UNIVERSITI PUTRA MALAYSIA

ISOLATION AND MOLECULAR CHARACTERIZATION OF EgCBF3 ENCODING OIL PALM CBF/DREB TRANSCRIPTION FACTOR AND EFFECTS OF ITS ECTOPIC EXPRESSION IN TOMATO (Solanum lycopersicum cv. MT1)

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By

MORTAZA EBRAHIMI

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

February 2015

Specially Dedicated

To my wife, children and parents

For their Love & Supports

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

ISOLATION AND MOLECULAR CHARACTERIZATION OF *EgCBF3* ENCODING OIL PALM CBF/DREB TRANSCRIPTION FACTOR AND EFFECTS OF ITS ECTOPIC EXPRESSION IN TOMATO (*Solanum lycopersicum* CV. MT1)

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One of the well understood mechanisms in plants to overcome biotic and abiotic stresses is mediated through transcription factors. The APETALA2/Ethylene Response Factor (AP2/ERF) is one of the plant specific transcription factors. They are categorized into three families, termed AP2, RAV and ERF. ERF family is divided into two major subfamilies; the Ethylene Responsive Factors (ERF) and the C repeat-binding factor/dehydration responsive element-binding factor (CBF/DREB). In this study, a new member of the *CBF/DREB* was isolated from oil palm (*Elaeis guineensis* var. Dura × Pisifera) ripening fruit and designated as *EgCBF3*. Bioinformatics analysis revealed that *EgCBF3* belongs to A-1 subgroup of CBF/DREB subfamily. The transcripts of *EgCBF3* were detected ubiquitously, in oil palm's root, leave and mesocarp tissue. This gene was responsive to the cold, ethylene, abscisic acid, NaCl and polyethylene glycol treatments. The *EgCBF3::mGFP* fusion protein was localized to the nucleus of onion epidermal cells. Using *in vitro* and *in vivo* DNA-protein binding assays it has been shown that *EgCBF3* was able to bind with DRE/CRT element. Expression pattern of *polygalacturonase* (*SlPG*) and *SlE8* two fruit ripening related genes were affected under transient overexpression of *EgCBF3* in tomato fruits at four different developmental stages. Two carotenoid biosynthesis-related genes *phytoene desaturase* (*SlPDS*) and *phytoene synthetase* (*SlPSY*) showed up-

regulation at four studied stages. Same result was observed for *9-cis-epoxycarotenoid dioxygenase* (*SlNCED1*). The ethylene biosynthesis related genes demonstrated an expression pattern related to the fruit developmental stages. These results predict that *EgCBF3* can mediate abiotic stress response in ripening fruits and regulates the ripening process through modulation of ethylene and abscisic acid biosynthesis. Functional characterization of *EgCBF3* was further performed using stable transformation of tomato cv. MT1. An *in vitro* technique was developed for efficient regeneration of transgenic tomato. Seed pretreatment with Thidiazuron (TDZ, 1 mg/l) enhanced organogenesis of the cotyledonary leaf with abaxial side down on MS medium supplemented with 2 mg/l Benzyl Amino Purine (BAP) and 0.02 mg/l Indole Acetic Acid (IAA). The *EgCBF3* tomatoes demonstrated dwarfism for the first few weeks, delayed leaf senescence and flowering time, increased chlorophyll content (~0.085 mg/cm^2) and abnormal morphology compare to wild type. *In vitro* studies of the transgenic lines confirmed that overproduction of *EgCBF3* can enhance drought, salt and cold tolerance in tomato. Expression of ethylene biosynthesis-related genes encoding *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS*) and *1-aminocyclopropane-1-carboxylic acid oxydase* (*ACO*) were down-regulated in transgenic lines. Also, the studied pathogenesis-related genes showed altered expression in wounded leaves of transgenic plants compared to wild types. These findings were consistent with the hypothesis that *EgCBF3* can modulate plant growth and development, as well plant biotic and abiotic stress tolerance through direct regulation of related regulons, and partly via ethylene regulatory pathway.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMENCILAN DAN PENCIRIAN MOLEKUL *EgCBF3* PENGEKOD FAKTOR TRANSKRIPSI CBF / DREB KELAPA SAWIT DAN KESAN PENGEKSPRESAN EKTOPIKNYA DI DALAM TOMATO (*Solanum lycopersicum* cv. MT1)

