

ANALYSIS OF EMBRYOGENIC CALLUS INDUCTION AND  
REGENERATION OF INDICA RICE VARIETY OF MALAYSIA

SURAIYA BINTE MOSTAFIZ

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## **DEDICATION**

I humbly dedicate this thesis to:  
My beloved family for their endless support and motivation

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## ABSTRACT

Rice is the main food-crop for more than half of the global population and its demand is increasing due to population growth. Different abiotic and biotic stresses are among the major reasons that lower the yield. Development of new rice varieties through *in vitro* somatic embryogenesis not only contributes to enhance the yield but also improves the quality of rice. However, exact timing of maintenance of embryogenic competent callus was yet to be established for *indica* rice, which is considered as a main barrier in genetic modification. Somatic embryogenesis receptor kinase (SERK1) gene is extensively used as an embryogenic marker in many plant species, which is expressed specifically in embryogenic callus. To obtain high callus induction, effect of plant growth regulators (i.e. 2,4-D and NAA), carbon sources (i.e. sucrose, maltose and sorbitol), basal media (i.e. MS, N<sub>6</sub> and LS), and pre-heat treatments (i.e. 35°C, 40°C, 45°C and 50°C) with different imbibition periods (3 days, 5 days and 7 days) were investigated for four Malaysian *indica* rice varieties (i.e. MR220, MR220-CL2, MR232 and Bario). SERK1 gene was quantified by real time PCR in differential stages of callus (14 days, 21 days, 28 days, 35 days and 42 days), different PGR (2,4-D, NAA, NAA+ 2,4-D), and pre-heat treatment. Plant regeneration was also optimised by using different concentrations of plant growth regulators. After regeneration, agronomic studies was carried out between control plant and treatment plant for all varieties. In the present study, highest percentage of callus induction was obtained for MR220 (96%), MR220-CL2 (100%) MR232 (100%) and Bario (95.7%) on MS media with 3 mg/L 2, 4-D and 3% maltose after 21 days of culture of pre-heat treated seed at 45°C for 3 days. The characteristics of embryogenic callus were found to be embryogenic from SEM and histology. Amplification of SERK1 cDNA was referred as detection of the gene of aged 21-days was successfully amplified in all four varieties. The phylogenetic tree analysis showed that SERK1 gene of all varieties were similar to the SERK1 of *Oryza sativa Japonica*. The Real Time PCR analysis revealed that SERK1 transcript was significantly higher at 21 days old callus on MS media with 2,4-D at 45°C pre-heat treated callus for all four varieties. Further, regeneration was tested for 21 days old callus, where the regeneration frequency were found to be 72%, 89%, 71% and 50% in MR220, MR220-CL2, MR232 and Bario respectively in optimised regeneration media (2mg/L BAP+ 2mg/L Kinetin+0.5mg/L NAA). Regenerated plants grew easily in the glasshouse with 90 –95% survival rate. Agronomic studies did not show any morphological variation but grain weight of *in vitro* raised plant was significantly higher than control plant in all tested varieties. These findings establish a suitable protocol for *in vitro* regeneration system to be used in genetic modification studies in *indica* rice in future.

