ความสัมพันธ์ระหว่างการแสดงออกของยีน *OsCam1-1* และ *OsNUC1* ในการตอบสนองต่อความเครียดจากความเค็มในข้าว *Oryza sativa* L.

นายธานินทร์ จันทรโชติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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RELATIONSHIP BETWEEN *OsCam1-1* AND *OsNUC1* GENE EXPRESSION

IN SALT-STRESS RESPONSE IN RICE *Oryza sativa* L.

Mr. Thanin Chantarachot

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Botany

Department of Botany

Faculty of Science

Chulalongkorn University

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Thesis Title	RELATIONSHIP BETWEEN *OsCam1-1* AND *OsNUC1* GENE EXPRESSION IN SALT-STRESS RESPONSE IN RICE *Oryza sativa* L.
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Field of Study	Botany
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ธานินทร์ จันทรโชติ : ความสัมพันธ์ระหว่างการแสดงออกของยีน *OsCam1-1* และ *OsNUC1* ในการตอบสนองต่อความเครียดจากความเค็มในข้าว *Oryza sativa* L. (RELATIONSHIP BETWEEN *OsCam1-1* AND *OsNUC1* GENE EXPRESSION IN SALT-STRESS RESPONSE IN RICE *Oryza sativa* L.) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.ศุภจิตรา ชัชวาลย์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.ธีรพงษ์ บัวบูชา, 130 หน้า.

นิวคลีโอลิน 1 (*OsNUC1*) เป็นยีนที่ตอบสนองต่อความเค็มและมีบทบาทในการทนเค็มของข้าว งานวิจัยนี้มุ่งศึกษาถึงกลไกที่เกี่ยวข้องกับการควบคุมการแสดงออกของยีน *OsNUC1*ภายใต้ภาวะเค็ม โดยได้โคลนชิ้นส่วนโปรโมเตอร์ของยีนดังกล่าวจากข้าว 4 พันธุ์/สายพันธุ์ คือ เหลืองประทิว 123 (LPT123) เหลืองประทิว 123 พันธุ์กลาย (LPT123-TC171) ขาวดอกมะลิ 105 (KDML105) และ FL530-IL พบว่าลำดับนิวคลีโอไทด์ในบริเวณโปรโมเตอร์ของยีน *OsNUC1* จากข้าวทั้ง 4 พันธุ์/สายพันธุ์มีบริเวณจดจำของทรานสคริปชันแฟกเตอร์ที่เกี่ยวข้องกับภาวะเครียดจากการขาดน้ำและความเค็มที่สำคัญ 4 ชนิดคือ MYB MYC DRE และ ABRE เมื่อเปรียบเทียบกันระหว่างข้าวแต่ละพันธุ์/สายพันธุ์ พบว่าลำดับนิวคลีโอไทด์ในบริเวณโปรโมเตอร์ของยีน *OsNUC1* ในข้าวพันธุ์ LPT123 และ KDML105 เหมือนกัน แต่แตกต่างจากข้าวสายพันธุ์ LPT123-TC171 และ FL530-IL ซึ่งมีตำแหน่งจดจำ MYB MYC และ ABRE มากกว่าข้าวพันธุ์ทั้งสอง ซึ่งอาจเป็นสาเหตุหนึ่งที่ทำให้ข้าวสายพันธุ์ LPT123-TC171 และ FL530-IL มีการแสดงออกของยีน *OsNUC1* ในภาวะเค็มสูงกว่าข้าวพันธุ์ LPT123 และ KDML105 ต่อมาได้ศึกษาอิทธิพลของยีนสร้างโปรตีนรับสัญญาณแคลเซียมชนิด *OsCam1-1* ต่อการแสดงออกของยีน *OsNUC1* ภายใต้ภาวะเค็มด้วยวิธี RT-qPCR พบว่ายีน *OsCam1-1* มีอิทธิพลในเชิงบวกต่อการแสดงออกของยีน *OsNUC1* ภายใต้ภาวะดังกล่าว ซึ่งชี้แนะว่าสัญญาณแคลเซียม และโปรตีน OsCaM1-1 มีส่วนควบคุมการแสดงออกของยีน *OsNUC1* ในภาวะเค็มด้วย นอกจากนี้ได้ตรวจหายีนสร้างโปรตีนทรานสคริปชันแฟกเตอร์ชนิด R2R3-MYB จากจีโนมข้าว พบยีนสร้างโปรตีนชนิดดังกล่าวที่มีศักยภาพในการจับกับโปรตีนคัลโมดูลิน จำนวน 7 ชนิด ในเบื้องต้นได้เลือกยีนสร้างโปรตีนเหล่านี้มา 4 ชนิดเพื่อทดสอบการเกิดปฏิสัมพันธ์โดยตรงกับโปรตีน OsCaM1-1 ด้วยวิธี yeast two-hybrid assay พบว่าไม่มีปฏิสัมพันธ์ระหว่างกัน

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THANIN CHANTARACHOT : RELATIONSHIP BETWEEN *OsCam1-1* AND *OsNUC1* GENE EXPRESSION IN SALT-STRESS RESPONSE IN RICE *Oryza sativa* L. ADVISOR : ASSOC. PROF. SUPACHITRA CHADCHAWAN, Ph.D., CO-ADVISOR : ASSOC. PROF. TEERAPONG BUABOOCHA, Ph.D., 130 pp.

Nucleolin1 (*OsNUC1*) is a salt stress-responsive gene of which the role in rice is implicated in salt tolerance. The principal objective of this study was to understand the regulation of *OsNUC1* gene expression under salinity stress. Promoter region of *OsNUC1* was cloned from two pairs of rice cultivars/lines; including a salt-sensitive cultivar, Leung Pra Tew 123 (LPT123), and its salt-resistant mutated line, Leung Pra Tew 123-TC171 (LPT123-TC171), as well as a salt-sensitive cultivar, Khao Dawk Mali 105 (KDML105) and its salt-resistant introgression line, FL530-IL. It was found that nucleotide sequence of *OsNUC1* promoter contained important *cis*-regulatory elements that are associated with dehydration/salt responsive gene expression, i. e. MYB and MYC recognition sites, dehydration responsive element (DRE), and ABA-responsive elements (ABREs). Sequence comparison among the four rice cultivars/lines revealed that *OsNUC1* promoter from the cultivar LPT123 and KDML105 were identical. However, They were different from those of LPT123-TC171 and FL530-IL in that the promoter of those lines exhibited greater number of MYB and MYC recognition sites and ABREs. These *cis*-element variations found on the gene promoter might be a cause for the distinct *OsNUC1* expression patterns/levels in response to salinity amongst the rice cultivars/lines. Furthermore, the effect of the salt-stress sensor *OsCam1-1* on *OsNUC1* gene expression under salinity was assessed. It was found that salt stress-responsive expression of *OsNUC1* was positively correlated with *OsCam1-1* transcript levels, suggesting the involvement of Ca^{2+}/calmodulin signaling in regulation of *OsNUC1* gene expression under salinity. Moreover, in this study, putative calmodulin-binding R2R3-MYB transcription factors were identified from the rice genome, seven of which showed potential to interact with calmodulin. Four of the putative R2R3-MYB proteins were selected for a test of interaction with the salt-stress sensor OsCaM1-1 by yeast two-hybrid assay. However, the assay revealed the absence of protein interaction.

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LIST OF ABBREVIATIONS

%	Percent
µg	Microgram
µl	Microliter
µM	Micromolar
3AT	3-aminotriazole
A	Adenine
ABA	Abscisic acid
bp	Base pair
°C	Degree Celcius
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
Ca^{2+}	Calcium ion
Ca^{2+}/CaM	Calcium-bound calmodulin
CaM	Calmodulin
CTAB	Cetyltrimethyl ammonium bromide
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2’-deoxynucleoside 5’-triphosphate
EDTA	Ethylenediamine tetraacetic acid
g	Gram
g	Gravity force
G	Guanine
h	Hour
Kb	Kilobase pair

KDML105	Khao Dawk Mali 105
l	Liter
IPTG	Iso-1-thio-beta-D-thiogalactopyranoside
LB medium	Luria-Bertani medium
LiAc	Lithium acetate
LPT123	Leung Pra Tew 123
LPT123-TC171	Lueng Pra Tew 123 isogenic line
M	Molar (mole per liter)
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MYB	Myeloblastosis viral oncogene homolog
N	Normal
NaOAc	Sodium acetate
ng	Nanogram
nm	Nanometer
ODx	Optical density at x nanometer
OsCam1-1	Rice *Oryza sativa Calmodulin1-1* gene
OsEF-1α	Rice *Oryza sativa Elongation Factor-1 alpha* gene
OsNUC1	Rice *Oryza sativa Nucleolin1* gene
PCR	Polymerase chain reaction
PEG-3350	Polyethylene glycol 3350
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
Sec	Second

SD-Leu/-Trp medium	Synthetic dropout medium lacking Leucine and Tryptophan
SD-Leu/-Trp/-His medium	Synthetic dropout medium lacking Leucine,Tryptophan and Histidine
SD-Leu/-Trp/-Ura medium	Synthetic dropout medium lacking Leucine,Tryptophan and Uracil
SDS	Sodium dodecyl sulfate
rpm	Revolution per minute
T	Thymine
TBE buffer	Tris-borate electrophoresis buffer
TF(s)	Transcription factor(s)
Tris	Tris (hydroxyl methyl) aminomethane
v/v	Volume by volume
w/v	Weight by volume
X-gal	5-bromo-4-chloro-3-beta-D-dalactopyranoside
YPDA medium	Yeast peptone dextrose adenine medium

CHAPTER I
INTRODUCTION

Soil salinity is one of the environmental stresses that impose detrimental effects on land plants worldwide. Infliction of salt-stress to plant cells primarily causes initial phase osmotic shock and later ion toxicity (Munns and Tester, 2008). Additionally, salt stress also leads to secondary oxidative stresses resulting from the production of reactive oxygen species (ROS) (Vinocur and Altman, 2005).

To enable their survival and reproductive success under such harmful conditions, plant species have evolved sophisticated resistant strategies to cope with the salt stress, arising from either escape, avoidance, or tolerance mechanisms (Peleg, Walia, and Blumwarld, 2012). Salt tolerance mechanism primarily relies on the concerted coordination of physiological and biochemical responses beginning at molecular level by transcriptional reprogramming of salt stress-responsive genes (Yamaguchi-Shinozaki and Shinozaki, 2006).

Amongst the cereals, rice (*Oryza sativa* L.) is considered as one of the most sensitive species to salinity (Munns and Tester, 2008), particularly during early seedling and reproductive stages (Suriya-arunroj et al., 2004; Singh et al., 2008). Owing to its significance as staple food consumed worldwide, enormous efforts have been put into understanding the underlying mechanisms of salt stress response/tolerance in rice, with the hope to find key components of salt tolerant traits and make use of them for the genetic improvement. During the past decades, an increasing number of genes that respond to salinity have been identified in rice and their roles in contributing to salt tolerance have later been intensively characterized (Todaka et al., 2012).

Rice *nucleolin1* (*OsNUC1*) was first discovered as a salt stress-associated gene from the differential display experiment in which the gene was found to be up-regulated in the mutated salt-tolerant rice line LPT123-TC171, compared to its original salt-sensitive cultivar LPT123 (Maneeprasopsuk, 2005). Later, it was suggested that expression level of *OsNUC1* under salt-stress condition is positively correlated with salt

tolerance of rice, when the rice lines with salt-tolerant phenotypes, LPT123-TC171 and FL530-IL, showed obviously higher level of the gene transcripts than their original salt-sensitive cultivar, Leung Pra Tew 123 (LPT123) and Khao Dawk Mali 105 (KDML105) (Sripinyowanich, 2010).

Subsequently, characterization of *OsNUC1* functions showed that the gene enhances tolerance to salinity in transgenic *Arabidopsis* with *OsNUC1* overexpression (Sripinyowanich, 2010). Apart from salt tolerance, the *OsNUC1* also promotes growth and development of the transgenic plants under normal condition, which differs from most of stress tolerant genes in that overexpression of those genes frequently results in growth retardation or yield penalty under normal condition (Kasuga et al., 1999; Vannini et al., 2004; Pasquali et al., 2008). For these reasons, *OsNUC1* is considered as a promising target to be used as a tool in rice genetic manipulation for salt-tolerance. While progress has been being made in understanding how the *OsNUC1* contributes to salt tolerance as well as growth promotion, however, mechanisms regarding the gene regulation, either transcriptional control or upstream signal transduction, remain largely unknown.

As regards signal transduction, calcium ions (Ca^{2+}) is a major cellular secondary-messenger that has been reported to play important roles in plant response to salt stress (Kim and Kim, 2006). Upon signal perception, salt stress signal transduction is initiated by the transient elevation of cytoplasmic Ca^{2+} with specific signature. The signal is then decoded by a specific group of Ca^{2+}sensor proteins, which finally leads to the proper response, transcription of downstream specific genes (Mahajan, Pandey, and Tuteja, 2008).

Calmodulin (CaM) is one of the eukaryotic Ca^{2+}-sensor proteins, of which functions have been implicated in a wide variety of physiological processes including salt stress response and tolerance (Snedden and Fromm, 2001; Phean-o-pas, Punteeranurak, and Buaboocha, 2005; Zhang et al., 2009; Xu et al., 2011; Parvin et al., 2012). CaMs lack their own catalytic activities or effector domains. Thereby, upon

binding with Ca^{2+}, they relay particular signals to their target effectors via direct/physical interaction (Snedden and Fromm, 2001).

A role for Ca^{2+}/CaM in transcriptional regulation of salt stress responsive genes has been reported in both direct and indirect manners (Kim et al., 2009). The most common, direct manner is achieved by the interaction of Ca^{2+}/CaM complex and their transcription factor (TF) targets, and the interaction alters functions of the particular TFs (Park et al., 2005; Yoo et al., 2005; Finkler, Padan, and Fromm, 2007). The other, indirect way for Ca^{2+}/CaM to regulate gene expression is by interacting with signaling cascade components such as CaM-binding kinase/phosphatase or TF-binding proteins which serve as regulators of transcription machinery of their target genes (Liu et al., 2007; Liu et al., 2008).

In regard to CaM-binding TFs, Yoo and colleagues (2005) have demonstrated that GmCaM1 and GmCaM4, two divergent salt stress-responsive CaM isoforms in soybean, are able to interact with a salt-associated R2R3-MYB TF, AtMYB2. Moreover, the two CaM isoforms differentially regulate the DNA-binding activity of AtMYB2: GmCaM4 promotes MYB2 target gene activation (*P5CS1*, *ADH1*, and *RD22*), whereas GmCaM1 inhibits its DNA-binding activity. As Ca^{2+}/CaM regulation of MYB-mediated gene expression is well characterized in dicotyledonous *Arabidopsis*, the question of whether this mechanism is also present in monocot rice species remains unanswered.

Amongst rice CaMs, *OsCam1-1* and *OsCam1-3* expression has been reported to be induced by dehydration and salt stresses (Phean-o-pas et al., 2005). Also, *OsCam1-1* shows higher expression in the salt-resistant rice line, FL530-IL, than in the salt-sensitive KDML105 cultivar under salinity (Saeng-ngam et al., 2012). Moreover, overexpression of *OsCam1-1* results in enhanced accumulation of abscisic acid (ABA) content in transgenic rice which in part results from the up-regulation of *ABA addehyle oxidase* (*OsAAO)* and *9-cis-epoxycarotenoid dioxygenase* (*OsNCED*), the genes encoding for enzymes in ABA biosynthesis pathway. However, what links the *OsCam1-1* with ABA biosynthesis pathway or the *OsCam1-1* interacting partners have never been unraveled.

Objectives of this study:

1. To clone and characterize the *OsNUC1* promoter from different rice cultivars/lines, including LPT123, LPT123-TC171, KDML105, and FL530-IL.
2. To determine the effect of *OsCam1-1* on *OsNUC1* expression under salt-stress condition.
3. To determine the physical interaction between the salt-stress sensor, OsCaM1-1, and putative CaM-binding R2R3-MYB TFs in rice.

CHAPTER II
LITERATURE REVIEWS

1. Rice

Domesticated from the wild glass, *Oryza rufipogon* Griff., 10,000 to 14,000 years ago (Zeigler and Barclay, 2008), rice today (*Oryza sativa* L.) belongs to the tribe Oryzeae under the sub-family Pooideae in the family Poaceae (or Gramineae). It is a diploid species (AA) with 24 chromosomes (Chang, Berdernas, and Rosario, 1965).

1.1. Botanical characteristics

In general, rice is considered a semi-aquatic annual grass, even though in the tropics it is capable of surviving as a perennial grass, generating new tillers from nodes. The mature plant consists of a major stem and several tillers. Each reproductive tiller produces a terminal flowering head (panicle). Their heights are in a range of 40 cm - 5 m, varying according to cultivars and environmental conditions. The morphology of rice is separately described between vegetative and reproductive organs (Maclean et al., 2002).

For vegetative organs, the roots are fibrous root system with rootlets and root hairs. The adventitious crown roots are developed from the underground nodes of the young culms and they are freely branched. Roots that originate from nodes above the soil surface are referred to as nodal roots. The culm, jointed stem of rice, is made up of a series of nodes and internodes. The node generates a leaf and bud. The mature internode is hollow and finely grooved. The internodes of a culm vary in range, generally increasing from the lower internode to the upper ones. The leaves are borne on the culm in two ranks, one at each node. The leaf consists of the sheath and blade. Auricles, ear-like appendages, are paired and borne on either side of the blade base. There is a membranous ligule at the junction of the blade and sheath on the inside. The number of leaves is highest in the main culm and decrease progressively with the rise in tillering order (Chang et al., 1965).

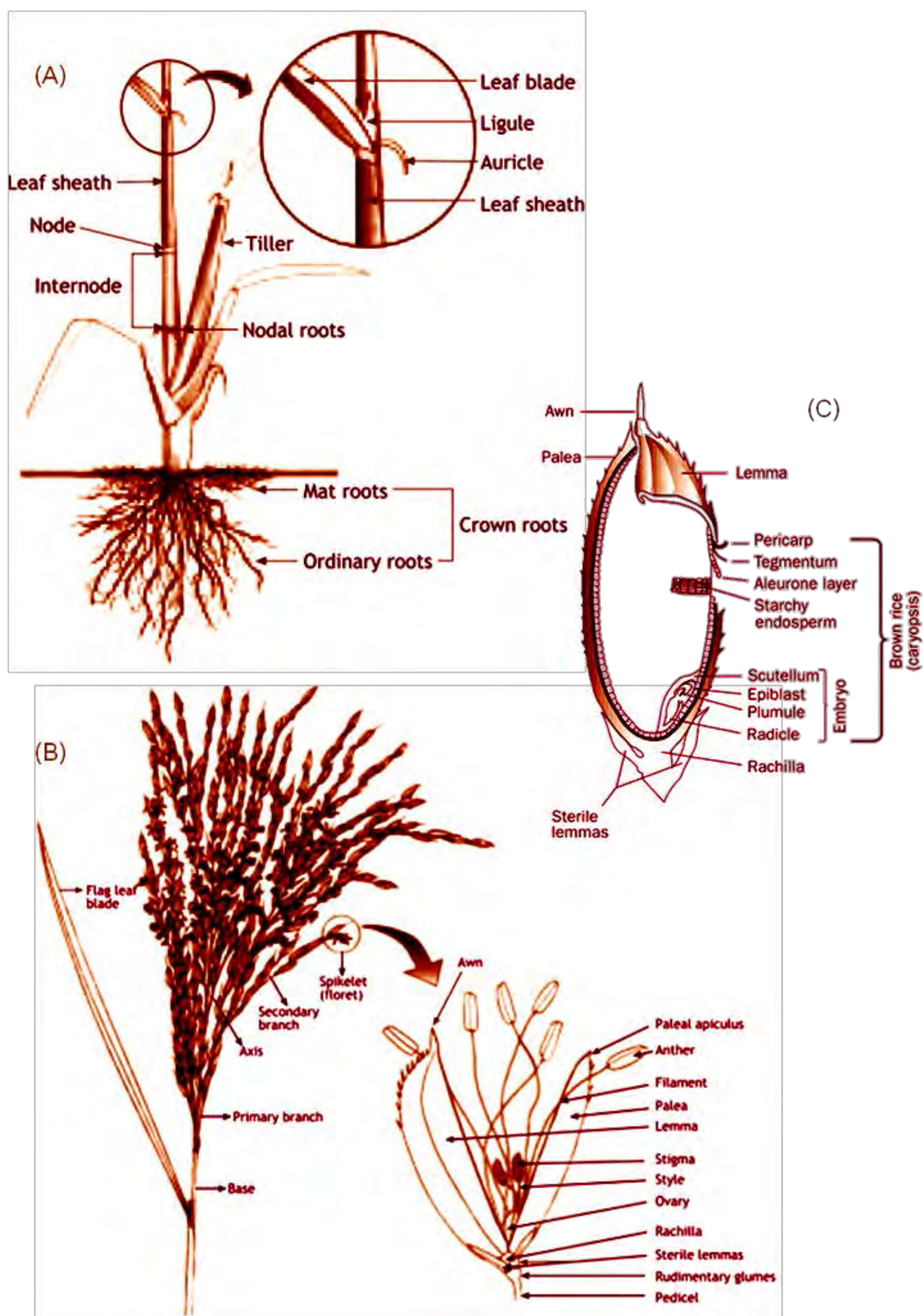


Figure 2.1 Morphology of rice (*Oryza sativa* L.)

A. vegetative organs, B. reproductive organs, C. cross section of the rice grain (Maclean et al., 2002)

For reproductive organs, the panicle is produced on the uppermost internode of the culm. It is a racemose type of branching, in which each node on the main axis gives rise to the primary branches and each of which in turn bears the secondary branches. The secondary branches produce the pediceled spikelets. The spikelet consists of two lower glumes, 2- ranked, sterile, and shorten than lemma and palea; each floret with lemma and palea, 6 stamens and 2- celled anthers, slender filament, superior ovary; bifurcate style and plumose stigma ; one ovule. (Chang et al., 1965).

The grain of rice is comprised of true fruit or brown rice (caryopsis) and hull. The brown rice has embryo and endosperm, which is surrounded with aleurone layer, tegmentum and pericarp, respectively. Palea, lemmas and rachilla constitute a hull of *indica* rice whereas a hull of *japonica* rice usually includes rudimentary glumes and perhaps a portion of the pedicel. Grain length, width, and thickness vary widely among rice cultivars. Hull weight averages about 20% of total grain weight (Maclean et al., 2002).

1.2. The importance of rice

As staple food, rice is one of the leading crops in the world, providing 21% of global human per capita energy and 15% of per capita proteins (Maclean et al., 2002). Rice consumption exceeds 100 kg per capita annually in several countries and it is the main food for most of the world's poorest people, especially in Asia. It is predicted that the demand for global production of rice in 10 years will be 80-100 million tons more than today's production, 600 million tons (Zeigler and Barclay, 2008). In addition to its dietary importance, therefore, it is unarguable that rice is also tightly tied with not only today's but also increasingly tomorrow's global food security and socioeconomic stability.

Apart from its social significance, rice manifests promise as an excellent model plant for monocot studies. Rice offers important advantages for basic research because of its small genome size, self fertilization, comparatively short life cycle, diploid

nature, transformability, close relationship to other important crops, and the availability of genetic and molecular resources. Rice also represents the only case where two subspecies (*japonica* and *indica*) have been sequenced and are publicly available (Jia, Clegg, and Jiang, 2004; Jung, An, and Ronald, 2008: Todaka et al., 2012).

1.3. Rice in Thailand

Rice is both culturally and economically important to Thailand. In economic aspect, Thailand is the world's top exporter, with 30.8 % market share in 2011 (International Rice Research Institute [IRRI], 2012: online). It is the fifth largest rice cultivator in the world, with 55% of its arable land being used for rice cultivation (IRRI, 2012: online). Also, rice is the staple food of population across the country. In cultural aspect, rice is deeply assimilated into the Thai culture, such as in the way of greeting, or explicitly the presence of ceremonies invoking rain and plentiful harvest performed by rice farmers before growing seasons.

Thai aromatic rice is well known for its aroma and soft texture cooked long-grain, making it desirable and popular worldwide. Khao Dawk Mali 105 (KDML105) is one of Thai Jasmine rice cultivars, of which fragrance smells like *Pandanus* leaf or bread flower. The cultivar KDML105 is a photo-sensitive variety, could be planted only once a year. It is also known as salt-sensitive, giving low yield and poor grain quality when it is grown under saline soils (Suriya-arunroj et al., 2004: Summart et al., 2010). The cultivation of KDML105 is mainly in the rainfed areas of Northeastern and Northern Thailand (Rice Department of Thailand, 2010). However, owing to geological characteristics of the Northeast that contain underground rock salt (NaCl), the areas over 2.8 million ha of Khorat basin (17% of the region) are affected by soil salinity, especially during dry season in which evaporation exceeds precipitation (Arunin, 1992). It has been shown that approximately 75% of the salt-inflicted lands in the region are under rainfed rice cultivation (Arunin, 1992) and the inflicted areas have been expanding primarily because of human activities including deforestation, irrigation, salt-

making, and construction of canals, reservoirs, and roads. Thus, rice production in the region is mainly limited by yield loss as a result of salt stress.

1.4. Rice as a salt-sensitive species

Rice is classified as a salt-susceptible species (Munns and Tester, 2008). Generally, the threshold for salt stress in rice is 3 dS/m, with a 12% of yield decrease per dS/m beyond this value (Maas, 1990). The sensitivity to salt stress of rice also depends on varieties and developmental stages (Khan, Hamid, and Karim, 1997; Lutts, Kinet, and Bouharmont, 1995; Shannon et al., 1998; Zeng and Shannon, 2000). Rice is tolerant to salt during germination, and then becomes susceptible at seedling stage. During vegetative growth, rice is relatively tolerant to salt. However, it becomes salt-sensitive again while entering reproductive phase. Salt stress primarily affects yield components of rice including panicle length, spikelet number per panicle, and grain quality (Zeng and Shannon, 2000). Furthermore, panicle initiation and flowering time are also delayed, and pollen viability is reduced as a result of salinity stress (Khatun and Flowers, 1995).

2. Salinity

According to Munns and Tester (2008), salinity is a soil condition characterized by a high concentration of soluble salts, which mostly refers to sodium chloride (NaCl) because it is the most soluble and abundant salt released. Soils are defined as saline when the electrical conductivity of the saturated paste extract (ECe) is 4 dS/m or more, which is equal to approximately 40 mM NaCl and creates an osmotic pressure of around 0.2 MPa. This definition of salinity derives from the ECe that significantly reduces the yield of most crops. As regards salinity, it not only constraints crop production but also leads to steady deterioration of soil and irrigation water, which subsequently affects crop plants in later generations(Bressan, Bohnert, and Hasegawa, 2008).

2.1. Salinity stress in plants

Salinity causes two main consecutive stresses to plant cells, namely the first, immediate osmotic stress because of high solute concentration in the soil and the second, ion-specific stresses owing to alteration of potassium/ sodium ion ratios (K^+/Na^+) as well as accumulation of toxic Na^+ and chloride (Cl^-) ions in the plant cells (Negrao et al., 2011).

2.1.1. Plant response to osmotic stress

In response to osmotic stress which occurs once the salt concentration around the roots reaches threshold level, plants manifest a rapid and temporary drop in shoot growth rate. New leaves emerge more slowly and the rate at which leaves expand is reduced. Lateral buds develop more slowly or remain quiescent, hence reducing the formation of new lateral shoots (Munns and Tester, 2008). In cereal species, the main effect of osmotic stress is a reduction in the number of tillers. In addition, osmotic stress leads to abscisic acid (ABA) accumulation which leads to stomatal closure (Negrao et al., 2011).

2.1.2. Plant response to ionic stress

Accumulation of toxic ions in leaf tissues leads to senescence of the old leaves. If the rate of old leaf senescence is greater than the production of new leaves, photosynthetic capacity of the plants will not be able to supply their carbohydrate requirement, further leading to the decline in their growth rate (Munns and Tester, 2008).

