ISOLATION AND CHARACTERIZATION OF cDNA SEQUENCES ENCODING FOR MADS-BOX TRANSCRIPTIONAL FACTORS FROM RICE LEAVES

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I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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CERTIFICATION

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF cDNA SEQUENCES ENCODING FOR MADS-BOX TRANSCRIPTIONAL FACTORS FROM RICE LEAVES

MADS-box genes are characterized by the conserved MADS domain and found in a wide range of eukaryotes including metazoans, fungi, slime mold, and green plants. Throughout plant evolution, MADS-box genes have been recruited as transcriptional regulators active in the development of diverse plant structures. In monocots, MADS-box genes have been identified in maize, rice, and orchids which play important roles during reproductive and vegetative developmental processes. Although rice MADS-box genes involved in flower and fruit development have been well characterized, rice MADS-box genes expressed in vegetative structures have yet to be explored. The aim of this project is to isolate and analyze the cDNA sequences encoding for putative MADS-box transcription factors from MR84 leaves. MR84 is currently the popular local cultivar which produced good quality and yield as well as confers resistance to Karah and Merah diseases in Malaysia. Besides that, this project also aimed to characterize leaf-expressed MADS-box genes in MR84 and to examine their expression in developing leaves. Poly-A^+ RNA was isolated from developing leaves at different stages using oligo-dT magnetic beads, and first strand cDNA was synthesized directly on the beads. A degenerate 5′ (forward) primer was designed against the conserved amino acid sequences in the MADS-box domain and an anchored dT_{18} as the reverse primer for the three-prime (3′)-RACE procedure. PCR using sscDNA as template resulted in an expected fragment size of approximately 1,013 bp. This PCR product was cloned in pGEM-T Easy plasmid vector and sequenced using forward and reverses universal M13 sequencing primers. The sequence analyses of the cloned product showed 94% similarity to other rice MADS-box cDNA. However, in order to obtain the full sequence, five-prime (5′)-RACE using inner 5′-RACE as forward primer and MADS-specific as reverse primer were used to obtain the remaining 284 bp of the cDNA. A BLAST search of the complete 1,297 bp full sequence confirmed that this was the MADS-box gene and it was designated as *OsMADS_UMS1*. The deduced amino acid sequence of *OsMADS_UMS1* contains the typical MADS-box cDNA namely, the M, I and K boxes as well as a C terminal variable domain. Phylogenetic analyses using the PHYLIP software showed that OsMADS_UMS1 belongs to the class F (AGL20 or TM3) subfamily. Real-time RT-PCR analysis shows gradual mRNA steady-state levels increment during leaf development. This preliminary result indicates a possible role for *OsMADS_UMS1* in leaf initiation and its further development. In the course of this study, another partial MADS-box cDNA was isolated designated OsMADS_UMS2. This cDNA was not further studied.

ABSTRAK

Gen-gen MADS-box dicirikan oleh kehadiran domain MADS dan terdapat pada eukariot termasuk metazoa, kulat dan tumbuhan hijau. Sepanjang evolusi tumbuhan, gen-gen MADS-box berfungsi sebagai pengawal transkripsi yang aktif dalam perkembangan struktur tumbuhan. Dalam tumbuhan monokot, gen-gen MADS-box telah dikenalpasti dalam jagung, padi dan orkid. Gen-gen MADS-box mempunyai fungsi penting ketika proses perkembangan vegetatif dan reproduktif. Walaupun gen-gen MADS-box padi yang terlibat dalam perkembangan bunga dan buah telah dicirikan dengan teliti, gen-gen MADS-box padi yang diekspreskan di struktur vegetatif masih perlu diterokai. Tujuan projek ini adalah untuk mengasingkan dan menganalisis jujukan cDNA yang mengkodkan faktor transkripsi MADS-box daripada daun MR84. MR84 adalah varieti padi tempatan yang popular dan mempunyai hasil dan kualiti yang baik serta tahan kepada serangan penyakit karah dan merah. Selain itu, ia juga bertujuan untuk mencirikan gen MADS-box yang diekspreskan pada daun MR84 dan seterusnya meneliti tahap pengekspresan dalam daun yang berkembang. RNA poly-A^+ diasingkan daripada daun yang berkembang menggunakan manik-manik magnet oligo-dT. Lima (5') primer hadapan yang degenerat dibina daripada jujukan asid amino dalam domain MADS-box dan dT_{18} sebagai primer belakang untuk prosedur tiga (3')-RACE. PCR daripada satu bebenang DNA komplementari menghasilkan kira-kira 1,013 pasangan bes seperti yang dijangka. Produk PCR ini diklonkan pada pGEM-T Easy vektor plasmid dan dijujukkan menggunakan primer penjujukan M13 universal hadapan dan belakang. Analisis jujukan klon menunjukkan 94% persamaan dengan cDNA MADS-box padi. Untuk mendapatkan jujukan lengkap, lima (5')-RACE digunakan untuk mendapatkan baki 284 pasangan bes komplementari DNA tersebut menggunakan primer dalaman 5'-RACE sebagai primer hadapan manakala primer spesifik untuk MADS-box sebagai primer belakang. Pencarian jujukan lengkap 1,297 pasangan bes tersebut menggunakan BLAST telah mengesahkan bahawa jujukan tersebut adalah gen MADS-box dan dinamakan sebagai OsMADS_UMS1. Jujukan asid amino OsMADS_UMS1 mengandungi domain tipikal gen MADS-box iaitu domain MADS, I, K dan C. Keputusan filogenetik menggunakan perisian PHYLIP menunjukkan OsMADS_UMS1 berada dalam kelas F (AGL20 atau TM3). Analisis RT-PCR secara langsung menunjukkan peningkatan tahap mRNA secara berperingkat ketika perkembangan daun. Keputusan awal ini menunjukkan kemungkinan OsMADS_UMS1 terlibat dalam permulaan pembentukan daun dan perkembangan daun yang seterusnya. Dalam kajian ini juga, MADS-box cDNA separa lengkap telah diasingkan dan dinamakan sebagai OsMADS_UMS2. Namun demikian, cDNA ini tidak terus dianalisis dalam kajian ini.