## ABSTRAK

Padi merupakan tanaman makanan utama kepada lebih daripada separuh populasi global dan permintaannya semakin meningkat disebabkan oleh peningkatan populasi. Persekitaran negatif abiotik dan biotik yang berbeza merupakan antara sebab utama yang mengurangkan hasil pokok padi. Penghasilan padi varieti baru melalui embriogenesis somatik *in vitro* bukan sahaja menyumbang kepada mempertingkatkan hasil tetapi juga memperbaiki kualiti beras. Walau bagaimanapun, masa yang tepat bagi pengendalian potensi kalus embriogeni untuk padi *indica* belum lagi ditentukan, yang dianggap sebagai halangan utama dalam pengubahsuaian genetik. Gen Kinase penerima embriogenesis somatik (SERK1) digunakan secara meluas dalam kebanyakan spesies pokok sebagai penanda embriogeni sel. Bagi mendapatkan induksi kalus embriogen yang tinggi, kesan pengawalatur pertumbuhan pokok (2,4-D dan NAA), sumber karbon (sukrosa, maltosa dan sorbitol), media basal (MS, N<sub>6</sub> and LS), dan prapemanasan (35°C, 40°C, 45°C dan 50°C) dengan tempoh berbeza (3, 5 dan 7 hari) diselidik bagi padi *indica* Malaysia varieti MR220, MR220-CL2, MR232 dan Bario. Menggunakan kaedah PCR masa sebenar, kandungan gen SERK1 dari umur kalus yang berbeza (14 hari, 21 hari, 28 hari, 35 hari dan 42 hari), kesan pra pemanasan, PGR (2,4-D, NAA, NAA+2,4-D) dan tempoh prapemanasan dianalisa. Pertumbuhan semula pokok dioptimumkan menggunakan pengawal atur pertumbuhan pokok dengan kepekatan yang berbeza. Selepas itu, agronomi pokok direkodkan diantara rawatan kawalan dan pokok yang dibesarkan secara *in vitro* untuk kesemua varieti. Peratusan induksi kalus 3 minggu yang tertinggi diperolehi untuk MR220 (96%), MR220-CL2 (100%) MR232 (100%), dan Bario (95.7%) dalam media MS dengan 3 mg/L 2, 4-D dan 3% maltosa selepas eksplan didedahkan kepada suhu 45°C selama 3 hari. Ciri-ciri kalus didapati embriogeni daripada SEM dan histologi. Amplifikasi gen SERK1 telah diperolehi dengan jayanya daripada cDNA kalus yang berusia 21 dari kesemua empat varieti. Analisis pokok filogenetik menunjukkan gen SERK1 bagi kesemua varieti serupa dengan gen SERK1 *Oryza sativa Japonica*. Dari analisis masa sebenar PCR mendedahkan bahawa transkrip SERK1 meningkat dengan ketara bagi kalus berusia 21 hari apabila dikultur atas media MS dengan 2,4-D pada suhu prapemanasan 45°C bagi kesemua empat varieti. Seterusnya, regenerasi kalus berusia 21 hari ini menunjukkan peratus pertumbuhan masing-masing sebanyak 72%, 89%, 71% dan 50% dalam MR220, MR220-CL2, MR232 dan Bario dalam media regenerasi optimum (2mg/L BAP+2mg/L Kinetin+0.5mg/L NAA). Pokok seterusnya tumbuh dengan kadar kemandirian 90–95%. Kajian agronomi tidak menunjukkan sebarang perbezaan morfologi tetapi berat bijian pokok dari sumber *in vitro* ketara lebih tinggi daripada pokok kawalan dalam kesemua varieti yang diuji. Kajian ini membuktikan protokol yang dibangunkan adalah sesuai untuk digunakan bagi kajian pengubahsuaian genetik pada masa akan datang.

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## LIST OF ABBREVIATIONS

ANOVA	-	Analysis of Variance
BAP	-	Benzoapyrene
BBM		Baby Boom
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base Pair
cDNA	-	complementary DNA
CIP	-	Callus Induction Percentage
cm	-	Centimetre
CTAB	-	Cetyltrimethylammonium Bromide
cps	-	Counts Per Second
Ct	-	Threshold Cycle
DEPC	-	Diethyl Pyrocarbonate
dH <sub>2</sub> O	-	Deionized Water
DNA	-	Deoxyribonucleic Acid
DNase 1	-	Deoxyribonuclease 1
dNTP	-	Deoxynucleotide Triphosphates
EC	-	Embryogenic Callus
EDTA	-	Ethylenediaminetetraacetic Acid
EtBr	-	Ethidium Bromide
g	-	Gram
HCl	-	Hydrochloride Acid
hr	-	Hours
i.e.	-	that is
K	-	Potassium
k	-	Kilo
kb	-	Kilobase

KOH	-	Potassium Hydroxide
L	-	Litre
LS		Linsmaier and Skoog
MgCl <sub>2</sub>	-	Magnesium Chloride
MgSO <sub>4</sub>	-	Magnesium Sulphate
MEGA		Molecular Evolutionary Genetics Analysis
min	-	Minute
ml	-	Millilitre
mm	-	Millimetre
mM	-	Millimolar
MMLV-RT	-	Maurine Moloney Leukemia Virus Reverse Transcriptase
mRNA	-	Messenger RNA
MS		Murasighe and Skoog
NaCl	-	Sodium Chloride
NaOH	-	Sodium Hydroxide
NE	-	Non-Embryogenic Callus
NAA		1-Naphthaleneacetic acid
ng	-	Nanogram
nt	-	Nucleotide
O <sub>2</sub>	-	Oxygen
OD	-	Optical density
OS	-	<i>Oryza sativa</i>
PAGE	-	Polyacrylamide Agarose Gel Electrophoresis
PCR	-	Polymerase Chain Reaction
pmol	-	Picomole
qRT-PCR	-	Quantitative Real-Time Polymerase Chain Reaction
RM	-	Regeneration Media
RNA	-	Ribonucleic Acid
RNase	-	Ribonuclease



