield. Plants carry out oxygenic photosynthesis, where the presence of water is critical as an initial electron donor to accept the photon and completing the light reaction in PSII and PSI which end up with the production of ATP and NADPH to form the glucose molecule in dark reaction (Nelson and Yocum, 2006). In other aspects, other than for plant survival, the enhancement of photosynthetic efficiency is a key component to maintain and boost the crop yield during stress condition.

Plants reacts to the stress differently according to their photosynthetic systems (C3 or C4), types or species of plants, as well as type, time and duration of the stress. As one of their adaptation mechanisms, plants respond to the stresses by coordinating their chloroplast and nuclear gene expression including by altering the level of genes and proteins related to photosynthesis. Chloroplast, thylakoid membrane and nucleus are the main targets of regulated proteins and metabolites associated with photosynthetic pathways. Rapid responses of plant cell metabolism and adaptation to photosynthetic machinery are key factors for survival of plants in a fluctuating environment (Nouri *et al*., 2015). Regarding that, the identification of 13 photosynthetic related transcripts that exhibited a higher level of expression in salt tolerant MR211 as compared to the sensitive MR220 had suggested the involvement of these genes in providing the tolerance adaptation during salinity stress.

Different varieties of plants such as rice differing in degree of salinity tolerance will exhibit different patterns of DNA methylation (Karan *et al*., 2012). Regarding to this, retrotransposon is one of the transcriptional units that were triggered in response to salt stress and may generates true genetic or epigenetic changes, thus lead into increasing plants adaptation to abiotic stresses (Reinders *et al*., 2009). We identified 6 retrotransposon that were found expressed specifically in MR211. Three were denoted as retrotransposon unclassified (XLOC_015326_4 and _5; FPKM=2.6, Os12t0142900-02; FPKM=1.5), whereas the other three were identified as retrotransposon ty3-gypsy subclass (Os04t0247700-00; FPKM=2.5, Os06t0626200-01; FPKM=36 and Os10t0198333-01; FPKM=1.6). Therefore, the elucidation of the epigenetic control of transcription and transposition of retrotransposons may provide a novel strategy to create genetic variation for developing stress tolerant crop plants in future (Mirouze and Paszkowski, 2011). The unique expression of retrotransposon in salt-tolerant variety, MR211 suggested the existence of relationship between DNA methylation and salinity tolerance. It also served as a novel strategy represented by MR211 for gene regulation and salt adaptation.

Based on the presence of 25 ncRNA (Table 3), it is interesting to see that 23 of the non-protein coding transcript (ncRNA) were expressed specifically in S211, thus suggests their involvement in salt-tolerant adaptation in MR211. ncRNA has been reported as the major products of plant transcriptional unit (Matsui *et al*., 2010; Wang *et al*., 2015). Previous studies have shown the ncRNA responsiveness and regulatory roles in numerous crucial biological processes in response to abiotic stresses including drought, heat, salinity and low temperature (Matsui *et al*., 2013; Khan *et al*., 2016; Zhao *et al*., 2016b). As listed in Table 3, the ncRNA accounted for one small ncRNAs (20-30 nt) and 24 medium ncRNA (50–200 nt). None of long ncRNA (> 200 nt) found in our libraries. Recent study had shown sncRNAs and lncRNAs were involved in the transcriptional

and post-transcriptional regulations of gene expression by modulation of RNA stability and translation under various physiological and stress conditions (Bokszczanin *et al*., 2015; Liu *et al*., 2015a, b). Unfortunately, there was no publication on medium ncRNA has been reported, thus further/specific characterization on the involvement of the generated ncRNA in salt stress adaptation cannot be figured out.

