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TRANSCRIPTOMIC ANALYSIS OF MALAYSIAN RICE SEEDLINGS (Oryza sativa L. ssp. indica) IN EARLY RESPONSES TO SALT-SHOCK

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By

NOR MUSTAIQAZAH BINTI MOHAMAD JURI

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Chairman : Noor Azmi Bin Shaharuddin, PhD
Faculty : Biotechnology and Biomolecular Sciences

Salinization of rice cultivation land is progressively enlarged thus negatively impair 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 was seen as a promiseable technology for a holistic understanding of salinity tolerance mechanisms in rice. Here, by using Illumina HiSeq 2000 sequencing platform, transcriptomes of salt-tolerant Malaysian rice variety MR211 and salt-sensitive MR220 were analyzed after nine hours of severe salinity stress (12 dS/m) treatment, labeled as S211 and S220, respectively. After trimming, a total of 76,456,236 (S211) and 57,323,996 (S220) high-quality reads were obtained. The assembly of these reads resulting a total of 20,853 (S211) and 19,315 (S220) genes. Through comparative expression between both samples, 252 significant genes were differentially expressed and were dominated by variety-induced genes (n=235; 93.3%) with majority of them (n=221; 88%) were categorized as uniquely expressed in salt-tolerant MR211. Further pathway based analysis on the DEGs that were categorized as "uniquely" and "higher expressed" in S211 when compared to S220 had assigned them to 33 KEGG pathways with the highest number of DEGs were accounted in purine metabolism and thiamine metabolism pathways. The functional annotation of these group of DEGs also 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 thus suggest how this salt tolerant genotype (MR211) gains its salt adaptation trait. The expression accuracy and reproducibility of the 252 DEGs identified from the RNA-seq experiment were further verified through RT-PCR followed by qRT-PCR analysis. Nine genes were selected as the representative with 4 of them namely *FER2*, *Thaumatin*, *VI* and *UBC* were in line with data generated from the RNA-seq analysis. The other 2 (*MT* and *HOX16)* showed a contradict trend of expression as compared to the RNA-seq data, whereas the other three candidate genes (*PSII*, *SAPK6* and *PAO*) had exhibited a similar (no difference)

level of expressions between S211 and S220. Next, the incorporation of control (untreated) cDNA samples (C211 and C220) in the expression analyses had revealed the expression of the genes in untreated plants as compared to after being subjected to salt stress. The expression analyses had highlighted *UBC* and *SPK6* genes as the most responsive towards salinity stress in MR211 and MR220, respectively thus might represent their uniqueness in response to salinity stress.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

ANALISIS TERHADAP RESPON AWAL TRANSKRIPTOM VARIATI PADI MALAYSIA (*Oryza sativa* L. ssp. *indica*) TERHADAP KEJUTAN KEMASINAN

