EMBRYOGENESIS PROTOCOL FOR GENETIC TRANSFORMATION AND FUNCTIONAL ANALYSIS OF JERF1 GENE IN DROUGHT-INDUCED MALAYSIAN RICE CULTIVAR MR219

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By

RAMBOD ABIRI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

"I don't know what your destiny will be, but one thing I know; the only ones among you who will be really happy are those who have sought and have found how to serve."

-Albert Schweitzer

This humble work is dedicated to:

My beloved wife, **Narges**, for your patience, love, friendship and humor, without

which I wouldn't have reached this present stage.

My parents who have supported me through all my various endeavors,

Louis and **MohammadReza**.

My parents in law who have prayed for me, **Forouz** and **Homayoun**.

My brother, **Ramin**, and my brother in law, **Ali** who have encouraged inspired me.

My sisters in law, **Jila** and **Armaghan,**

My nephew**, Shervin**

Thank you for understanding that distance can help one improve his knowledge, even though 6,615 km is a long way from home.

I decided to devote my life to telling the truth,
because having survived I owe something to the dead and anyone who does not remember them betrays them,
my dearest brothers,
Navid and **Amir**,
who I have lost them, may their souls continue to rest in perfect peace.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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September 2018

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Faculty: Biotechnology and Biomolecular Sciences

In Malaysia, severe drought stresses have affected residents and agricultural crops, especially rice, in various regions of the country and it is likely to continue and may worsen in the future. To mitigate the problems, new tolerant plant varieties to drought must be developed. The main objective of the current research was to develop a suitable *in vitro* protocol and elucidate the response of Malaysian rice cultivar MR219 to *Jasmonate and Ethylene Response Factor 1* (*JERF1*) gene in drought condition. The best recipe of callogenesis for MR219 was MS media added with 2 mg L^{-1} of 2,4-D and root was the prominent explant. The best priming conditions were seen at 18 hours of hydropriming and 50 mg L^{-1} of abscisic acid. For the proliferation phase, the highest efficiency was observed at week four in the MS media supplemented with 2 mg L^{-1} of 2,4-D, 2 mg L^{-1} of kinetin, 50 mg L^{-1} of L-proline, 100 mg L^{-1} of casein and 30 mg L^{-1} of silicon. MS media with 3 mg L^{-1} of KIN, 30 mg L^{-1} of silicon and 2 mg L^{-1} of NAA was selected as the best media for regeneration. To promote the roots of the regenerated explants, 0.4 mg L^{-1} of IBA was found to be the best activator. TRIzol protocol was found to be an appropriate method for isolating high quality RNA from the tomato leaves and fruits. Four hours of NaCl and ABA treatments on the tomato prior to the nucleic acids extractions produced the highest *JERF1* expression and led to the successfully isolating the *JERF1* gene. The entry and the expression vectors were constructed using Gateway® Technology. *Agrobacterium*-mediated transformation method was used to transform *JERF1* gene to MR219. Transgenicity of the transformed plants was evaluated using polymerase chain reaction, q-PCR and High-Performance Liquid Chromatography. The result of functional study has shown that the average width of the wild type seeds was significantly higher than transgenic seeds. Meanwhile, the seeds ratio of wild type was higher in comparison to transgenic in the second generation. The morpho- and physiological results of the two-week-old rice seedlings had shown that the responses of both wild type and transgenic rice in terms of shoot and root length and plumule fresh and dry weight were significantly different ($p \leq 0.01$). The responses of both wild type and transgenic rice in terms of the shoot and root length,

leaf proline, root proline, chlorophyll a and b, total chlorophyll and carotenoid and other pigments were significantly different ($p \leq 0.01$) in three-week-old rice. These results have confirmed the significant differences ($p \leq 0.05$) between wild type and transgenic plants in terms of the ratio of root to shoot proline in three-week-old rice. The concentrations of some amino acids such as aspartic acid, serine, glycine, proline and cysteine were significantly different between wild type and transgenic plants. Real-time PCR confirmed the over-expression of *OsLTP1*, *OsCDPK13*, *OsP5CS* and *OsSPDS2* by *JERF1* genes in transgenic plants. To conclude, the results of this experiment confirmed the high efficiently of somatic embryogenesis protocol and potential of *JERF1* as a prominent gene under drought stress condition.