Without putting aside of other pathways, the abundant number of differential transcripts (11 transcripts) in purine and thiamine metabolism suggest the significant role of these pathways for the salt adaptation mechanism in MR211. How would purine metabolisms provide salt tolerance in plants? It is widely agreed that the intermediates of purine catabolism, ureides, allantoin and allantoate are responsible in providing plant resistance in various abiotic stresses such as dark stress and drought, as well as reducing the effect of plant senescent (Brychkova *et al*., 2008). However, how these intermediates contribute to stress adaptation has been largely underexplored. So far, two hypotheses have been made. First, allantoin has been proposed to function in quenching the deleterious effects of reactive oxygen species (ROS) (Werner and Witte, 2011; Takagi *et al*., 2016). A recent study also showed that increasing accumulation of allantoin would result in up-regulation of stress-related genes which subsequently resulting in plants tolerance to drought-shock and osmotic stresses. This phenotype enhancement has been found to be caused by specific effects of allatoin in activating abscisic acid (ABA) metabolism. There are two mechanisms of ABA activation by allantoin. First through increasing the transcription of *NCED3*, encoding a key enzyme in ABA biosynthesis and second, through formation a high-molecular weight complex of β-glucosidase hydrolysing glucose-conjugated ABA, known as BGI (Watanabe *et al*., 2014). The same trend of enriched purine metabolism in various plants responses to biotic and abiotic stress has also widely reported in *Zea mays* (Peterson *et al*., 1988), *Phaseolus vulgaris* (Yesbergenova *et al*., 2005) as well as in tomato and *A. thaliana* (Alamillo *et al*., 2010).

Meanwhile, thiamine or also known as vitamin B1 is a crucial element for the proper operation of all living organisms. The biosynthesis and further phosphorylation/dephosphorylation of free thiamine along this pathway generate an active form of thiamine, known as thiamine diphosphate (TDP). It works as universal coenzyme that involves in major metabolic pathways including the ones that deal with stress factors such as the oxidative pentose phosphate pathway (Baxter *et al*., 2007) and the ethanolic fermentation (Ku¨rsteiner *et al*., 2003), Calvin-Benson cycle (CBC), the pentose phosphate pathway (PPP), the tricarboxylic acid cycle (TCAC) and the isoprenoid phosphate biosynthesis pathway (IPP) which in turn facilitates the activation of certain defense mechanisms and the production of stress protecting molecules. Other than that, thiamine itself can also work as important source of antioxidants and also take parts as stress alarmone, a form of intracellular signal molecule that is produced due to harsh environmental factors (Bettendorff and Wins, 2009).

As been showed in our data, the enrichment of thiamine metabolism in salt-treated MR211 proposed the involvement of this pathway as salt-stress adaptation strategy of this rice variety. This is consistent with the studies that were reported by Rapala-Kozik *et al*. (2008, 2012), where up-regulation of free thiamine, TDP biosynthesis and thiamine biosynthetic genes (THIC, THI1, TH1 and TPK) had been detected in *Zea mays* and *Arabidopsis thaliana*, mediated by drought, salinity and oxidative stresses. However, none of genes involved in thiamine biosynthesis were found in our DEGs which might be due to time-dependent response of those genes in MR211 seedlings in response to salt stress which creates no significant different with MR220.

RNA-Seq is massively used in the expression profiling of a wide dynamic range of transcriptional units of an organism which includes genes, novel transcripts and splicing events. However, several issues regarding on the design and analyses of RNA-Seq experiments might affect the validity of the identified DEGs and their expression profiling during comparative expressions. Currently, a dozen of reports have highlighted on the substantial role played by the 'number of biological replicates' and the 'types of bioinformatics tool used' for a valid interpretation of *in silico* differential expression analyses. As suggested by others, more than six biological replicates were required to minimize the true and false positive identification of significant DEGs (Schurch *et al*., 2016). However, due to financial constraint, only one biological replicates of S211 and S220 of RNA samples were preceded for the RNA-Seq. The expression accuracy and reproducibility of the 252 DEGs identified from the RNA-Seq experiment were validated using semi-quantitative RT-PCR and real-time PCR. Nine genes were selected as the representative for this validation stage. They were *UBC, FER2, HOX16, MT, VI, PSII, SAPK6, PAO* and *Thaumatin*.

By comparing on the resulted expression profiling of candidate genes via RNA-Seq (Table 4), semi-quantitative RT-PCR (Fig. 10) and real-time PCR (Fig. 11), differences in the expression value and fold of DEGs expression between S211 and S220 were exhibited between these three gene expressions profiling method. These differences might have caused by several factors. It has been suggested that the differences of sensitivity and normalization methods used in RNA-Seq, semi-quantitative RT-PCR and real-time PCR is one of the factors (Ho *et al*., 2016). Furthermore, the choice of data analysis software used and the number of sample pooling/ biological replicates used during RNA-Seq analyses will significantly affect the rate of false-positivity and false-negativity in DEGs detection. Meanwhile, Rajkumar *et al*. (2015) reported that Cuffdiff program that was used in our RNA-Seq data analyses had resulted in the highest rate of false positivity compared to other three tested

programs including edgeR, DESeq2 and Two-stage Poisson Model (TSPM). Among them, edgeR was a relatively more true-positive DEGs and high specificity. Another important factor is a number of samples pooled in the RNA-Seq analysis. A higher number of biological replicates will significantly reduce the biasness in estimating the DEGs expression. It has been reported that RNA-pool with eight biological replicates significantly improved the correlation between the fold of expression (Log_2FC) and the corresponding individual sample.

