UNIVERSITI PUTRA MALAYSIA

CLONING AND In Silico ANALYSES OF MADS-BOX GENES ISOLATED FROM CALYX TISSUES OF Hibiscus sabdariffa L. var. UMKL

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By

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CLONING AND *In Silico* ANALYSES OF MADS-BOX GENES ISOLATED FROM CALYX TISSUES OF *Hibiscus sabdariffa* L. var. UMKL

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Chair : Christina Yong Seok Yien, PhD
Faculty: Science

Flower organ development is mainly controlled by genetic factor. MADS-box transcription factor genes play a crucial role in controlling the development of calyx in flowering plants. *Hibiscus sabdariffa* L. var. UMKL (roselle) is cultivated in Malaysia mainly for its calyx which is high with vitamin C and anthocyanin content. Unfortunately, the genetic information regarding the flowering pathway of roselle is very scarce. It is critical to understand the genetics underlying roselle's flower developmental process by studying on genes related to its agronomic trait for the development of genetic markers and improvement of planting material. Hence, two MADS-box genes, designated as *HsMADS1* and *HsMADS2* were isolated from the young and mature calyx tissues of *H. sabdariffa* using 3'- RACE PCR and primer walking approaches. Both *HsMADS1* and *HsMADS2* CDS were consisted of 951 bp and 981 bp nucleotides, and encoded for putative proteins of 244 amino acids, respectively. The assembled nucleotides sequence of *HsMADS1* and *HsMADS2* genes obtained were 3,050 bp and 2,791 bp nucleotides, respectively. *HsMADS1* was predicted to have 7 exons, 6 introns and *HsMADS2* was consisted of 8 exons, 7 introns. Interproscan analysis showed that the deduced amino acids sequences of *HsMADS1* and *HsMADS2* possessed the MADS and K-box domains. The prediction of the distinct K-domain provided a strong indication that *HsMADS1* and *HsMADS2* belong to the type II (MIKC-type) of MADS-box gene. Specific motifs predicted at the C-terminal of their protein sequences suggested that *HsMADS1* and *HsMADS2* might belong to two different subfamilies of SEPALLATA and AGAMOUS-like 6, respectively under the same Class E of MADS-box superfamily. The function of the genes based on the phylogenetic analysis suggested that *HsMADS1* possibly involved in the expression of *SEP* gene in stem, leaf, bud and flower organs of roselle, whereas *HsMADS2* may probably involve in the late expression of floral tissue for stem branching. Template identification results showed that HsMADS1 and HsMADS2 were respectively 43% and 38% accurate to Crystal structure of MADS-box/Myocyte Enhancer Factor-2 from *Homo sapiens* (PDB:1N6J). The homology modeling of HsMADS1 and HsMADS2 demonstrated that both genes shared the same topology of β-α-β-α-α and suggesting that the molecular functions of both proteins may be involved in DNA binding and also act as transcriptional repressor or activator in a calcium-dependent manner. The differences in the nucleotide sequences, protein sequences, primary structures,

secondary structures and 3D protein structures of *HsMADS1* and *HsMADS2* allows the assumption that both genes might be different genes involved in the development of young and mature calyx tissues of roselle. Nevertheless, further characterizations need to be carried out in order to confirm the functions of both genes.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGKLONAN DAN *In Silico* ANALISIS GEN MADS-BOX DIASINGKAN DARIPADA TISU KALIKS DARIPADA *Hibiscus sabdariffa* L. KEPELBAGAIAN UMKL

Oleh

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Perkembangan organ bunga secara utamanya dikawal oleh faktor genetik. faktor transkripsi gen MADS-box memainkan peranan penting dalam mengawal perkembangan kaliks dalam tumbuh-tumbuhan berbunga. *Hibiscus sabdariffa* L. var UMKL (roselle) ditanam di Malaysia terutamanya untuk kaliks yang mana mengandungi kandungan vitamin C dan anthocyanine yang tinggi. Malangnya, maklumat genetik mengenai laluan proses pembungaan roselle adalah sangat terhad. Ianya sangat kritikal untuk memahami genetik yang menjadi asas untuk proses perkembangan bunga roselle dengan mengkaji gen yang berkaitan dengan sifat agronominya untuk tujuan pembangunan penanda genetik dan peningkatan bahan tanaman. Oleh itu, dua gen MADS-box ditetapkan sebagai *HsMADS1* dan *HsMADS2* telah diasingkan daripada tisu-tisu kaliks muda dan matang dari *H. sabdariffa* menggunakan 3'- RACE PCR dan penjanaan primer. CDS untuk *HsMADS1* dan *HsMADS2* masing-masing terdiri daripada 951 bp dan 981 bp nukleotida dan dikodkan untuk protein daripada 244 asid amino. Kumpulan urutan nukleotida daripada gen *HsMADS1* dan *HsMADS2* yang diperoleh adalah masing-masing sebanyak 3050 bp dan 2791 bp nukleotida. *HsMADS1* telah diramalkan mempunyai 7 ekson, 6 intron manakala *HsMADS2* telah terdiri daripada 8 ekson, 7 intron. Analisis Interproscan menunjukkan bahawa dapat disimpulkan amino asid urutan *HsMADS1* dan *HsMADS2* memiliki Mads dan K-box domain. Kehadiran K-domain memberi petanda yang kuat bahawa *HsMADS1* dan *HsMADS2* tergolong dalam jenis II (jenis-MIKC) daripada gen MADS-box. Spesifik motif di C-terminal dalam jujukan protein mencadangkan bahawa *HsMADS1* dan *HsMADS2* mungkin tergolong dalam dua subfamili berbeza iaitu SEPALLATA dan AGAMOUS-like 6, yang masing-masingnya di bawah Kelas E MADS-box yang sama. Fungsi gen berdasarkan keputusan analisis filogenetik mencadangkan *HsMADS1* mungkin terlibat dalam ekspresi gen *SEP* pada organ batang, daun, putik dan bunga dalam roselle, manakala *HsMADS2* mungkin terlibat semasa lewat perkembangan untuk tisu pembungaan demi percambahan batang. Pengenalpastian templat menunjukkan *HsMADS1* dan *HsMADS2* masing-masing 43% dan 38% tepat kepada struktur kristal daripada MADS-box/Myocyte Enhancer Faktor-2 daripada *Homo sapiens* (PDB: 1N6J). Pemodelan homologi HsMADS1 dan HsMADS2 menunjukkan bahawa kedua-dua gen yang berkongsi topologi yang sama β-α-β-α-α-α dan berkemungkinan bahawa fungsi molekul kedua-dua protein terlibat dalam pengikatan