oleh

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Salah satu mekanisma yang difahami secara mendalam adalah perantaraan melalui faktor transkripsi. 'APETALA2/Ethylene Response factor' (AP2/ERF) adalah salah satu faktor transkripsi khusus tumbuhan. Mereka dikategorikan kepada tiga famili iaitu AP2, RAV dan ERF. Famili ERF dibahagikan kepada dua subfamili utama; 'Ethylene Responsive Factors' (ERF) dan 'repeat-binding factor/dehydration responsive element-binding factor' (CBF/DREB). Dalam kajian ini, ahli baru CBF/DREB telah dipencilkan daripada buah kelapa sawit (*Elaeis guineensis* var. Dura × Pisifera) masak dan dinamakan sebagai *EgCBF3*. Analisis bioinformatik mendedahkan *EgCBF3* adalah kepunyaan subfamili CBF/DREB kumpulan A-1. Traskrip *EgCBF3* dikesan merata dalam akar, daun, dan tisu mesokarp. Gen ini responsif pada rawatan sejuk, etilina, asid absisik, NaCl dan polietilina glikol. Protein gabungan GFP *EgCBF3* tersasar dalam nukleus sel epidermis bawang. Asai pengikat DNA-protein *in vitro* dan *in vivo* menunjukkan bahawa EgCBF3 mengikat elemen DRE/CRT. Corak pengekspresan polygalacturonase (SIPG) dan SIE8 dua gen kemasakan buah responsif etilina menunjukkan perubahan pengekspresan dalam pengekspresan transien EgCBF3 dalam buah tomato pada empat peringkat perkembangan berlainan. Dua gen berkaitan biosintesis karotenoid, phytoene desaturase (SIPDS) dan phytoene synthetase (SIPSY) menunjukkan peningkatan tahap pengekspresan dalam

keempat peringkat yang dikaji. Keputusan yang sama telah dilihat untuk *9-cis-epoxycarotenoid dioxygenase* (*SINCED1*). Gen biosintesis etilina menunjukkan corak pengekspresan bertalian dengan peringkat perkembangan buah. Keputusan ini mencadangkan *EgCBF3* menjadi perantara tindakbalas tekanan abiotik pada peringkat kemasakan buah dan mengawal proses peranuman melalui modulasi biosintesis etilina dan asid absisik. Pencirian kefungsian *EgCBF3* selanjutnya dibuat melalui transformasi kekal menggunakan tomato cv. MT1. Teknik *in vitro* telah dibangunkan untuk kecekapan dalam pertumbuhan semula tomato transgenik. Prarawatan biji benih dengan TDZ (1 mg/L) meningkatkan organogenesis daun kotiledon dengan bahagian abaksial di bawah di dalam media MS yang ditambah dengan 2 mg/L BAP dan 0.02 mg/L IAA.. Tomato *EgCBF3* menunjukkan sifat kerdil untuk beberapa minggu, penangguhan senesens dan pembungaan, peningkatan kandungan klorofil (~0.085 mg/cm^2) dan morfologi bunga yang tidak normal berbanding tomato liar. Kajian *in vitro* ke atas lajur transgenik mengesahkan bahawa penghasilan berlebihan *EgCBF3* boleh meningkatkan toleransi terhadap kekeringan, kemasinan dan kesejukan dalam tomato. Ekspresi gen biosintesis etilena yang mengekodkan *1-aminocyclopropane-1-carboxylic acid synthase* (ACS) dan *1-aminocyclopropane-1-carboxylic acid oxidase* (ACO) telah menurun. Juga gen pengekspresan gen patogenesis yang dikaji menunjukkan perubahan dalam tisu daun transgenik yang cedera berbanding jenis liar. Penemuan ini adalah konsisten dengan hipotesis yang *EgCBF3* boleh mengawal pertumbuhan dan perkembangan tumbuhan, juga toleransi terhadap tekanan biotik dan abiotik melalui kawalan terus regulon dan sebahagiannya melalui tapak jalan peagawalaturan etilina.