Conclusion

The data generated from this study will serve as an invaluable genomic reference to further our knowledge about the molecular and cellular events that specifically occur in rice seedlings during their early response to severe salinity stress. Not only constricted as the candidate genes for rice improvements through genetic engineering, the potential salt-tolerant genes can also be utilized as DNA markers to fasten the selection process during conventional breeding.

Acknowledgements

This work was supported by the Ministry of Education Malaysia under the Fundamental Research Grant Scheme (FRGS-2013-2-5524334). N-M Juri was supported by Graduate Research Fellowship (GRF) and MyBrain15 Scholarship. We would like to acknowledge Malaysian Agricultural Research and Development Institute (MARDI) for providing the MR211 and MR220 seeds.

References

Alamillo, J.M., J.L. Díaz-Leal, M.V. Sánchez-Moran and M. Pineda, 2010. Molecular analysis of ureide accumulation under drought stress in *Phaseolus vulgaris* L. *Plant Cell Environ.*, 33: 1828–1837

Allotey, D.F.K., R.D. Asiamah, C.D. Dedzoe and A.L. Nyamekye, 2008. Physico-chemical properties of three salt-affected soils in the lower volta basin and management strategies for their sustainable utilization. *West Afr. J. Appl. Ecol.*, 12: 163–182

Amudha, J. and G. Balalubramani, 2011. Recent molecular advances to combat abiotic stress tolerance in crop plants. *Biotechnol. Mol. Biol. Rev.*, 6: 31–58

André, S., H.C. Siebert, M. Nishiguchi, K. Tazaki and H.J. Gabius, 2005. Evidence for lectin activity of a plant receptor-like protein kinase by application of neoglycoproteins and bioinformatic algorithms. *Biochim. Biophys. Acta*, 1725: 222–232

Antiabong, J.F., M.G. Ngoepe and A.S. Abechi, 2016. Semi-quantitative digital analysis of polymerase chain reaction electrophoresis gel: Potential applications in low-income veterinary laboratories. *Vet. World*, 9: 935–939

Baxter, C.J., H. Redestig, N. Schauer, D. Repsilber, K.R. Patil, J. Nielsen, J. Selbig, J. Liu, A.R. Fernie and L.J. Sweetlove, 2007. The metabolic response of heterotrophic Arabidopsis cells to oxidative stress. *Plant Physiol*., 143: 312–325

Begley, T.P., C. Kinsland and E. Strauss, 2001. The biosynthesis of Co-enzyme A in bacteria. *Vitam. Horm*., 61: 157–171

Bettendorff, L. and P. Wins, 2009. Thiamin diphosphate in biological chemistry: New aspects of thiamin metabolism, especially triphosphate derivatives acting other than as cofactors. *FEBS J.*, 276: 2917–2925

Bokszczanin, K.L., N. Krezdorn, S. Fragkostefanakis, S. Müller, L. Rycak, Y. Chen, K. Hoffmeier, J. Kreutz, M.J. Paupière, P. Chaturvedi, R. Iannacone, F. Müller, H. Bostan, M.L. Chiusano, K.D. Scharf, B. Rotter, E. Schleiff and P. Winter, 2015. Identification of novel small ncRNAs in pollen of tomato. *BMC Genomics*, 16: 714

Bolger, A.M., M. Lohse and B. Usadel, 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, 30: 2114–2120

Bouwmeester, K. and F. Govers, 2009. Arabidopsis L-type lectin receptor kinases: Phylogeny, classification and expression profiles. *J. Exp. Bot*., 60: 4383–4396

Brychkova, G., Z. Alikulov, R. Fluhr and M. Sagi, 2008. A critical role for ureides in dark and senescence-induced purine remobilization is unmasked in the *Atxdh1 Arabidopsis* mutant. *Plant J*., 54: 496–509