DNA dan juga bertindak sebagai represor atau pengaktif transkripsi yang sangat bergantung kepada kalsium. Perbezaan dalam urutan nukleotida dan susunannya protein, struktur utama, struktur menengah dan struktur protein 3D HsMADS1 dan HsMADS2 membenarkan andaian bahawa kedua-dua gen mungkin gen berbeza yang terlibat dalam perkembangan tisu kaliks roselle semasa muda dan matang. Walau bagaimanapun, pencirian lanjut perlu dilakukan untuk mengesahkan fungsi kedua-dua gen.

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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 the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µl	Microlitre
%	Percentage
°C	Centrugade Celcius
1X	One time
AGL6	Agamous-Like 6
aa	Amino acid
bp	Base pair
BLAST	Basic Local Alignment Search Tool
CDS	Coding sequence
cm	centimetre
DNA	Deoxyribonucleic acid
dNTPS	Dinucleotide triphosphate
DEPC	Diethyl pyrocarbonate
DOPE	Discrete Optimized Protein Energy
EDTA	Ethylene diamine tetra acetic Acid
EtBr	Ethidium Bromide
EtOH	Ethanol
GSP	Gene Specific Primer
g	Gram
HCL	Hydrocloric
LB	Luria-bertani
L	Litre
MADS	MINICHROMOSOME MAINTENANCE, AGAMOUS, DEFICIENS, SERUM RESPONSE FACTOR (S)
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
MIKC	MADS-domain, I-domain, K-domain, C-domain
ml	mililitre
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaOAc	Sodium Acetate
NaOH	Sodium hydroxide

ng	Nano gram
NCBI	National Center for Biotechnology Information
ORF	Open Reading Frame
PCR	Polymerase Chain reaction
RACE	Rapid Amplification of Complementary DNA ends
rpm	Rotation per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
SEP	SEPALLATA
SQUA	SQUAMOSA
TAE	Tris-acetate-EDTA
TD-PCR	Touchdown PCR
U	Unit
UTR	Untranslated region
V	Volt
var.	variety

CHAPTER I

INTRODUCTION

1.1 Introduction

Hibiscus sabdariffa L. var. UMKL is widely utilised in food, beverages and pharmaceutical industries as synonym with its high content of vitamin C and anthocyanin (Mohamad *et al.*, 2009). Calyx, as one part of the flower's organ is incontrovertibly the most profitable and considerable organ in roselle plant. MADS box genes had been reported to play critical roles in plant developments and flower formation. Extensive studies on the characterization and expression of MADS-box genes involved in flower developmental of other economically important plant species (Tani *et al*., 2009) such as *Coffea arabica*, L. (de Oliveira *et al*., 2014), *Crocus sativus* (Tsaftaris *et al*., 2005), wheat (*Triticum aestivum* L.) (Zhao *et al*., 2006), *Silene latifolia* (Matsunaga *et al.*, 2004) and *Alpinia hainanensis* (Song *et al*., 2010) had been conducted. However, there are no reports yet regarding MADS-box genes in *H. sabdariffa* despite the commercial potentials this plant has.

In Malaysia many studies have been conducted on roselle but they were mainly focussing on physico-chemical properties and antioxidant content of the calyx. Apart from the study on mutation breeding, there is a lack of molecular study regarding the isolation of flowering genes in *H. sabdariffa.* Very limited knowedge is known regarding the genes that may be involved in the flowering pathway of *H. sabdariffa*. Furthermore, most MADS-box genes isolated from the different tissues of various species of angiosperms are present in isoforms, but the knowledge on whether the MADS-box genes isolated from the same type of tissue at different developmental stages present in isoforms or not is still uncertain. The fundamental knowledge such as the primary structures, domains functional regions, protein structural motifs and the protein structures of the MADS-box genes are crucially needed for the understanding of the genes functions in flowering pathway of *H. sabdariffa*. Therefore, isolation of the MADS-box genes from the roselle in this study provided basic genetic information that are useful for further study aiming at improving crop yields through genetic modification.

It was hypothesised that the two MADS-box genes isolated from the calyx tissues in roselle at different developmental stages might have differences in their nucleotide and amino acid sequences. There is a possibility that the two MADS-box genes isolated from the *H. sabdarifa* might be involved in different functions during the flower development in roselle.

1.2 Objectives of The Research

Since MADS-box genes are crucial in the flowering pathway of the flower organ in an angiosperm, this study aimed to isolate the first two MADS-box genes from roselle plant. Therefore, two objectives that needed to be achieved in this study are;

1. To isolate and clone the MADS-box genes from young and mature calyx tissues of *Hibiscus sabdariffa* L. var. UMKL.

2. To analyse the sequences and to predict the three dimensional (3D) structure of the MADS-box proteins using bioinformatic tools.

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