2.2. Cellular responses to salinity

2.2.1. Osmotic adjustment

To prevent cellular dehydration, protein denaturation and destabilization of cellular structures under salinity stress, plant cells accumulate nontoxic osmotically active compounds, or compatible solutes, in the cytoplasm. These compounds help adjust osmotic potential between the cytosol and apoplastic solution by lowering the cytosol water potential (Singh et al., 2008). A number of metabolites, such as sugars (glucose, fructose and sucrose), complex sugars (trehalose, raffinose

and fructans), sugar alcohols (mannitol and glycerol) and amino acid and derivatives (proline, glycine-betaine, and proline-betaine) have been reported to serve this function (Bohnert, Nelson, and Jensen, 1995; Flowers and Colmer, 2008).

2.2.2. Na^+ exclusion and K^+ homeostasis

To reconstruct the cellular ion homeostasis which is required for cellular enzyme activities and plant growth under salt stress, plants employ three main strategies for the maintenance of high cytosolic K^+/Na^+ concentration ratio. These strategies include exclusion of Na^+ entry, extrusion of Na^+ out of the cells, and vacuolar compartmentalization of Na^+ (Negrao et al., 2011).

2.2.3. Regulation of antioxidant system

Reduced availability of CO_2 as a result of stomatal closure under salt stress condition leads to electron transfer from photosystem I to oxygen, forming O_2^- which subsequently produces more cytotoxic reactive oxygen species (ROS). Over-production of harmful ROS causes oxidative damages to a wide range of cellular components, such as membrane lipids, nucleic acids and proteins (Mittler, 2002). To cope with such damages, plants up-regulate not only non-enzymatic antioxidant compounds such as ascorbic acid and reduced glutathione, but also antioxidant enzymes such as catalase, superoxide dismutase, glutathione-*S*-transferase, etc (Sairam and Tyagi, 2004).

2.3. salinity-induced changes in gene expression in plants

There is tremendous evidence indicating that changes in gene expression occur in plants during an exposure to salt stress (Kawasaki et al., 2001; Krebs et al., 2002). A large number of salt-responsive genes have been identified and functionally characterized. Salt-responsive genes are thought to play a role not only in protecting the cells from damages, but also in the regulation of genes for signal transduction in stress response and tolerance (Shinozaki and Yamaguchi-Shinozaki, 1997). The functions of these gene products are categorized into two major groups. The first group is involved in stress tolerance including water channel proteins, enzymes for the biosynthesis of compatible solutes, proteins that protect other macromolecules and membranes and

detoxification enzymes, etc. The second group is comprised of proteins that are associated with further regulation of signal transduction and gene expression for stress responses. This group includes transcription factors (TFs), protein kinases, and enzymes for lipid metabolism (Yamaguchi-Shinozaki and Shinozaki, 2005). There are many transcriptional regulatory systems involved in stress-responsive gene expression, depending on different sets of transcription factors and their corresponding *cis*-elements on the promoter of target genes (reviewed by Yamaguchi-Shinozaki and Shinozaki, 2006).

3. Calcium and salt stress signaling

Calcium ion (Ca^{2+}) is a major secondary signaling molecule playing crucial roles in plant growth and development, not only under normal but also stress conditions (Mahajan et al., 2008). After perception of the signal by membrane receptors, stress signaling pathway for stress tolerance is initiated by a specific spatio-temporal pattern of cytosolic Ca^{2+} perturbation (Tracy et al., 2008). The unique signal is then precisely decoded by specific Ca^{2+} sensor proteins, resulting in further signal relay or appropriate responses, such as regulation of salt-stress responsive gene expression.

Ca^{2+} sensor proteins are generally classified as either sensor relays or sensor responders (Sanders et al., 2002). On the one hand, sensor responder proteins such as Ca^{2+}-dependent protein kinases (CDPKs) contain both sensing and catalytic/effector domains. On the other hand, sensor relays such as calcineurin B-like proteins (CBLs) and calmodulins (CaMs) contain only Ca^{2+} binding, sensing domains, lacking other effector domains. To transmit the Ca^{2+} signal, sensor relays must hence interact with target proteins and regulate their activities (Dodd, Kudla, and Sanders, 2010).

3.1. Calmodulin

Calmodulin (CaM) is a small acidic Ca^{2+} sensor protein (16-18 kD) present in all eukaryotes. The CaM prototype is composed of 148 amino acids arranged in two globular domains linked with a long flexible helix. Each globular domain contains

a pair of intimately connected EF hands. One EF hand motif comprises a specialized helix-loop-helix structure that binds to a single molecule of Ca^{2+} (Bouche et al., 2005).

CaM has no catalytic or effector domain. It relays signal by physically interacting with its target proteins. By either using bioinformatics tools or screening cDNA expression libraries, a number of CaM targets with diverse functions have been unraveled from plant species (Liao and Zielinski, 1995; Reddy, Ali, and Reddy, 2002; Choi et al., 2005). Interestingly, despite the high amino acid sequence conservation among CaMs, CaM-binding site of their targets does not show a correspondingly strong sequence homology (Rhoads and Friedberg, 1997). The majority of known target sites for CaM interaction are generally composed of an amino acid stretch of 12-30 contiguous residues with positively charged amphiphilic characteristics and propensity to form an α-helix upon binding to CaM (Snedden and Fromm, 2001). Accordingly, Rhoads and Friedberg (1997) have classified the sequence motifs for calmodulin recognition into three classes. One is a class of Ca^{2+}-independent CaM-binding motif. The other two are 1-8-4 and 1-5-10 types of Ca^{2+}-dependent binding motifs, of which the names are designated based on the position of conserved hydrophobic residues essential for the hydrophobic interaction with CaMs.

3.2. Calmodulin-binding Transcription factors

A number of TFs from different families are known to be interacting targets of CaMs. For instance, CaM-binding transcription activator (CAMTA), AtGT-2 (GT-element-binding protein/ GTL), MYB TF, zinc-finger-type WRKY TF, NAC domain-containing CBNAC/NT9 TF, bZIP (TGA) TF, Scarecrow/Scarecrow-like TF, MADS-box (AGL) protein, no apical meristem (NAM) TF, auxin-responsive protein (SAUR_B) and AUX/IAA TF families (Park et al., 2005; Yoo et al., 2005; Finkler et al., 2007; Kim et al., 2007; Popescu et al., 2007; Galon, Finkler, and Fromm, 2010). They are involved in almost all biological processes, some of which is associated with salt stress response and tolerance.

AtGTL2, a member of plant specific GT-2 subfamily of GT factors, is a Ca^{2+}/CaM-binding TF involved in plant response to cold and salt stress. AtGTL2 interacts with CaM in Ca^{2+}-dependent manner, with the CaM-binding motif located in its carboxyl-terminal DNA-binding domain. *AtGTL2* expression is induced by cold, NaCl, and ABA treatments. Overexpression of *AtGTL2* contributes to up-regulation of the cold- and salt-inducible marker genes, *RD29A* and *ERD10* (Xi et al., 2012).

AtMYB2, a transcriptional regulator of salt- and dehydration-responsive genes (Abe et al., 1997; Hoeren et al., 1998; Yoo et al., 2005), is known as one of the CaM-binding R2R3 MYB TFs. Two divergent salt-upregulated soybean CaM isoforms, GmCaM1 and GmCaM4, are reported to bind with AtMYB2 in Ca^{2+}-dependent manner. The motif for CaM interaction of AtMYB2 is classified as the 1-5-10 type as it contains hydrophobic residues; leucine, valine, and isoleucine at position 1, 5, and 10, respectively. Moreover, binding with GmCaM4 promotes AtMYB2 DNA-binding activity. In contrast, GmCaM1 inhibits this activity. Furthermore, overexpression of *GmCaM4* results in the up-regulation of AtMYB2 target genes, including *P5CS*, *ADH1* and *RD22*, which finally leads to enhanced salt tolerance in the transgenic plants (Yoo et al., 2005). Apart from AtMYB2, other R2R3-MYB TFs are also reported to interact with CaMs. For example, *Arabidopsis* MYB62 and MYB78 interacted with both GmCaM1 and GmCaM4 *in vitro* (Yoo et al., 2005).

In addition, a protein microarray experiment using *Arabidopsis* CaMs and calmodulin-like proteins (CMLs) as probes identifies R2R3-MYB members, AtMYB14, AtMYB70 and TRF-like7, as CaM-interacting partners (Popescu et al., 2007).

3.3. Rice calmodulin

There are five and thirty-two CaM- and CML-encoding genes, respectively, in the rice genome (Boonburapong & Buaboocha, 2007). Rice CaM isoforms are implicated in salt stress signaling and tolerance (Kawasaki et al., 2001; Phean-o-pas et al., 2005: Saeng-ngam et al., 2012), one of which is OsCaM1-1. Expression of *OsCam1-1* is induced by salinity. Comparison of *OsCam1-1* gene

transcript between the salt sensitive rice, KDML105, and its salt-tolerant isogenic line, FL530-IL, indicates that salt stress-responsive expression of *OsCam1-1* in the FL530-IL line is more rapid and much higher than in the KDML105. Comparing to the wild type and the control transgenic line, overexpression of *OsCam1-1* in KDML105 leads to higher accumulation of ABA in the transgenic rice both in normal and salt stress conditions. Higher ABA accumulation in the *OsCam1-1* overexpressors is correlated with the elevated expression of ABA biosynthesis genes, *ABA aldehyde oxidase* (*AAO*) and *9-cis-epoxycarotenoid dioxygenase* (*NCED*). Concomitantly, the transgenic lines overexpressing *OsCam1-1* gene display enhanced tolerance to salinity (Saeng-ngam et al., 2012).

Moreover, a novel CML-encoding gene, *OsMSR2*, is isolated from the rice cultivar Pei'ai 64S. Expression of *OsMSR2* is strongly up-regulated by a wide spectrum of stresses, including cold, drought, and heat. Expression of *OsMSR2* confers enhanced tolerance to high salt and drought in *Arabidopsi*s together with altered expression of stress and ABA-responsive genes (*RD29A*, *P5CS1*, and *ABI3*). The transgenic plants also exhibit hypersensitivity to ABA (Xu et al., 2011).

4. Nucleolin

4.1. General characteristics

Nucleolin is one of the most abundant non-ribosomal proteins in the nucleolus. It is the major component, accounting for about 10%, of all the nucleolar proteins (Bicknell et al., 2005). On the one hand, nucleolin is stable in actively proliferating cells. On the other hand, it undergoes auto-degradation in quiescent cells (Chen, Chiang, and Yeh, 1991; Pederson, 1998). Nucleolin is primarily responsible for rRNA synthesis and the biogenesis of ribosome. In addition, it is also implicated in many biological processes including cell growth and proliferation, cell cycle regulation, nucleo-cytoplasmic transport, chromatin structure, gene silencing, rDNA transcription, rRNA maturation as well as mRNA stabilization (Ginisty et al., 1999; Olson, Dundr, and Szebeni, 2000; Zhang, Tsaprailis, and TimBowden, 2008).

Nucleolin can be found in all eukaryotes, ranging from yeast to plant and human (Lee, Xue, and Melese, 1991; Martin et al., 1992; Tuteja et al., 1995). Even though nucleolin from yeast and plants shows the similar structural organization with those from animals, it is found that they share low sequence homology. Therefore, yeast and plant nucleolins are classified as nucleolin-like proteins (Tajrishi, Tuteja, and Tuteja, 2011).

4.2. Nucleolin structure

The primary sequence of nucleolin is generally comprised of three domains with different structures and functions, including amino-terminal (N-terminal), central, and carboxyl-terminal (C-terminal) domains (Ginisty et al., 1999) (Figure 2.2).

The N-terminal portion of nucleolin is comprised of several acidic sequence repeats interspersed with basic segments. The number of acidic stretches varies from species to species. These acidic residues serve as phosphorylation sites by casein kinase 2 (CK2) and cyclin dependent kinase 1 (CDK1) (Mongelard and Bouvet, 2006). The sequence downstream of acidic stretches is a bipartite nuclear localization signal (NLS), responsible for targeting the protein to nucleus (Tong et al., 1997). However, it is found that the N-terminus is not crucial for nucleolar accumulation of nucleolin (Tuteja and Tuteja, 1998).

The central domain of nucleolin contains RNA recognition motifs (RRMs), or consensus RNA-binding domain (CS-RBD) (Serin et al., 1997). The role of RRMs is involved in heterogeneous RNA packaging, pre-mRNA splicing, pre-ribosome assembly, poly (A) tail synthesis and maturation, transcriptional control and mRNA stability (Bourbon et al., 1983; Amrein, Gorman, and Nothiger, 1988; Dreyfuss et al., 1993; Zhang et al, 1993; Naranda et al., 1994). This domain is also found to interact and specifically bind with short RNA stem-loop structure of 18S and 28S ribosomal RNA (Serin et al., 1997).

The C-terminal proximal region of nucleolin is composed of glycine- and arginine-rich (GAR) repeat segments. The length of GAR domain is variable among

species (Bogre et al., 1996). This domain facilitates the interaction of nucleolin RRMs with targets located within large and complex RNA, including rRNA (Heine, Rankin, and Dimario, 1993). The GAR domain tends to primarily function in protein-protein interactions, such as the interaction with ribosomal proteins (Bouvet et al., 1998).

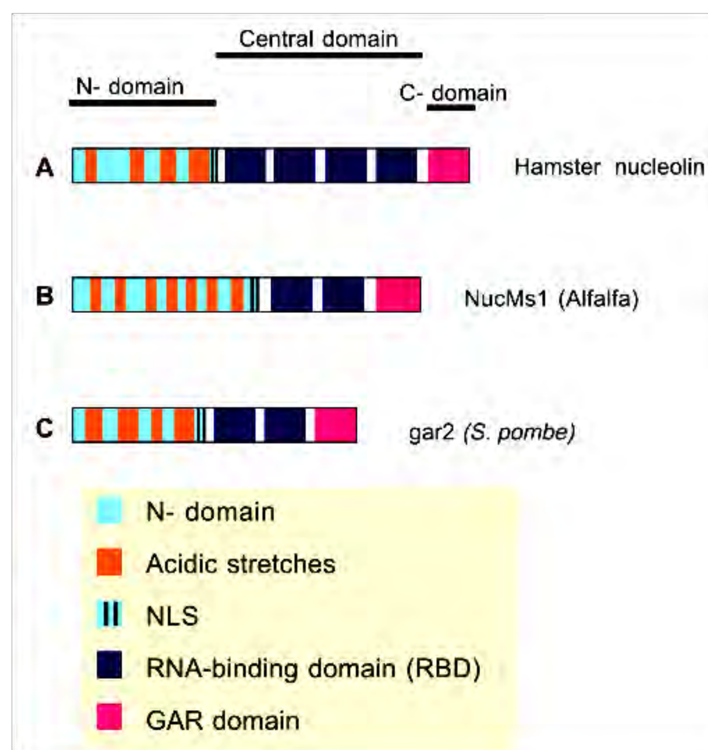


Figure 2.2 Schematic representations for structural organization of nucleolin and nucleolin-like proteins (Ginisty et al., 1999)

(A) Organization of animal nucleolin (hamster)

(B) Organization of plant nucleolin-like proteins (alfalfa)

(C) Organization if yeast nucleolin-like proteins

(Gar2 of *Saccharomyces pombe*)

4.3. Plant nucleolin-like protein

Plant nucleolin-like protein was first identified in onion root meristem. It is involved in chromatin decondensation process, rDNA transcription and early steps of pre-rRNA processing (Martin et al., 1992).

In alfalfa, expression of *Nucleolin* is up-regulated at G1 phase of cell cycle and the protein level corresponds with cell proliferation (Bogre et al., 1996).

In pea, *Nucleolin* transcript is responsive to light. *Nucleolin* transcription is induced by red light treatment at 1 h before rRNA synthesis and correlates with increased cell division rates (Tong et al., 1997).

Arabidopsis genome contains two nucleolin-like proteins, namely AtNUC-L1 and AtNUC-L2. The *AtNUC-1L* is solely expressed in normal condition, responsible for normal growth and development. Disruption of *AtNUC-L1* affected plant growth and development; *Atnuc-L1* mutants grow slower than the wild type, their newly-emerged leaves are small, pointed, irregular and scrunched. Morphological aberrations are also observed in flower, sepals, anthers and siliques of the *Atnuc-L1* mutants (Pontvianne et al., 2007).

Rice (*Oryza sativa* L.) genome contains two nucleolin-like protein-encoding genes, i.e. *OsNUC1* and *OsNUC2*. A study on *OsNUC1* expression indicates that the gene transcript is mainly detected in roots, flowers, and seeds (Sripinyowanich, 2010). Moreover, *OsNUC1* expression is found to be induced by salinity stress. Comparison of gene expression under salt stress shows that *OsNUC1* transcript levels in salt-tolerant rice lines, LPT123-TC171 and FL530-IL, are higher than in their isogenic salt-susceptible cultivars, LPT123 and KDML105. Interestingly, genome database reports two species of *OsNUC1* cDNAs. One, the longer species, encodes for all of the three domains, while the other species encodes for only the central and C-terminal domains. Overexpression of the latter, comprising of the central and C-terminal domains, in *Arabidopsis* shows that root growth and leaf number of the transgenic plants are greater than the wild type both in normal and salt-stress conditions. Furthermore, endogenous salt stress responsive genes, *AtP5CS1* and *AtSOS1*, are up-

regulated in the transgenic plants, suggesting a role for *OsNUC1* in salt stress response and tolerance (Sripinyowanich, 2010).

5. MYB transcription factors

5.1. Structure and classification

MYB genes comprise one of the largest transcription factor (TF) families in plants. They are named according to the first identified *v-MYB* oncogene of avian myeloblastosis virus (Du et al., 2009). The MYB family is defined by the presence of a highly conserved MYB DNA-binding domain. This domain is generally comprised of one to four imperfect amino acid sequence repeats (R), each of which usually consists of 50-53 amino acids with regularly spaced tryptophan residues, and builds three α-helices. In each repeat, the second and third helices form a helix-turn-helix (HTH) structure (Figure 2.3). The third helix of each repeat serves as “recognition helix”, responsible for DNA interaction (reviewed by Dubos et al., 2010).

The members of MYB family are classified into four classes according to the number of their adjacent repeats (R) (Figure 2.3). The three repeats of c-Myb, which is a prototype of MYB proteins, are referred to as R1, R2, and R3. Repeats of other MYB proteins are designated according to their similarity to the R1, R2 or R3 of c-Myb. All four classes have been identified in plants. Four-repeat-containing, 4R-MYB is the smallest group, usually present in only single gene in each species. Three repeats, R1R2R3-class (3R-MYB) usually contains five members (Stracke, Weber, and Weisshaar, 2001; Yanhui et al., 2006; Matus, Aquea, and Arce-Johnson, 2008). They have been detected in most eukaryotic genomes, representing a conserved class mostly responsible for cell cycle control (Ito, 2005). One repeat-containing 1R-MYB and MYB-related genes are diverse, and can be further classified into many subclasses (Rosinski and Atchley, 1998). They are associated with the control of secondary metabolism and cellular morphogenesis (Simon et al., 2007; Matsui, Umemura, and Ohme-Takagi, 2008). The last class, two-repeat-containing R2R3 MYB proteins, constitutes the largest group of MYB TFs, with 126 and 109 members in *Arabidopsis*

and rice, respectively (Stracke et al., 2001; Yanhui et al., 2006). This class is believed to evolve from an R1R2R3-MYB gene ancestor by losing the R1 repeat and subsequently expanding of the gene family (Rosinski and Atchley, 1998).

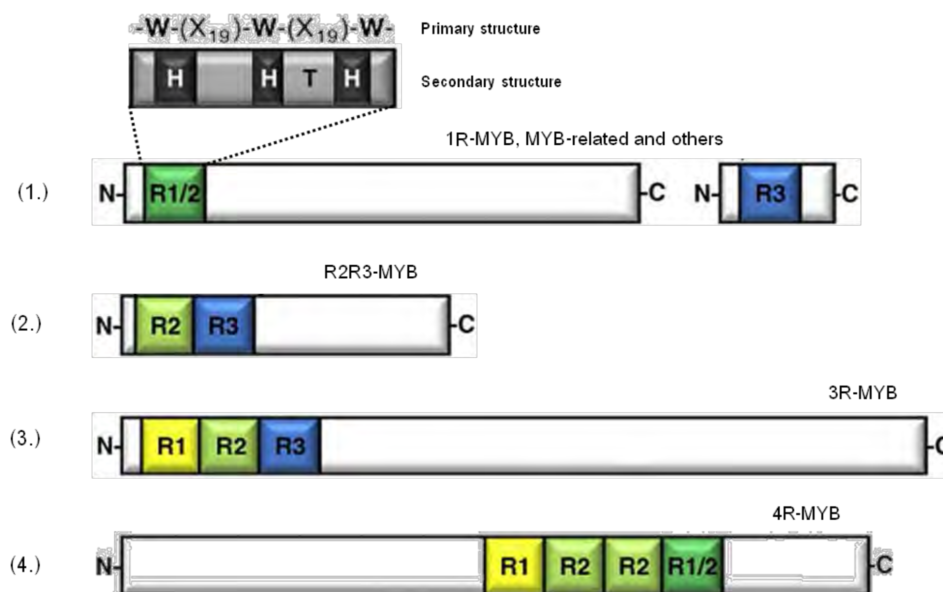


Figure 2.3 Schematic representations for structural organization of plant MYB TF classes (Dubos et al., 2010)

(1) One repeat-MYB-domain containing, 1R-MYB and MYB-related proteins

(2) Two repeat-MYB-domain containing, R2R3-MYB TFs

(3) Three repeat-MYB-domain containing, 3R-MYB TFs

(4) Four repeat-MYB-domain containing, 4R-MYB TFs

The primary and secondary structures of the MYB domain are present. H, helix; T, turn; W, tryptophan; X, amino acid (X).

5.2. R2R3-MYB Transcription factors

5.2.1. Structure

The members of R2R3-MYB class have a modular structure, with an N-terminal MYB DNA-binding domain and an activation/repression domain embedded on C-terminus. Other regions other than the MYB domain are highly variable, even in the proteins with similar functions (Matus et al., 2008). R2R3-MYB TFs in *Arabidopsis* have been classified into functional subgroups according to the conservation of the MYB domain and of sequence motifs in their C-terminal regions (Stracke et al., 2001).

5.2.2. Functions

R2R3-MYB proteins are associated with plant-specific processes; including (1) the regulation of primary and secondary metabolism such as flavonol, tannin and anthocyanin biosynthesis (Stracke et al., 2007; Gonzalez et al., 2008; Gonzalez et al., 2009), (2) control of cell fate and identity such as trichome initiation and root hair patterning (Kang et al., 2009), (3) regulation of plant development by controlling shoot morphogenesis and leaf patterning (Byrne et al., 2000), as well as (4) plant responses to abiotic and biotic stresses including ABA signaling and hypersensitive cell death (Cominelli et al., 2005; Raffaele et al., 2008; Li et al., 2009).

5.2.3. R2R3-MYB TFs and abiotic stress response and tolerance

The functions of R2R3 MYB TFs have been well characterized in the plant model *Arabidopsis*, and many of them are associated with abiotic stress signaling pathways, including dehydration, salinity, and low temperature (Yamaguchi-Shinozaki and Shinozaki, 2005). The followings are examples of R2R3-MYB genes that play a role in dehydration/salt stress;

AtMYB2 gene expression is up-regulated by dehydration, salt stress, and ABA treatment. The AtMYB2 protein specifically binds to a consensus MYB recognition sequence (5'-TAACTG-3') (Urao et al., 1993) as well as GT-motif (5'-TGGTTT-3') in the promoter of *ADH1* (Hoeren et al., 1998). AtMYB2 together with a basic-helix-loop-helix MYC2 functions as a transcriptional activator in the dehydration- and ABA-inducible expression of *rd22*, through MYB recognition site on the gene promoter (Abe et al., 1997; Abe et al., 2003). Overexpression of *AtMYB2* alone as well

as *AtMYB2* together with *AtMYC2* leads to ABA hypersensitivity in transgenic plants, and enhanced ABA-induced expression of *rd22* and *ADH1*. Moreover, the transgenic plants overexpressing both *AtMYB2* and *AtMYC2* show increased expression of several ABA-inducible genes (Abe et al., 2003).

HOS10, considered as an R2R3-MYB protein-encoding gene found in *Arabidopsis*, is an important coordinator for plant growth and development and abiotic stress response. *hos10-1* mutants exhibit developmental alteration such as early flowering and reduction in plant size and fertility. In the same time, the mutant is extremely sensitive to freezing temperature and hypersensitive to NaCl. Also, induction of *NCED3* by dehydration as well as ABA accumulation are reduced by this mutant (Zhu et al., 2005).

MYB15 is a part of transcriptional network controlling the expression of CBFs and other genes in response to cold stress. The *MYB15* gene transcript is up-regulated by cold stress. MYB15 protein interacts with a bHLH-type transcription factor, and binds to MYB recognition sequence in the promoters of CBF genes. The *myb15* mutant shows increased expression of CBF genes and is tolerant to freezing stress, whereas its overexpression results in reduced expression of CBF genes and freezing tolerance (Agarwal et al., 2006). Furthermore, *AtMYB15* is involved in the regulation of ABA biosynthesis, signaling, and responsive genes (*ABA1*, *ABA2*, *ABI3*, *ADH1*, *RD22*, *RD29B* and *EM6*). Its transcript is highly up-regulated by ABA, drought, and salt treatment. Overexpression of AtMYb15 leads to improved survival and reduced water loss under dehydration, and contributes to salt tolerance (Ding et al., 2009).

AtMYB44 transcript is induced by ABA treatment as well as various abiotic stresses including dehydration, low temperature, and salinity. Transgenic *Arabidopsis* with *AtMYB44* overexpression is more sensitive to ABA, and shows more rapid ABA-induced stomatal closure response than wild type plants, thus reducing the rate of water loss and increasing tolerance to drought and salinity. The function of *AtMYB44* in drought/salt tolerance is thought to confer by transcriptional suppression of

a group of serine/threonine protein phosphatase 2C genes which are negative regulators of ABA signaling (Jung et al., 2008).

AtMYB96 is a molecular link between ABA and auxin signaling during drought stress, and control lateral root growth as an adaptive strategy under dehydration. *Arabidopsis AtMYB96* overexpressors exhibit enhanced drought tolerance, while the gene knock-out mutant is more sensitive to drought when compared to the wild type (Seo et al., 2009).

AtMYB102 is reported to be an integrator of wounding and osmotic stress signals. Transcription of *AtMYB102* is rapidly induced by ABA, osmotic stress as well as wounding. 5'-Untranslated region of the gene which contains W-box and MYB-binding element is essential for osmotic-and wounding-induced expression (Denekamp and Smeekens, 2003).

In resurrection plant *Craterostigma plantagineum*, *CpMYB10* up-regulation is specifically under dehydrated status and by exogenous ABA application. The recombinant CpMYB10 protein binds to mybRE elements within the *LEA*, *Cp11-12*, and *CpMYB10* promoters. Overexpression of the gene in *Arabidopsis* indicates that *CpMYB10* mediates stress tolerance, and alters ABA and glucose signaling (Villalobos, Bartels, and Iturriaga, 2004).

In barrel medic, *Medicago truncatula*, a number of R2R3-MYB genes such as *MtMYB119*, *MtMYB634*, *MtMYB636* and *MtMYB1070* are rapidly up-regulated in root apexes by salt stress (Gruber et al., 2009).

Amongst 156 MYB-encoding genes in soybean genome, transcript levels of 43 MYB genes are altered by ABA treatment, salt, drought and/or cold stress. Of these, *GmMYB76*, *GmMYB92* and *GmMYB177* are induced by various abiotic stresses but are not significantly affected by ABA. One of them, *GmMYB76* is a close homolog of *AtMYB2*. Intensive characterization of the three genes reveals that all of them can bind to AtMYB2-recognition sequence, TATAACGGTTTTTT. All the three *GmMYB* genes promote the expression of *DREB2A*, *RD17*, *P5CS* genes (Liao et al., 2008).

In wheat plant, a good number of R2R3-MYB TFs have been implicated in drought and salinity response/tolerance. *TaMYB32* transcript is induced by salt stress (Li-chao et al., 2009). Besides, *TaMYB33* gene expression is induced by NaCl, PEG ad ABA treatments. Its promoter sequence contains putative ABRE, MYB and other abiotic stress related *cis*-elements. Ectopic over-expression of *TaMYB33* in *Arabidopsis* remarkably enhances tolerance of the transgenic plants to drought and NaCl stresses, partly by their superior ability for osmotic balance reconstruction and ROS detoxification (Qin et al., 2012).