Chun, J., F.S. Li, Y. Ma, S.H. Wang and F. Chen, 2014. Cloning and characterization of a *SnRK2* gene from *Jatropha curcas* L. *Genet. Mol. Res.*, 13: 10958–10975

Crouzet, J., J. Roland, E. Peeters, T. Trombik, E. Ducos, J. Nader and M. Boutry, 2013. NtPDR1, a plasma membrane ABC transporter from *Nicotiana tabacum*, is involved in diterpene transport. *Trends Biochem. Sci*., 82: 181–192

Deng, K., Q. Wang, J. Zeng, X. Guo, X. Zhao, D. Tang and X. Liu, 2009. A lectin receptor kinase positively regulates ABA response during seed germination and is involved in salt and osmotic stress response. *J. Plant Biol*., 52: 493–500

Diego, N.D., M.C. Sampedro, R.J. Barrio, I. Saiz-Fernandez, P. Moncalean and M. Lacuesta, 2013. Solute accumulation and elastic modulus changes in six radiata pine breeds exposed to drought. *Tree Physiol*., 33: 69–80

Dievart, A., C. Perin, J. Hirsch, M. Bettembourg, N. Lanau and F. Artus, 2016. The phenome analysis of mutant alleles in leucine-rich repeat receptor-like kinase genes in rice reveals new potential targets for stress tolerant cereals. *Plant Sci*., 242: 240–249

F.A.O., Food and Agricultural Organization, 2016. STAT online database. Available at: http://www.fao.org/about/meetings/agroecology-symposium-china/en/ (Accessed: 15 March 2017)

Fei, G., Z. YiJun, H. LingYun, H.E. DaCheng and Z. GenFa, 2008. Proteomic analysis of long- term salinity stress responsive proteins in *Thellungiella halophila* leaves. *Chin. Sci. Bull*., 53: 3530–3537

Ferreira, L.J., V. Azevedo, J. Maroco, M.M. Oliveira and A.P. Santos, 2015. Salt tolerant and sensitive rice varieties display differential methylome flexibility under salt stress. *PLoS One*, 10: 1–19

Garcia-Hernandez, M., T.Z. Berardini, G. Chen, D. Crist, A. Doyle, E. Huala, E. Knee, M. Lambrecht, N. Miller and L.A. Mueller, 2002. TAIR: A resource for integrated *Arabidopsis* data. *Funct. Integr. Genomics*, 2: 239–253

Hakim, M.A., A.S. Juraimi, M.M. Hanafi, E. Ali, M.R. Ismail, A. Selamat and S.M. Karim, 2014. Effect of salt stress on morpho-physiology, vegetative growth and yield of rice. *J. Environ. Biol.*, 35: 317–326

Hakim, M.A., A.S. Juraimi, M. Begum, M.M. Hanafi, M.R. Ismail and A. Selamat, 2010. Effect of salt stress on germination and early seedling growth of rice (*Oryza sativa* L.). *Afr. J. Biotechnol*., 9: 1911–1918

Hartmann, M.D., C.T. Mendler, J. Bassler, I. Karamichali, O. Ridderbusch, A.N. Lupas and B.H. Alvarez, 2016. α/β coiled coils. *ELife*, 5: 1–23

He, X.J., Z.G. Zhang, D.Q. Yan, J.S. Zhang and S.Y. Chen, 2004. A salt-responsive receptor-like kinase gene regulated by the ethylene signaling pathway encodes a plasma membrane serine/threonine kinase. *Theor. Appl. Genet*., 109: 377–383

Ho, C.L., Y.C. Tan, K.A. Yeoh, A.K. Ghazali, W.Y. Yee and C.C. Hoh, 2016. *De novo* transcriptome analyses of host- fungal interactions in oil palm (*Elaeis guineensis* Jacq.). *BMC Genomics*, 17: 66

Hove, C.A.T., Z. Bochdanovits, V.M.A. Jansweijer, F.G. Koning, L. Berke, G.F. Sanchez-Perez, B. Scheres and R. Heidstra, 2011. Probing the roles of *LRR-RLK* genes in *Arabidopsis thaliana* roots using a custom T-DNA insertion set. *Plant Mol. Biol*., 76: 69-83

Illumina, 2011. Technical note: Sequencing. Quality scores for next-generation sequencing: assessing sequencing accuracy using Phred quality scoring. Available at: http://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf. (Accessed: 23 June 2018)