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LIST OF ABBREVIATIONS

aa	Amino acid
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CIAP	Calf intestinal alkaline phosphatase
DNA	Deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylenediaminetetra-acetate
EtBr	Ethidium bromide
EBI	European Bioinformatics Institute
g	Gram
HE	Highly expressed
H_20	Water
HCl	Hydrochloric acid
h	Hour
kb	Kilobase pairs
LE	Lowly expressed
LB	Luria-Bertani
M	Molar
mg	Miligram
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Mililitre
mM	Milimolar
mm	Milimeter
U	Unit
µg	Microgram
µl	Microlitre

μM	Micromolar
NCBI	National Center for Biotechnology Information
NaOAc	Sodium acetate
ng	Nanogram
NLS	Nuclear localization signal
nm	Nanometer
NO_3^-	Nitrate
nts	Nucleotides
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
RACE	Rapid Amplification of cDNA Ends
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
sdH_2O	Sterile distilled water
SAM	Shoot apical meristem
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sec	Seconds
TAP	Tobacco acid pyrophosphatase
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris-EDTA
TSS	Transcription start site
UTR	Untranslated region
UV	Ultraviolet
V	Voltage

LIST OF SYMBOLS

~	Approximately
/	*Per*
<	Less than
>	More than
≤	Less than or equal to
'	Prime
%	Percent
α	Alpha
β	Beta
δ	Delta
^0C	Degree celcius

CHAPTER ONE

INTRODUCTION

MADS-box transcription factors are named after the initials of the four originally identified members. The yeast MINICHROMOSOME MAINTAINANCE 1 (MCM1) protein regulates mating-type-specific gene expression, the *Arabidopsis* AGAMOUS (AG) and *Antirrhinum* DEFICIENS (DEF) proteins play regulatory roles in specifying the identity of floral organs, and the human serum response factor (SRF) is involved in the transcriptional regulation of the protooncogene, *cfos*. After the isolation of the first plant MADS-box genes, *DEF* and *AG* from plants (Sommer *et al.,* 1990; Yanofsky *et al.,* 1990), a large number of MADS-box genes have been sequentially identified in different species of angiosperms, and the orthologues of floral homeotic MADS-box genes have also been found in gymnosperm (Becker *et al.,* 2000; Munster *et al.,* 1997). MIKC type MADS-box genes were also cloned from a number of species outside the seed plant lineage including ferns, mosses, lycopids and from the closest relatives of land plants, the fresh water green algae charophyceans (Tanabe *et al.,* 2005; Svensson and Engstrom, 2002; Henschel *et al.,* 2002; Hasebe *et al.,* 1998; Munster *et al.,* 1997).

MADS-box genes are characterized by the conserved MADS domain and found in a wide range of eukaryotes including metazoans, fungi, slime mold, and green plants. The gene family can be divided into two main lineages, referred to as type I and type II, both of which are present in plants, animals and fungi (Alvarez-Buylla *et al.,* 2000a). All members of the family possess the 180-nucleotide long (on average) MADS box. In plants, type II MADS-domain proteins, referred to as MIKC-type proteins, possess three additional functional domains: a well-conserved K (keratin) domain, a less-well-conserved I (intervening) domain, and a variable C-terminal region.

In contrast to type II genes, which have been the subject of extensive research, not much is known about the type I genes in plants. Except for the MADS box, the type I genes share no sequence similarity with type II genes. However, some type I genes share conserved C-terminal motifs with each other (De Bodt *et al.,* 2003b; Parenicova *et al.,* 2003). In addition, a third group of genes has recently been identified that possesses only half of the MADS box or are overall highly divergent. These are referred to as MADS-like genes (De Bodt *et al.,* 2003b).