In rice, *OsMYB*2 expression is up-regulated by salinity, low temperature, and dehydration. Transgenic rice overexpressing *OsMYB2* gene is more tolerant to salt, cold, and dehydration stresses, and more sensitive to ABA than the wild type. The transgenic rice also shows normal growth and development under normal condition. The *OsMYB2* overexpressing plants exhibit the increase in accumulation of proline and soluble sugar and proline synthase and transporter gene expression are also enhanced. The transgenic plants show higher activities of antioxidant enzyme peroxidase, catalase, and superoxide dismutase corresponding with the lower H_2O_2 and malondialdehyde (MDA) content. Moreover, a number of stress-related genes including *OsLEA3*, *OsRab16A* and *OsDREB2A* are up-regulated in the *OsMYB2*-overexpressing plants (Yang, Dai, and Zhang, 2012).

In ABA-dependent pathway of transcriptional regulatory network for gene expression under dehydration and salinity stress, MYB TFs are regarded as master switches for the expression of dehydration-and salt-stress responsive genes through their corresponding *cis*-regulatory elements on the gene promoter (Yamaguchi-Shinozaki and Shinozaki, 2005). Consistently, direct interaction between MYB proteins with the Ca^{2+} signal transducer CaMs connects MYB TFs with Ca^{2+}/CaM signaling pathway. Amongst salinity-responsive MYB TFs in rice, one might be a target of the salt-induced rice CaM isoforms and possibly salt stress-responsive expression of *OsNUC1* is regulated by Ca^{2+}/CaM mediated salt stress signaling through a group of transcription factor recognizing the gene promoter, such as MYB TFs.

CHAPTER III
MATERIALS AND METHODS

I. MATERIALS

1. Plant materials (*Oryza sativa* L.)

1.1. 'Khao Dawk Mali 105' ('KDML105') rice and the salt resistant introgression line, FL530-IL (Saeng-ngam et al., 2012)

Seeds of Thai rice cultivar/line KDML105 and FL530-IL were kindly provided by Rice Gene Discovery Unit (RGDU), Kasetsart University Kamphangsaen Campus, Kamphangsaen, Thailand.

Seeds of the transgenic rice lines, overexpressing *OsCam1-1* gene as well as the T-DNA insertion control lines with KDML105 background (Saeng-ngam et al., 2012) were kindly provided by Associate Professor Dr. Teerapong Buaboocha, Department of Biochemistry, Faculty of Science, Chulalongkorn University.

1.2. 'Leung Pra Tew 123' ('LPT123') rice and its mutated line, Leung Pra Tew 123-TC171 (LPT123-TC171)

Seeds of Thai rice cultivar LPT123 were kindly provided by the Rice Department, Ministry of Agriculture and Cooperatives of Thailand.

LPT123-TC171 rice is a salt-resistant mutated line of 'LPT123' generated from somaclonal variation, variegated cell lines with the selection under salt stress (Vajrabhaya and Vajrabhaya, 1991; Klomsakul, 2004; Maneeprasopsuk, 2004; Thikart et al., 2005; Udomchalothorn et al., 2009; Sripinyowanich, 2010). The seeds in the F7 generation used in this study were provided by the Environment and Plant Physiology Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University.

2. Bacterial and yeast strains

Bacterial cells, *Escherichia coli* strain DH5α, were provided by the Environment and Plant Physiology Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University. The genotype of DH5α was presented in Table 3.1.

Yeast cells of *Saccharomyces cerevisiae* strain MaV203 were obtained from the National Laboratory for Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University, China. The genotype of MaV203 was shown in Table 3.1.

Table 3.1 Genotype of bacterial and yeast strains

Organism (strain)	Genotype
E. coli (DH5α)	*deo*R, *end*A1, *gyr*A96, *hsd*R17 (rk-mk+), *rec*A1, *rel*A1, *sup*E44, *thi*-1, F- (Woodcock et al., 1989)
S. cerevisiae (MaV203)	MATα, *leu*2-3,112, *trp*1-901, *his*3Δ200, *ade*2-101, *gal4*Δ, *gal*80Δ, *SPAL*10::*URA*3, *GAL*1::*lacZ*, *HIS*3UAS GAL1::*HIS*3@*LYS*2, *can*1R , *cyh*2R (Vidal, 1997)

3. **Equipments**

-20°C freezer (SANYO Biomedical Freezer, Japan)

-80°C deep freezer (Thermo-Scientific, USA)

4°C refrigerator (SANYO, Japan)

Micropipette (Gilson, France)

Balances (METTLER TOLEDO AG285, Switzerland)

Cuvette (Agilent Technology, Germany)

Incubator (GEMMYCO, USA)

Gel documentation system (The Discoveries™ Quantity One D-1 Analysis software, BIO-RAD, USA)

Gel electrophoresis syetem (MiniRun GE-100, Hangzhou Bioer Technology, China)

Laminar flow (Astec Microflow, Bioquell Medical Limited, USA)

Microcentrifuge (SORVALL Biofuge, Kendro, Germany)

PCR thermal cycler (PTC-100™, Peltier Thermal Cycler, MJ Research, USA)

pH meter (Sartorius Professional Meter PP-50, USA)

Refrigerated centrifuge (Universal16, Hettich, Germany)

Real-time PCR (CFX96™ Real-Time PCR Detection System, BIO-RAD, USA)

Spectrophotometer (Agilent Technology, USA)

Shaking incubator (Memmert Lab Shaking Incubator Model IN-666)

Vortex (Labnet, USA)

Water bath (New Brunswick Scientific, USA)

4. Chemicals and reagents

All chemicals and reagents used in this study were molecular biological or analytical grade, and purchased from different companies as followed:

2-mercaptoethanol (Acros Organics, USA)

3AT (Sigma, USA)

Acetic acid (QRëC, Malaysia)

Adenine sulfate (Sigma, USA)

Agarose gel (Research Organics, USA)

Ampicillin (Meiji, Thailand)

Bacto-agar (Becton, France)

Bacto-tryptone (Himedia, India)

Bacto-yeast extracts (Difco Laboratories, USA)

Boric acid (CARLO ERBA, Italy)

Bromophenol blue (LABCHEM, Australia)

Calcium chloride dihydrate (MERCK, Germany)

Chloroform (RCI LABSCAN, Thailand)

CTAB (Amersham, USA)

DEPC (Bio Basic, Canada)

Disodium hydrogen phosphate (M&B, England)

DMSO (Sigma, USA)

dNTPs (Roche, Germany)

EDTA (Bio Basic INC, Canada)

Ethanol (MERCK, Germany)

Ethidium bromide (Gibco BRL, USA)

Gentamycin (Meiji, Thailand)

Glucose (CARLO ERBA, Italy)

Glycene (BDH biochemicals, England)

Glycerol (UNIVAR, Australia)

Hydrochloric acid (MERCK, Germany)

IPTG (Sigma, USA)

Isoamyl alcohol (UNIVAR, Australia)

Isopropanol (UNIVAR, Australia)

Kanamycin (Meiji, Thailand)

Lauryl sarcosinate (Sigma, USA)

Lithium acetate (Fluka, USA)

Lithium chloride (Ajex Fine Chem, Australia)

Magnesium sulfate (TOYOBO, Japan)

Oligo (dT) 15 primer (Promega, USA)

Phenol (Merck, Germany)

PEG-3350 (Sigma, USA)

Potassium acetate (Unilab, Australia)

Potassium chloride (UNIVAR, Australia)

PVP (Fluka, Switzerland)

RNaseA (Sigma, USA)

SD-Leu/-Trp (ClonTech, Takara, USA)

SD-Leu/-Trp/-His (ClonTech, Takara, USA)

SD-Leu/-Trp/-Ura (ClonTech, Takara, USA)

SDS (Ajex Fine Chem, Australia)

Sodium acetate (Fisher Scientific, UK)

Sodium chloride (UNIVAR, Australia)

Sodium dihydrogen phosphate (UNIVAR, Australia)

Sodium hydroxide (UNIVAR, Australia)

Standard 1 kb DNA marker (Fermentas, USA)

Tris base (Affymetrix, USA)

X-gal (Sigma, USA)

Xylene cyanol F (LABCHEM, Australia)

5. DNA-modifying enzymes and commercial kits

CWBIO DNA purification kit (CoWin Biotech, China)

DNaseI (Takara, Japan)

Gateway® LR Clonase™ Enzyme Mix (Invitrogen, USA)

KOD-Plus-Neo DNA polymerase (TOYOBO, Japan)

Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, USA)

pENTR Directional TOPO® Cloning Kits (Invitrogen, USA)

Pfx50™ DNA polymerase (Invitrogen, USA)

ProQuest™ Two-Hybrid System (Invitrogen, USA)

Restriction endonuclease (*Eco*RV, *Eco*RI and *Xho*I) (NEB, USA)

SsoFast™ EvaGreen® Supermix (BIO-RAD, USA)

T4 DNA ligase (Takara, Japan)

UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories, Inc, USA)

6. cDNA clones

Full-length cDNA clones of the genes encoding rice R2R3-MYB TFs used in the yeast two-hybrid assay (AK120551, AK103455, AK069082, and AK099283) were purchased from the Rice Genome Resource Center, National Institute of Agrobiological Sciences of JAPAN through the Knowledge-based *Oryza* Molecular Biological Encyclopedia (KOME: http://cdna01.dna.affrc.go.jp/cDNA/).

Full-length cDNA of *OsCam1-1* (AU081299) was kindly provided by Associate Professor Dr. Teerapong Buaboocha, Department of Biochemistry, Faculty of Science, Chulalongkorn University.

7. **Supplies**

8-well real-time PCR strips (BIO-RAD, USA)

Aluminum foil

Petri dish (Pyrex, USA)

Glass bottles

Liquid nitrogen (TIG, Thailand)

Microcentrifuge tubes (Extragene, USA)

Mortars and pestles

Parafilm (Whatman®, GE healthcare, USA)

PCR tubes (Axygen Inc., USA)

Pipette tips (Extragene, USA)

Plastic trays (Local market, Thailand)

Sand

Whatman® 541 filter paper (GE healthcare, USA)

II. METHODS

1. Cloning and *in silico* characterization of *OsNUC1* promoter

1.1. Plant growing condition and tissue collection

Seeds of rice cultivar/line LPT123, LPT123-TC171, KDML105, and FL530-IL were soaked in carbon filtered water for 2 nights for imbibitions, and were subsequently germinated and grew on sand supplemented with a modified WP nutrient solution (Vajrabhaya and Vajrabhaya, 1991; Udomchalothorn et al., 2009) in a greenhouse at natural light and ambient temperature. Leaves of two-week-old seedlings were harvested for genomic DNA extraction.

1.2. Isolation of genomic DNA

The CTAB extraction method (Doyle and Doyle, 1987) was employed for rice genomic DNA extraction. A hundred milligrams of rice leaves were ground in liquid nitrogen with cooled mortar and pestle. The ground tissue was transferred to a microcentrifuge tube containing 500 µl of CTAB extraction buffer (140 mM NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 2% (v/w) PVP, 4% (v/w) CTAB and 0.2% (w/w) 2-mercaptoethanol). The sample was incubated at 65°C for 15-30 min with occasionally mixing by inversion. An equal volume of phenol:chloroform (1:1, v/v) was subsequently added to the sample and mixed thoroughly for 5 min, followed by centrifugation at 13,000 rpm for 5 min at 4°C. After centrifugation, the upper aqueous phase was collected and extracted once again with an equal volume of chloroform. After addition of 0.6 volumes of cooled isopropanol and 0.1 volumes of 3 M NaOAc, the DNA was allowed to precipitate overnight at -20°C. The DNA was recovered by centrifugation at 13,000 rpm for 10 min at 4°C. The pellet was washed with cooled 70% (v/v) ethanol and air-dried at room temperature. Finally, the pellet was resuspended in 250 µl of TE buffer.

To eliminate a trace of RNA that may be present in the sample, RNase A was added to reach the concentration of 20 µg/ml prior to incubation at 37°C for an hour. The RNase A was then removed from the sample by addition of an equal volume of phenol:chloroform (1:1, v/v). After centrifugation at 10,000 rpm for 5 min at 4°C, the DNA-containing supernatant was transferred to a new microcentrifuge tube. The DNA was precipitated with 2 volumes of absolute ethanol and 0.1 volumes of 3 M NaOAc at 4°C overnight. The DNA was harvested by centrifugation at 13,000 rpm for 10 min at 4°C and washed with cooled 70% ethanol. After air-dried, the DNA was dissolved in 20-30 µl of TE buffer, depending on the size of DNA pellet.

The quality of DNA was evaluated by agarose gel electrophoresis and OD260/OD280 ratio. The quantity of DNA was estimated by the optical density at 260 nm and calculated according to the following equation:

[DNA, μg/ml] = OD260 x dilution factor x 50*

*The concentration of DNA with an OD260 of 1.0 is approximately 50 μg/ml. (Sambrook and Russell, 2001).

1.3. Primer design

The putative promoter region of *OsNUC1* gene was identified by aligning a 120-bp sequence of the first exon of *OsNUC1* cDNA (accession no. AK103446) with *Oryza sativa* (*indica* cultivar-group) chromosome 4 (GeneBank accession no. CM000129.1) in NCBI database. The sequence located on 5'-upstream of the cDNA was considered as *OsNUC1* promoter. A pair of specific primers was designed, with the forward primer located at 1,353 bp up-stream of *OsNUC1* transcription start site and the reverse primer resided on in the first-intron region (218 bp down-stream of the transcription start (Figure 3.1). The primer sequences were presented in Table 3.2.

Table 3.2 A primer pair used for cloning of *OsNUC1* promoter

Primer name	Primer sequence (5' → 3')	Tm (°C)	Expected product size (bp)
p*OsNUC1*-F	5'-GCCCTTGCTTTATACTGCGGA-3'	59.8	1,571
p*OsNUC1*-R	5'-CCTAAACCCTAGCCTTACCAACC-3'	62.4	

1.4. Cloning of *OsNUC1* promoter

1.4.1. Amplification of *OsNUC1* promoter by polymerase chain reaction (PCR)

The 50-μl PCR reaction was comprised of 1x Pfx^{TM} PCR mix, 2.5 units of Pfx50^{TM} DNA polymerase (Invitrogen, USA), 0.3 mM dNTP mix (Roche, Germany), 0.3 μM of each primer, and 300 ng of genomic DNA. The PCR conditions were performed as followed; initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 68°C for 2 min. Finally, final extension was performed at 68°C for 5 min.

```
                                        pOsNUC1-F
CM000129    30500878 GGGGGGGGGGGGAGGTCGCCCTTGCTTTATACTGCGGAGAGGCGCCTCAC   30500928

AK103446           1 --------------------------------------------------          0

CM000129    30500929 AAAGTCACAATCCGCGGTCCCGACATGTCCGCCCGCGCGCGGGAACGACG   30500978

AK103446           1 --------------------------------------------------          0

CM000129    30500979 ACGACGACGAAGCCATTCTTCCTCGCTACGTCACGTCACCCGTTTGATCG   30501028

AK103446           1 --------------------------------------------------          0

CM000129    30501029 ATCGCTTGTGGATTTTTTTTTACATTTTAGCCCTTTTTGAAAACTTATTT   30501078

AK103446           1 --------------------------------------------------          0
                                          .
                                          .
                                          .
                                          .
CM000129    30501979 CGAGTCAAATTGCACCGGTTTCAATAGTTCGGGAGTCGGGATATCCGAAT   30502028

AK103446           1 --------------------------------------------------          0

CM000129    30502029 TGTAGATTAGGGATACAAATCATATCCACTCACTTATTAAGGGACCCAAA   30502078

AK103446           1 --------------------------------------------------          0

CM000129    30502079 GTGAACTTATTCCCCGATGAAACCTACGTGAGGAGGTCCAGCCCGGTCTG   30502128

AK103446           1 --------------------------------------------------          0

CM000129    30502129 GGCCGGCCCAAGCTACAGCCCACAGACACTGGTGGCCCCGTGGCCTGGCG   30502178

AK103446           1 --------------------------------------------------          0

CM000129    30502179 GCGGGCAGGGACAACCCTAGCAGCAGGTGCCGCCATATAAAAGGATCGCC   30502228

AK103446           1 --------------------------------------------------          0

CM000129    30502229 CGTGCGGTCTCGCCTCCCCCATTACCTCCCCGCCCGCGCACCTCGAGCCC   30502278
                                        .||||||||||||||||||||||||||||||
AK103446           1 -------------------GATTACCTCCCCGCCCGCGCACCTCGAGCCC         31

CM000129    30502279 CCAGGTCCGCCTCGCCTCTCCGTCGCTTGCCGCCGCCCGCAGTGGTCGCC   30502328
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
AK103446          32 CCAGGTCCGCCTCGCCTCTCCGTCGCTTGCCGCCGCCCGCAGTGGTCGCC         81

CM000129    30502329 GGCCGCGCGGGGCTTCGCCGGTGTCGCTTCCTCGTTTGGGTAAGTCGCTC   30502378
                     |||||||||||||||||||||||||||||||||||||||
AK103446          82 GGCCGCGCGGGGCTTCGCCGGTGTCGCTTCCTCGTTTGG-----------        120

CM000129    30502379 CTCACTCCCGCGTGACTCTACTTCGGTGAATTTTAGGTGTGGGGTTGCCG   30502428

AK103446             --------------------------------------------------
                                           pOsNUC1-R
CM000129    30502429 GGGGGGGGGGGGGGGGGTTGGTAAGGCTAGGGTTTAGGGTTTATGTGAAT   30502478

AK103446             --------------------------------------------------
```

Figure 3.1 A nucleotide alignment of the *OsNUC1* first exon (AK103446) with the rice chromosome 4 (CM000129). Grey boxes indicate the sites for forward and reverse primers.

1.4.2. Agarose gel electrophoresis

Separation of the PCR products was performed by agarose gel electrophoresis. The 0.8% (w/v) agarose gel was prepared in 0.5x TBE buffer (45 mM Tris-borate, 1 mM EDTA; pH 8.0). Before loaded into a well, 5 volumes of DNA solution was mixed with 1 volume of 6x gel-loading buffer, which contains 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 30% (v/v) glycerol in water. Standard 1 kb marker (Fermentas, USA) was used for the size estimation of the DNA fragments. Electrophoresis was done at constant voltage of 100 Volt. To visualize the DNA, the gel was stained with 0.5 mg/l of ethidium bromide for 5-10 min, placed on a UV transilluminator and photographed using a gel documentation system (The Discoveries™ Quantity One D-1 Analysis software, BIO-RAD, USA).

1.4.3. Purification of DNA fragments from agarose gel

The UltraClean™ 15 DNA Purification Kit (MO BIO, USA) was used to purify the DNA fragments from agarose gel. The desired DNA fragments was excised from agarose gel by using a sterilized blade, and weighed before transferred to a microcentrifuge tube. After added and mixed well with 0.5 volumes of ULTRA MELT and 4.5 volumes of ULTRA SALT, the sliced gel was incubated at 55°C for 5 min with occasional mixing to ensure the gel solubilization. Subsequently, (5+x) µl of ULTRA BIND, where x is the amount of the DNA (in µg) expected to be recovered, was added to the dissolved gel mixture and mixed well by flicking the tube for 5 min. After impulse at 13,000 rpm for 5 sec, the supernatant was discarded and 1 ml of ULTRA WASH was added and mixed well by vortexing. After impulse at 13,000 rpm for 5 sec, the supernatant was completely discarded. The pellet was air-dried and subsequently resuspended in 2*(5+x) µl of sterile distilled water, and mixed well by pipetting. The mixture was incubated at room temperature for 5 min prior to centrifugation at 13,000 rpm for 1 min. The DNA-containing supernatant was collected and transferred to a new microcentrifuge tube.

1.4.4. Ligation of PCR product

A plasmid pBluescript (Figure 3.2) was used as cloning vector to clone blunt-ended PCR products produced by Pfx50™ DNA polymerase as indicated in 1.4.1. The plasmid was blunt-ended linearized by *Eco*RV digestion (NEB, USA) according to the manufacturer's instructions. The ligation reaction was composed of 1x ligation buffer, 175 Weiss units of T4 DNA ligase (Takara, Japan), 25 ng of linearlized pBluescript plasmid, and 62.5 ng of purified PCR product (5:1 molar ratio of insert:vector). The reaction was incubated overnight at 4°C before used for transformation.

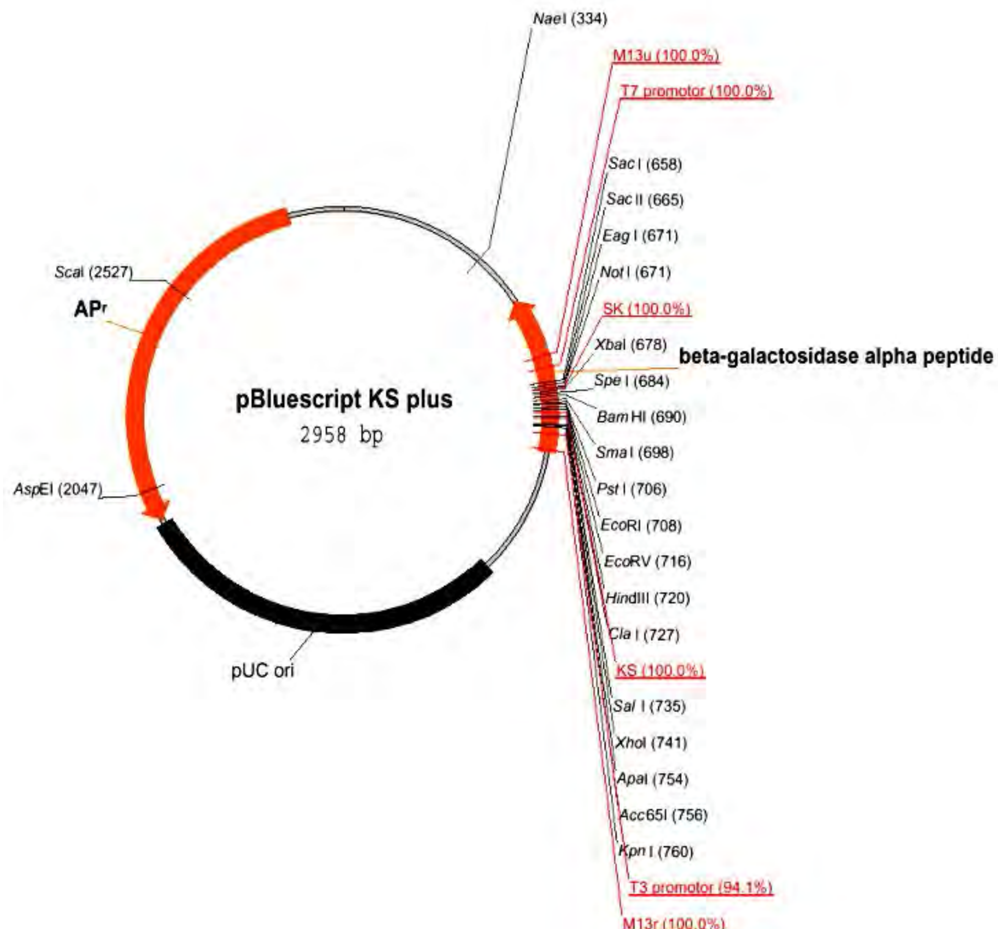


Figure 3.2 Map of pBluescript KS plus vector and its restriction sites represented common characteristics of the pBluescript plasmid family (http://www.xenbase.org/other/vectorAction.do?method=displayVectorSummary&vectorId=1221299)

1.4.5. Preparation of *E. coli* competent cells

Frozen *E. coli* strain DH5α was streaked on LB medium (1% (v/w) bacto-tryptone, 0.5% (v/w) bacto-yeast extract, 1% (v/w) NaCl, and 2% (v/w) bacto agar, pH 7.0) prior to incubation overnight at 37°C. A single colony was inoculated in 10 ml of LB broth before incubated with shaking (220-230 rpm) at 37°C for 16-18°C. Subsequently, 250 µl of the culture was transferred to a new 25 ml of LB broth followed by incubation with shaking at 37°C for 2.5 h (or, until the OD600 reached 0.6-0.8). The culture was then transferred to a new cooled 50 ml centrifuge tube and kept on ice for 30 min. After centrifugation at 1,600x*g* for 7 min at 4°C, LB medium was removed and the cell pellet was resuspended in 5 ml of ice cold 100 mM $CaCl_2$. The cell mixture was then centrifuged at 1,100x*g* for 5 min at 4°C. After removal of supernatnat, the pellet was dissolved in 5 ml of cooled 100 mM $CaCl_2$ followed by incubation on ice for 30 min. After centrifugation at 1,100x*g* for 5 min at 4°C, the supernatant was removed and 1 ml of ice cold 100 mM $CaCl_2$ was added and mixed well by pipetting. DMSO was added to a final concentration of 7% (v/v) and mix well by pipetting. The cell suspension was divided into 100 µl aliquots for transformation, or for later use by storing at -80°C.

1.4.6. Bacterial transformation and selection of positive clones

Two microliters of ligation reaction was added to 100 µl of *E. coli* DH5α competent cells. The mixture was incubated on ice for 30 min and heated at 42°C for 90 sec before placed on ice immediately. After incubation on ice for 3 min, 400 µl of LB broth was added and incubated with shaking (220 rpm) at 37°C for an hour. After incubation, 4 µl of IPTG (200 mg/ml) and 40 µl of X-gal (20 mg/ml) were added. The culture was finally spread on LB agar supplemented with 100 µg/ml of ampicillin prior to incubation at 37°C overnight.

1.4.7. Characterization of the selected positive clones

Bacterial cells capable of growing on ampicillin-supplemented medium with a white colony were considered as putative positive clones, harboring pBluescript plasmids with PCR insert. The putative positive colonies was inoculated in

10 ml of LB broth containing 100 µg/ml ampicillin, followed by incubation with shaking at 37°C overnight.

1.4.7.1. Plasmid DNA extraction

The overnight culture (1.5 ml) was transferred to a microcentifuge tube and centrifuged at 7,000 rpm for 5 min at room temperature. The pellet was collected, resuspended in 100 µl of RNase A-containing (20 µg/ml), ice-cooled solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) and mixed well by vortexing. Two hundred microliters of freshly prepared solution II (0.2 N NaOH, 1% SDS) was then added and gently mixed by inverting the tube. After incubation on ice for 5 min, 150 µl of solution III (3 M potassium acetate, 5 M acetic acid) was added and gently mixed by inverting the tube, and then it was stored on ice for 5 min. After centrifugation at 13,000 rpm for 5 min at room temperature, the plasmid DNA-containing supernatant was transferred to a new microcentrifuge tube. Precipitation of the DNA was done by addition of 800 µl of ice-cold 95% ethanol and stored on ice for 10 min. The DNA was pelleted by centrifugation at 13,000 rpm for 5 min at room temperature. The DNA pellet was washed with 70% ethanol, air-dried, and subsequently resuspended in 15-20 µl of distilled water.

1.4.7.2. Plasmid digestion by restriction endonucleases

The presence of PCR insert in the selected positive clones was verified by plasmid digestion. *Eco*RI and *Xho*I (NEB, USA) were used to estimate the size of the insert released from the vector. The digestion reaction contained 5 µg of plasmid DNA, 1X NEBuffer, 1X BSA (NEB, USA), and 5 units of each of the restriction enzymes in 25-µl total volume. The reaction was incubated at 37°C for an hour prior to analysis of digested products by agarose gel electrophoresis.

1.4.7.3. Sequencing of plasmid DNA

Plasmid DNA of the positive clones, which was also confirmed by digestion as indicated in 1.4.7.2., was subjected to DNA sequencing by a service of Pacific Science Company, Ltd. The DNA sequencing was performed by using a pair of

the universal M13 forward- and reverse-primers. For each rice cultivar/line, the sequences of at least two clones were determined.

1.5. *In silico* characterization of *OsNUC1* promoter

Putative *cis*-acting regulatory elements on the promoter of *OsNUC1* gene from each of rice cultivars/lines were annotated and compared by using PLACE (http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al., 2002) algorithms.

2. The effect of *OsCam1-1* on *OsNUC1* gene expression under salt-stress condition

To determine the effect of a stress sensor, *OsCam1-1* gene, on salt-stress-responsive expression of *OsNUC1*, the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to compare expression levels of *OsNUC1* among different rice lines with KDML105 genetic background, including the wild type, the T-DNA insertion control transgenic line (T1), and the transgenic lines with *OsCam1-1* over-expression, 35SCaM1-1T1, 35SCaM1-1T2, and 35SCaM1-1T3.