I.R.R.I., 2002. *Standard evaluating score for rice (SES).* Los Banos. Philippines: International Rice Research Institute (IRRI), Vol. 4, pp: 15–16

Jianyan, H., Z. Xiaobo, Y. Huihui, O. Yidan, W. Lei and Z. Qifa, 2009. The ankyrin repeat gene family in rice: Genome-wide identification, classification and express ion profiling. *Plant Mol. Biol*., 71: 207–226

Joseph, E.A. and K.V. Mohanan, 2013. A study on the effect of salinity stress on the growth and yield of some native rice cultivars of Kerala state of India. *Agric. For. Fish*, 2: 141–150

Joshi, A., D.Q. Hung, N. Vaid and N. Tuteja, 2010. Pea lectin receptor-like kinase promotes high salinity stress tolerance in bacteria and expresses in response to stress *in planta*. *Glycoconj. J.*, 27: 133–150

Karan, R., T. DeLeon, H. Biradar and P.K. Subudhi, 2012. Salt stress induced variation in DNA methylation pattern and its influence on gene express ion in contrasting rice genotypes. *PLoS One*, 7: 1–10

Khan, Y.S., H. Tyagi, A.K. Sigh and S. Magadum, 2016. Abiotic stress tolerance in soybean: Regulated by ncRNA. *J. Agric. Search*, 3: 1–6

Kohorn, B.D., 2000. Plasma membrane-cell wall contacts. *Plant Physiol.* 124: 31–38

Kosová, K., I.T. Prášil and P. Vítámvás, 2013. Protein contribution to plant salinity response and tolerance acquisition. *Intl. J. Mol. Sci*., 14: 6757–6789

Ku¨rsteiner, O., I. Dupuis and C. Kuhlemeier, 2003. The pyruvate decaroboxylase1 gene of *Arabidopsis* is required during anoxia but not other environmental stresses. *Plant Physiol*., 132: 968–978

Kulik, A., I. Wawer, E. Krzywińska, M. Bucholc and G. Dobrowolska, 2011. SnRK2 protein kinases—Key regulators of plant response to abiotic stresses. *OMICS J. Integr. Biol*., 15: 859–872

Lally, D., P. Ingmire, H.Y. Tong and Z.H. He, 2001. Antisense expression of a cell wall-associated protein kinase, WAK4, inhibits cell elongation and alters morphology. *Plant Cell*, 13: 1317–1331

Li, S., C. Fan, Y. Li, J. Zhang, J. Sun, Y. Chen, C. Tian, X. Su, M. Lu, C. Liang and Z. Hu, 2016. Effects of drought and salt-stresses on gene expression in *Caragana korshinskii* seedlings revealed by RNA-seq. *BMC Genomics*, 17: 200

Liu, J., H. Wang and N.H. Chua, 2015a. Long noncoding RNA transcriptome of plants. *Plant Biotechnol. J*., 13: 319–328

Liu, X., L. Hao, D. Li, L. Zhu and S. Hu, 2015b. Long non-coding RNAs and their biological roles in plants. *Genom. Proteom. Bioinform.*, 3: 137–147

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, 25: 402–408

Lorenzo, L.D., F. Merchan, P. Laporte, R. Thompson, J. Clarke, C. Sousa and M. Crespi, 2009. A novel plant leucine-rich repeat receptor kinase regulates the response of *Medicago truncatula* roots to alt stress. *Plant Cell*, 21: 668–680

Maksup, S., K. Supaibulwatana and G. Selvaraj, 2013. High-quality reference genes for quantifying the transcriptional responses of *Oryza sativa* L. (sspp. *indica and japonica*) to abiotic stress conditions. *Chin. Sci. Bull*., 58: 1919–1930

Matsui, A., A.H. Nguyen, K. Nakaminami and M. Seki, 2013. Arabidopsis non-coding RNA regulation in abiotic stress responses. *Intl. J. Mol. Sci*., 14: 22642–22654

Matsui, A., J. Ishida, T. Morosawa, M. Okamoto, J.M. Kim, Y. Kurihara, M. Kawashima, M. Tanaka, T.K. To and K. Nakaminami, 2010. Arabidopsis tiling array analysis to identify the stress-responsive genes. *Meth. Mol. Biol*., 639: 141–155