Abstrak tesis yang dikemukakan kepada Senate Universiti Putra Malaysia sebagai memeuhi keperluan untak ijazah Doktor Falsafah

PROTOCOL EMBRIOGENESIS UNTUK ANALISIS TRANSFORMASI GENETIk DAN FUNGSI GEN *JERF1* DI DALAM PADI MALAYSIA KULTIVAR MR219 TERARUH-KEMARAU

Oleh

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Di Malaysia, kemarau yang teruk telah menjejaskan kehidupan dan tanaman pertanian, terutamanya padi, di seluruh negara ini dan ia mungkin akan berterusan dan boleh menjadi lebih buruk pada masa akan datang. Untuk mengatasi masalah ini, variati tumbuhan toleran yang rintang kemarau mesti dibangunkan. Objektif utama penyelidikan ini adalah untuk membangunkan protokol *in vitro* dan mengkaji tindak balas kultivar padi MR219 kepada gen *Jasmonate dan Ethylene Response Factor 1* (*JERF1*) dalam keadaan kemarau. Resipi yang terbaik untuk proses kallogenesis untuk MR219 adalah media MS yang ditambah dengan 2 mg L^{-1} 2,4-D dengan akar sebagai explan yang paling sesuai. Kondisi proses pemula yang terbaik ialah pada 18 jam pemula-hidro di dalam 50 mg L^{-1} asid absisik. Untuk fasa proliferasi, keeffisyenan tertinggi didapati pada minggu keempat dalam media MS yang ditambah dengan 2 mg L^{-1} 2,4-D, 2 mg L^{-1} kinetin, 50 mg L^{-1} L-prolin, 100 mg L^{-1} kasein dan 30 mg L^{-1} silikon. Media MS dengan 3 mg L^{-1} KIN, 30 mg L^{-1} silikon dan 2 mg L^{-1} NAA dipilih sebagai media terbaik untuk proses percambahan. Untuk menggalakkan pertumbuhan akar pada eksplan, 0.4 mg L^{-1} IBA didapati sebagai pengaktif terbaik. Kaedah TRIzol merupakan kaedah yang sesuai untuk memencilkan RNA berkualiti tinggi dari daun dan buah tomato. Rawatan NaCl dan ABA pada tomato selama empat jam sebelum pengekstrakan asid nukleik, telah menghasilkan ekspresi *JERF1* tertinggi dan menyumbang kepada kejayaan pemencilan gen *JERF1*. Vektor ungkapan dibina dengan menggunakan teknologi Gateway®. Kaedah transformasi agrobakterium telah digunakan untuk memindahkan gen *JERF1* kepada MR219. Transgenisiti pada padi transgenik dinilai menggunakan tindak balas rantaian polimerase, q-PCR dan Kromatografi Cecair Berprestasi Tinggi. Hasil kajian fungsional telah menunjukkan bahawa lebar purata biji padi jenis liar adalah jauh lebih tinggi daripada biji transgenik. Sementara itu, nisbah benih liar lebih tinggi berbanding dengan transgenik pada generasi kedua. Hasil morfologi dan fisiologi dari anak benih padi berusia dua minggu telah menunjukkan perbezaan yang sinifikan ($p \leq 0.01$) pada tindakbalas padi liar dan padi transgenik dari segi pucuk dan panjang akar dan plumul berat segar dan kering.

Respons terhadap kedua-dua jenis liar dan padi transgenik dari segi pucuk dan panjang akar, proline daun, proline akar, klorofil a dan b, jumlah klorofil dan karotenoid dan pigmen lain adalah sangat berbeza ($p \leq 0.01$) dalam padi yang berusia tiga minggu. Hasil ini telah mengesahkan perbezaan ketara ($p \leq 0.05$) antara padi jenis liar dan padi transgenik dari segi nisbah prolin pada akar-pucuk dalam padi berusia tiga minggu. Kepekatan beberapa asid amino seperti asid aspartik, serin, glisin, prolin dan sistin sangat berbeza antara padi liar dan padi transgenik. Analisis qRT-PCR telah mengesahkan pengekspresan lampau gen-gen *OsLTP1*, *OsCDPK13*, *OsP5CS* dan *OsSPDS2* oleh gen *JERF1* dalam padi transgenik. Kesimpulannya, hasil eksperimen ini telah berjaya menghasilkan protokol embriogenesis somatik yg berkeeffisyenan tinggi dan mengesahkan potensi *JERF1* sebagai gen yang utama dalam keadaan kemarau.