2.1. Plant growing conditions and tissue collection

Seeds of *indica* rice cultivar KDML105 including the wild type, the T-DNA insertion control line, and the transgenic lines over-expressing *OsCam1-1* gene were imbibed in carbon-filtered water for 2 nights. After imbibition, the seeds were transferred to damp sand and allowed to germinate for a week. The 1-week old seedlings were subsequently transferred to a modified WP nutrient solution (Vajrabhaya and Vajrabhaya, 1991; Udomchalothorn et al., 2009) and were grown for 2 weeks. In the course of time, the nutrient solution was renewed once a week and adjusted to the same level every day by addition of carbon-filtered water.

Salt-stress treatment was performed on 3 week-old seedlings by adding NaCl to the nutrient solution to the final concentration of 0.5% (w/v). Rice seedlings at

the same age grown in modified WP solution without added NaCl were used as a control group. Leaf tissues were harvested and immediately frozen in liquid nitrogen after 0, 0.5, 1, 2 and 3 day of the treatment.

The experiment was conducted with a completely randomized design (CRD) with three biological replicates, each of which was a pool of three individual seedlings. The whole process was done in a transgenic greenhouse under natural light condition and ambient temperature.

2.2. Plant total RNA isolation

The total RNA was isolated by standard hot-phenol method according to Thikart et al. (2005) with some modifications. Rice tissues were ground in liquid nitrogen using chilled mortars and pestles. The powdery, ground tissues were then added to an equal volume of hot phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and RNA extraction buffer (100 mM Tris-HCl pH 9.0, 100 mM NaCl, 20 mM EDTA pH 8.0, 1% (w/v) lauryl sarcosinate, 0.1% (v/v) 2-mercaptoethanol and 0.1% (v/v) DEPC), mixed homogeneously, and incubated on ice. The tissue mixture was centrifuged at 12,000 rpm for 10 min at 4°C and the upper, aqueous phase was transferred into a new microcentrifuge tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) was subsequently added, mixed gently before centrifuged at 12,000 rpm for 5 min at 4°C. After centrifugation, the upper-phase RNA was transferred into a new microcentrifuge tube, and then precipitated by 2 volumes of absolute ethanol at -20°C for 30 min. The RNA was recovered by centrifugation at 12,000 rpm for 10 min at 4°C. The RNA pellet was washed with 80% (v/v) ethanol before air-dried at room temperature. The pellet was resuspended in 160 µl DEPC-treated TE buffer. Forty microliters of 10M LiCl were subsequently added and mixed gently before stored at -20°C overnight. After incubation, the RNA was harvested by centrifugation at 12,000 rpm for 10 min at 4°C. The pellet was washed once with 80% (v/v) ethanol before air-dried at room temperature. Finally, the RNA was resuspended in 20 µl DEPC-treated TE buffer and stored at -80°C.

The quality of RNA was assessed by agarose gel electrophoresis. The quantity of RNA was determined by the optical density at 260 nm and calculated according to the following equation:

[RNA, µg/ml] = OD260 x dilution factor x 40*

*The concentration of RNA with an OD260 of 1.0 is around 40 µg/ml. (Sambrook and Russell, 2001).

2.3. Preparation of DNA-free RNA

To eliminate DNA residues contaminated in RNA samples, 10 µg of the total RNA was added to DNaseI reaction mixture, containing 1x DNaseI buffer and 10 units of recombinant DNaseI (Takara, Japan). The reaction was incubated at 37°C for an hour. After incubation, 200 µl of DEPC-treated distilled water and 250 µl of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) was added, and mixed gently before centrifuged at 12,000 rpm for 5 min at 4°C. After centrifugation, the upper phase RNA solution was collected and transferred into a new centrifuge tube. The RNA was subsequently precipitated by 0.1 volumes of 3 M NaOAc, (pH 5.2) and 0.6 volumes of isopropanol at -20°C for 30 min. The DNA-free RNA was harvested by centrifugation at 12,000 rpm for 10 min at 4°C. Subsequently, the RNA pellet was washed with 80% ethanol and air-dried at room temperature. Finally, the DNA-free RNA was dissolved in 10 µl of DEPC-treated distilled water and kept at -80°C.

2.4. First-strand cDNA synthesis

The first-strand cDNA synthesis was performed by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT). The reaction containing 2 µg of total RNA and 0.5 µg oligo(dT) 15 primer (Promega, USA) was incubated at 70°C for 5 min, cooled on ice immediately, and spun briefly. After addition of 1x of M-MVL reaction buffer, 0.6 mM dNTP mix (Roche, Germany), and 200 units of M-MLV RT (Promega, USA), the reaction was incubated at 42°C for three hours. Finally, the reaction was terminated by heating at 70°C for 15 min.

2.5. RT-qPCR

The real-time quantification of cDNA targets was performed with the CFX96™ Real-Time PCR Detection System (BIO-RAD, USA). The *OsNUC1* gene-specific primers used for RT-qPCR (Table 3.3) were previously designed by Sripinyowanich (2010). The 10-µl PCR reaction contained 1x SsoFast™ EvaGreen® Supermix (BIO-RAD, USA), 0.2 µM of each primer, and 2 µl of a 1:5 dilution of first-strand cDNA reaction. The PCR conditions were programmed as followed: initial denaturation at 98°C for 30 sec, followed by 40 cycles of 2-step PCR as denaturation at 98°C for 5 sec and annealing as well as extension at 58.0°C for 10 sec. Finally, the melting curve analysis of the PCR product was performed to verify the product specificity.

For normalization of *OsNUC1* gene expression, a parallel amplification of an internal control gene, *OsEF-1α* was performed. The 10-µl PCR reaction comprised 1x SsoFast™ EvaGreen® Supermix (BIO-RAD, USA), 0.25 µM of each of *OsEF-1α* gene specific primers (Table 3.3) (Saeng-ngam et al., 2012), and 1 µl of a 1:5 dilution of first-strand cDNA reaction. The PCR was in the same conditions as that of *OsNUC1*; except for the annealing temperature which was adjusted to 58.3°C.

The data were collected as Cq values (cycle number at which the fluorescence signal passes over the threshold) during the extension step. For each cDNA sample, the PCR was done with 3 technical replicates. Relative expression level of *OsNUC1* gene in each sample was calculated by normalized expression (ΔΔCq) method with the expression of *OsEF-1α* as a reference. The expression level of *OsNUC1* in the wildtype KDML105 at day0 (without salt-stress treatment) was set as a control. Inter-run calibration and PCR efficiencies of both *OsNUC1* and *OsEF-1α* genes were included in the calculation. Calculation of gene expression was performed by Gene Study module of Bio-Rad CFX Manager 2.1 software (BIO-RAD, USA).

Table 3.3 Primers used for RT-qPCR

Primer name	Primer sequence (5' → 3')	Tm (°C)	Expected product size (bp)
OsNUC1-F	5'-ATGGATCTGACCTCGGTGGA-3'	57.4	123
OsNUC1-R	5'-GTCTTCCTCCTCTCCCACTG-3'	61.4	
OsEF-1α-F	5'-ATG GTT GTG GAG ACC TTC-3'	53.7	127
OsEF1-α-R	5'-TCA CCT TGG CAC CGG TTG-3'	58.2	

3. **Direct interaction between OsCaM1-1 and putative rice R2R3-MYB TF proteins**

3.1. Identification of putative CaM-binding R2R3-MYB TFs in rice

3.1.1. Functional search of the rice genome

Amino acid sequence of AtMYB2 (accession no. NP_182241.1) was used as a query to search against the MSU Rice Genome Annotation Database (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml; Ouyang et al., 2007). Proteins with the highest sequence similarities (>40%) to AtMYB2, together with their nucleotide and deduced amino acid sequences as well as related information, were collected. Amino acid sequences of the putative proteins were submitted to the Pfam protein families database (http://pfam.sanger.ac.uk/; Finn et al., 2010) to confirm the presence of MYB-DNA binding domains.

3.1.2. Sequence Alignment and helical wheel prediction

Amino acid sequences of the putative proteins were aligned with that of AtMYB2 using ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Based on sequence comparison, moreover, an 18-amino acid region of the CaM-binding motif in the putative proteins was predicted. The helical wheel projection of the predicted motif was performed by submitting the 18-amino acid sequence region to a program on the website http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html.

3.1.3. Phylogenetic tree reconstruction

181 MYB TF-encoding genes were obtained from the rice genome by a keyword search in the MSU Rice Genome Annotation Database (Ouyang et al., 2007). The corresponding amino acid sequence of each gene was then submitted to the Pfam protein families database (Finn et al., 2010) for domain prediction. Consequently, only 107 MYB proteins with two MYB DNA binding domains (R2R3) were collected for further analysis. Complete amino acid sequences of 125 R2R3-MYB transcription factors, which were previously identified by Stracke et al. (2001), were downloaded from The *Arabidopsis* Information Resource (TAIR) (http://www.arabidopsis.org). Using MEGA5.05 software (Tamura et al., 2011), an alignment of rice and *Arabidopsis* R2R3-MYB protein sequences was performed by ClustalW algorithm, and a phylogenetic tree was constructed. Options for tree construction were as followed: statistical method, maximum likelihood; phylogeny test, bootstrap method with 1,000 replicates; substitution model, Poisson; Rates among sites, uniform rates; gaps treatment, partial deletion with 95% cutoff.

3.1.4. Putative functions of the identified R2R3-MYB genes

Putative functions in abiotic stress response of the identified R2R3-MYB TFs were evaluated by their expression profiles from microarray experiments in the Rice Oligonucleotide Array Database (http://www.ricearray.org/; Jung et al., 2008).

3.2. Determination of protein-protein interaction by yeast two-hybrid assay

The ProQuest™ Two-Hybrid System (Invitrogen, USA), a genetic method for detecting *in vivo* protein interactions in the yeast *S. cerevisiae*, was employed to test the physical interaction between four of the identified rice R2R3-MYB TFs and OsCaM1-1.

3.2.1. Construction of yeast expression vectors

3.2.1.1. GAL4 DNA-Binding Domain (DBD)/OsCaM1-1 construct

OsCaM1-1 was expressed as bait protein by fusing with the GAL4-DBD. The OsCaM1-1 coding region was amplified PCR with *OsCam1-1* cDNA-specific primers (Table 3.4). 25-µl PCR reaction was composed of 1x PCR buffer for KOD-Plus-Neo, 0.5 unit of KOD-Plus-Neo DNA polymerase (TOYOBO, Japan), 0.2 mM of each of dNTPs, 1.5 mM $MgSO_4$, 5% (v/v) DMSO, 0.3 µM of each of primers, and 50 ng of plasmid DNA template (AU081299). PCR amplification was performed as followed: initial denaturation at 94°C for 2 min; followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 58°C for 30 sec, and extension at 68°C for 20 sec; and final extension at 68°C for 10 min. PCR products were separated by agarose gel electrophoresis. The PCR product with expected size was isolated from agarose gel, and was purified according to the manufacturer's protocol of CWBIO DNA purification kit (CoWin Biotech, China). The purified PCR product was then ligated into pENTR/D-TOPO® vector (Figure 3.3) using the pENTR Directional TOPO® Cloning Kits (Invitrogen, USA). A 3-µl TOPO® reaction containing 0.5µl of Salt Solution, 0.3 µl of TOPO® vector, and 1 ng of purified PCR product (molar ratio of insert : vector is 1:1) was incubated at 20-25°C for 30 min prior used for transformation into *E. coli* DH5α competent cells. The transformed cells were spread on LB medium supplemented with 50 µg/ml kanamycin, and incubated overnight at 37°C. Bacterial colonies capable of growing on the medium were subjected to colony PCR to confirm the presence of the insert. A colony-PCR positive clone, called entry clone, was finally confirmed by plasmid DNA sequencing.

The coding sequence of *OsCam1-1* in the entry clone was subsequently fused in-frame with GAL4-DBD in pDEST32 destination vector (Figure 3.4) by means of recombination using Gateway® LR Clonase™ Enzyme Mix (Invitrogen,

USA). The 5-µl LR reaction comprised of 1x LR Clonase™ Reaction buffer, 20 ng of entry clone, and 75 ng of pDEST32 destination vector(molar ratio of entry clone : destination vector is 1:1) was incubated at 25°C for 3 h. The LR reaction was then transformed into *E. coli* DH5α competent cells. The transformed cells were grown on LB medium containing 50 µg/ml gentamycin, and incubated overnight at 37°C. Bacterial colonies capable of growing on the gentamycin-supplemented medium were subjected to colony PCR to confirm the presence of *OsCam1-1* coding region. A colony-PCR positive clone was finally confirmed by plasmid DNA sequencing.

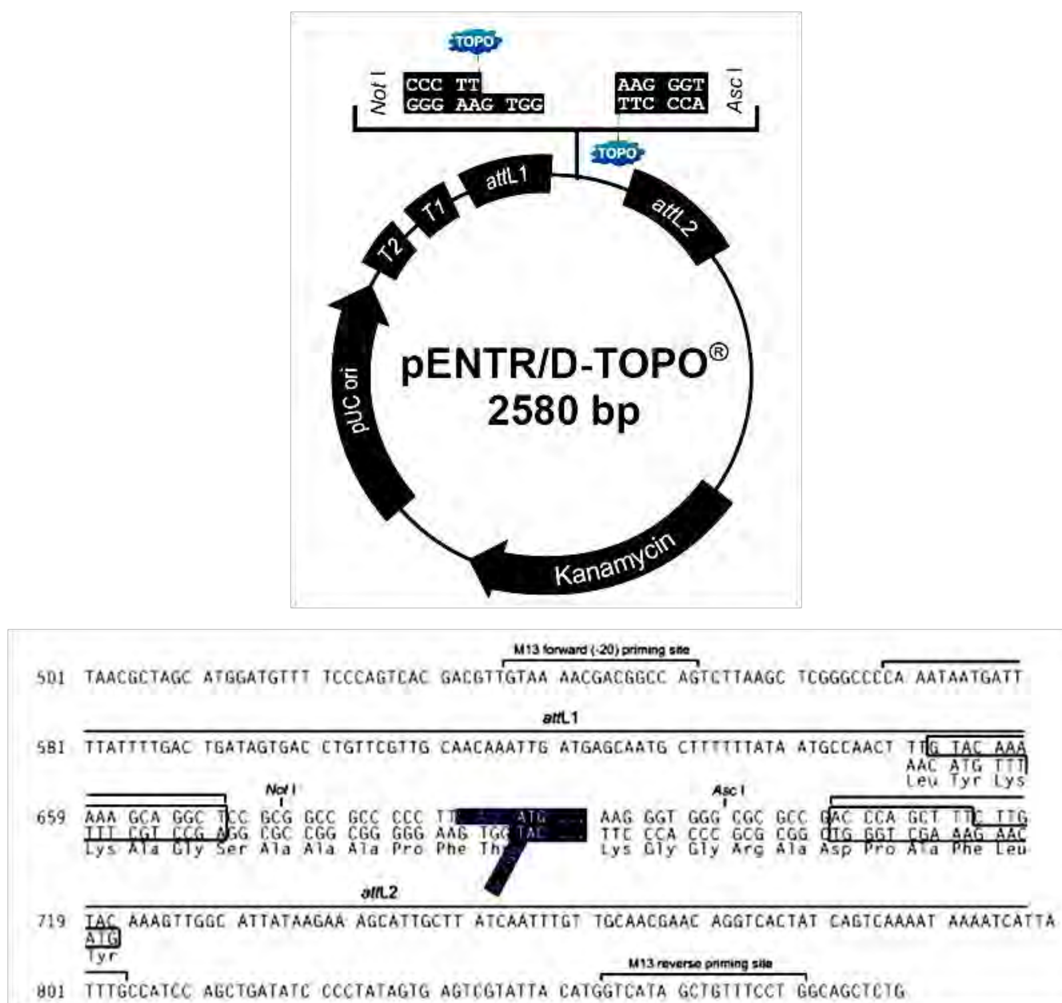


Figure 3.3 Map of pENTR/D-TOPO® vector and its TOPO® cloning site

(Invitrogen, USA)

3.2.1.2. GAL4 Activation Domain (AD)/ OsR2R3-MYB constructs

Coding sequences of four of the putative CaM-binding R2R3-MYB TFs, namely LOC_Os03g20090.1, LOC_Os07g48870.1, LOC_Os11g45740.1, and LOC_Os02g40530.1, were each fused to the GAL4-AD in pDEST22 to serve as preys. Coding regions of the putative TFs were amplified from their corresponding cDNA clones (as shown in Table 3.5) by PCR. Each of the 25-μl PCR reactions contained of 1x PCR buffer for KOD-Plus-Neo, 0.5 unit of KOD-Plus-Neo DNA polymerase (TOYOBO, Japan), 0.2 mM of each of dNTPs, 1.5 mM $MgSO_4$, 5% (v/v) DMSO, 0.3 μM of each of each gene-specific primers (see Table 3.4), and 50 ng of cDNA template corresponding to each of the genes. PCR amplification was performed in the same conditions as that of *OsCam1-1*, except that the extension time was raised to 30 sec and that the annealing temperature for LOC_Os03g20090.1 was changed to 62°C (Table 3.4). PCR products were separated by agarose gel electrophoresis. The expected PCR product was excised from agarose gel, and was purified with CWBIO DNA purification kit (CoWin Biotech, China) following the manufacturer's guideline. Generation and confirmation of an entry clone for each gene was conducted in the same way as that of *OsCam1-1*.

The coding region of each *OsR2R3-MYB TF* in its entry clone was then fused in-frame with GAL4-AD in pDEST22 destination vector (Figure 3.4), using Gateway® LR Clonase™ Enzyme Mix (Invitrogen, USA). The 5-μl LR reaction containing 1x LR Clonase™ Reaction buffer, 30 ng of entry clone, and 75 ng of pDEST32 destination vector (molar ratio of entry clone : destination vector is 1:1) was incubated at 25°C for 3 h. The LR reaction was subsequently transformed into *E. coli* DH5α competent cells. The transformed cells were spread on LB medium containing 50 μg/ml ampicillin, and incubated overnight at 37°C. Bacterial colonies capable of growing on the ampicillin-supplemented medium were subjected to colony PCR to confirm the presence of their respective gene-coding regions. In-frame recombination of the colony-PCR positive clone was finally confirmed by plasmid DNA sequencing.

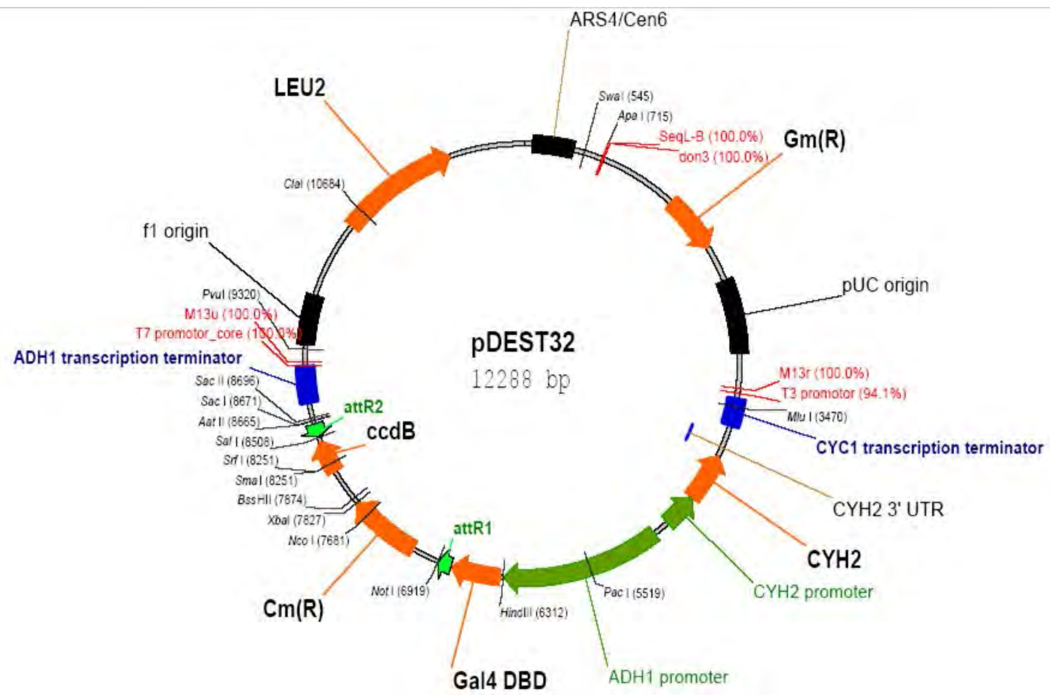
Table 3.4 cDNA clones, PCR primers and conditions used in the construction of yeast expression vectors

Gene (cDNA clone No.)	Primers	Primer sequence (5’ → 3’)	Annealing temperature (°C)
LOC_Os03g20090.1 (AK120551)	Forward	5’-CACCATGGACATGGCGCACGAGAG-3’	62.0
	Reverse	5’-ACTTTTCACACGGCGGCCTG-3’	
LOC_Os07g48870.1 (AK103455)	Forward	5’-CACCATGATGATGGCGCGAGAGGT-3’	58.0
	Reverse	5’-TTCACAAGCCAAGCATTTGCGT-3’	
LOC_Os11g45740.1 (AK069082)	Forward	5’-CACCATGGAGATGGTGCTGCAGA-3’	58.0
	Reverse	5'-TGCCTTCCTTGATGTCTCTCTA-3'	
LOC_Os02g40530.1 (AK099283)	Forward	5’-CACCATGGAAGGGCAGCAGTTC-3’	58.0
	Reverse	5'-ATAGCTAGTAGAAAGCGAGGC-3'	
OsCam1-1 (AU081299)	Forward	5’-CACCATGGCGGACCAGCTCACC-3’	58.0
	Reverse	5'-TCACTTGGCCATCATGACCTTG-3'	

3.2.2. Preparation of yeast competent cells

The competent MaV203 cells were prepared according to the manufacturer’s protocol (Invitrogen, USA). In brief, a colony of MaV203 was inoculated in 10 ml of YPDA medium (1% (v/w) bacto-yeast extract, 2% (v/w) tryptone, 2% (v/w) glucose, 0.01% (v/w) adenine sulfate) and cultured at 30°C with shaking overnight. The overnight culture was diluted to an OD600 of 0.4 in a new 50 ml of YPDA solution, and grown for an additional 3 hours. After centrifugation at 2,500 rpm for 5 min at room temperature, the cell pellet was resuspended in 40 ml of 1xTE followed by centrifugation at 2,500 rpm for 5 min at room temperature. The cells were finally resuspended in 2 ml of 1xLiAc/0.5xTE and incubated at room temperature for 10 min prior to transformation.

(A)



(B)

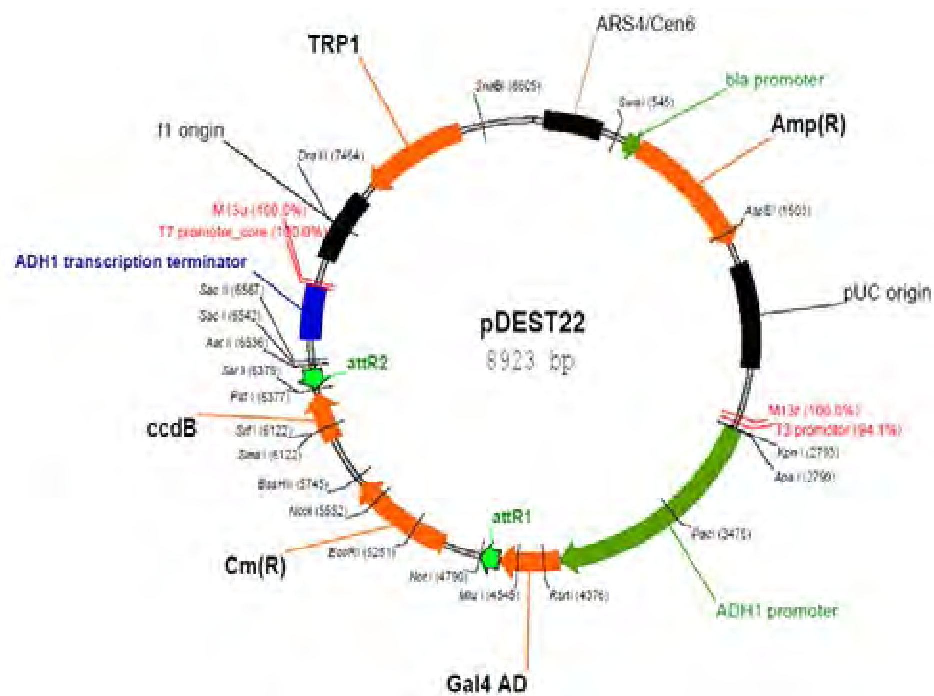


Figure 3.4 Maps of pDEST32 (A) and pDEST22 (B)

(http://www.imagenes-bio.org/info/vectors)

3.2.3. Yeast transformation and selection

100 µl of competent yeast cell suspension was mixed with 1 µg of each of bait and prey plasmids (Table 3.5). The mixture was subsequently added with 700 µl of 1xLiAc/40% PEG-3350/1xTE, and mixed well by pipetting followed by incubation at room temperature for 30 min. The mixture was then subjected to heat shock at 42°C for 7 min. After centrifugation at 8,000 rpm for 10 sec, the supernatant was removed and the pellet was resuspended in 1 ml of 1xTE. The cell suspension was re-pelleted and then resuspended in 50 µl of 1xTE before plated on a Synthetic Dropout medium lacking leucine and tryptophan (SD-Leu/-Typ) (Clontech, USA) for the selection of yeast transformants.

Along with each combination of bait and prey plasmids to be tested for protein-protein interaction, transformations of positive-and negative-interaction control vectors, as well as self-activation control experiments were also (Table 3.5) performed so as to validate the employed system.

3.2.4. Test of protein interaction

Yeast transformants which were able to grow on SD-Leu/-Typ were considered to harbor both bait and prey plasmids. Physical interaction between bait and prey proteins in the nucleus brought about the expression of reporter genes, including *URA3*, *HIS3* and *LacZ*. Hence, protein interaction in the transformants could be detected by evaluating the reporter gene expression. At least 20 clones were selected for the following analyses.

3.2.4.1. *URA3* gene expression

The expression of the *URA3* reporter was tested by transferring yeast transformants to a Synthetic Dropout medium lacking leucine, tryptophan, and uracil (SD-Leu/-Typ/-Ura) (Clontech, USA). Yeast cells containing plasmids that express interacting bait and prey proteins were able to grow and form colonies.

Table 3.5 Combination of plasmid vectors used for transformation into yeast cells

No.	Combination		Purpose
	Bait plasmid	Prey plasmid	
1.	None	None	Negative transformation control
2.	pEXT32/Krev1	pEXT22/RalGDS-wt	Strong positive interaction control
3.	pEXT32/Krev1	pEXT22/RalGDS-m1	Weak positive interaction control
4.	pEXT32/Krev1	pEXT22/RalGDS-m2	Negative interaction control
5.	pDEST32	pDEST22	Negative self-activation control
6.	pDEST32/OsCam1-1	pDEST22	Test of self-activation
7.	pDEST32	pDEST22/ LOC_Os03g20090.1	Test of self-activation prey
8.	pDEST32	pDEST22/ LOC_Os07g48870.1	Test of self-activation prey
9.	pDEST32	pDEST22/ LOC_Os11g45740.1	Test of self-activation prey
10.	pDEST32	pDEST22/ LOC_Os02g40530.1	Test of self-activation prey
11.	pDEST32/OsCam1-1	pDEST22/ LOC_Os03g20090.1	Test of interaction
12.	pDEST32/OsCam1-1	pDEST22/ LOC_Os07g48870.1	Test of interaction
13.	pDEST32/OsCam1-1	pDEST22/ LOC_Os11g45740.1	Test of interaction
14.	pDEST32/OsCam1-1	pDEST22/ LOC_Os02g40530.1	Test of interaction

3.2.4.2. *HIS3* gene expression

The expression of the *HIS3* reporter was determined by transferring yeast transformants to a Synthetic Dropout medium lacking leucine, tryptophan, and histidine (SD-Leu/-Typ/-His) (Clontech, USA) supplemented with appropriate concentration of 3-aminotriazole (3AT) (Sigma, USA). The 3AT was added to the medium in order to suppress selt-activation of the reporter gene *HIS3*, thus inhibiting false-positive interaction. Yeast cells harboring plasmids that express true interacting bait and prey proteins were able to grow and form colonies on this medium.