Throughout plant evolution, MADS-box genes have been recruited as transcriptional regulators active in the development of diverse plant structures. Since the discovery of the first MADS-box genes more than a decade ago, biologists have made great progress in elucidating the roles of these genes in plant development. Expression studies and mutant analyses on MADS-box genes in diverse plant species revealed the crucial importance of MADS-box genes in the regulation of both reproductive (flower, seed, fruit) (Vrebalov *et al.,* 2002; Nesi *et al.,* 2002; Liljegren *et al.,* 2000; Fernandez *et al.,* 2000; Mao, 2000; Western and Haughn,1999; Gu *et al.,* 1998) and vegetative (root, leaf) development (Rosin *et al.,* 2003; Prakash and Kumar, 2000; Garcia-Maroto *et al.,* 2000; Heuer *et al.,* 2001, 2000; Zhang and Forde, 1998). Furthermore, MADS-box genes used in the control of floral patterning form the ideal genetic toolkit to study the diversification of flower architecture (Pinyopich *et al.,* 2003; Goto *et al.,* 2001; Theissen, 2001; Theissen and Saedler, 2001; Pelaz *et al.,* 2001, 2000; Alvarez and Smyth, 1999; Colombo *et al.,* 1995; Coen and Meyerowitz, 1991).

In monocots, MADS-box genes have been identified in maize, rice, and orchids (Lee *et al.,* 2003a; Pelucchi *et al.,* 2002; Heuer *et al.,* 2001; Yu and Goh, 2000). The fact that MADS box genes also function in monocotyledons is of great interest because of the agronomic importance of the species that belong to this group, not least among which are the cereals. MADS-box genes may thus provide a tool for elucidating the complex genetic system controlling development in cereals.

However, the structural and functional characterization of new MADS-box genes in cereals is required to further our understanding of this complex family of regulatory genes and their involvement in controlling the developmental processes in

cereals. Rice was chosen because of its importance as a worldwide crop and, additionally, because of its advantages as a biological model system for cereals in general, its genetics is well developed, extremely detailed genetic linkage maps are available and it can be efficiently transformed (Greco *et al.,* 1997).

MADS-box gene family in rice has resulted in the discovery of non-redundant 35 MADS-box genes (Yamaguchi *et al.,* 2006; Lee *et al.,* 2003a). The MIKC-type MADS box genes in *Japonica* rice subspecies is shown in Table 2.1. Although rice MADS-box genes involved in flower and fruit development have been well characterized, rice MADS-box genes expressed in vegetative structures have yet to be explored. Much work is still needed to uncover the molecular mechanisms of vegetative development.

Therefore, the aim of this project is to isolate and analyze the cDNA sequences encoding for putative MADS-box transcription factors from MR84 leaves. MR84 is currently the popular local cultivar which produced good yield and quality and confers resistance to Karah and Merah diseases as well. Besides that, it also aimed to characterize leaf-expressed MADS-box genes and to examine their expression in developing leaves.

Objectives of the study:

a. To isolate cDNA of MADS-box gene in rice leaves
b. To characterize the cDNA sequence using bioinformatic applications (BLAST search, CLUSTALW multiple alignment, exon/intron and RNA motifs/elements prediction and phylogenetic analysis)
c. To investigate the expression level of the MADS-box gene during rice leaves development

CHAPTER TWO

LITERATURE REVIEW

2.1 THE MADS-BOX GENE FAMILY

The MADS box is a highly conserved sequence motif found in a family of transcription factors. The conserved domain was named after the first four members of the family, which were MCM1, AGAMOUS, DEFICIENS and SRF. The name MADS was constructed form the "initials" of these four "founders".

2.1.1 The Modular Structure of Plant MADS-Box Proteins

Typical plant transcription factors consist of a DNA-binding region, an oligomerization site, a transcription regulation domain and a nuclear localization signal although some lack either a transcription regulation domain or a specific DNA-binding domain (Liu *et al.,* 1999). By mutational and functional analysis it has been demonstrated that MADS-box proteins consist of a DNA-binding region, a region which serves as an interface for dimerization and interactions with other proteins, and sometimes contain a transcriptional activation domain. There is a considerable overlap between these functional domains and the M, I, K and C structural domains, although none of the functions can exclusively be assigned to just one sin gle domain. Such modular organization is common to many eukaryotic transcription factors.

a. DNA Binding

DNA binding by plant type II MADS-box proteins to *cis*-acting promoter elements, named the CArG-box, is mediated by the MADS-domain and the K-box is believed to contribute to the binding specificity. Studies to identify the minimal DNA-binding domain of the *Antirrhinum* MADS-box proteins SQUAMOSA (SQUA) and PLENA (PLE) demonstrated that the MADS- and I-domains are sufficient to permit sequence-specific DNA binding by the proteins (West *et al.,* 1998). Similar results were

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