RT	-	Room Temperature
SD	-	Standard Deviation
SDS	-	Sodium Dodecyl Sulphate
sec	-	Seconds
SE	-	Standard Error
SEM	-	Standard Error of the Mean
SERK	-	Somatic Embryogenesis Receptor Kinase
SPSS		Statistical Package for the Social Sciences
T	-	Thiamine
TAE	-	Tris-Acetate-EDTA

## LIST OF SYMBOLS

%	-	Percentage
$\alpha$	-	Alpha
$\beta$	-	Beta
$\lambda$	-	Lambda
$^{\circ}\text{C}$	-	Degree Celsius
$\mu\text{g}$	-	Microgram
$\mu\text{l}$	-	Microliter
$\mu\text{M}$	-	Micromolar

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# CHAPTER 1

## INTRODUCTION

### 1.1 Background of the Study

Rice (*Oryza sativa* L.) is an important food-crop among 70 species and 11 genera of tribe Oryzaeae. It is the second most widely cultivated crop in the world after wheat (Sandhu and Kumar, 2017) and is a model monocot system for genetic and functional genomics. Rice (*Oryza sativa* L.) belongs to the large and economically important Gramineae family, which includes a variety of species such as *japonica*, *javanica*, and *indica* (Azizi et al, 2015). These rice species are found throughout the tropical and temperate regions while *indica* subspecies is the most widely cultivated in South and Southeast Asian countries (Khush, 2005).

Rice is consumed by more than half (i.e. 70%) of the world population as their major source of energy. Total annual rice production of the world was 758.9 million tonnes on 165 million hectares in 2016 with a fluctuating trend during the last five years (FAO Statistics Division, 2017). Similar to other South Asian countries, rice is the staple food-crop in Malaysia. It is estimated that 97% population of Malaysia take rice as a major source of their carbohydrate. In Malaysia, the production of rice was 3.1 million tonnes on 695 hectares of land in 2017. Domestic consumption of rice in Malaysia is projected to increase from 2.75 million tonnes in 2016/17 to 2.8 million tonnes in 2017/18 according to the increasing population growth (Wahab, 2017).

Malaysian rice is grown both in wetland and upland areas of the country. Although the yield of wetland rice is comparatively higher than upland rice, upland rice is advantageous due to its low-cost of production and low-irrigation requirements (Fageria and Baligar, 2003). The upland rice Bario is also beneficial for its sticky texture, fine elongated grains, mild pleasant aroma and exquisite taste (Wong et al., 2009) and could be promoted as a health food for its low glycaemic index (Nicholas et al., 2014). For increasing yield, Malaysian Agricultural Research and Development Institute (MARDI) has released several hybrid rice varieties including MR220, MR220-CL2, and MR232. These hybrid rice varieties are prime in terms of their quality and comparatively higher yields. Even though, these varieties are said to be high-yielding, but their plants are susceptible to flooding, drought, and low resistance to diseases and pests (Libin et al, 2012; Kevin et al, 2007; Naeg, 2012).

Malaysia sets to achieve food security up to 80% by 2020, which eventually increases the food demand. Several measures have been taken by the government to stabilize the rice supply, however, the food security only reached to 72% as of 2017. Nearly 700 hectares of paddy field were flooded which had affected reaching the self-sufficiency target (80%) (Shabery, 2017). Furthermore, the area of land available for cultivation of crop is decreasing rapidly because of urbanization as well as decreasing the area of fertile land (Kumar, 2017). Previous reports showed that rice production has been adversely affected by abiotic stress and high vulnerability to weather changes (Bzour et al, 2018; Azmi et al., 2012; Zulkarnain et al, 2013; FAO Statistics Division, 2017; Wahab, 2017). The increasing demand for rice earnestly requires increasing the yield to bridge the gap between demand and production.