Mirouze, M. and J. Paszkowski, 2011. Epigenetic contribution to stress adaptation in plants. *Curr. Opin. Plant Biol*., 14: 267–274

Mohanty, S., R. Wassmann, A. Nelson, P. Moya and S.V.K. Jagadish, 2013. Rice and climate change: Significance for food security and vulnerability. *Intl. Rice Res. Inst.*, 14: 1–18

Muthayya, S., J.D. Sugimoto, S. Montgomery and G.F. Maberly, 2014. An overview of global rice production, supply, trade and consumption. *New York Acad. Sci*., 1324: 7–14

Nelson, N. and C. Yocum, 2006. Structure and function of photosystems I and II. *Annu. Rev. Plant Biol*., 57: 521–565

Nishiguchi, M., K. Yoshida, T. Sumizono and K. Tazaki, 2002. A receptor-like protein kinase with a lectin-like domain from Lombardy poplar: Gene expression in response to wounding and characterization of phosphorylation activity. *Mol. Genet. Genomics*, 267: 506–514

Nouri, M.Z., A. Moumenu and S. Komatsu, 2015. Abiotic Stresses: Insight into gene regulation and protein express ion in photosynthetic pathways of plants. *Intl. J. Mol. Sci*., 16: 20392–20416

Oliveira, A.B.D., N.L.M. Alencar and E. Gomes-Filho, 2013. Comparison between the water and salt stress effects on plant growth and development. *In: Responses of organisms to water stress*. Sener, A. (Ed.). InTech doi: 10.5772/5422

Park, Y.W., K. Baba, Y. Furuta, I. Iida, K. Sameshima, M. Arai and T. Hayashi, 2004. Enhancement of growth and cellulose accumulation by overexpression of xyloglucanase in poplar. *FEBS Lett.*, 564: 183–187

Park, Y.W., R. Tominaga, J. Sugiyama, Y. Furuta, E. Tanimoto, M. Samejima, F. Sakai and T. Hayashi, 2003. Enhancement of growth by expression of poplar cellulase in *Arabidopsis thaliana*. *Plant J*., 33: 1099–1106

Patishtan, J., T.N. Hartley, R.F.D. Carvalho and F.J. Maathuis, 2017. Genome wide association studies to identify rice salt-tolerance markers. *Plant Cell Environ*., 41: 970–982

Peterson, T.A., C.J. Lovatt and R.C. Nieman, 1988. Salt stress causes acceleration of purine catabolism and inhibition of pyrimidine salvage in *Zea mays* root tips. *J. Exp. Bot.*, 39: 1389–1395

Rahnama, A., R.A. James, K. Poustini and R. Munns, 2010. Stomatal conductance as a screen for osmotic stress tolerance in durum wheat growing in saline soil. *Funct. Plant Biol*., 37: 255–263

Rajkumar, A.P., P. Qvist, R. Lazarus, F. Lescai, J. Ju, M. Nyegaard, O. Mors, A.D. Børglum, Q. Li and J.H. Christensen, 2015. Experimental validation of methods for differential gene expression analysis and sample pooling in RNA-seq. *BMC Genomics*, 16: 548

Rapala-Kozik, M., N. Wolak, M. Kudja and A.K. Banas, 2012. The upregulation of thiamine (vitamin B1) biosynthesis in *Arabidopsis thaliana* seedlings under salt and osmotic stress conditions is mediated by abscisic acid at the early stages of this stress response. *BMC Plant Biol.*, 12: 2

Rapala-Kozik, M., E. Kowalska and K. Ostrowska, 2008. Modulation of thiamine metabolism in *Zea mays* seedlings under conditions of abiotic stress. *J. Exp. Bot*., 59: 4133–4143

Rayon, C., H.L. Gall, F. Philippe, J. Domon, F. Gillet and J. Pelloux, 2015. Cell wall metabolism in response to abiotic stress. *Plants*, 4: 112–166

Reinders, J., B.B.H. Wulff, M. Mirouze, A. Marı-Ordonez and M. Dapp, 2009. Compromised stability of DNA methylation and transposon immobilization in mosaic Arabidopsis epigenomes. *Genes Dev*., 23: 939–950

Riou, C., C. Hervé, V. Pacquit, P. Dabos and B. Lescure, 2002. Express ion of an *Arabidopsis* lectin kinase receptor gene, *lecRK-a1*, is induced during senescence, wounding and in response to oligogalacturonic acids. *Plant Physiol. Biochem*., 40: 431–438