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

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A. tumefaciens	*Agrobacterium tumefaciens*
aa	Amino acid
AA	Ascorbic acid
ABA	Abscisic Acid
Ala	Alanine
AP2/ERF	APETALA 2/ethylene-responsive
Arg	Arginine
Asp	Aspartic acid
B.C.	Before Christ
BAP	(benzylamino) purine
Bp	Base Pairs
Ca^{2+}	Calcium ions
CaMV35s	Cauliflower mosaic virus
cDNA	Complementary DNA
Ch.a	chlorophyll a
Ch.b	chlorophyll b
CTAB	Hexacetyltrimethyl ammonium bromide
DEPC	Diethyl pyrocarbonate
Dicamba	3,6-dichloro-o-anisic acid
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPS	deoxynucleotides
DRE/CRT	Dehydration-Responsive Clement/C-Repeat
Ds	Double-stranded
ER	Endoplasmic reticulum
ERF	Ethylene-responsive
ET	Ethylene
EtBr	Ethidium bromide
FAO	Food and Agriculture Organization
G	Gram
g (rcf)	Gravity
G%	The germination percentage
GA3	Gibberellic acid
GFP	Green fluorescent protein
GI	Gibberellins
Glu	Glutamic acid

Gly	Glycine
GM	Genetic modification
GVI	Germination Vigor Index.
GVI	The germination vigor index
H	Hour
HCl	Hydrochloric acid
His	Histidine
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Abscisic acid
Ile	Isoleucine
JA	Jasmonic acid
JERF1	*Jasmonate and Ethylene Response Factor 1*
K_2SiO_3	Potassium metasilicate
Kb	Kilo base-pair
KIN	Kinetin
L	Litre
LB	Luria-bertani
LB	Lysogeny Broth
Leu	Leucine
LiCl	Lithium chloride
LP	Proline of root
Lys	Lysine
M	Molar
MARDI	Malaysian Agricultural Research and Development Institute
Met	Methionine
Mg	Milligram
mg g^{-1}	Milligram per gram
MGT	Mean germination time
MGT	Mean Germination Time,
Min	Minute
µg	Microgram
µg μL^{-1}	Microgram per microliter
µL	Microliter
mL	Millilitre
mM	Millimolar
MR219	MARDI 219

mRNA	Messenger RNA
MS	Murashige and Skoog
MSO	MS media free hormone
NAA	1-Naphthaleneacetic acid
NaCl	Sodium chloride
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
Ng	Nanogram
NOA	Naphthoxyacetic acid
ORF	Open reading frame
p-CPA	Para-chlorophenoxyacetic acid
PCR	Polymerase chain reactions
PEG	polyethylene glycol
%	Percentage
PGRs	Plant Growth Regulators
Phe	Phenylalanine
Picloram	4-amino-3, 5,6- tricholoropyridinecarboxylic acid
PR	Pathogenesis-related
Pro	Proline
PVP	Polyvinylpyrrolidone
PVPP	Polyvinyl polypyrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
RP	Proline of root
RP/SP	Proline root to shoot ratio
RT	Room Temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Salicylic Acid
SDS	Sodium dodecyl sulphate
Sec	Second
Ser	Serine
Spp	Species
SS	Single-stranded
T.G	Total Germination
T50	50 Percentage of Germination,

T50	Time to 50% germination
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
T-DNA	Transferred DNA
TDZ	Thidiazuron
TE	Tris-EDTA
Thr	Threonine
Total chi	Total chlorophyll.
Tyr	Tyrosine
Val	Valine
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
zeatin	6-(4-hydroxy-3- methyl but-2-enyl amino)-purine)]