3.2.4.3. *LacZ* gene expression

Yeast transformants were transferred onto sterile Whatman filter paper laid on SD-Leu/-Typ medium. The yeast cells were cultured on the filter paper at 30°C for 48h. The expression of *LacZ* reporter gene was then evaluated by X-gal assay. Using forceps, the yeast cell-containing filter paper was gently removed from the surface of the medium and completely immersed in liquid nitrogen for 90 sec. After complete evaporation of liquid nitrogen, the immersion was repeated twice to ensure that yeast cells were completely lysed. The filter was then colony-side-up placed on top of two-stack filter paper soaked with X-gal assay solution (1 mg/ml X-gal; 0.006% (v/v) 2-mercaptoethanol; 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1mM $MgSO_4$, pH 7.0). After removal of air bubbles and access solution, the filter paper was incubated at 30°C. The appearance of blue color was monitored over a 24-h period.

CHAPTER IV
RESULTS

1. Cloning and *in silico* characterization of *OsNUC1* promoter

According to Sripinyowanich (2010), there is an obvious difference, both in patterns and levels of the *OsNUC1* expression in response to salt stress between different rice cultivars/lines. The salt-resistant lines, LPT123-TC171 and FL530-IL, show higher levels of *OsNUC1* expression than the salt-susceptible cultivars, LPT123 and KDML105, suggesting some differences in transcriptional gene regulation between them. This distinction in *OsNUC1* expression profiles may result from variations in either *cis*- or *trans*-acting factors that regulate the gene expression. In this regard, characterization of the gene promoter is the most practical way so as to start scrutinizing this question. In this study, therefore, the promoter of *OsNUC1* gene was cloned from each of the rice cultivars/lines, which includes LPT123, LPT123-TC171, KDML105 and FL530-IL.

The *OsNUC1* promoter region was predicted by aligning the first exon of *OsNUC1* full-length cDNA with the chromosome 4 of *indica* rice. Accordingly, the *OsNUC1* transcription start site was located at the region 30502248 of the chromosome 4. Consequently, the sequence 5'-up-stream of this point considered as the gene promoter was retrieved. A pair of specific primers for the amplification of *OsNUC1* promoter was designed according to this sequence, with forward primer located approximately on 1,300 bp up-stream of the transcription start and reverse primer resided on the first intron, 200 bp downstream the transcription start.

The putative *OsNUC1* promoter was amplified by PCR, using leaf tissue-derived genomic DNA from each of the rice cultivars/lines as template. An expected 1,500-bp product was selected and isolated from agarose gel. After purification, the product was ligated into the plasmid pBluescript, and transformed into *E. coli* DH5α (Figure 4.1). The resulting putative positive clones were determined by plasmid digestion (Figure 4.2),

and finally the clones with the expected insert size were subjected to nucleotide sequencing.

The cloned sequences of *OsNUC1* promoter from the four rice cultivars/lines were compared by sequence alignment in order to determine whether there was variation in their promoter architectures. Together, these sequences were analyzed for the published *cis*-regulatory elements by PLACE and PlantCARE algorithms. As presented in Figure 4.3, the 1,355-bp sequence upstream of the transcription start site in the rice cultivar LPT123 was identical to that in the cultivar KDML105. They also shared high sequence similarity with those in LPT123-TC171 and FL530-IL lines; with only one deletion site and a small number of nucleotide mismatches.

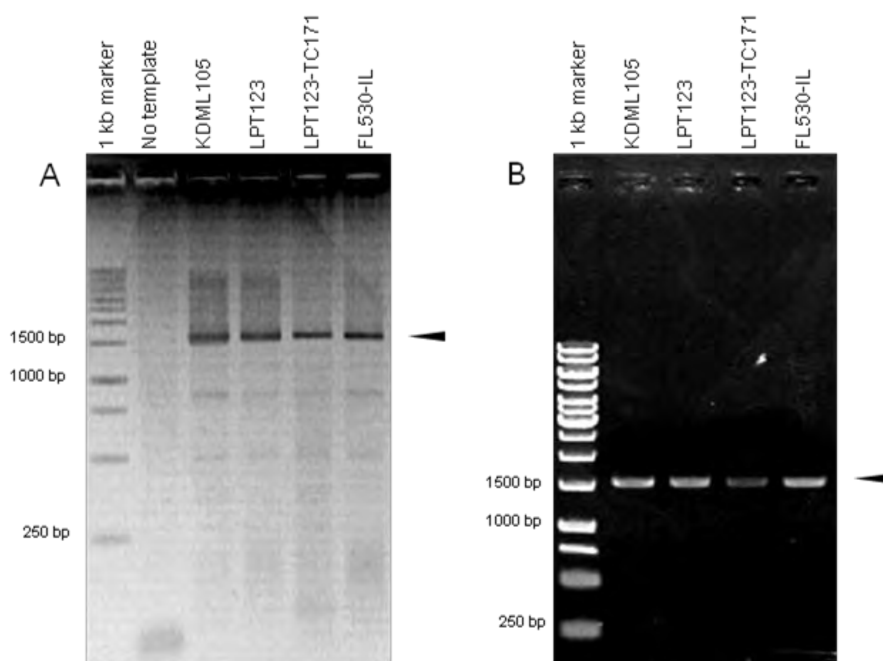


Figure 4.1 PCR amplification of *OsNUC1* promoter from each of the rice cultivars/lines. As indicated by arrow heads, the expected product of ~1,500 bp in (A) was isolated and purified from agarose gel to use for cloning. The purified PCR products are presented in (B). The sizes of molecular weight marker are indicated on the left of each panel.

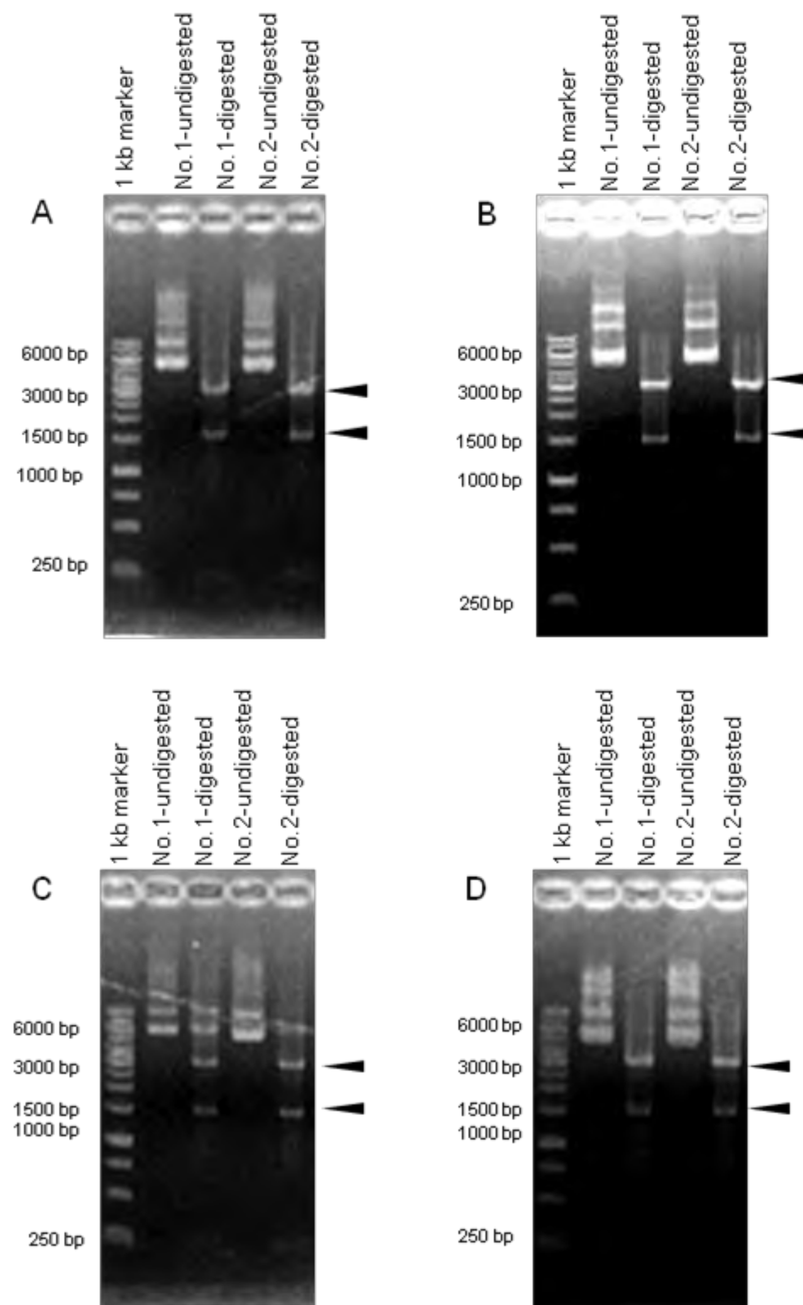


Figure 4.2 Estimation of the insert sizes in positive clones by *Eco*RI and *Xho*I digestion. Panel (A) to (D) represent the clones of *OsNUC1* promoter from the cultivar/line LPT123, LPT123-TC171, KDML105 and FL530-IL, respectively. Digested products were visualized on 0.8% agarose gel. The sizes of molecular weight marker are indicated on the left of each panel. Upper and lower arrow heads in each panel indicate the size of the emptied vector (~3,000 bp) and released inserts (~1,500 bp), respectively.

On the one hand, the deletion site found in a poly(T) region 1,202 bp upstream of the transcription start site in the cultivar LPT123 and KDML105 did not give rise to any element difference. On the other hand, 19 nucleotide mismatches led to the higher number of *cis*-elements on the promoter of LPT123-TC171 and FL530-IL lines, respectively (Figure 4.3).

A change from thymine, at position -278 in the cultivar LPT123 and KDML105, to cytosine in LPT123-TC171 and FL530 contributed to the presence of a MYB recognition site (5'-AAACCA-3') (Abe et al., 2003) in the line LPT123-TC171 and FL530 themselves.

Changes in nucleotide sequence, from guanine (-445) in the cultivar/line LPT123, KDML105 and FL530-IL to adenine in LPT123-TC171 as well as from thymine (-899) in LPT123 and KDML105 to cytosine in the other two lines, resulted in the occurrence of MYC recognition sites (5'-CAGATG-3' and 5'-CATTTG-3', respectively) (Abe et al., 2003; Chinnusamy et al., 2003; Hartmann et al., 2005; Agarwal et al., 2006) on those regions.

Furthermore, the presence of "ACGT" sequence at position -598 to -601 in the line LPT123-TC171 and FL530-IL brought about the core of ABA responsive elements (ABREs) (Simpson et al., 2003; Suzuki, Ketterling, and McCarty, 2005).

Moreover, the presence of a nucleotide guanine (-1059) in the line LPT123-TC171 and FL530-IL, instead of adenine in KDML105 and FL530-IL, conferred the "CWWWWWWWWG" motif; a binding site of MADS domain protein AGL15 (AGAMOUS-like15) (Tang and Perry, 2003).

Lastly, the occurrence of guanine at position -1,164 in the line LPT123-TC171 contributed to the ARR1-binding motif (5'-AGATT-3') (Sakai, Aoyama, and Oka, 2000; Ross, et al., 2004).

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LPT123-TC171    GCCCTTGCTTTATACTGCGGAGAGGCGCCTCACAAAGTCACAATCCGCGGTCCCGACATG -1296
FL530-IL        GCCCTTGCTTTATACTGCGGAGAGGCGCCTCACAAAGTCACAATCCGCGGTCCCGACATG -1296
LPT123          GCCCTTGCTTTATACTGCGGAGAGGCGCCTCACAAAGTCACAATCCGCGGTCCCGACATG -1295
KDML105         GCCCTTGCTTTATACTGCGGAGAGGCGCCTCACAAAGTCACAATCCGCGGTCCCGACATG -1295
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LPT123-TC171    TCCGCCCGCGCGCGGGAACGACGACGACGACGAAGCCATTCTTCCTCGCTACGTCACGTC -1236
FL530-IL        TCCGCCCGCGCGCGGGAACGACGACGACGACGAAGCCATTCTTCCTCGCTACGTCACGTC -1236
LPT123          TCCGCCCGCGCGCGGGAACGACGACGACGACGAAGCCATTCTTCCTCGCTACGTCACGTC -1235
KDML105         TCCGCCCGCGCGCGGGAACGACGACGACGACGAAGCCATTCTTCCTCGCTACGTCACGTC -1235
                ************************************************************

LPT123-TC171    ACCCGTTTGATCGATCGCTTGTGGATTTTTTTTTTACATTTAGCCCTTTTTGAAAACTTA -1176
FL530-IL        ACCCGTTTGATCGATCACTTGTGGATTTTTTTTTTACATTTAGCCCTTTTTGAAAACTTA -1176
LPT123          ACCCGTTTGATCGATCGCTTGTGGATTTTTTTTT-ACATTTAGCCCTTTTTGAAAACTTA -1176
KDML105         ACCCGTTTGATCGATCGCTTGTGGATTTTTTTTT-ACATTTAGCCCTTTTTGAAAACTTA -1176
                ****************.***************** *************************

LPT123-TC171    TTTTATAATTAGATTCTTAAATAAACTTATTATGTTAACACCGAGGTTCAATATCTCACC -1116
FL530-IL        TTTTATAATTAAATTCTTAAATAAACTTATTATGTTAACACCGAGGTTCAATATCTCACC -1116
LPT123          TTTTATAATTAAATTCTTAAATAAACTTATTATGTTAACACCGAGGTTCAATATCTCACC -1116
KDML105         TTTTATAATTAAATTCTTAAATAAACTTATTATGTTAACACCGAGGTTCAATATCTCACC -1116
                ***********.************************************************

LPT123-TC171    CTCATTCTCCTATAGAAATTAAGGACTTGTTTGGTTTAGTACCCTAACAATATATTGGTA -1056
FL530-IL        CTCATTCTCCTATAGAAATTAAGGACTTGTTTGGTTTAGTACCCTAACAATATATTGGTA -1056
LPT123          CTCATTCTCCTATAGAAATTAAGGACTTGTTTGGTTTAGTACCCTACCAATATATTAATA -1056
KDML105         CTCATTCTCCTATAGAAATTAAGGACTTGTTTGGTTTAGTACCCTACCAATATATTAATA -1056
                **********************************************.*********..**

LPT123-TC171    GTGCTAAACTCTAGGCAATTTTTGGGACTATCAATAGTTTGGTAAGGTATAATTGTAAAT -996
FL530-IL        GTGCTAAACTCTAGGCAATTTTTGGGACTATCAATAGTTTGGTAACGTATAATTGTAAAT -996
LPT123          GTGCTAAACTCTAGGCAATTTTTGGAACTATCAATAGTTTGGTAAGGTATAATTGTAAAT -996
KDML105         GTGCTAAACTCTAGGCAATTTTTGGAACTATCAATAGTTTGGTAAGGTATAATTGTAAAT -996
                *************************.******************* **************

LPT123-TC171    GAGTCTTTTTGGATTTATGCCAATGAGAGCCAAATTTCAAATTGTGGTGAAGAATTTTCA -936
FL530-IL        GAGTCTTTTTGGATTTATGCCAATGAGAGCCAAATTTCAAATTGTGGTGAAGAATTTTTA -936
LPT123          AAGTCTTTTTGGATTTATGCCAATGAGAGCCAAATTTCAAATTGTGGTGAAGAATTTTCA -936
KDML105         AAGTCTTTTTGGATTTATGCCAATGAGAGCCAAATTTCAAATTGTGGTGAAGAATTTTCA -936
                .********************************************************* *

LPT123-TC171    AAATTTGTGCCAAATTAAGTATAGACATTACCAAACATTTGGCTTCAAACCAAATCATCA -876
FL530-IL        AAATTTGTGCCAAATTAAGTATAGACATTACCAAACATTTGGCTTCAAACCAAATCATCA -876
LPT123          AAATTTGTGCCAAATTAAGTATAGACATTACCAAATATTTGGCTTCAAACCAAATCATCA -876
KDML105         AAATTTGTGCCAAATTAAGTATAGACATTACCAAATATTTGGCTTCAAACCAAATCATCA -876
                *********************************** ************************

LPT123-TC171    CATTTACTATTTAAACTACCAAAAGATTGGTATGATAACATTTTGGTATCAAACCAAAAA -816
FL530-IL        CATTTACTATTTAAACTACCAAAAGATTGGTATGATAACATTTTGGTATCAAACCAAAAA -816
LPT123          CATTTACTATTTAAACTACAAAAAGATTGGTATGATAACATTTTGGTATCAAACCAAAAA -816
KDML105         CATTTACTATTTAAACTACAAAAAGATTGGTATGATAACATTTTGGTATCAAACCAAAAA -816
                *******************.****************************************

LPT123-TC171    GCCCCTAAGTCACATATGTAAAACTAAGCCAATGTCACTGGCGCCAAAGACACTAGCATA -756
FL530-IL        GCCCCTAAGTCACATATGTAAAACTAAGCCAATGTCACTGGCGCCAAAGACACTAGCATA -756
LPT123          GCCCCTAAGTCACATATGTAAAACTAAGCCAATGTCACTGGCGCCAAAGACACTAGCATA -756
KDML105         GCCCCTAAGTCACATATGTAAAACTAAGCCAATGTCACTGGCGCCAAAGACACTAGCATA -756
                ************************************************************

LPT123-TC171    AACGACCTGTTTGAAGATTTTTTTTTACATTTAATCCTTTTTATTTTTTTACAAATAGAT -696
FL530-IL        AACGACCTGTTTGAAGATTTTTTTTTACATTTAATCCTTTTTATTTTTTTACAAATAGAT -696
LPT123          AACGACCTGTTTGAAGATTTTTTTTTACATTTAGTCCTTTTTATTTTTTTACAAATAGGT -696
KDML105         AACGACCTGTTTGAAGATTTTTTTTTACATTTAGTCCTTTTTATTTTTTTACAAATAGGT -696
                *********************************.************************.*

LPT123-TC171    CCTCAACACCGATTCTCTTAACATTAGTGACGTAGCTTCTACGGAAAGGGAGGGTGTCGG -636
FL530-IL        CCTCAACACCGATTCTCTTAACATTAGTGACGTAGCTTCTACGGAAAGGGAGGGTGTCGG -636
LPT123          CCTCAACACCGATTCTCTTAACATTAGTGACGTAGCTTCTGCGGAAAGGGAGGGTGTCGG -636
KDML105         CCTCAACACCGATTCTCTTAACATTAGTGACGTAGCTTCTGCGGAAAGGGAGGGTGTCGG -636
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LPT123-TC171    TGCCAATATCATTAAGACAGTGACATGAACCTCAACGTCGGGGTCACTGACACCGAGAGA -576
FL530-IL        TGCCAATATCATTAAGACAGTGACATGAACCTCAACGTCGGGGTCACTGACACCGAGAGA -576
LPT123          TGCCAATATCATTAAGACAGTGACATGAACCTCAACATCGGGGTCACTGACACCGAGAGA -576
KDML105         TGCCAATATCATTAAGACAGTGACATGAACCTCAACATCGGGGTCACTGACACCGAGAGA -576
                ************************************.***********************

LPT123-TC171    AGGGTTCATTTCTTTAATAAATTTTTCAAGAGTCTATTTGTAAAATACTACCTCCGTTCC -516
FL530-IL        AGGGTTCATTTCTTTAATAAATTTTTCAAGAGTCTATTTGTAAAATACTACCTCCGTCCC -516
LPT123          AGGGTTCATTTCTTTAATAAATTTTTCAAGAGTCTATTTGTAAAATACTACCTCCGTCCC -516
KDML105         AGGGTTCATTTCTTTAATAAATTTTTCAAGAGTCTATTTGTAAAATACTACCTCCGTCCC -516
                ********************************************************* **

LPT123-TC171    ATTTTAAATGCGGTTATAGATTTTCGTCTCCAACGCTTGACCGTCCGTTTTATTTGTCAA -456
FL530-IL        ATTTTAAATGCGGTTATAGATTTTCGTCTCCAACGCTTGACCGTCCGTTTTATTTGTCAA -456
LPT123          ATTTTAAATGCGGTTATAGATTTTCGTCTCCAACGCTTGACCGTCCGTTTTATTTGTCAA -456
KDML105         ATTTTAAATGCGGTTATAGATTTTCGTCTCCAACGCTTGACCGTCCGTTTTATTTGTCAA -456
                ************************************************************

LPT123-TC171    CAAGAATGGCAGATGGGGCCCATATGTCAGTGGGTTCCACAATTTTTTCTCGTGCGAATG -396
FL530-IL        CAAGAATGGCGGATGGGGCCCATATGTCAGTGGGTTCCACGATTTTTTCTCGTGCGAATG -396
LPT123          CAAGAATGGCGGATGGGGCCCATATGTCAGTGGGTTCCACAATTTTTTCTCGTGCGAATG -396
KDML105         CAAGAATGGCGGATGGGGCCCATATGTCAGTGGGTTCCACAATTTTTTCTCGTGCGAATG -396
                **********.*****************************.*******************

LPT123-TC171    ACAAATGAGTCCCACATATTTTTTTTAAATTCTAATATCACATAAACACCACGCCAAACG -336
FL530-IL        ACAAATGAGTCCCACATATTTTTTTTAAATTCTAATATCACATAAGTGCCACGCCAAACG -336
LPT123          ACAAATGAGTCCCACATATTTTTTTTAAATTCTAATATCACATAAGCGCCACGCCAAACG -336
KDML105         ACAAATGAGTCCCACATATTTTTTTTAAATTCTAATATCACATAAGCGCCACGCCAAACG -336
                *********************************************. .************

LPT123-TC171    CCACATGAGACGAACACCAGGTCAACAACGTTACGTTAGCGAAACCGTTCTCCAAAACCA -276
FL530-IL        CCACATGAGACGAACACCAGGTCAACAACGTCACGTTAGCGAAACCGTTCTCCAAAACCA -276
LPT123          CCACATGAGACGAACACCAGGTCAACAACGTCACGTCAGCGAAACCGTTCTCCAAAATCA -276
KDML105         CCACATGAGACGAACACCAGGTCAACAACGTCACGTCAGCGAAACCGTTCTCCAAAATCA -276
                ******************************* **** ******************** **

LPT123-TC171    TCGAGCGAGTCAAACTGCACCGGTTTCAATAGTTCGGGAGTCGGGATATCCGAATTGTAG -216
FL530-IL        TCGAGCGAGTCAAATTGCACCGGTTTCAATAGTTCGGGAGTCGGGATATCCGAATTGTAG -216
LPT123          TCGAGCGAGTCAAATTGCACCGGTTTCAATAGTTCGGGAGTCGGGATATCCGAATTGTAG -216
KDML105         TCGAGCGAGTCAAATTGCACCGGTTTCAATAGTTCGGGAGTCGGGATATCCGAATTGTAG -216
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LPT123-TC171    ATTAGGGATACAAATCATATCCACTCACTTATTAAGGGACCCAAAGTGAACTTATTCCCC -156
FL530-IL        ATTAGGGATACAAATCATATCCACTCACTTGTTAAGGGACCCAAAGTGAACTTATTCCCC -156
LPT123          ATTAGGGATACAAATCATATCCACTCACTTATTAAGGGACCCAAAGTGAACTTATTCCCC -156
KDML105         ATTAGGGATACAAATCATATCCACTCACTTATTAAGGGACCCAAAGTGAACTTATTCCCC -156
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LPT123-TC171    GATGAAACCTACGTGAGGAGGTCCAGCCCGGTCTGGGCCGGCCCAAGCTACAGCCCACAG -96
FL530-IL        GATGAAACCTACGTGAGGAGGTCCAGCGCGGTCTGGGCCGGCCCAAGCTACAGCCCACAG -96
LPT123          GATGAAACCTACGTGAGGAGGTCCAGCCCGGTCTGGGCCGGCCCAAGCTACAGCCCACAG -96
KDML105         GATGAAACCTACGTGAGGAGGTCCAGCCCGGTCTGGGCCGGCCCAAGCTACAGCCCACAG -96
                *************************** ********************************

LPT123-TC171    ACACTGGTGGCCCCGTGGCCTGGCGGCGGGCAGGGACAACCCTAGCAGCAGGTGCCGCCA -36
FL530-IL        ACACTGGTGGCCCCGTGGCCTGGCGGCGGGCAGGGACAACCCTAGCAGCAGGTGCCGCCA -36
LPT123          ACACTGGTGGCCCCGTGGCCTGGCGGCGGGCAGGGACAACCCTAGCAGCAGGTGCCGCCA -36
KDML105         ACACTGGTGGCCCCGTGGCCTGGCGGCGGGCAGGGACAACCCTAGCAGCAGGTGCCGCCA -36
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LPT123-TC171    TATAAAAGGATCGCCCGTGCGGTCTCGCCTCCCCCATTACCTCCCCGCCCGCGCACCTCG +25
FL530-IL        TATAAAAGGATCGCCCGTGCGGTCTCGCCTCCCCCATTACCTCCCCGCCCGCGCACCTCG +25
LPT123          TATAAAAGGATCGCCCGTGCGGTCTCGCCTCCCCCATTACCTCCCCGCCCGCGCACCTCG +25
KDML105         TATAAAAGGATCGCCCGTGCGGTCTCGCCTCCCCCATTACCTCCCCGCCCGCGCACCTCG +25
                ************************************************************
                                                  (+1)
LPT123-TC171    AGCCCCCAGGTCCGCCTCGCCTCTCCGTCGCTTGCCGCCGCCCGCAGTGGTCGCCGGCCG +85
FL530-IL        AGCCCCCAGGTCCGCCTCGCCTCTCCGTCGCTTGCCGCCGCCCGCAGTGGTCGCCGGCCG +85
LPT123          AGCCCCCAGGTCCGCCTCGCCTCTCCGTCGCTTGCCGCCGCCCGCAGTGGTCGCCGGCCG +85
KDML105         AGCCCCCAGGTCCGCCTCGCCTCTCCGTCGCTTGCCGCCGCCCGCAGTGGTCGCCGGCCG +85
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LPT123-TC171      CGCGGGGCTTCGCCGGTGTCGCTTCCTCGTTTGGGTAAGTCGCTCCTCACTCCCGCGTGA +145
FL530-IL          CGCGGGGCTTCGCCGGTGTCGCTTCCTCGTTTGGGTAAGTCGCTCCTCACTCCCGCGTGA +145
LPT123            CGCGGGGCTTCGCCGGTGTCGCTTCCTCGTTTGGGTAAGTCGCTCCTCACTCCCGCGTGA +145
KDML105           CGCGGGGCTTCGCCGGTGTCGCTTCCTCGTTTGGGTAAGTCGCTCCTCACTCCCGCGTGA +145
                  ************************************************************

LPT123-TC171      CTCTACTTCGGTGAATTTTAGGTGTGGGGTTGCCCGGGGGGGGGGGGGGGTTGGTAAGGC +205
FL530-IL          CTCTACTTCGGTGAATTTTAGGTGTGGGGTTGCCGGGGGGGGGGGG----TTGGTAAGGC +201
LPT123            CTCTACTTCGGTGAATTTTAGGTGTGGGGTTGCCGGGGGGGGGGGGG---TTGGTAAGGC +202
KDML105           CTCTACTTCGGTGAATTTTAGGTGTGGGGTTGCCGGGGGGGGGGGG----TTGGTAAGGC +201
                  ********************************** ***********    **********

LPT123-TC171      TAGGGTTTAGG +216
FL530-IL          TAGGGTTTAGG +212
LPT123            TAGGGTTTAGG +213
KDML105           TAGGGTTTAGG +212
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Figure 4.3 Comparison of nucleotide sequences of *OsNUC1* promoter form the rice cultivar/line LPT123, LPT123-TC171, KDML105 and FL530-IL. The alignment was performed by ClustalW program. Asterisks and dots represent identical residues and nucleotide mismatches, respectively. Grey boxes represent *cis*-acting elements that originate from the nucleotide mismatches. The bold character-region indicates the first exon while italic characters represent the first-intron nucleotides. +1 stands for the transcription start.

Based on sequence comparison, the largest number of *cis*-acting elements on the *OsNUC1* promoter was detected in the line LPT123-TC171. Furthermore, the obvious response to salinity of *OsNUC1* gene expression was also observed in this line (Sripinyowanich, 2010). Therefore, the *OsNUC1* promoter region form LPT123-TC171 was chosen as a representative to enumerate all of its potential regulatory elements (Table 4.1).