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I certify that a Thesis Examination Committee has met on to conduct the final examination of Mortaza Ebrahimi on his thesis entitled " **ISOLATION AND MOLECULAR CHARACTERIZATION OF OIL PALM CBF/DREB TRANSCRIPTION FACTOR, EGCBF3 AND EFFECTS OF ITS ECTOPIC EXPRESSION IN TOMATO (*Solanum lycopersicum* cv. MT1)**" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Pertanian Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the relevant degree of Doctor of Philosophy.

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LIST OF ABBREVIATIONS

AP2/ERF	APETALA2/Ethylene Response Factor
CBF/DREB	C-Repeat-Binding Factor/Dehydration Responsive Element-Binding Factor
RAV	Related to ABI3/VP1
w.a.a	Week After Anthesis
kb	Kilo Base-Pair
bp	Base Pairs
CDS	Coding Region
cDNA	Complementary DNA
CTAB	Hexacetyltrimethyl Ammonium Bromide
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
DNase	Deoxyribonuclease
RNase	Ribonuclease
$dNTP_S$	Deoxynucleotides
ss	Single-Stranded
ds	Double-Stranded
LiCl	Lithium Chloride
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
DRE/CRT	Dehydration Response Element/C-Repeat
TF	Transcription Factor
rpm	Round per minute
TE buffer	Tris-EDTA buffer
PCR	Polymerase Chain Reactions
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
NaCl	Sodium Chloride
SDS	Sodium Dodecyl Sulphate
NaOH	Natrium Hydroxide
LB	Luria-Bertani
S.O.C	Super Optimal Broth
OD	Optical Density
NCBI	National Center For Biotechnology Information
DFCI	Dana-Farber Cancer Institute
BLAST	Basic Local Alignment Search Tool
WU-BLAST	Washington University-Basic Local Alignment Search Tool
3D	3 Dimensional
Phyer	**P**rotein **H**omology/**A**nalog**Y** **R**ecognition Engine
MES	2-(N-morpholino)ethanesulfonic acid
EMSA	Electrophoretic Mobility Shift Assay
LiAc	Lithium Acetate
PEG	Polyethylene Glycol

PVP	Polyvinylpyrrolidone
ABA	Abscisic Acid
pI	Isoelectric point
KDa	Kilodalton
ACS	*1-aminocyclopropane-1-carboxylic acid synthase*
ACO	*1-aminocyclopropane-1-carboxylic acid oxydase*
PG	*polygalacturonase*
PDS	*phytoene desaturase*
PSY	*phytoene synthetase*
NCED	*9-cis-epoxycarotenoid dioxygenase*
w/v	Weight/volume
v/v	Volume/volume
ANOVA	Analysis of variance
mRNA	Messenger RNA
TDZ	Thidiazuron
BAP	Benzyl amino purine
Kin	Kinetin
2iP	6-(gamma,gamma-Dimethylallylamino)purine
IAA	Indole Acetic Acid
NAA	Naphthalene Acetic Acid
CaMV	Cauliflower Mosaic Virus
WT	Wild Type

CHAPTER 1

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a perennial monocot crop originated from West and Central Africa (Soh et al., 2009). Oil palm trees produce oil rich fruits and it is the highest yielder among oil producing crops. This tree is cultivated in 16.4 million hectares of agricultural lands worldwide (FAO, 2013), and Malaysia is undoubtedly one of the biggest producers and exporters of the oil palm products, by having about 17.6 million tons (24.1%) of the total global palm oil trade (Oil World, 2013). It is reported that 77% of the arable land (15% of the total land) in Malaysia is devoted to oil palm (MPOB 2012). The most economic part of this crop is the fruit, in which two types of vegetable oil are produced. Almost 95% of total oil of the fruit is crude palm oil (CPO) produced from the mesocarp tissue, and about 5% of oil is the non-edible palm kernel oil (PKO).