In order to ensure food security, the country needs to develop new high yielding rice varieties (FAO, 2015) through available options such as molecular breeding and biotechnology (Thuy and Saitoh, 2017). Conventional breeding, genetic engineering and *in vitro* tissue culture methods usually used to develop new rice varieties (Gosal and Kang, 2012). Although conventional breeding methods improve rice variety, their progress rates are very slow (Wang et al, 2011).

*In vitro* method is known to be an efficient method for developing improved rice variety (Kalhori et al, 2017; Kumar et al, 2010). New varieties through genetic engineering plants with resistance to various stresses, both biotic and abiotic, require a detail understanding of the cellular and functional features of the plant's genes (Lin et al, 2017). However, the lack of efficient tissue culture protocols is also one of the main barriers to breeding improvement and biotechnological studies. One of the main objectives of plant genetic modification is cultivar development, which can be accomplished through plant regeneration by using somatic embryogenesis (Azizi et al, 2015). Therefore, the availability of an efficient *in vitro* regeneration protocol is an essential requisite prior to genetic modification program.

The recalcitrance of *indica* rice has been attributed to low callusing and regenerating abilities (Silva, 2010) compared to *japonica* subspecies (Kalhori et al, 2017) in particular to various conditions of *in vitro* tissue culture. Eventually, within *indica* subspecies, significant variation was also found with *in vitro* culture response in different genotypes (Rahman et al, 2010). However, the lack of potential tissue culture method in certain rice species to regenerate the healthy plantlets remains the main hindrance for genetic modification of a wide range of plant species (Uddain, 2015).

The earliest stage of *in vitro* callus induction draws attention due to the determination of embryonic cells, which provides us the information of the mechanism about cell development as well as the regeneration potential that are used in plant biotechnology (Zimmerman, 1993; Wójcikowska and Gaj, 2017). Thus, the information of exact timing of maintenance of embryogenic callus and its effect on embryogenic quality are inadequate of all types of *indica* rice varieties. Therefore, marker base studies could be an alternative solution for evolving a new protocol. Molecular and morphological marker production of embryogenic competent cell in certain developmental ages could support to develop new regeneration protocol of Malaysian *indica* rice for most of the genotypes.

Molecular changes of somatic embryogenesis involve different gene expression patterns which are triggered by a series of signal cascades. Five somatic embryogenesis receptor kinase gene (SERK) types have been identified in

*Arabidopsis*, which are responsible for development, stress tolerance and organ differentiation (Salaj et al, 2008). So, it is important to identify the specific gene which is involved in the molecular regulation of somatic embryogenesis in plant species (Talapatra et al, 2013). Among all the gene involved in the procurement of embryogenic potential, somatic embryogenesis receptor kinase1 (SERK1) gene has been used as a marker of somatic embryogenesis in different plant species (Hu et al, 2005; Talapatra et al, 2013; Podio et al, 2014).

Knowledge of the molecular mechanisms that operate in the signal transduction pathway of cellular response to somatic embryogenesis of Malaysian *indica* rice is still quite unavailable. This project has performed a comprehensive study on SERK1 to identify the certain-stage embryogenic callus induction which is potential for regeneration. Although several plant species including *japonica* and wild rice were studied and SERK1 was identified as an embryogenic marker. However, SERK1 was not yet studied in Malaysian *indica* rice cultivar. This study conducts to identify putative SERK1 homologs from the embryogenic callus and its expression as a potential embryogenic marker for selected Malaysian *indica* rice.

## **1.2 Problem Statement**

To ensure the sufficient rice production and meet up the demand of rice consumption, several new varieties had been developed and introduced to the farmers in Malaysia through the breeding technology. Hence, to fulfill the self-sufficiency in rice production, Malaysia still had to imports rice from neighboring countries such as Thailand, Vietnam, China, Pakistan and India.

Though MR220, MR232, and MR220-CL2 are newly developed rice varieties, they do not fulfill the expected predicted yields due to their vulnerability to environmental stress and adverse soil condition. In addition, Bario is popular upland rice variety in Sarawak. However, its production is very low due to soil salinity, narrow range of genetic variability and lack of sufficient information to improve the quality and yield of rice (Hoang et al, 2016). Therefore, new hybrid rice variety by



improving their quality and yield through genetic modification techniques and technologies can meet the targeted demand for rice in Malaysia.