CHAPTER 1

INTRODUCTION

After wheat, rice (*Oryza sativa* L.) is the second most important plant in the world (Al-Amin and Ahmed, 2016). Fluctuation and scarcity of agricultural products lead to spike in prices of rice in recent years (Tadasse *et al*., 2016; Torero, 2016). In this regard, environmental stresses have been identified as the top reasons for the price spikes by affecting on both food security and livelihoods (De La Fuente *et al*, 2013). Despite the increasing demands for rice products, this plant is exposed to the wide spectrum of environmental stresses that negatively effects on its growth, function and productivity (Bajaj and Mohanty, 2005). Drought and water deficit have been extensively reported as the most hazardous stresses to rice (Wang *et al*., 2012).

Drought tolerance mechanism is observed in all crops but its extent varies from one species to another species and within species (Jaleel *et al*., 2009). Tolerance to drought is a complex mechanism, due to interactions between different undesirable factors and various molecular, biochemical and physiological phenomena affecting plant growth as well as development (Razmjoo *et al*., 2008). Drought defense mechanisms in plants can be classified into four types, including drought -recovery, -escape, -tolerance and -avoidance. Different physiological indicators have been applied to evaluate the tolerance of plants under drought conditions such as plant growth regulators contents, amino acid level, water potential, cell membrane stability, leaf and root traits as well as adjustment capabilities. During the years, researches have assessed the molecular and genetic mechanisms of drought resistance plants to obtain the drought-related genes with regard to drought- tolerance and -avoidance. With increasing knowledge of drought resistance mechanisms in model plants, it still a matter of concern to improve drought- resistance and water-saving (Fang and Xiong, 2015; Zheng *et al*., 2009). A better conceptual understanding of the plant mechanisms could be used to create or select resistance plant varieties, which may obtain a more tolerant plant under drought stress (Jaleel *et al*., 2009). Under drought stress and water deficit, complex signaling networks have been elaborated in rice. These networks perceives the stress signals and modulates the resistance genes expression (Xiong *et al*., 2002). Rice reaction to drought differs considerably at diverse organ levels depending upon stress duration and intensity as well as crop growth stage and species (Jaleel *et al*., 2009).

Genetic modification (GM) is a popular method that researchers have been using to increase the yield of plant products by improving certain traits, including the responses of plants to abiotic and biotic stresses (Ashraf *et al*., 2008). Success in genetic engineering process depends upon selecting the proper method of transformation to introduce desirable traits into the host genome while concentrating on preserving the individual characteristics of the plant (Abiri *et al*., 2015). In gene transformation processes, the gene(s) of interest of donor plants, bacteria or viruses are transferred to host plants using various methods (Rivera *et al*., 2012). In rice, the engineered cultivars were produced through poly ethylene glycol, particle bombardment and electroporation during the 1980s- 1990s (Birch and Franks, 1991). However, the advantages of

Agrobacterium-mediated transformation encouraged researchers to use in the rice genetic transformation (Hiei *et al*., 1994).

The most important gene family involves in stress responses consists of transcription factors (sequence-specific DNA-binding factors) that play vital roles in influencing or controlling several biological processes. Among the stress- responsive transcription factor-encoding genes, the APETALA 2/ethylene- responsive (AP2/ERF) family genes have been described as the main stress- responsive genes in various physiological networks in rice. *Jasmonate and Ethylene Response Factor 1* (*JERF1*) is a tomato protein containing a conserved ERF DNA- binding motif. The *JERF1* overexpression enhanced the tobacco resistance to low temperature, salt concentration and osmotic stress (Zhang *et al*., 2004). The general objective of the current research was to develop a suitable *in vitro* protocol as well as to elucidate the response of Malaysian rice cultivar MR219 to *JERF1* gene in drought-induced condition. Therefore, the specific objectives of this study were:

1. To investigate the effect of different priming factors in germination and establish the most suitable media formulations *in vitro* condition.
2. To isolate the drought stress tolerance gene (*JERF1*) from the most suitable explants of Malaysian tomato (*Solanum lycopersicum*) cultivar MT1 and construct the expression vector.
3. To transfer the *JERF1* gene to MR219, determine the function of *JERF1* in MR219 rice cultivar and evaluate *JERF1* by computational analysis.

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