Rubio, S., L. Whitehead, T.R. Larson, I.A. Graham and P.L. Rodriguez, 2008. The coenzyme A biosynthetic enzyme phosphopantetheine adenylyltransferase plays a crucial role in plant growth, salt/osmotic stress resistance and seed lipid storage. *Plant Physiol*., 148: 546–556

Sakai, H., S.S. Lee, T. Tanaka, H. Numa, J. Kim, Y. Kawahara, H. Wakimoto, C.C. Yang, M. Iwamoto, T. Abe, Y. Yamada, A. Mutu, H. Inokuchi, T. Ikemure, T. Matsumoto, T. Sasaki and T. Itoh, 2013. Rice Annotation Project Database (RAP-DB): An integrative and interactive database for rice genomics. *Plant Cell Physiol*., 54: e6

Sakamoto, H., J. Tochimoto, S. Kurosawa, M. Suzuki and S. Oguri, 2014. A novel gene encoding ankyrin-repeat protein, *SHG1*, is indispensable for seed germination under moderate salt stress. *Intl. J. Biol. Biomol. Agric. Food Biotechnol. Eng*., 8: 234–237

Schneider, C.A., W.S. Rasband and K.W. Eliceiri, 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Meth.*, 9: 671–675

Schurch, N.J., P. Schofield, M. Gierliński, C. Cole, A. Sherstnev and V. Singh, 2016. How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use? *RNA*, 22: 839–851

Sengupta, S. and A.L. Majumder, 2009. Insight into the salt tolerance factors of wild halophytic rice, *Porteresia coarctata*: A physiological and proteomic approach. *Planta*, 229: 911–929

Seong, E.S., D. Choi, H.S. Cho, C.K. Lim, H.J. Cho and M.H. Wang, 2007a. Characterization of a stress-responsive ankyrin repeat-containing zinc finger protein of *Capsicum annuum* (CaKR1). *J. Biochem. Mol. Biol*., 40: 952–958

Seong, E.S., H.S. Cho, D. Choi, Y.H. Joung, C.K. Lim, J.H. Hur and M.H. Wang, 2007b. Tomato plants overexpressing *CaKR1* enhanced tolerance to salt and oxidative stress. *Biochem. Biophys. Res. Commun*., 363: 983–988

Shankar, R., A. Bhattacharjee and M. Jain, 2016. Transcriptome analysis in different rice cultivars provides novel insights into desiccation and salinity stress responses. *Sci. Rep*., 6: 1–15

Shavrukov, Y., 2012. Salt stress or salt shock: Which genes are we studying? *J. Exp. Bot.*, 64: 119–127

Sivaguru, M., B. Ezaki, Z.H. He, H. Tong, H. Osawa, F. Baluska, D. Volkmann and H. Matsumoto, 2003. Aluminum induced gene expression and protein localization of a cell wall-associated receptor protein kinase in *Arabidopsis thaliana*. *Plant Physiol.*, 132: 2256–2266

Song, X., X. Yu, C. Hori, T. Demura, M. Ohtani and Q. Zhuge, 2016. Heterologous overexpression of poplar *SnRK2* genes enhanced salt stress tolerance in *Arabidopsis thaliana*. *Front. Plant Sci*., 7: 612–623

Sun, X.L., Q.Y. Yu, L.L. Tang, W. Ji, X. Bai, H. Cai, X.F. Liu, X.D. Ding and Y.M. Zhu, 2012. Gs SRK, a G-type lectin S-receptor-like serine/threonine protein kinase, is a positive regulator of plant tolerance to salt stress. *J. Plant Biol*., 170: 505–515

Takagi, H., Y. Ishiga, S. Watanabe, T. Konishi, M. Egusa, N. Akiyoshi, T. Matsuura, I.C. Mori, T. Hirayama, H. Kaminaka, H. Shimada and A. Sakamoto, 2016. Allantoin, a stress -related purine metabolite, can activate jasmonate signaling in a MYC2-regulated and abscisic acid-dependent manner. *J. Exp. Bot*., 67: 2519–2532

Tanaka, H., Y. Osakabe, S. Katsura, S. Mizuno, K. Maruyama, K. Kusakabe, J. Mizoi, K. Shinozaki and K. Yamaguchi-Shinozaki, 2012. Abiotic stress-inducible receptor-like kinases negatively control ABA signaling in *Arabidopsis*. *Plant J*., 70: 599–613