The major drawbacks of *in vitro* culture of *indica* rice are low percentage of callus induction, somatic embryogenesis and plant regeneration compared to *japonica* rice variety (Hoque and Mansfield, 2004; Abiri et al, 2017). These issues are considered as the main barriers in the genetic modification of *indica* rice (Azizi et al, 2015). Additionally, limited information was found on the exact age of maintenance of embryogenic callus and its effect on embryogenic quality. The expressions of SERK1 gene by real time PCR during SE were yet not determined in Malaysian rice callus produced for different varieties such as MR220, MR220-CL2, and MR232 and Bario rice cultivars.

Based on the above research problems, the present study determines the high callus induction and regeneration performance of MR220, MR232, Bario and MR220-CL2 on these growth media which are better than control treatment. Expression of SERK1 gene during somatic embryogenesis of four *indica* rice varieties was evaluated using different plant growth regulators, different age callus, pre-heat treatment. Therefore, using this information, the understanding of the fundamental molecular events that trigger somatic embryogenesis, which guides the development of propagation practises, for those plants that are recalcitrant to *in vitro* somatic embryogenesis. In addition, the present study proposed the prediction three-dimensional structures of *Oryza sativa* SERK1 using bioinformatics tools. These would broaden the horizon of biotechnological advancements in the field of crop science.

### 1.3 Objectives

The objectives of this research are stated below:

- (a) To determine the effects of different basal media and plant growth regulators, pre-heat treatments on callus induction.
- (b) To characterize SERK1 gene based on the structure and phylogenetic relationship.
- (c) To quantify the expression of SERK1 gene in different varieties and their developmental ages, pre-heat treatment, and plant growth regulators.
- (d) To determine the effects of pre-heat treatment on regeneration.

### 1.4 Scope of the Study

Establishment and optimization parameters affect somatic embryogenesis (callus induction of different basal media and plant growth regulators, carbon sources, gelling agent, and pre-heat treatment) of wetland rice varieties (MR220, MR220-CL2, MR232) and upland *indica* rice variety of Bario. For callus induction, seed were cultured on MS media, N<sub>6</sub> media, and LS media supplemented with different concentration of plant growth regulator (auxin) such as 2,4-dichlorophenoxyacetic acid (2,4-D),  $\alpha$ -Naphthaleneacetic Acid (NAA) alone or in combination. The morphology of embryogenic and non-embryogenic callus was observed and recorded (callus percentage). Then, the ultrastructure and cell composition were examined through scanning electron microscopy (SEM) and histology respectively.

RNA extraction was employed and reverse transcriptase polymerase chain reaction (RT-PCR) was conducted to synthesize the complementary DNA (cDNA). The *Oryza sativa* was subsequently authenticated using the BLASTn and Neighbor-

joining (NJ) tree analysis in software MEGA 6 (Tamura et al, 2013). The expression of the gene was quantified by real-time PCR (Rotor- gene-Q, Qiagen). Quanti Nova SYBR gene PCR kit was used for real-time PCR. Fold change of the gene were quantified by using the Bioinformatics software of Gene Globe (Qiagen).

The present study was also conducted regeneration work from callus of these rice varieties by using different combinations of plant growth regulators (cytokinin and auxin) such as 6-Benzylaminopurine, benzyl adenine (BPA), Kinetin (Kin), and NAA. Germination test was conducted and seed weight from the *ex-vitro* plants was evaluated. The evaluation was compared from control plant and *ex-vitro* plant.

## 1.5 Significance of the Study

The success in establishing a standard callus induction and regeneration method for some important Malaysian *indica* rice varieties such as MR220, MR220-CL2, MR232 and Bario will assist in overcoming the issues of insufficiency of regeneration method for variety development which is extensively used in genetic modification research.

This study has focused on getting highly efficient callus induction media for Malaysian *indica* rice. The molecular studies were therefore intended on identifying the SERK1 gene during somatic embryogenesis in MR220, MR232, MR220-CL2, and Bario. Moreover, the expression of SERK1 gene from different developmental ages of somatic embryogenesis were not yet identified in MR220, MR220-CL2, MR232 and Bario cultivar. This project has performed a comprehensive study on SERK1 to identify the certain age of embryogenic callus induction which is potential for regeneration. Besides, to my best knowledge, pre-heat treatment of rice seeds contributed to high throughput callus induction had not yet studied in detail.

Therefore, this study helps to formulate a new approach for regenerating of *indica* rice. Additionally, the knowledge of SERK1 gene as embryogenic marker could widen the specific target of competency callus before establishment of any

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