The oil palm fruit, like tomato and many other fruits where there is a burst in ethylene production during the ripening stage (Tranbarger et al., 2011), is categorized as a climacteric fruit. This gaseous hormone plays a key regulatory role in the ripening process in these fruits. This is clearly indicated through comparative transcriptome analysis where 37% of the differentially expressed genes during fruit development and ripening stages in tomato are influenced by ethylene (Alba et al., 2005). Expression of proteins involved in fruit development and ripening is modulated by regulatory proteins and transcription factors (TF) especially play a major role in transcriptional regulation of these genes. Extensive studies have been made on characterization of different classes of TFs regulating the fruit ripening process, upstream as well as downstream of the ethylene signaling pathway, however function of the APETALA2/Ethylene Response Factors (AP2/ERF) proteins have received very little attention. This AP2/ERF superfamily is one of the largest plant specific TFs. The AP2/ERF is classified into three subfamilies: the APETALA2 (AP2) family with two AP2/ERF domains; the RAV (Related to ABI3/VP1) family with a B3 domain and an AP2/ERF domain; and Ethylene Response Factor (ERF) family with one AP2/ERF domain. There are two major subfamilies of the ERF; the Ethylene Responsive Factors (ERF) and the C-repeat-binding factor/dehydration responsive element-binding factor (CBF/DREB). It has been well documented that members of AP2/ERF act as key regulators in plant developmental processes, plant architecture (Chung et al., 2010), and biotic and abiotic stress tolerance in plants (Mei et al., 2007). These proteins are major regulators of fruit ripening via regulation of ethylene biosynthesis and the signaling pathway (Karlova et al., 2011; Tiznado-hernández and Mattoo 2012). Despite the economic importance of oil palm fruits, functions of the regulatory proteins involved in different aspects of fruit ripening process are not thoroughly understood.

Fruit ripening transcriptome analysis for lipid and carotenoid metabolism in oil palm was reported by Tranbarger et al. (2011). Although, their study provided a better understanding of the molecular mechanisms regulating fruit ripening in oil palm, but the mechanism is not fully discovered. Tranbarger et al. (2011) reported that a member of type VII and a type IX transcription factor of AP2/ERF superfamily showed up-regulation at the transition stage from system I of ethylene production to system II (Tranbarger et al., 2011) in the mesocarp tissue of oil palm fruits. Data mining of

transcriptomic datasets provided by Xu et al. (2011), Bourgis et al. (2011) and Tranbarger et al. (2011) showed no evidence of expression of *CBF/DREB* in oil palm ripening fruit.

Although, it has been shown that the CBFs play an important role in plant resistance to abiotic stresses, especially freezing tolerance, their function in biotic stress tolerance, as well as fruit ripening process is still under investigation (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997; Thomashow, 1999; Medina et al., 2011; Zhang et al., 2009b; Li et al., 2011). Alongside the *in vitro* DNA-protein binding assay demonstrating preference of the CBFs for the DRE/CRT element, there are some recent finding verifying possible affinity of this protein with both DRE/CRT and GCC-box elements in *in vivo* condition (Gutha and Reddy, 2008; Li et al., 2011).

Among the different biotic and abiotic stresses, freezing condition is a key factor with adverse effects on plant yield and geographical distribution. However, several reports showed contradictory role of ethylene in plant cold acclimation. While cold acclimation in tomato and tobacco was reported to be enhanced by ethylene (Ciardi et al. 1997; Zhang and Huang 2010), new recent findings indicate negative effects of ethylene in plant freezing tolerance (Shi et al., 2012; Zhao et al., 2014). In addition, more recent report indicated that the freezing tolerance in *Arabidopsis* was negatively affected by suppression of CBF through ethylene production (Shi et al., 2012).

Ethylene biosynthesis related enzymes contain different *cis* elements like GCC-box and DRE/CRT on their promoter sequences (Zhang et al., 2009b). So, it is hypothesized that a mechanism for the CBFs, to regulate chilling tolerance in plants is mediated partly through ethylene biosynthesis pathway and as a result, it can regulate plant growth, development and disease resistance, either directly by binding to the related *cis*-elements on different target genes or indirectly by ethylene biosynthesis pathway. So, the main objectives of this study were:

1. To isolate and clone the oil palm *EgCBF3* expressed in ripening oil palm fruit
2. To determine the *EgCBF3* responsiveness to different hormonal and stress treatments in oil palm mesocarp tissue
3. To characterize the DNA-protein binding and trans-activation abilities of *EgCBF3* using *in vitro* and *in vivo* assays.
4. To characterize the possible function of *EgCBF3* in regulating ethylene biosynthesis-related genes, pathogenesis-related genes and abiotic stress tolerance in tomato cv. MT1 using transgenic approaches.

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