According to PLACE and PlantCARE searches, the upstream elements for class II promoter, CAAT motifs (Rieping and Schoffl, 1992), were found on the *OsNUC1* promoter. Likewise the core promoter element TATA box which is essential for basal transcription (Tjaden, Edwards, and Coruzzi, 1995) was also present at around 30 bp upstream of transcription start.

Many tissue- and organ-specific elements were present on the *OsNUC1* promoter. Tetranucleotide (CACTFTPPCA1) and GATABOX motifs, which are responsible for mesophyll-specific gene expression (Lam and Chua, 1989; Gowik et al., 2004), were found along the 1.3 kb-length of *OsNUC1* promoter. Furthermore, motifs associated with meristermatic gene expression, including CCGTCC-box, site II element (SITEIIATCYTC) (Welchen and Gonzalez, 2005), and LEAFYATAG (Kamiya et al., 2003), were found in the promoter region as well. Considering the regulation of *OsNUC1* gene expression in root tissues, a number of root- and root hair-associated *cis*-acting elements were revealed. These included the root-apical meristem-specific LEAFYATAG motif (Kamiya et al., 2003), nodulin consensus sequences (NODCON1GM and NODCON2GM) (Stougaard et al., 1990), OSE1ROOTNODULE and OSE2ROOTNODULE motifs (Vieweg et al., 2004; Fehlberg et al., 2005), root hair-specific *cis*-element RHERPATEXPA7 (Kim et al., 2006), ROOTMOTIFTAPOX1 box (Elmayan and Tepfer, 1995), and SPBF binding- SP8BFIBSP8BIB element (Ishiguro and Nakamura, 1992). In addition, anther- and pollen-specific elements, namely GTGA motif (GTGANTG10) (Rogers et al., 2001), POLLEN1LELAT52 box (Bate and Twell, 1998; Filichkin et al., 2004), and quantitative-element (QELEMENTZMZM13) (Hamilton, Schwarz, and Mascarenhas, 1998), were also present. Moreover, it was found that some *cis*-elements associated with seed and embryo specificity were located on the *OsNUC1* promoter as well. These elements comprised -300ELEMENT (Thomas and Flavell, 1990), core of (CA)n element (CACTFTPPCA1) (Ellerstrom et al., 1996), cereal glutenin box (CEREGLUBOX2PSLEGA) (Shirsat et al., 1989), DPBF-1, 2 binding core sequence (DPBFCOREDCDC3) (Kin, Chug, and Thomas, 1997), and SEF4 binding site (SEF4MOTIFGM7S) (Allen et al., 1989; Lessard et al., 1991).

A large number of abiotic stress-related *cis*-regulatory elements were present on the *OsNUC*1 promoter. MYB TF-binding sites, including 3 elements of MYB1AT (Abe et al., 2003) and 1 element of MYBCORE (Urao et al., 1993; Solano et al., 1995), were found. Concomitantly, MYC recognition sites, including 1 element of MYCATRD22 (Abe et al., 1997) and 7 elements of MYCCONSENSUSAT (Abe et al., 2003; Chinnusamy et

al., 2003; Hartmann et al., 2005), were also detected on the *OsNUC1* promoter. Moreover, one dehydration responsive element (DRE1COREZMRAB17) (Kizis and Pages, 2002) was detected on the *OsNUC1* promoter as well. In addition to drought and salt stresses, some *cis*-elements associated with cold signaling, namely HEXMOTIFTAH3H4 motif and LTRECOREATCOR15 motif (Terada et al., 1995; Shimizu et al., 2005), were also found on the *OsNUC1* promoter. Besides, there were some elements involved in anaerobic stress-related gene expression on this proximal promoter regoin. Such elements included ANAERO2CONSENSUS, ANAERO3CONSENSUS, and ARE (Mohanty et al., 2005).

Concerning plant mineral and nutrition, several nutrient- associated elements were predicted. Examples of these elements were sugar starvation-associated CGACG element (CGACGOSAMY3) (Hwang et al., 1998), copper response element (CURECORECR) (Quinn et al., 2000), PHR1-binding sequence (P1BS) associated with phosphate starvation-responsive gene expression (Schunmann et al., 2004), and core of sulfur-responsive element (SURECOREATSULTR11) related to sulfur-deficiency response (Maruyama-Nakashita et al., 2005).

Almost every class of phytohormone-regulated elements was found on the *OsNUC1* promoter. ABRELATERD1, an ABA responsive element (ABRE) usually present on the promoter of ABA-responsive genes (Simpson et al., 2003; Nakashima et al., 2006) was detected. Furthermore, 7 boxes of the ACGT sequence (ACGTATERD1) which serves as the core element of ABREs (Suzuki et al., 2005) were present as well (Simpson et al., 2003). *Cis*-acting elements involved in auxin signaling and responses found on the *OsNUC1* promoter included ASF-1 binding site (ASF1MOTIFCAMV), NDE element (CATATGGMSAUR), and TGA-element (Xiang, Miao, and Lam, 1997; Xu, Hagen, and Guilfoyle, 1997; Klinedinst et al., 2000; Redman et al., 2002). For cytokinin responsiveness, ARR1-binding elements (ARR1AT) were detected (Sakai et al., 2000; Ross et al., 2004). GA responsive elements found on *OsNUC1* promoter were Pyrimidine box (PYRIMIDINEBOXOSRAMY1A) and "TATCCA" element (TATCCAOSAMY) (Morita et al., 1998; Mena et al., 2002; Isabel-LaMoneda et al., 2003). Methyl jasmonate

responsive elements included CGTCA- and TGACG-motifs. Concomitantly, ethylene responsive element associated with senescense –related gene expression and circadian control, the ERELEE4 motif, were also present (Itzhaki, Maxson, and Woodson, 1994; Rawat et al., 2005).

A lot of pathogen/elicitor- and wounding-associated motifs was found on the promoter region of *OsNUC1*. Variants of w-box motifs (ELRECOREPCRP1, WBOXNTERF3, WRKY71OS) recognized by WRKY TFs were present (Eulgem et al., 1999; Chen and Chen, 2000; Rushton et al., 2002; Nishiuchi, Shinshi, and Suzuki, 2004; Zhang et al., 2004). Additionally, the core of GCC-box (GCCCORE) and the binding sites of rice BIHD1 factor (BIHD1OS), which are related to jasmonate-mediated gene expression (Brown et al., 2003; Chakravarthy et al., 2003) and disease resistance responses (Luo et al., 2005), respectively, were also located on the promoter.

A number of light- and phytochrome-regulated *cis*-elements were abundantly found on the *OsNUC1* promoter region. For example, CIACADIANLELHC motif necessary for circadian expression of tomato *Lhc* gene (Piechulla, Merforth, and Rudolph, 1998), initiator elements (INRNTPSADB) (Nakamura, Tsunoda, and Obokata, 2002), sequences over-represented in light-induced promoters (SORLIP1AT & SORLIP2AT) (Hudson and Quail, 2003), Box A consensus (PALBOXAPC) (Logemamm, Parniske, and Hahlbrock, 1995), etc.

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
1. Promoter consensus			
CAATBOX1	10 sites: -246/-249, -413/-416, -629/-632, -783/-786, -792/-795, -1021/-1024, -1037/-1040, -1065/-1068, -1124/-1127, -1312/-1315	CAAT	CAAT promoter consensus sequence
CAAT-box	2 sites: -412/-416, -1036/-1040	CAATT	*Glycine max* CAAT-box/ Promoter consensus
CAAT-box	7 sites: -201/-205, -390/-394, -700/-704, -881/-885, -921/-925, -954/-958, -961/-965	CAAAT	*Brassica rapa* CAAT-box/ Promoter consensus
TATABOX5	3 sites -461/-466, -710/-715, -1173/-1178	TTATTT	*Pisum sativum* TATA box/ Promoter consensus

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
TATA-box	9 sites: -369/-373, -464/-468, -510/-514, -705/-709, -713/-717, -723/-727, -730/-734, -1194/-1198, -1201/-1205	TTTTA	*Lycopersicon esculentum* TATA-box/ Core promoter element
TATA-box	7 sites: -498/-501, -913/-916, -1005/-1008, -1061/-1065, -1101/-1105, -1169/-1172, -1342/-1345	TATA	*Arabidopsis thaliana* TATA-box/ Core promoter element
TATA-box	1 site: -1098/-1107	CCTATAAAAA	*Arabidopsis thaliana* TATA-box/ Core promoter element
TATA-box	1 site: -30/-35	TATAAA	*Arabidopsis thaliana* TATA-box/ Core promoter element
TATA-box	1 site: -1061/-1066	ATATAT	*Brassica napus* TATA-box/ Core promoter element
TATA-box	1 site: -359/-363, -557/-561	TAATA	*Glycine max* TATA-box/ Core promoter element

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
TATA-box	1 site: -31/-36	ATATAA	*Brassica oleracea* TATA-box/ Core promoter element
TATABOXOSPAL	1 site: -862/-868	TATTTAA	Binding site for TATA binding protein
2. Tissue-/ Organ-specific expression			
-300ELEMENT	1 site: -531/-537	TGHAAARK	-300 element/ Endosperm-specific expression
BOXIINTPATPB	1 site: -1099/-1104	ATAGAA	Box II/ Plastid genes expression
CACTFTPPCA1	9 sites: -91/-94, -187/-190, -191/-194, -527/-530, -588/-591, -762/-765, -777/-780, -868/-871, -1340/-1343	YACT	Tetranucleotide (CACT)/ Key component of Mesophyll expression module1, Mesophyll-specific gene expression
CANBNNAPA	1 site: -319/-325	CNAACAC	Core of (CA)n element/ Embryo- and endosperm-specific expression
CEREGLUBOX2PSLEGA	1 site: -1178/-1185	TGAAAACT	Cereal glutenin box/ Seed-specific expression
CCGTCC-box	1 site: -470/-475	CCGTCC	*Arabidopsis thaliana* CCGTCC-box/ Meristem-specific gene activation

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
DPBFCOREDCDC3	3 sites: -89/-95, -316/-322, -760/-766	ACACNNG	DPBF-1, 2 binding core sequence/ Embryo-specific expression, ABA responsiveness
GATABOX	3 sites: -209/-206, -228/-231, -839/-842	GATA	GATA box/ Trichome-, vascular element-, epidermal and mesophyll cells-specific expression
GTGANTG10	5 sites: -140/-143, -167/-170, -613/-616, -666/-669, -946/-949	GTGA	GTGA motif/ Pollen-specific expression
LEAFYATAG	1 site: -781/-787	CCAATGT	Root apical meristem-specific expression
NODCON1GM	1 site: -849/-854	AAAGAT	Nodulin consensus sequence/ Root-nodule specific gene expression
NODCON2GM	1 site: -677/-681	CTCTT	Nodulin consensus sequence/ Root-nodule specific gene expression
OSE1ROOTNODULE	1 site: -849/-854	AAAGAT	Organ-specific elements/ Root-nodule- and arbuscule-containing cell-specific expression
OSE2ROOTNODULE	1 site: -677/-681	CTCTT	Organ-specific elements/ Root-nodule- and arbuscule-containing cell-specific expression

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
POLLEN1LELAT52	1 site: -1098/-1102	AGAAA	Anther- and pollen-specific expression
QELEMENTZMZM13	1 site: -312/-317	AGGTCA	Quantitative-element/ Pollen-specific expression
RHERPATEXPA7	1 site: -1237/-1242	KCACGW	Root Hair-specific *cis*-element
ROOTMOTIFTAPOX 1	2 sites: -376/-380, -1060/-1064	ATATT	Root-specific gene expression
SEF4MOTIFGM7S	1 site: -1032/ -1038	RTTTTTR	SEF4 binding site/ Embryo development
SITEIIATCYTC	1 site: -117/-122	TGGGCY	Site II element/ Anther- and meristem-specific gene expression
SP8BFIBSP8BIB	1 site: -865/-871	TACTATT	SPBF binding site/ Regulation of tuberous-root-portein-encoding gene
3. Abiotic and mineral stresses			
ANAERO2CONSEN SUS	1 site: -47/-52	AGCAGC	Anaerobic-stress responsiveness
ANAERO3CONSEN SUS	1 site: -875/-881	TCATCAC	Anaerobic-stress responsiveness
ARE	1 site: -1079/-1084	TGGTTT	*Zea mays* ARE element/ *cis*-acting regulatory element essential for the anaerobic induction
ARE1	1 site: -660/-670	RGTGACNNNG C	Antioxidant response element/ Oxidative-stress responsiveness

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
CGACGOSAMY3	4 sites: -1264/-1268, -1267/-1271, -1270/-1274, -1273/-1277	CGACG	CGACG element/ Sugar starvation
CURECORECR	1 site: - 1074/-1077	GTAC	The core of copper-response element/ Copper- and oxygen-responsive expression
DRE1COREZMRAB 17	1 site: -578/-584	ACCGAGA	Core element of DRE1/ Drought-responsiveness
EECCRCAH1	1 site: -491/-497	GANTTNC	MYB TF-binding site/ Low CO_2-responsiveness
HEXMOTIFTAH3H4	2 sites: -1235/-1240, -1240/-1245	ACGTCA	Cold signaling/ Replication dependent expression of *histone H3* gene in meristems
LTRECOREATCOR1 5	1 site: -1299/-1303	CCGAC	Core of low temperature responsive element / Cold- and drought-induced gene expression, ABA responsiveness
MYB1AT	3 sites: -276/-281, -820/-825, -884/-889	WAACCA	MYB recognition site/ Drought- and ABA-induced gene expression
MYBCORE	1 site: -500/-505	CNGTTR	MYB recognition site/ Drought- and salt-stress responsiveness, ABA induction, secondary metabolism

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
MYCATRD22	1 site: -329/-334	CACATG	Binding site for AtMYC/ Drought- and ABA-regulated gene expression
MYCCONSENSUSAT	7 sites: -42/-47, -329/-334, -389/-394, -430/-435, -441/-446, -798/-803, -895/-900	CANNTG	MYC recognition site/ Dehydration responsiveness, cold regulation CBF genes, light-responsive and tissue-specific activation of phenylpropanoid biosynthetic genes
P1BS	1 site: -225/-232	GNATATNC	PHR1-binding sequence/ Phosphate starvation-responsive gene expression
SURECOREATSULTR11	1 site: -325/-329	GAGAC	Core of sulfur-responsive element / Sulfur-deficiency response
4. Hormone responses			
ABRELATERD1	1 site: -140/-146	ACGTG	ABRE-like sequence/ Etiolation-induced expression, drought- and ABA-responsive expression
ACGTATERD1	7 sites: -141/-145, -299/-303, -305/-308, -598/-601, -663/-666, -1237/-1240, -1242/-1245	ACGT	ACGT sequence/ Core element of ABREs/ Etiolation-induced expression, drought- and ABA-responsive expression

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
ARR1AT	8 sites: -213/-217, -494/-498, -682/-686, -737/-741, -848/-852, -981/-985, -1161/-1165, -1209/-1213	NGATT	ARR1-binding element/ Cytokinin-regulated gene expression
ASF1MOTIFCAMV	1 site: -664/-668	TGACG	TGACG motif/ ASF-1 binding site/ Auxin and/or salicylic acid-activated gene expression, light regulation, abiotic- and biotic-stress responsiveness
CATATGGMSAUR	2 sites: -430/-435, -798/-803	CATATG	NDE element/ Auxin responsiveness
CGTCA-motif	2 sites: -1235/-1239, -1240/-1244	CGTCA	*Hordeum vulgare* CGTCA-motif/ Methyl jasmonate-responsiveness
ERELEE4	1 site: -955/-962	AWTTCAAA	Ethylene responsive element/ Ethylene responsiveness, senescence-related expression, circadian control
PYRIMIDINEBOXOSRAMY1A	2 sites: -715/-720, -1186/-1191	CCTTTT	Pyrimidine box/ GA-responsiveness, sugar-repressed gene expression

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
TATCCAOSAMY	1 site: -193/-198	TATCCA	"TATCCA" element/ Binding sites of OsMYBS1, OsMYBS2 and OsMYBS3/ GA response and sugar starvation
TGA-element	3 sites: -750/-755, -1274/-1279, -1275/-1279	AACGAC	*Brassica oleracea* auxin-responsive element
5. Pathogen and wounding			
AT-rich sequence	1 site: -527/-535	TAAAATACT	Element for maximal elicitor-mediated activation in *Pisum sativum*
BIHD1OS	3 sites: -427/-431, -457/-461, -779/-783	TGTCA	Binding site of OsBIHD1/ Disease resistance responses
ELRECOREPCRP1	1 site: -474/-479	TTGACC	Elicitor Responsive Element/ WRKY-binding site/ Pathogen elicitor and wounding responsiveness, salicylic acid-induced expression
GCCCORE	1 site: -37/-42,	GCCGCC	Core of GCC-box / Pathogen-responsive and jasmonate-mediated gene expression
WBOXNTERF3	1 site: -474/-478,	TGACY	W-box/ Wounding

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
WRKY71OS	5 sites: -394/-397, -475/-478, -585/-588, -612/-615, -665/-668	TGAC	A core of TGAC-containing W-box/ Binding site for rice WRKY71/ Biotic stress, GA and ABA signaling
6. Light responsiveness			
Box I	2 sites: -934/-940, -955/-961	TTTCAAA	*Pisum sativum* Box I/ Light responsive element
CIACADIANLELHC	2 sites: -274/-283, -695/-704	CAANNNNATC	Circadian clock-regulated expression
G-box	1 site: -141/-146	TACGTG	*Daucus carota cis*-acting regulatory element involved in light responsiveness
G-box	1 site: -1236/-1241	CACGTC	*Zea mays cis*-acting regulatory element involved in light responsiveness
INRNTPSADB	2 sites: -564/-571, -1108/-1115	YTCANTYY	Initiator elements / Light-responsive transcription
LAMP-element	1 site: -276/-284	CCAAAACCA	Part of a light responsive element in *Spinacia oleracea*
PALBOXAPC	1 site: -470/-475	CCGTCC	Box A Consensus/ Elicitor- or light-mediated gene activation
REALPHALGLHCB21	2 sites: -819/-824, -883/-888	AACCAA	Phytochrome regulation

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
Sp1	1 site: -3/-8	CC(G/A)CCC	*Zea mays* light responsive element
SORLIP1AT	1 site: -332/-336	GCCAC	Sequences Over-Represented in Light-Induced Promoters/ Phytochrome A-regulated gene expression
SORLIP2AT	2 sites: -117/-121, -436/-440	GGGCC	Sequences Over-Represented in Light-Induced Promoters/ Phytochrome A-regulated gene expression
7. Miscellanies			
ACGTTBO	1 site: -304/-309	AACGTT	T-box/ Plant bZIP proteins-binding site
ATGCAAAT motif	1 site: -201/-208	ATACAAAT	*Cis*-acting regulatory element associated with the TGAGTCA motif in *Oryza sativa*
AT-rich element	1 site: -1004/-1104	ATAGAAATCAA	binding site of AT-rich DNA binding protein (ATBP-1) in *Glycine max*
CARGCW8GAT	2 sites: -28/-37, -1059/-1068	CWWWWWWWWG	A variant of CArG motif/ Plant MADS domain proteins-binding site/ embryo development
CARGNCAT	1 site: -27/-38	CCWWWWWWWWGG	A relevant *cis* element for AGL15/ Plant MADS domain proteins-binding site/ embryo development

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
CGCGBOXAT	4 sites: -1281/-1286, -1283/-1288, -1285/-1290, -1306/-1311	VCGCGB	CGCG box/ AtSRs-binding site/ $Ca^{2+/}$CaM -mediated signal transduction
CTAG-motif	1 site: -755/-764	ACTAGCAGAA	*Avena sativa* CTAG-motif
DOFCOREZM	7 sites: -28/-31, -170/-173, -648/-651, -767/-770, -815/-818, -851/-854, -1319/-1322	AAAG	Dof TFs-binding site/ Carbon metabolism, endosperm-specific expression
E2FCONSENSUS	1 site: -155/-162	WTTSSCSS	E2F consensus sequence/ Cell cycle regulation, DNA replication, and chromatin dynamics
MARTBOX	2 sites: -706/-715, -708/-717	TTWTWTTWTT	T-Box
MYBST1	2 sites: -206/-210, -228/-232	GGATA	MYB recognition site
POLASIG1	2 sites: -555/-560, -1151/-1156	AATAAA	PolyA signal

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

Table 4.1 *In silico* characterization of the *OsNUC1* promoter region (continued)

Element ID/ Name	Sites: Positions	Core sequence (5'→3')	Descriptions/ Functions
RAV1AAT	3 sites: -309/-313, -454/-458, -688/-692	CAACA	RAV1-binding site
TATCCAYMOTIFOSRAMY3D	1 site: -192/-198	TATCCAY	TATCCAY motif
TGACGTVMAMY	1 site: -663/-668	TGACGT	TGACGT motif
UP1ATMSD	1 site: -431/-439	GGCCCAWWW	Up1 motif/ Axillary bud outgrowth
WBOXATNPR1	1 site: -475/-479	TTGAC	W-box

*R=A/G, Y= C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

2. **The effect of *OsCam1-1* on *OsNUC1* gene expression under salt-stress condition**

The effect of the salt-stress signal transducer, *OsCam1-1* gene, on expression of the *OsNUC1* gene was determined in rice by comparing the amount of *OsNUC1* transcripts under salt-stress condition between three independent lines of transgenic rice constitutively expressing the *OsCam1-1* gene (35SCaM1-1T1, 35SCaM1-1T2, and 35SCaM1-1T3), the T-DNA insertion control line (without the transgene *OsCam1-1*) (T1) and the wild type KDML105, using RT-qPCR as a tool.

The calculation of *OsNUC1* transcripts was carried out by means of normalized expression (ΔΔCq), using the housekeeping gene *OsEF-1*α as an internal control. Amplification efficiency of the PCR reaction for both *OsNUC1* and *OsEF-1*α genes was first determined by standard curve analysis, using 4 concentrations of 5-fold dilutions of cDNA templates. Real-time PCR efficiency (E) was calculated according to Pfaffl (2001); $E = 10^{-1/slope}$. In this experiment, the amplification efficiency (E-1) of the *OsNUC1* and *OsEF-1*α were 108.5% ($r^2 = 0.996$) and 105.5% ($r^2 = 0.999$), respectively.

According to the RT-qPCR analysis, it was found that the *OsNUC1* expression patterns in all of the rice lines/cultivars tested, both in normal and salt-stress conditions, were rather similar to one another. In every line tested, the *OsNUC1* transcripts reached the highest levels at 12 h after the beginning of the treatment and started declining to its basal levels afterwards (Figure 4.4).

In the wild type line, KDML105, expression levels of *OsNUC1* between the salt-stressed and the control groups were not different at any time point of the experiment (Figure 4.4-A), which indicates that the *OsNUC1* gene in this cultivar is not responsive well to salinity. In contrast to the wild type, however, the T-DNA insertion control line (T1) demonstrated a significantly higher level of *OsNUC1* transcripts since at the beginning (0h) of the treatment when compared to the wild type. Moreover, the T1 line in the salt-treated group showed significantly higher level of *OsNUC1* transcript than the control at 2 days after the treatment.

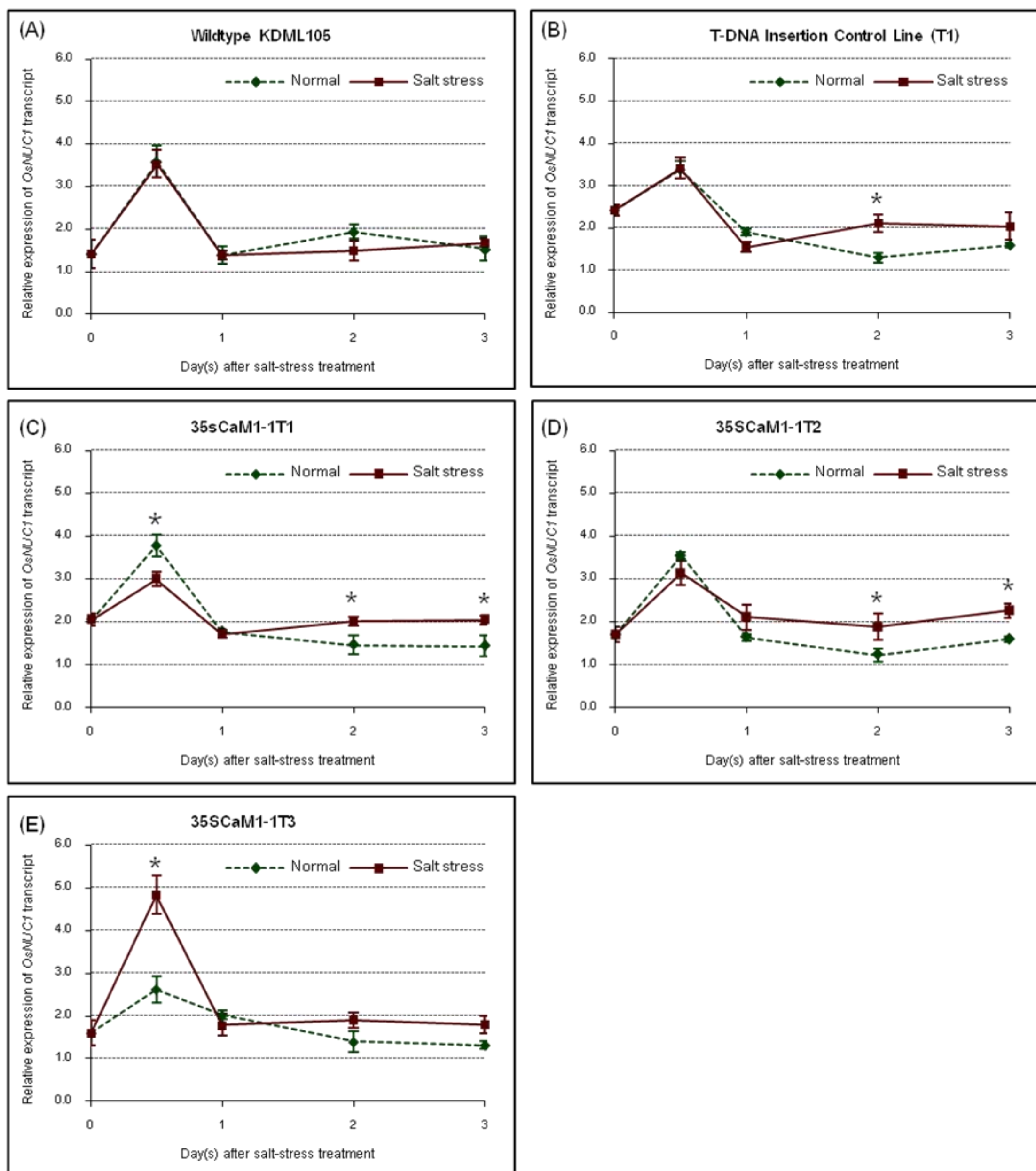

Figure 4.4 Salt-stress responsive expression patterns of the *OsNUC1* in the rice KDML105 (A), the T-DNA insertion line (B), and three lines of the transgenic rice constitutively overexpressing *OsCam1-1* gene (C-E). The presented data are means of three biological replicates and SE. Asterisks indicate the significant difference of transcript levels between normal and salt-stress conditions at each time point. (DMRT, ANOVA; $P < 0.05$)

In regard to three lines of the transgenic rice constitutively expressing *OsCam1-1* gene, it was found that the *OsNUC1* expression of the transgenic 35SCaM1-1T1 in the salt-inflicted group was higher than in the control at 2 and 3 days after the salt-stress treatment (Figure 4.4-C). The expression profiles of the transgenic 35SCaM1-1T2 were in the same fashion as that of the 35SCaM1-1T1, showing the significantly higher *OsNUC1* transcripts after 2 and 3 days of salt imposition (Figure 4.4-D). Surprisingly, the transgenic 35SCaM1-1T3 exhibited the distinct patterns of gene expression in that the significant up-regulation of *OsNUC1* transcripts in salt-stressed group was detected since 12 h of the treatment (Figure 4.4-E).

Taken together, there was at least one time point of the treatment that the transgenic lines overexpressing *OsCam1-1* genes showed up-regulation of *OsNUC1* transcript by salinity whereas the wild type KDML105 showed no response to salinity, which indicated that *OsNUC1* expression under salt-stress condition was positively affected by *OsCam1-1* expression levels.