Oleh

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Proses pemasinan tanah berlaku dengan progresif di kawasan-kawasan penanaman padi dan telah menjejaskan bekalan beras dunia. Walaubagaimanapun, penghasilan varieti padi yang mempunyai sifat ketahanan yang tinggi terhadap kemasinan telah menjadi satu cabaran yang besar disebabkan oleh mekanisme ketahanan tumbuhan yang bersifat poligenik dan kompleks. Oleh itu, pemprofilan transkriptom dilihat sebagai satu teknologi baru yang menjanjikan pemahaman terhadap mekanisme pertahanan padi secara holistik. Dalam kajian ini, transkriptom dua varieti padi Malaysia, MR211 yang mempunyai ketahanan yang tinggi terhadap kemasinan dan MR220 yang sensitif terhadap kemasinan telah dianalisa selepas diberikan rawatan kemasinan yang tinggi (12 dS/m) selama 9 jam dan masing-masing dilabel sebagai S211 dan S220. Sejumlah 76,456,236 (S211) dan 57,323,996 (S220) bacaan berkualiti tinggi telah diperoleh, dan perhimpunan bacaan ini menghasilkan sejumlah 20,853 (S211) dan 19,315 (S220) gen. Melalui perbandingan pengekspresan yang dibuat di antara S211 dan S220, sebanyak 252 gen yang signifikan telah diekspres secara berbeza (DEGs). DEGs ini secara keseluruhannya telah didominasi oleh gen-gen yang dikspres secara spesifik pada satu variati sahaja (n=235, 93.3%) dengan majoriti daripadanya diekspres secara unik hanya pada MR211. Analisis lanjutan yang dibuat terhadap DEGs yang diekspres secara "unik" dan "lebih tinggi" pada S211 apabila dibandingkan dengan S220 telah menunjukkan bahawa kumpulan gen ini terlibat di dalam 33 laluan KEGG, dengan bilangan gen yang tertinggi terlibat di laluan metabolisme purin dan tiamin. Anotasi fungsian kumpulan DEGs ini juga menunjukkan kehadiran gen-gen pengawalaturan seperti faktor transkripsi (TFs), protein kinase dan protein fosfatase, serta gen berfungsi yang terlibat dalam pelbagai mekanisme penyesuaian terhadap tekanan seperti sokongan mekanikal, sistem penghapus ROS, pengeksklusian ion dan pemetakan intrasel, yang dengan itu memberi petunjuk bagaimana pokok MR211 memperoleh ciri kerintangan terhadap tekanan kemasinan. Ketepatan dan kebolehulangan pengekspresan 252 DEGs yang diperoleh dari analisis RNA-seq disahkan melalui analisis RT-PCR dan qRT-PCR. Sembilan gen wakilan dipilih dengan 4 daripadanya iaitu *FER2, Thaumatin, VI* dan *UBC* menunjukkan pengekspresan yang sejajar dengan

data yang dihasilkan dari analisis RNA-seq. *MT* dan *HOX16* walaubagaimanapun memperlihatkan trend pengekspresan yang bercanggah berbanding dengan data RNA-seq, manakala tiga lagi gen (*PSII, SAPK6* dan *PAO*) memperlihatkan tahap ekspresi yang sama (tidak ada perbezaan) antara S211 dan S220. Seterusnya, penggabungan sampel cDNA yang tidak dikenakan rawatan kemasinan (kawalan) (C211 dan C220) dalam analisis pengekspresan melalui RT-PCR dan qRT-PCR telah menonjolkan *UBC* dan *SPK6* masing-masing sebagai gen yang paling responsif terhadap tekanan kemasinan oleh MR211 dan MR220, sekali gus mewakili keunikan tindak balas kedua-dua varieti padi Malaysia ini terhadap tekanan kemasinan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of Supervisory Committee were as follows:

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and S211. *HNR* and *EP* were used as internal control, whereas C211 was used as the control sample. White bar represent the expression of target gene in C211 cDNA template whereas the red bar represent the expression of target gene in S211 cDNA

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LIST OF ABBREVIATIONS

NGS	Next Generation Sequencing
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
qRT-PCR	Real-time Reverse Transcription Polymerase Chain Reaction
ε	Extinction Coefficient
EDTA	Ethylenediamine Tetraacetic Acid
TAE	Tris-Acetat-EDTA
Tris	Tris(Hydroxymethyl) Aminomethane
× g	Gravitational Constant
kDa	KiloDalton
NCBI	National Centre for Biotechnology Information
nt	Nucleotide
QC	Quality Control

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Rice (*Oryza sativa* L.) is a critical crop for global food security. It is ranked as one of the most important crops of the world, primarily in Asian and certain part of Latin America and African countries (Muthayya *et al.,* 2014). It feeds almost half of world population which represented by more than 3.5 billion of peoples, as well as an important source of employment and incomes for the rural farmers. Over the years, a massive growth of world population has led to the increment of global rice demand. It has been estimated that an additional of 114 million tons of rice is required in order to meet global rice demand in 2035 (FAO, 2016; Mohanty *et al.*, 2013).

Unfortunately, rice is prone to a wide range of environmental constraint. A stagnation of rice yield in many Asian countries has been recorded due to various abiotic stress factors (Redfern *et al.,* 2012). Among them, salt stress has been represented as a significant threat for rice growth and development (Munns 2011; Kumar and Khare, 2016), accounted in 30% to 50% (Islam *et al.,* 2007; Joseph and Mohanan, 2013) or even higher losses of rice yield (Michael *et al.,* 2004; Zeng and Shannon, 2000).