Umezawa, T., N. Sugiyama, M. Mizoguchi, S. Hayashi, F. Myouga and K. Yamaguchi-Shinozaki, 2009. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, 106: 17588–17593

Verica, J.A., L. Chae, H. Tong, P. Ingmire and Z.H. He, 2003. Tissue-specific and developmentally regulated expression of a cluster of tandemly arrayed cell wall-associated kinase-like kinase genes in *Arabidopsis*. *Plant Physiol.*, 133: 1732–1746

Vlad, F., S. Rubio, A. Rodrigues, C. Sirichandra, C. Belin and N. Robert, 2009. Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in *Arabidopsis*. *Plant Cell*, 21: 3170–3184

Wagner, T. and B. Kohorn, 2001. Wall-associated kinases are expressed throughout plant development and are required for cell expansion. *Plant Cell*, 13: 303–318

Wang, H., H. Wang, H. Shao and X. Tang, 2016. Recent advances in utilizing transcription factors to improve plant abiotic stress tolerance by transgenic technology. *Front. Plant Sci.*, 7: 67–80

Wang, J., B. Li, Y. Meng, X. Ma, Y. Lai, E. Si, K. Yang, P. Ren, X. Shang and H. Wang, 2015. Transcriptomic profiling of the salt-stress response in the halophyte *Halogeton glomeratus*. *BMC Genomics*, 16: 169

Wang, X., Z. Liu and Y. He, 2008. Responses and tolerance to salt stress in bryophytes. *Plant Signal. Behav.*, 3: 516–518

Wassmann, F., N.X. Hien, C.T. Hoanh and T.P. Tuong, 2004. Sea level rise affecting the Vietnamese Mekong Delta: Water elevation in the flood season and implications for rice production. *Clim. Change*, 66: 89–107

Watanabe, S., M. Matsumoto, Y. Hakomori, H. Takagi, H. Shimada and A. Sakamoto, 2014. The purine metabolite allantoin enhances abiotic stress tolerance through synergistic activation of abscisic acid metabolism. *Plant Cell Environ*., 37: 1022–1036

Werner, A.K. and C.P. Witte, 2011. The biochemistry of nitrogen mobilization: Purine ring catabolism. *Trends Plant Sci*., 16: 381–387

Yang-Rong, C., C. Hao-Wei, L. Zhi-Gang, T. Jian-Jun, M. Biao, W.K. Zhang, S.Y. Chen and J.S. Zhang, 2015. Tobacco ankyrin protein NEIP2 interacts with ethylene receptor NTHK1 and regulates plant growth and stress responses. *Plant Cell Physiol*., 56: 803–818

Yesbergenova, Z., G. Yang, E. Oron, D. Soffer, R. Fluhr and M. Sagi, 2005. The plant Mo-hydroxylases aldehyde oxidase and xanthine dehydrogenase have distinct reactive oxygen species signatures and are induced by drought and abscisic acid. *Plant J*., 42: 862–876

Yoshida, S., D.A. Forno and J.H. Cook, 1976. *Laboratory manual for physiological studies of rice*, pp: 61–65. International Rice Research Institute, Manila, Philippines

Yuen, B. and L. Kong, 2009. Climate change and urban planning in Southeast Asia. *Surv. Perspect. Integr. Environ. Soc.*, 2: 1–11

Zhang, H., X. Mao, R. Jing, X. Chang and H. Xie, 2011. Characterization of common wheat (*Triticum aestivum* L.) *TaSnRK2.7* gene involved in abiotic stress responses. *J. Exp. Bot*., 62: 975–988

Zhao, J., Q. He, G. Chen, L. Wang and B. Jin, 2016a. Regulation of non-coding RNAs in heat stress responses of plants. *Front. Plant Sci*., 7: 1213

Zhao, Q., J. Suo, S. Chen, Y. Jin, X. Ma, Z. Yin, Y. Zhang, T. Wang, J. Luo, W. Jin, X. Zhang, Z. Zhou and S. Dai, 2016b. Na_2CO_3-responsive mechanisms in halophyte *Puccinellia tenuiflora* roots revealed by physiological and proteomic analyses. *Sci. Rep*., 6: 32717

[Received 09 Jan 2019; Accepted 29 Jul 2019; Published (online) 22 Dec 2019]