3. Direct interaction between OsCaM1-1 and putative rice R2R3-MYB TF proteins

To inspect the existence of CaM-MYB protein interaction in rice, the potential CaM-binding MYB TFs were identified from the rice genome and were tested for their interactions with CaM.

3.1. Identification of putative CaM-binding R2R3-MYB TFs in rice

Functional search against the rice genome, using AtMYB2 protein sequence as the query, yielded a number of loci that encode for putative MYB proteins similar to the AtMYB2. Of those, 9 loci with the corresponding proteins of highest similarities to the AtMYB2 (E value from 4.8×10^{-62} to 1.1×10^{-41}) were selected as candidates. As shown in Table 4.2, the open reading frames of these genes were between 687-1,053 bp in length, yielding the proteins of 228-350 amino acids which shared 44.8-52.4% of sequence similarities with AtMYB2.

Table 4.2 Characteristics of the identified rice putative CaM-binding MYB TF genes and their encoded proteins

Locus Name	ORF length (bp)	Amino acids	%Similarity with AtMYB2	No. of MYB domains*	Transcript evidence[#]	
					No. of ESTs	Full-length cDNA clones
LOC_Os05g04210.1	933	310	51.3%	2	6	AK243659
LOC_Os03g20090.1	990	329	50.3%	2	36	AK120551, CT830663, CT834128
LOC_Os07g48870.1	903	300	50.6%	2	15	AK103455
LOC_Os01g19330.1	882	293	51.7%	2	4	AK241615
LOC_Os11g45740.1	858	285	52.4%	2	14	AK069082, AY026332
LOC_Os01g03720.1	1,053	350	44.8%	2	3	AK107424
LOC_Os12g37690.1	903	300	46.2%	2	12	AK072403
LOC_Os01g45090.1	687	228	47.9%	2	3	-
LOC_Os02g40530.1	870	289	47.1%	2	10	AK111807, AK099283

* The presented no. of MYB domains was according to Pfam database (http://pfam.sanger.ac.uk/)

[#] Transcript evidence presented in the table was summarized from the Rice Genome Browser (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/)

Their deduced amino acids were also screened for functional domains in Pfam database. The results from Pfam searches showed that all of the proteins contained two MYB DNA-binding, R2 and R3 domains.

Furthermore, the verification of transcript evidence of the identified R2R3-MYB genes was done by searching for their reported ESTs and cDNAs. As shown in Table 4.2, all of the selected MYB TF-producing genes showed their respective EST and/or cDNA clones.

The homology of the identified rice R2R3-MYBs and the *Arabidopsis* CaM-binding TFs was confirmed by phylogenetic analysis. A Maximum Likelihood tree was reconstructed from all of the R2R3-MYB proteins from both the rice and *Arabidopsis* genomes. As presented in Figure 4.5, the phylogenetic tree grouped seven of the nine rice R2R3-MYB proteins in the same clade with the *Arabidopsis* CaM-binding MYB2, MYB62 and MYB78, which were previously classified to the subgroup 20 (Kranz et al., 1998; Stracke et al., 2001). Aside from two highly conserved MYB domains at the amino-terminus (N-terminus), these seven R2R3-MYB TFs shared the same WxPRL motif with those *Arabidopsis* members at the carboxy-terminus (C-terminus). On the other hand, the other two (LOC_Os01g45090.1 and LOC_Os02g40530.1) proteins did not contain any motif at their C-termini, hence not being classified into any subgroup (Figure 4.5).

Possible roles of the identified rice R2R3-MYB TFs in abiotic stress responses were also evaluated by their expression profiles from microarray experiments in the Rice Oligonucleotide Array Database (Jung et al., 2008). It was found that six of the identified rice R2R3-MYB TFs encoding genes including LOC_Os03g20090.1, LOC_Os07g48870.1, LOC_Os01g19330.1, LOC_Os11g45740.1, LOC_Os12g37690.1, and LOC_Os02g40530.1 were up-regulated under drought and/or salt stresses (Figure 4.6; Appendix B), five of which belonged to subgroup 20.

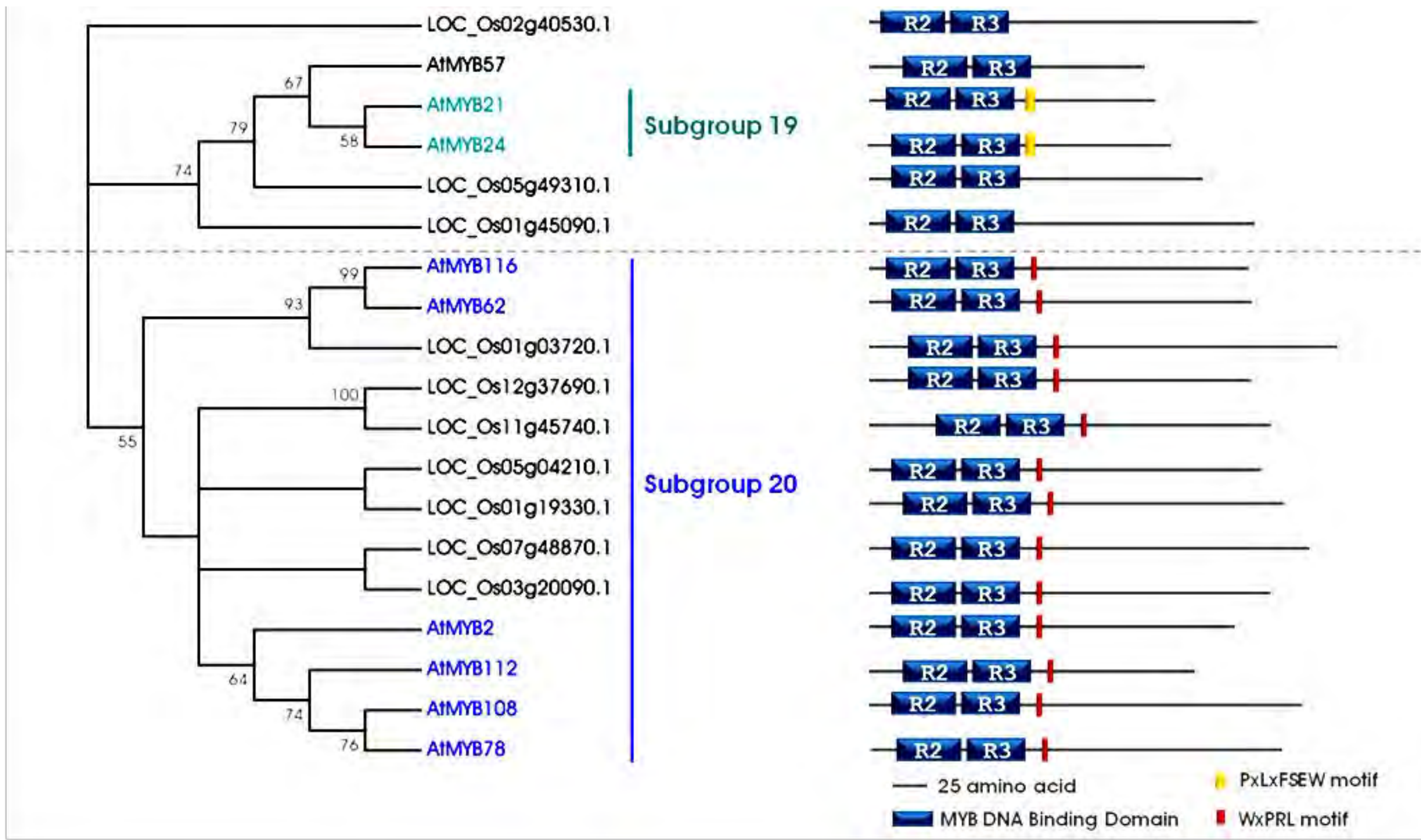

Figure 4.5 Schematic representations of the protein structures and phylogenetic relationships between the identified rice R2R3-MYB TFs with the members of *Arabidopsis* R2R3-MYB Subgroup 19 and 20.

The tree was inferred using the maximum likelihood method and 1000 bootstraps with putative amino acid sequences of rice and *Arabidopsis* R2R3-MYB TFs. Bootstrap values lower than 50 were omitted.

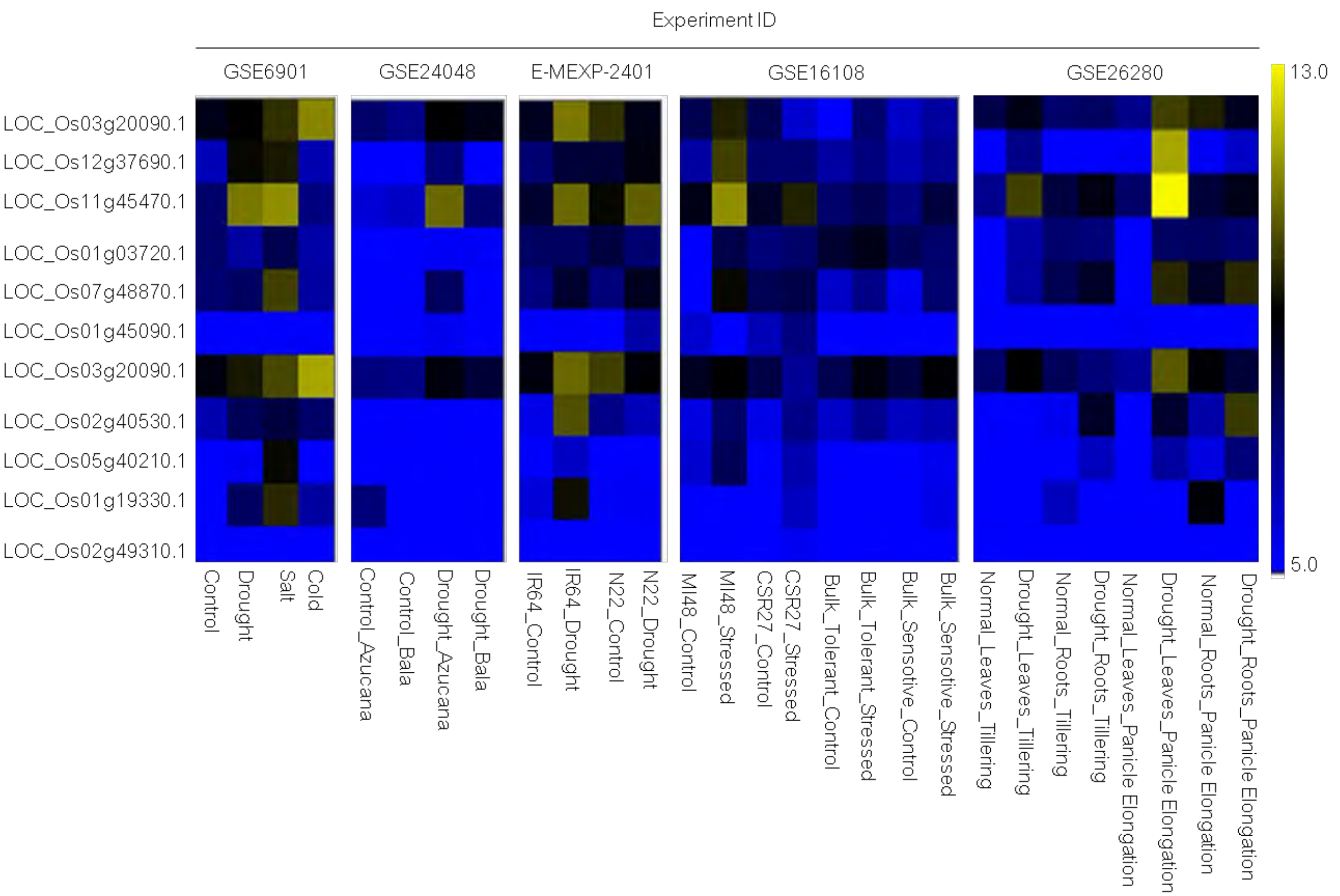

Figure 4.6 Heat map representing gene expression levels of the identified rice R2R3-MYB TFs under abiotic stresses.

The gene expression data were downloaded from Rice Oligonucleotide Array Database (http://www.ricearray.org/)

The motif for CaM recognition of *Arabidopsis* MYB TFs has been experimentally identified in AtMYB2 (Yoo et al., 2005). It stretches over 18 contiguous amino acids with an amphipathic characteristics, a separation of basic and polar amino acids on one side but hydrophobic residues on the other side (Figure 4.7-A). CaM-binding motifs of the rice R2R3-MYB TFs were predicted and compared to those of *Arabidopsis* CaM-binding R2R3-MYBs. As presented by sequence comparison in Figure 4.7-B, most of the predicted motifs of the rice MYB TFs showed similar patterns to the 1-5-10 motif of AtMYB2.

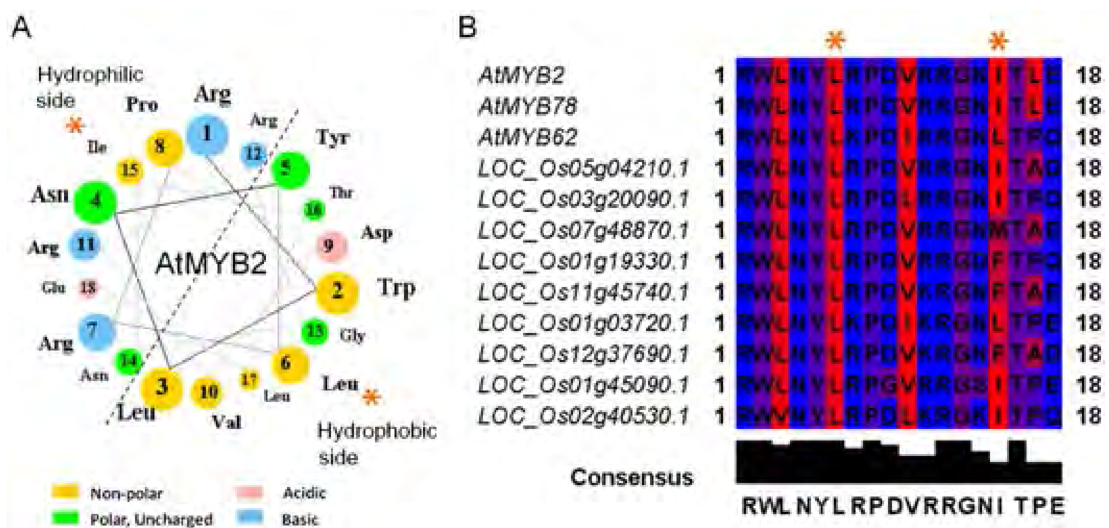

Figure 4.7 CaM-binding motif of AtMYB2 (A) and comparison of the putative motifs from rice R2R3-MYB TFs with *Arabidopsis* CaM-binding MYB TFs (B). The CaM-binding motif of AtMYB2 in (A) is presented in the form of helical wheel projection. In (B), the most hydrophobic residues, according to the Kyte-Doolittle hydrophobicity scale (Kyte and Doolittle, 1982), are colored red and the most hydrophilic ones are colored blue. Asterisks indicate the key residues for CaM-interaction (Yoo et al., 2005).

3.2. Determination of protein-protein interaction by yeast two-hybrid assay

In this study, the GAL4-based yeast two-hybrid system was used to assess physical interactions between the putative R2R3-MYB TFs, which were previously identified in 3.1., with the salt-stress responsive OsCaM1-1. Four of the identified rice R2R3-MYB TFs was selected for the test by using sequence similarity, availability of full-length cDNA clones, and a propensity to form a CaM-binding motif as criteria. These selected genes included LOC_Os03g20090.1, LOC_Os07g48870.1, LOC_Os11g45740.1, and LOC_Os02g40530.1.

Each of the four R2R3-MYB proteins was individually expressed as prey, a GAL4-AD fusion protein, whereas OsCaM1-1 fused to GAL4-DBD served as bait. Yeast transformants capable of growing on SD-Leu-Trp medium were analyzed for protein interaction on two selective media (SD-Leu/-Trp/-Ura and SD-Leu/-Trp/-His+3AT) and by X-gal assay.

As shown in Figure 4.8, results from two independent experiments showed that the yeast harboring each combination of bait and prey vectors neither grew on SD-Leu/-Trp/-Ura or SD-Leu/-Trp/-His+3AT media nor displayed β-galactosidase activity, whereas the positive controls did, indicating the absence of protein interaction.

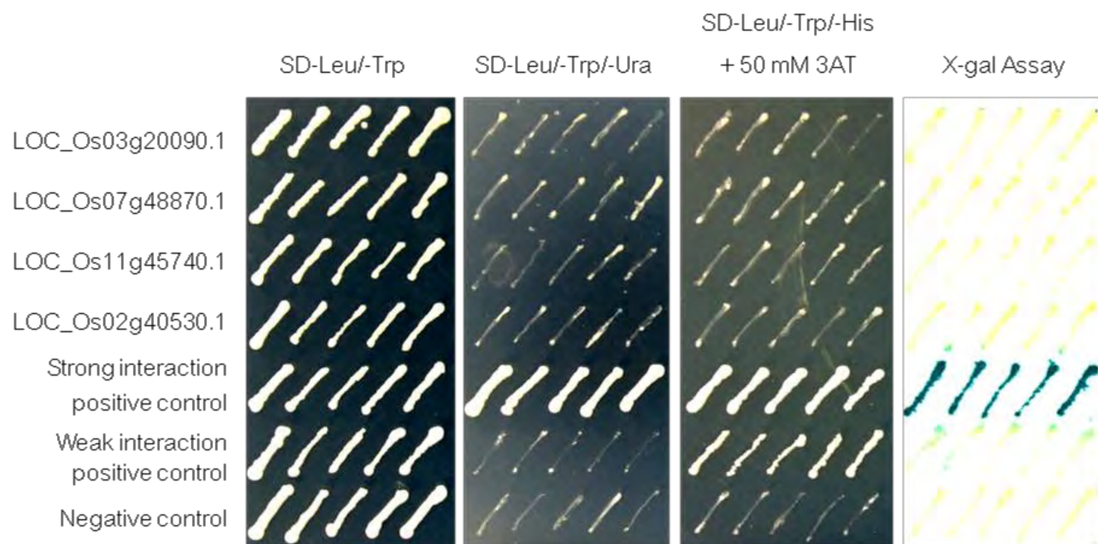

Figure 4.8 Yeast two-hybrid assay for the interaction between OsCaM1-1 and the putative CaM-binding R2R3-MYB TFs. The growth of yeast cells on SD-Leu/-Trp/-Ura and SD-Leu/-Trp/-His + 50 mM 3AT was determined after 4 and 3 days, respectively. Five independent clones of the yeast transformants are presented.

CHAPTER V
DISCUSSIONS

1. Cloning and *in silico* characterization of *OsNUC1* promoter

In the present study, the promoter region of *OsNUC1* gene was successfully cloned from two pairs of rice cultivars/lines, each of which has similar genetic backgrounds but different salt-tolerant phenotypes (Suriya-arunroj et al., 2004; Sripinyowanich, 2010; Saeng-ngam et al., 2012) and salt-responsive *OsNUC1* expression profiles (Chamnanmanoontham, 2009; Sripinyowanich, 2010). The four cultivars/lines included LPT123, KDML015 (salt-sensitive, low *OsNUC1* expression), LPT123 and FL530-IL (salt tolerance, high *OsNUC1* expression).

The promoter sequence from each of the rice cultivars/lines was analyzed for its containing *cis*-acting elements. It was found that the line LPT123-TC171and FL530-IL, respectively, contained greater number of *cis*-regulatory elements than the other two cultivars, and most of which are associated with dehydration and salt-stress responsive gene expression. Figure 5.1 summarized dehydration/salt-specific *cis*-regulatory elements embedded on the *OsNUC1* promoter in each of the rice cultivars/lines. The line LPT123-TC171 and FL530-IL contained four elements of MYB-recognition motifs, while only three of the motifs were found in the cultivar LPT123 and KDML105. Similarly, MYC-recognition motifs were found on seven and six locations in the line LPT123-TC171 and FL530-IL, respectively, while these motifs were found only on five sites in the cultivar LPT123 and KDML105. Likewise, the number of “ACGT” sequence, a core of ABA-responsive elements (ABREs) (Suzuki et al., 2005), in the line LPT123-TC171 and FL530-IL was found one motif higher than in the other two cultivars.

It has been reported in *Arabidopsis* that expression of a stress-responsive gene, *rd22*, is coordinately regulated by MYB and MYC TFs. Two closely located MYB and MYC recognition sites in a 67-bp region between the position -141 and -207 of *rd22* promoter were sufficient for the gene induction by dehydration and ABA, and MYB

(AtMYB2) and MYC (rd22BP1 or AtMYC2) proteins activated *rd22* transcription through these respective elements (Abe et al., 1997). As presented in Figure 5.1, it should be noted that there was no closely embedded MYB and MYC elements present on the proximal region of *OsNUC1* promoter in the cultivar LPT123 and KDML105, while they were present in the line LPT123-TC171 and FL530-IL. Likewise, the distal *OsNUC1* promoter of the line LPT123-TC171 and FL530-IL contained two pairs of closely located MYB and MYC elements but only one pair was present in the cultivar LPT123 and KDML105. If it was the case, as in *Arabidopsis*, that MYB- and MYC-class proteins are the specific regulator of *OsNUC1* expression in salt stress, these differences in number and distributions of MYB- and MYC- recognition motifs on the gene promoter might be able to account for the higher levels of *OsNUC1* expression occurred in the line LPT123-TC171 and FL530-IL.

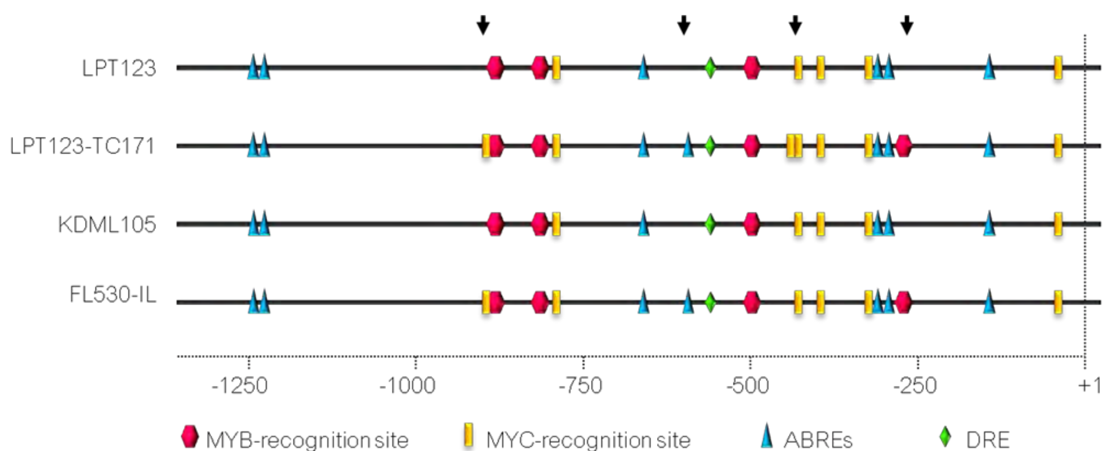

Figure 5.1 A schematic diagram representing the dehydration- and salt-associated *cis*-regulatory elements found on the *OsNUC1* promoter

Arrows indicate the elements that are different between rice cultivars/lines.

The negative numbers represent upstream end of the promoter region.

Apart from the differences in promoter elements found in this study that supported differential expression between each of the rice cultivars/lines, it should be noted that 5'-untranslated region (UTR) also plays crucial roles in gene regulation. Sequence characteristics of 5'-UTR, including presented regulatory *cis*-elements, intron sequence, or secondary structure can have either positive or negative effects on gene expression (Curie and McCormick, 1997). It was reported in poplar plant that a 309-bp, 5'-UTR of the *PtDrl02* gene, including the exon1 and the leading intron, was responsible for the gene regulation by repressing transcription/ destabilizing the gene mRNA (Zheng et al., 2009). Similarly, the translation start of *OsNUC1* was located 1,764 bp downstream the transcription start site. The role of this 5'-UTR region on O*sNUC1* gene regulation should be further investigated.

Looking in details for each group of *cis*-regulatory elements presented on the *OsNUC1* promoter, the analysis of promoter motifs disclosed a number of tissue-and organ-specific *cis*-elements. The presence of root hair-, and root-specific motifs on the *OsNUC1* promoter was consistent with Sripinyowanich's experiment (2010) indicating that the highest level of *OsNUC1* transcript was detected in root tissues. Likewise, in the same experiment, it was found that high levels of the gene expression were also detected in flowers and seeds, which was in agreement with the presence on the promoter of anther- and pollen-related elements, and seed-and embryo-specific elements, respectively. Another group of *cis*-acting elements that supported expression levels of *OsNUC1* in different rice organs in Sripinyowanich's experiment (2010) was a group of meristem-specific elements which were also found on its promoter. Roots, flowers, seeds and young leaves which showed high *OsNUC1* expression levels, were mainly comprised of vigorously dividing meristematic zones. Thereby, it might be these meristem-specific elements that control *OsNUC1* expression in these particular regions, leading to high levels of gene expression in the whole particular organs.

The presence in large number of light- and phytochrome-regulated *ci*s-elements on the *OsNUC1* promoter might perhaps contribute to the expression of the gene. As

suggested by Chamnanmanoontham (2009), the expression of *OsNUC1* was likely to rhythmically change, with distinctively high expression levels in the evening (12 h after the commencement of salt-stress treatment). Moreover, a study on nucleolin-like protein-encoding gene in pea also indicated that the gene was responsive to light, of which the regulation was mediated by phytochrome (Tong et al., 1997).

Most importantly, this study provided us with solid evidence supporting the up-regulation of *OsNUC1* expression under salt-stress condition as a number of crucial dehydration/salt-associated *cis*-acting factors as well as stress hormone-related ABREs on the gene promoter were revealed (Figure 5.1). According to the *cis*-regulatory elements found on its promoter, it is possible that the specific regulators of *OsNUC1* expression in salt stress belonged to one (or more) of the following families; the helix-turn-helix DNA-binding MYB family, the basic helix-loop-helix (bHLH) leucine zipper DNA-binding MYC proteins, the dehydration responsive element-binding proteins (DREBs) of AP2/ERF family, and the basic leuzine zipper domain (bZIP)-containing protein family.

The possibility that the *OsNUC*1 is regulated by MYB-type TFs was supported by the presence of MYB1AT (Abe et al., 2003) and MYBCORE (Urao et al., 1993; Solano et al., 1995) elements on the promoter. These elements were experimentally demonstrated to be recognized by MYB2 and MYB15, which are the regulators of dehydration and cold responses, respectively (Urao et al., 1993; Agarwal et al., 2006). Consistently, a great deal of *Arabidopsis* R2R3-MYB TFs has been reported to be involved in salt/drought stress as well as ABA response. For instance, *AtMYB2, AtMYB13, AtMYB15, AtMYB33, AtMYB41, AtMYB44, AtMYB60, AtMYB73, AtMYB77, AtMYB96, AtMYB101, AtMYB102,*and *AtMYB108* (*BOS1*) (Hoeren et al., 1998; Kirik et al., 1998; Denekamp and Smeekens, 2003; Mengiste, 2003; Cominelli et al., 2005; Zhu et al., 2005; Reyes and chua, 2007; Jung et al., 2008; Ding et al., 2009; Lippold et al., 2009; Seo et al., 2009). Amongst 185 MYB and MYB-related genes in the rice genome (Yanhai et al., 2006), a number of them were reported to be associated with dehydration and

salinity response/tolerance. For example, a 3-R MYB *OsMYB3R-2* was induced by cold-, drought-, and salt-stress. Overexpression of the gene resulted in tolerance to the respective stresses in transgenic plants (Dai et al., 2007). An R2R3-MYB TF, *OsMYB2*, was demonstrated to play a pivotal role in drought, salt, and cold tolerance in rice and served as a master regulator of hundreds of stress-associated genes (Yang et al., 2012).