Soil is categorized as saline once its Electrical Conductivity (EC) reading reached 4 dS/m or higher, with pH less than 8 and Exchangeable Sodium Percentage (ESP) less than 15 (Allotey *et al.*, 2008). A continuous salinization process is naturally occurring through release of soluble salts from parental rocks and deposition of oceanic salts carried in wind and rain in coastal areas. In agricultural land, application of chemical fertilizer and irrigation of brackish water itself are the common source of salinization (FAO, 2005; Petronia 2011; Munns and Tester, 2008). More than that, the effect of climate change such as increasing of annual temperature, decreasing of rainfall and rise in global sea level have led to a tremendous effect of soil salinization (Brinkman 1980; Hakim *et al.,* 2013).

In a scientific assessment conducted by Yuen & Kong in 2009, the coastlines of Southeast Asia that made up of the regions which serve as 'world rice bowl' are highly vulnerable to the effects of climate change. In Malaysia, Indonesia and Vietnam for example, thousands square kilometer (km^2) loss of land were expected due to the 30-50 cm increment of sea level (Wassmann *et al.,* 2004; UNEP, 2006; IPCC, 2001). For granary lands which especially located on delta and coastal areas (Nguyen *et al.,* 2014; Brinkman 1980), rising of sea level will lead into sea water intrusion and submersion of the fertile fields under sea thus makes it unsuitable for rice planting. Depending on its concentration and duration, salinity stress invokes various changes in physiological and

metabolic events in plants, in which, ultimately inhibit their production and survival (Rahnama *et al.,* 2010; Rozema & Flowers, 2008).

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 in soil around plant root are triggered into the decreasing of cellular osmotic potential and ultimately lead to cell dehydration. Meanwhile ionic effect occurs when the salt ion penetrate and accumulate in cell cytoplasm via plasma membrane (James *et al.,* 2011; Rahnama *et al.,* 2010; Kosová *et al.,* 2013). 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 and reduction of cell division and expansion (Munns, 2002; Rahnama *et al.,* 2010; Zhu, 2007).

As in other plant species, responses of rice plant to salt stress vary with varieties and growth stages. It has been stated that rice is very sensitive to salinity throughout young seedling growth stage (Heenan *et al.,* 1988; Lutts *et al.,* 1996). Based on standard evaluating score (SES) in rating the visual symptoms of salt toxicity (IRRI, 2002; Gregorio *et al.,* 1997), varieties of rice can be differentiated as highly tolerant, tolerant, moderately tolerant and susceptible. As being applied by Hakim *et al.,* (2010), the screening of eight Malaysian rice varieties using the SES had identified MR211 as the most tolerant while MR220 as the most susceptible variety among others varieties.

Salinity tolerance is a quantitative trait controlled by many genes that involve in different pathways. Therefore, a full understanding on the molecular responses of rice plants to varying conditions and identification of genes that 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 program through 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 as it is time consuming (Miah *et al.,* 2012).

Plant biotechnology through genetic engineering and molecular breeding approaches offers much rapid development in a crop improvement program 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 "omicss" method 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 (Kosová *et al.,* 2013).

Here in present work, 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 that were differentially expressed between both varieties where further analyses had identified candidate genes related to salt tolerant.

The data generated from this study will serve as an invaluable genomic reference to further our knowledge on 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 in conventional breeding.

1.2 Research objectives

The main aim of this project is to study salt-shock adaptation pathways exhibited by Malaysian rice varieties, MR211 and MR220 in response to severe salinity stress treatment. Therefore the specific objectives of this work were:

1. To identify the early responses salt-shock differentially expressed genes (DEGs) of MR211 and MR220 seedlings via comparative RNA-seq transcriptomic analysis.

2. To validate the expression profile of salt-shock DEGs in MR211 and MR220 identified from the RNA-seq analysis through semi-quantitative reverse transcription-PCR and quantitative real-time PCR analyses.

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