Along with MYB-binding sites, a number of MYCATRD22 (Abe et al., 1997) and MYCCONSENSUSAT (Abe et al., 2003; Chinnusamy et al., 2003; Hartmann et al., 2005) motifs, which have been reported to be recognized by drought-, salt- and cold-responsive AtMYC2, were also embedded on the *OsNUC1* promoter. These motifs were found on the promoters of such drought- and ABA-responsive genes as *rd22* and *ADH1* (Abe et al., 2003), and of the cold stress-responsive *CBF3* gene, which serves as the regulator of CBF/DREB1 regulon (Chinnusamy et al., 2003; Hartmann et al., 2005; Agarwal et al., 2006). In a Rat1 cell line, it was experimentally demonstrated that MYC TFs activated transcription of *Nucleolin* via an E-box (5'-CACGTG-3') (Greasley, Bonnard, and Amati, 2000), which was resemble the MYCCONSENSUSAT found here on the promoter of *OsNUC1*. Apart from AtMYC2, only a few members of bHLH-MYC protein family were intensively characterized for their regulatory roles in drought and salt stresses. In wild rice species, *Oryza rufipogon bHLH2* (*OrbHLH2*) gene was reported to be induced by salt stress (Li et al., 2010). Overexpression of the gene also contributed to increased salt and osmotic stress tolerance in transgenic plants (Zhou et al., 2009).

One dehydration responsive element (DRE1COREZMRAB17) found on the *OsNUC1* promoter gives rise to the potential of DREB proteins to serve as the *trans*-acting regulator of *OsNUC1.* This element was first reported to be recognized by DBF1 protein, of which the function is involved in desiccation, NaCl and ABA responses (Kizis and Pages, 2002). Regulatory functions of DREB proteins in osmotic stress have been very well characterized. More than one hundred genes have been discovered as DREB-targets (Yamaguchi-Shinozaki and Shinozaki, 2006). The rice genome comprises at

least ten *DREB1*- and four *DREB2*-type genes, respectively (Todaka et al., 2012). Of these, *OsDREB1A* and *OsDREB1B* expressions were up-regulated under cold stress condition. Transgenic rice plants with overexpression of these genes were tolerant to drought, salt, and cold stress (Ito et al., 2006). Similarly, *OsDREB1F* transcript was induced by salinity, drought, cold stresses as well as ABA induction. The gene also enhanced tolerance to the respective stresses in transgenic plants (Wang et al., 2008). *OsDREB2A* and *OsDREB2B* expression was shown to be up-regulated by salt, drought, and high temperature stresses, and transgenic *Arabidopsis* constitutively expressing the *OsDREB2B* gene also showed improved drought and heat stress tolerance (Matsukura et al., 2010).

Another class of TFs that might participate in the regulation of *OsNUC1* expression is bZIP-containing TFs that recognized ABREs and/or "ACGT" sequence (ACGTATERD1) on the gene promoter. ABREs were required for the ABA-responsive expression of *erd1, rd29A, rd29B* and other ABA-responsive genes in *Arabidopsis* (Simpson et al., 2003; Nakashima et al., 2006). Similarly, the "ACGT" sequence was known as a core of ABREs (Suzuki et al., 2005) found in a large number of ABA responsive genes. This sequence is required for expression of the *Arabidopsis erd1* (*early responsive to dehydration1*), a gene involved in dehydration stress and dark-induced senescence (Simpson et al., 2003). A great number of rice bZIP-encoding genes were reported to be associated with salinity and dehydration. A survey of bZIP factors in the rice genome revealed that 26 and 11 *OsbZIP* genes were respectively up- and down-regulated under drought, salt, and/or low temperature stresses (Nijhawan et al., 2008). *OsABI5*, one of the bZIP-type TFs, was also shown to be induced by salt stress and ABA treatment (Zuo et al., 2008). Moreover, *OsbZIP23* was up-regulated by salt, drought and ABA application (Xiang et al., 2008), and transgenic rice with *OsbZIP23* overexpression also showed enhanced tolerance to drought and salinity stress.

2. The effect of *OsCam1-1* on *OsNUC1* gene expression under salt-stress condition

Several lines of evidence point to a role for Ca^{2+} and CaMs in transcriptional regulation of salt stress-responsive genes (Yang and Poovaiah, 2002; Yoo et al., 2005; Kim and Kim, 2006). Ca^{2+}/CaMs can regulate gene expression either by directly interacting with and activating/inhibiting transcriptional activities of their target TFs, or indirectly by relaying the salt stress signal through signaling cascades, and finally regulating gene expression (Kim et al., 2009).

Interestingly, PLACE and PlantCARE search on the *OsNUC1* promoter revealed a number of *cis*-regulatory elements that are associated wtih Ca^{2+}/CaMs signaling. MYB and WRKY proteins, which correspond to MYB1AT and MYBCORE elements and W-box on the *OsNUC1* promoter, respectively, have been reported to be direct targets of CaMs (Park et al., 2005; Yoo et al., 2005). Moreover, CGCG boxes found on the *OsNUC1* promoter were also associated the Ca^{2+}/CaMs signaling components (Yang and Poovaiah, 2002). Either directly or indirectly, it might be possible that salt stress-related CaMs are upstream regulators of *OsNUC1* expression. Concomitantly, OsCaM1-1 is a Ca^{2+} signal transducer, the functions of which have been implicated in salt-stress signaling and tolerance (Phean-o-pas et al., 2005; Saeng-ngam et al., 2012). Therefore, in this experiment, we determined whether the salt stress sensor *OsCam1-1* affected the expression of *OsNUC1* under salt stress condition, by comparing *OsNUC1* transcript levels between three independent lines of transgenic rice constitutively over-expressing the *OsCam1-1* gene (35SCaM1-1T1, 35SCaM1-1T2, and 35SCaM1-1T3), the T-DNA insertion control line (without the transgene *OsCam1-1*) (T1), and the wild type KDML105.

In this study, the wild type KDML105 and the T-DNA insertion line (T1) were used as control plants. Nevertheless, comparison of *OsNUC1* transcript level between these two lines at the beginning of the experiment (0h) revealed that their basal levels of *OsNUC1* expression were significantly different. Without salt stress imposition, The T1 line showed evidently higher level of *OsNUC1* transcript than the wild type, indicating

that insertion of the T-DNA alone in the rice genome increased the expression of *OsNUC1*, which implied that the T1 line was not a suitable control plant in this experiment. However, it has been reported that ABA content and transcript levels of ABA-biosynthesis gene, *AAO* and *NCED*, in this T-DNA insertion line were not different from the wild type (Saeng-ngam et al., 2012), indicating that the up-regulation of *OsNUC1* found in this rice line was ABA independent.

The highest level of *OsNUC1* transcripts detected at 12 h after the beginning of salt stress might result from other factors rather than the given treatment because the similar response was also detected in the unstressed control groups of almost every line tested, especially the 35SCaM1-1T1 and 35SCaM1-1T2 lines which was in higher levels than the salt-treated groups. The highest levels of *OsNUC1* gene expression detected at 12 h in this study were similar to those in an early response analysis of *OsNUC1* reported by Chamnanmanoontham (2009). It was shown that the highest levels of daily expression of *OsNUC1* were detected at the same time, not only in KDML105 but in FL530-IL, LPT123 and LPT123-TC171 as well. In this case, it might be the effect of changes in temperature or light intensity that induced the gene expression. The possibility that this phenomenon was a result of light-associated factors correlated with the presence of light- and light-related *cis*-regulatory elements on the *OsNUC1* promoter.

However, it was interesting that the salt-stressed group of the 35SCaM1-1T3 line exhibited significantly higher levels of *OsNUC1* expression than the control group at 12 h after the treatment, which was in the opposite fashion from the other two *OsCam1-1*-overexpressing lines, 35SCaM1-1T1 and 35SCaM1-1T2. It is well perceived that different levels of transgene expression between individual transformants are commonly found as a result of position effect (Peach and Velten, 1991) and this effect might be the reason for the distinctive *OsNUC1* expression pattern in the 35SCaM1-1T3 line.

As demonstrated by significant up-regulation of *OsNUC1* transcript after 2 and 3 days of salt-stress treatment in the 35SCaM1-1T1 and 35SCaM1-1T2 lines as well as

after 12 h of the treatment in the line 35SCaM1-1T3, compared to the wild type of which *OsNUC1* expression was unchanged for the whole course of salt stress treatment, it was convincing that *OsCam1-1* enhanced the expression of *OsNUC1* in response to salinity, and salt stress-responsive expression of *OsNUC1* is regulated by Ca^{2+}/CaM signaling.

3. Direct interaction between OsCaM1-1 and putative rice R2R3-MYB TF proteins

With regard to the characterization of Ca^{2+}/CaM-mediated transcriptional regulation of salt-stress responsive genes in the dicotyledonous *Arabidopsis* model, it was revealed that one of the interacting targets for signal relays of CaMs is R2R3-MYB-type TFs (Yoo et al., 2005). However, it is still unclear whether this interaction is present in the monocot rice plant. Therefore, in this study, potential CaM-binding R2R3-MYB TFs were identified in rice, by using existing information from *Arabidopsis* together with bioinformatics tools. Yeast two-hybrid assay was carried out to test interaction of these TFs and OsCaM1-1 protein.

3.1. Identification of putative CaM-binding R2R3-MYB TFs in rice

In this experiment, nine putative MYB TFs were retrieved from the rice genome based on their sequence homologies with the known CaM-binding R2R3-MYB protein from *Arabidopsis*, AtMYB2 (Yoo et al., 2005). Structural analysis of the identified proteins by Pfam scan indicated that all of the proteins posses two repeats of MYB-type DNA binding domain in their N-terninus. They were thus classified to the same R2R3 class of MYB protein family as AtMYB2. Sequence comparisons between the identified proteins and AtMYB2 showed that they shared only 44.8-52.4% similarity with AtMYB2. Low degree of amino acid sequence similarities was due the high levels of sequence variation in the C-terminus, which is characteristic of MYB family (Jiang, Gu, and Peterson, 2004).

Furthermore, the presences of gene products, in either the form of ESTs and/or cDNAs, proved that the identified rice R2R3-MYB genes are real functional genes, displaying *in vivo* expression.

Based on the conservation of the MYB-DNA-binding domains and of amino acid motifs in the C-terminal region, 125 R2R3-MYB proteins in *Arabidopsis* have been divided into 23 functional subgroups (Kranz et al., 1998; Stracke et al., 2001). Interestingly, three of the reported CaM-binding R2R3-MYB TFs (AtMYB2, AtMYB62 and AtMYB78) were clustered in the same subgroup, subgroup 20. In this regard, the phylogenetic tree combining all of the R2R3-MYB proteins from rice and *Arabidopsis* genomes was constructed. The tree classified seven out of the proteins, including LOC_Os05g04210.1, LOC_Os03g20090.1, LOC_Os07g48870.1, LOC_Os01g19330.1, LOC_Os11g45740.1, LOC_Os01g03720.1 and LOC_Os12g37690.1, in the same clade with the *Arabidopsis* R2R3-MYB subgroup 20, sharing the WxPRL motif at their C-termini. As structures implicate functions, this classification buttressed the possibility that the identified protein may have similar functional properties with their *Arabidopsis* counterparts.

Interestingly, the functions of *Arabidopsis* R2R3-MYB TF subgroup 20 have been implicated in stress response (Dubos et al., 2010). Of its six members, only three have been functionally characterized, and all of which are associated with abiotic stresses; AtMYB2 regulates the ABA-mediated expression of salt and dehydration responsive genes (Abe et al., 2003), AtMYB62 is involved in the response to phosphate starvation (Devaiah et al., 2009), and AtMYB108 in both biotic and abiotic stress responses associated with jasmonate signaling pathway (Mengiste et al., 2003). To assess putative functions of the identified rice R2R3-MYB genes whether they are involved in abiotic stress, the public cDNA microarray data (Jung et al., 2008) was employed. It was found that gene expression of five of the identified genes that belonged to subgroup 20 (LOC_Os03g20090.1, LOC_Os07g48870.1, LOC_Os01g19330.1, LOC_Os11g45740.1 and LOC_Os12g37690.1) were up-regulated

under salt and drought stress. Most recently, the *OsMYB2* gene, which corresponds to the LOC_Os03g20090.1 in this study, was reported to play a critical regulatory role in abiotic stress tolerance in rice (Yang et al., 2012). The *OsMYB2* gene expression was responsive to dehydration, salt and cold stresses. Without growth retardation in normal condition, over-expression of the *OsMYB2* gene in transgenic rice conferred higher ABA sensitivity and tolerance to dehydration, salt and cold stresses; evidenced by the enhanced proline and soluble sugars contents as well as antioxidant enzyme activities. Moreover, a good number of stress-associated genes, such as *OsLEA3*, *OsRab16A* and *OsDREB2A*, were up-regulated in *OsMYB2*-overexpressing transgenic plants. Taken together, this information supported and proved the potential of the identified R2R3-MYB genes to be involved in abiotic stress like their *Arabidopsis* counterparts.

Sequence motifs for CaM interaction have been reported and classified into three classes based on experimental investigation. The three classes of CaM recognition motifs includes one class of Ca^{2+}-independent CaM binding motifs and two classes of Ca^{2+}-dependent binding motifs, designated the 1-8-4 and 1-5-10 motifs according to the position of conserved hydrophobic residues (Rhoads and Friedberg, 1997). CaM binding motif of AtMYB2 was classified as the 1-5-10 type (Yoo et al., 2005) as it contained leucine, valine, and isoleucine at position 1, 5, and 10, respectively. Comparisons of the putative 1-5-10 type CaM-binding motif in the rice R2R3-MYB TFs with those in the *Arabidopsis* CaM-binding MYB proteins showed that they had similar motif patterns. This was due to the predicted CaM-binding site is located in the conserved region of DNA-binding domains. However, looking at the position 10 (Ile15) which was reported to be one of the key residues for CaM-interaction (Yoo et al., 2005), there were amino acid variations at this residue though they still have the same hydrophilic property. Consequently, this residue might affect CaM-binding affinity of the respective proteins.

3.2. Determination of protein-protein interaction by yeast two-hybrid assay

Four of the identified R2R3-MYB proteins, including three of subgroup 20 member, LOC_Os03g20090.1, LOC_Os07g48870.1, LOC_Os11g45740.1, and one out-group, LOC_Os02g40530.1, were chosen for the initial test of their interactions with the salt-stress transducer, OsCaM1-1. However, the test of CaM-interaction by yeast two-hybrid assay revealed the absence of protein interaction in all of the selected proteins.

Two hypotheses were raised to account for the lack of interaction with OsCaM1-1 by the four selected rice R2R3-MYB TFs in the two-hybrid assay. First, the R2R3-MYB TFs might not serve as the interacting-partners of OsCaM1-1. In an *Arabidopsis* protein microarray using three CaMs and four Calmodulin-like proteins (CMLs) as probes (Popescu et al., 2007), it was found that only approximately 25% of 173 CaM/CML-interacting proteins interacted with all CaMs/CMLs while the remaining 75 % interacted with only one or a few CaMs/CMLs. This evidence indicated that CaM-target proteins are specific to only one or a few CaM/CMLs and that different CaM family members function through different targets. There are five CaMs and thirty two CMLs in the rice genome (Boonburapong and Buaboocha, 2007), each of which may specifically function through different targets. The binding partner of R2R3-MYB TFs in rice might not be OsCaM1-1, but rather another CaM or CML.

Likewise, there is specificity in the interaction of MYB proteins with their targets. A study on the interaction between MYB TFs and WD40 domain-containing OsTTG1 and basic helix loop helix (bHLH) OsGL3 proteins in rice by yeast two-hybrid assay showed that of 30 OsMYB TFs tested, only three interacted with the OsTGG1 and five interacted with the OsGL3, respectively (Kim, Kim, and Ahn, 2012).

In nine proteins identified from the rice genome, seven of them were classified to the same clade with the members of *Arabidopsis* R2R3-MYB subgroup 20. In this study, only three proteins in the clade were selected. It might be these remaining proteins that interact with OsCaM1-1.

Following this hypothesis, therefore, the CaM-R2R3-MYB TF interaction should be investigated for a larger number of both CaM/CML and R2R3-MYB proteins.

The other hypothesis is that R2R3-MYB TFs and OsCaM1-1 do interact in rice, but this interaction could not be detected by the yeast two-hybrid assay. Though yeast two-hybrid has been successfully used for the study of CaM protein interaction (Vanoosthuyse et al., 2003; Yamaguchi et al., 2005; Yoo et al., 2005), Reddy and colleagues (2011) claimed that the yeast-two hybrid assay might not be an ideal system for CaM-protein interaction because the interaction of CaM with its targets is normally dependent on Ca^{2+}. Hence, the unexpected result may be due to the different cellular Ca^{2+} signatures between plant and yeast cells. It is difficult to manipulate calcium concentration in yeast cells during this process.

Moreover, it was possible that plant proteins produced in yeast might not be as functionally active as those in plants. An assay of 96 *Arabidopsis* protein kinases produced by yeast revealed that only 3-5% of the proteins were active, while 82% were active when expressed *in planta* (Popescu et al., 2007). Hence, to ensure that the recombinant proteins produced by yeast two-hybrid system are in their active forms, a pair of known CaM-interacting positive controls, such as GmCaM4 and AtMYB2, should be provided in the experiment.

Alternatively, plant-based expression system such as co-immunoprecipitation (CoIP), which enable *in vitro* Ca^{2+} manipulation and have been successfully used for studies of CaM interaction (Takahashi et al., 2011), is another prospective approach for verification of the protein interaction.

CHAPTER VI
CONCLUSION

1. Cloning and *in silico* characterization of *OsNUC1* promoter

The *OsNUC1* promoter contained a number of dehydration/salt stress-related *cis*-regulatory elements, including dehydration responsive element (DRE), binding sites of MYB and MYC transcription factors, and ABA-responsive elements (ABREs). One (or more) of these elements might be responsible for the elevation of *OsNUC1* expression during salt-stress condition.

Sequence variations were present on the *OsNUC1* promoter of four rice cultivars/lines studied. The line LPT123-TC171 and FL530-IL exhibited higher number of dehydration/salt-related *cis*-regulatory elements than the cultivar LPT123 and KDML105. These variations might contribute to the distinction in salt-stress responsive expression of the *OsNUC1* gene observed in each pair of the rice cultivars/lines.

Apart from stress-associated elements, groups of *cis*-acting elements detected on the *OsNUC1* promoter included organ-specific, phytohormone and light/phytochrome-responsive elements which also support the potential role of *OsNUC1* in plant development.

2. The effect of *OsCam1-1* on *OsNUC1* gene expression under salt-stress condition

Transgenic rice lines with the constitutive overexpression of *OsCam1-1* gene showed significant up-regulation of *OsNUC1* transcripts at 2 and 3 days of salt-stress treatment, while the *OsNUC1* transcripts in the wildtype KDML105 were unchanged. This indicated that *OsNUC1* expression was positively affected by the salt-stress transducer *OsCam1-1*, suggesting the involvement of *OsNUC1* and Ca^{2+}/CaM salt-stress signaling pathway.

3. Direct interaction between OsCaM1-1 and putative rice R2R3-MYB TF proteins

Seven R2R3-MYB TFs with the potential to serve as targets of CaM were identified from the rice genome as they showed the highest sequence similarities with the known CaM-binding R2R3-MYB TF in *Arabidopsis*, sharing the same C-ternimal WxPRL motif. Their putative CaM-interacting motifs were also similar to those of *Arabidopsis* CaM-binding proteins. All of the identified proteins were functional and associated with abiotic stresses like their *Arabidopsis* counterparts.

However, the initial test of CaM interaction between four of the putative R2R3-MYB proteins and the salt stress-related OsCaM1-1 by yeast two-hybrid assay revealed the lack of protein interaction.

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APPENDICES

Appendix A

Modified WP nutrient solution

(Vajrabhaya and Vajrabhaya, 1991; Udomchalothorn et al., 2009)

The solution 1 liter contains;

Macroelements:

KNO_3	580 mg
$CaSO_4$	500 mg
$MgSO_4.7H_2O$	450 mg
Triple super phosphate	250 mg
$(NH_4)_2SO_4$	100 mg

Microelements:

Na_2EDTA [a]	160 mg
$FeSO_4.7H_2O$ [a]	120 mg
$MnSO_4.H_2O$	15 mg
H_3BO_3	5 mg
$ZnSO_4.7H_2O$	1.5 mg
KI	1 mg
$Na_2MoO_4. 2H_2O$	0.1 mg
$CuSO_4. 5H_2O$	0.05 mg
$CoCl_2. 6H_2O$	0.05 mg

Appendix B

Gene expression of the identified rice R2R3-MYB transcription factors from Rice Oligo Array Database (http://www.ricearray.org/)

Experiment ID: GSE6901

Overall design: Seven-day-old, light-grown rice seedlings grown under controlled conditions and those subjected to various abiotic stress conditions were used for RNA extraction and hybridization on Affymetrix microarrays. Three biological replicates of each sample were used for microarray analysis. For salt treatment, the rice seedlings were transferred to a beaker containing 200 mM NaCl solution for 3 h. For desiccation, rice seedlings were dried for 3 h between folds of tissue paper at 28° C, in a culture room. For cold treatment, the seedlings were kept at 4 degree C for 3 h. The seedlings kept in water for 3 h, at 28°C, served as control.

Locus	Expression			
	Control	Drought	Salt	Cold
Os05g40210.1	3.8891	5.2414	9.1262	2.3587
Os03g20090.1	8.7046	9.1058	9.8698	11.1573
Os03g20090.1	8.8072	9.4208	10.1507	11.7388
Os07g48870.1	7.269	7.5641	10.107	6.5287
Os01g19330.1	4.0711	7.5834	9.6315	6.5896
Os11g45470.1	7.463	10.8913	11.3613	7.0725
Os01g03720.1	7.4018	6.5058	7.8725	6.4268
Os12g37690.1	6.2255	9.1285	9.3976	6.3454
Os01g45090.1	5.0485	3.5492	3.6198	3.8261
Os02g40530.1	6.2896	7.7169	8.0313	7.4767

Experiment ID: GSE24048

Overall design: Two rice cultivars, Bala and Azucena, were grown in 1.2 m^2 plots under flooded conditions. Starting at 59 days after sowing, drought was imposed by withholding water, while a set of control plots had continued flooding conditions. At 2 pm on the 83rd day after sowing (after 24 days of drought) the second youngest fully expanded leaf was taken off three plants in two plots per block, the leaves had the top and bottom 4 cm removed and the central portion of the leaf was placed in a bag and then into liquid N_2. For the controls there was only one plot of the genotypes per block. There was one bag for each block and three replicate blocks. A total of 6 droughted leaf samples (3 Bala and 3 Azucena) and six control leaf samples (3 Bala and 3 Azucena) were collected for RNA extraction and hybridization on Affymetrix microarrays.

Locus	Expression			
	Control Azucena	Control Bala	Drought Azucena	Drought Bala
Os05g40210.1	3.4576	3.4365	2.448	2.158
Os03g20090.1	7.4795	6.9739	9.1091	8.6824
Os03g20090.1	7.0502	6.8827	8.8105	8.2409
Os07g48870.1	2.9582	5.105	7.5403	4.9078
Os01g19330.1	7.2175	4.4229	3.1416	3.1643
Os11g45470.1	5.6311	5.996	10.649	7.315
Os01g03720.1	1.1909	3.1533	5.2369	5.1825
Os12g37690.1	2.0261	4.3982	7.2041	3.3355
Os01g45090.1	2.7423	3.4105	5.3387	3.5059
Os02g40530.1	4.1604	3.3687	3.7728	3.7726

Experiment ID: E-MEXP-2401

Overall design: Comparative analysis of drought responsive transcriptome between *indica* rice genotypes with contrasting drought tolerance was performed. High quality RNA was extracted from the whole seedlings (Combined root and leaf samples) using TRI Reagent (Ambion, Inc. USA) and pooled from 12 independent stressed and non-stressed plant samples separately.

Locus	Expression			
	IR64_control	IR64_drought	N22_control	N22_drought
Os05g40210.1	4.2387	5.9061	3.426	4.8189
Os03g20090.1	8.5506	10.9095	9.7958	8.6593
Os03g20090.1	8.8347	10.7259	10.0715	8.9866
Os07g48870.1	7.1006	8.2703	7.1818	8.5966
Os01g19330.1	5.7037	9.3045	5.1471	5.2241
Os11g45470.1	8.3639	10.6959	9.2063	10.6152
Os01g03720.1	7.5269	7.5736	8.0444	7.2599
Os12g37690.1	7.3385	8.2265	8.2249	8.7955
Os01g45090.1	2.4863	4.6288	4.5653	6.4073
Os02g40530.1	5.3868	10.2801	6.9148	6.1951

Experiment ID: GSE16108

Summary: The aim of this study was to minimize the number of candidate genes responsible for salt tolerance between a pair of rice varieties (CSR27 and MI48) with contrasting level of salt tolerance by bulked segregant analysis of their recombinant inbred lines.

Overall design: RNA from CSR27, MI48, tolerant bulk and susceptible bulk grown under control and salt stress conditions were analysed in two different biological replications making total sixteen samples

Locus	Expression							
	MI48 control	MI48 stressed	CSR27 control	CSR27 stressed	Bulk tolerant control	Bulk tolerant stressed	Bulk sensitive control	Bulk sensitive stressed
Os05g40210.1	5.7996	7.6979	3.3437	6.2735	3.1289	2.6524	2.5532	5.2988
Os03g20090.1	8.6113	9.0635	7.9095	6.7053	7.8344	8.797	8.0146	8.9201
Os03g20090.1	7.7723	9.5141	7.7679	5.8687	4.8585	7.3425	5.9156	6.5749
Os07g48870.1	4.9833	9.199	7.7519	7.947	5.9492	6.9489	5.3423	7.3533
Os01g19330.1	4.7643	4.8495	4.9742	6.0492	2.4983	4.8781	4.1458	5.4429
Os11g45470.1	8.6468	11.3383	8.3762	9.6058	7.4002	7.7772	7.0351	8.0935
Os01g03720.1	4.6188	7.8786	7.1456	7.4216	8.3528	8.6261	7.7255	7.4947
Os12g37690.1	6.5515	10.0748	7.3356	7.3573	6.6086	7.0078	6.3544	6.8697
Os01g45090.1	5.8377	4.9505	6.2236	7.0271	4.0337	3.9131	3.3084	2.7115
Os02g40530.1	5.5476	7.4742	5.4892	6.9456	5.9121	6.7554	6.0463	6.2889

Experiment ID: GSE26280

Overall design: The gene expression patterns across four tissues including leaves and roots at tillering stage and panicle elongation stage were characterized by using the Affymetrix rice microarray platform based on a drought tolerant rice line derived from IR64.

Locus	Expression							
	normal leaves tillering	Drought leaves tillering	normal roots tillering	Drought roots tillering	normal leaves panicle elongation	Drought leaves panicle elongation	normal roots panicle elongation	Drought roots panicle elongation
Os05g40210.1	2.483	3.3564	3.6065	6.027	4.2627	6.5602	5.5977	7.232
Os03g20090.1	7.4804	9.0209	7.6763	7.2083	6.6869	10.4082	8.9864	8.2037
Os03g20090.1	7.9696	8.7056	7.3862	7.5019	6.9705	10.1001	9.6179	8.5162
Os07g48870.1	2.6638	6.5753	7.7726	8.4589	2.6612	9.7062	8.4091	9.6561
Os01g19330.1	5.0874	4.574	6.0057	2.7213	3.7347	4.9807	8.9565	4.5936
Os11g45470.1	6.8402	10.0287	8.1167	8.8229	7.4438	12.9636	8.1326	8.6665
Os01g03720.1	3.7448	6.4337	7.361	7.1633	2.4596	7.5581	7.5318	7.3386
Os12g37690.1	4.0795	6.9942	4.4651	3.6263	5.2142	11.6666	5.2487	5.9313
Os01g45090.1	3.7221	2.8662	3.6658	4.9055	5.232	4.1285	3.6186	3.6248
Os02g40530.1	4.9709	3.8056	5.3211	8.4343	5.1162	8.2551	6.4827	9.9245

BIOGRAPHY

Mr. Thanin Chantarachot was born on October 18, 1986 in Songkhla Province, Thailand. After finishing his high school study in 2005, he was enrolled in Biology program at the Department of Biology, Faculty of Science, Prince of Songkla University and graduated with the Bachelor Degree of Science in Biology (1st Class Honors) in 2009. Upon completion of his Bachelor's degree, Mr. Chantarachot continued his study in the Master of Science Program in Botany at the Department of Botany, Faculty of Science, Chulalongkorn University.

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In 2012, Mr. Chantarachot was a recipient of a scholarship from the Department of Botany, Faculty of Science, Chulalongkorn University to participate in the 6th Botanical Conference of Thailand at Prince of Songkla University, Hat Yai, Thailand.