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CHARACTERIZATION OF THE NOVEL SALT STRESS RESPONSIVE GENE IN RICE *Oryza sativa* L.

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งานวิจัยนี้มีจุดมุ่งหมายหลักสองประเด็น ประเด็นแรกคือศึกษาลักษณะสมบัติของยีนพบใหม่ คือยีนนิวคลีโอลิน1 (*OsNUC1*) ซึ่งตอบสนองต่อความเครียดจากความเค็มในข้าว ทั้งนี้เพื่อระบุหน้าที่ของยีนนี้ในแง่ของการเป็นยีนที่ชักนำให้พืชต้านทานต่อความเครียดจากความเค็มได้ ประเด็นที่สองเพื่อเพิ่มความเข้าใจเกี่ยวกับกลไกการชักนำให้พืชทนต่อความเครียดจากความเค็มของยีน *OsNUC1* และ *OsP5CS1* (ยีนหลักในการกระบวนการสังเคราะห์โพรลีน) ในแง่ของความสัมพันธ์กับกระบวนการส่งสัญญาณของกรดแอบไซซิก (ABA)

ยีนนิวคลีโอลินในข้าวเป็นสมาชิกของกลุ่มยีนขนาดเล็กในข้าว ซึ่งประกอบด้วย 2 ยีน คือ *OsNUC1* ที่โครโมโซม 4 และ *OsNUC2* ที่โครโมโซม 8 โดยโปรตีน OsNUC1 ประกอบด้วยบริเวณอนุรักษ์ 3 โดเมน ที่มีการอนุรักษ์ในสิ่งมีชีวิตชนิดอื่น ๆ โดยบริเวณอนุรักษ์ดังกล่าว ได้แก่ 1) acidic glycine rich domain และ nuclear localization signal (NLS) เป็นโดเมนทางปลายอะมิโน 2) โดเมนของ RNA recognition motif (RRM) ซึ่งมี 2 โมทีฟอยู่บริเวณส่วนกลางสายพอลิเปปไทด์ และ 3) โดเมนที่มีไกลซีนและอาร์จินีน (glycine-and arginine-rich (GAR) domain) ที่ปลายคาร์บอกซิล จากฐานข้อมูลจีโนมข้าวพบ cDNA clone แบบสมบูรณ์ 2 แบบ คือ สายโพลิเปปไทด์ที่ประกอบด้วยทั้งสามบริเวณ และสายโพลิเปปไทด์ที่ประกอบด้วยบริเวณส่วนกลางและปลายคาร์บอกซิล การทดลองตรวจสอบตำแหน่งของโปรตีนภายในเซลล์ โดยใช้เซลล์เยื่อผิวของหัวหอม ด้วยการใช้ GFP-Nucleolin fusion protein พบว่า GFP-three-domain Nucleolin1 พบในนิวเคลียสเท่านั้น ในขณะที่ GFP-two-domain Nucleolin1 ซึ่งเชื่อมต่อระหว่าง GFP และบริเวณส่วนกลางและปลายคาร์บอกซิลของ Nucleolin1 กระจายอยู่ทั้งในนิวเคลียสและไซโตพลาสซึม แสดงให้เห็นว่า บริเวณโดเมนทั้งสองของ Nucleolin1 นี้สามารถถูกขนส่งเข้าสู่นิวเคลียสโดยไม่ต้องอาศัยบริเวณ NLS ที่ปลายอะมิโนได้ นอกจากนี้ยังพบการแสดงออกของยีน *OsNUC1* ในเนื้อเยื่อใบ ราก เมล็ด และดอก โดยระดับการแสดงออกที่สูงที่สุดพบได้ในเนื้อเยื่อดอกและราก จากการตรวจสอบการแสดงออกของยีนด้วยเทคนิค quantitative real-time PCR พบว่าความเครียดจากความเค็มสามารถชักนำการแสดงออกของยีน *OsNUC1* ได้ถึง 9 วัน ในข้าวสายพันธุ์ทนเค็ม LPT123-TC171 และ FL530-IL และยังพบการแสดงออกของยีน *OsNUC1* ในระดับที่สูงกว่าเมื่อเปรียบเทียบกับการแสดงออกของ *OsNUC1* ในข้าวสายพันธุ์ไม่ทนเค็มคือ LPT123 และ KDML105 ซึ่งมีพื้นฐานทางพันธุกรรมเดียวกันกับสายพันธุ์ทนเค็มข้างต้น *OsNUC1* อาจทำหน้าที่เป็นยีนที่ต้านทานความเครียดจากความเค็มในข้าว การแสดงออกของ cDNA clone ที่แปลรหัสให้บริเวณส่วนกลางและปลายคาร์บอกซิล Arabidopsis ดัดแปรพันธุกรรม สามสายพันธุ์ซึ่งมีระดับการแสดงออกของยีน *OsNUC1* แตกต่างกันถูกนำมาใช้ในการศึกษาผลกระทบของการแสดงออกของยีน *OsNUC1* โดยพบว่าการแสดงออกของยีน *OsNUC1* สามารถกระตุ้นการเจริญของราก และเพิ่มจำนวนใบทั้งในภาวะปกติและภาวะเครียดจากความเค็ม ผลการทดลองนี้ยืนยันสมมติฐานที่ว่า *OsNUC1* เกี่ยวข้องกับกลไกการต้านทานความเครียดจากความเค็มในพืช นอกจากนี้ *OsNUC1* ยังสามารถกระตุ้นการแสดงออกของยีนที่ต้านทานต่อความเครียดจากความเค็มได้อย่างน้อยสองยีน คือ *AtSOS1* และ *AtP5CS1* ซึ่งสนับสนุนบทบาทของ *OsNUC1* ในการทำหน้าที่เป็นยีนต้านทานความเครียดจากความเค็ม การให้กรดแอบไซซิคจากภายนอกสามารถชักนำการแสดงออกของยีน *OsNUC1* ในข้าว สอดคล้องกับการพบ ABRE-*cis* element ในบริเวณโปรโมเตอร์ของ *OsNUC1* อย่างไรก็ตามการให้สารยับยั้งการสังเคราะห์กรดแอบไซซิค (abamineSG) ภายใต้ภาวะเครียดจากความเค็มไม่มีผลกระทบต่อการแสดงออกของ *OsNUC1* ซึ่งชี้ให้เห็นว่า กรดแอบไซซิคไม่จำเป็นต่อการแสดงออกของยีน *OsNUC1*

การตอบสนองต่อความเครียดจากความเค็มโดยการส่งสัญญาณผ่านกรดแอบไซซิค ยังเกี่ยวข้องกับกลไกการสังเคราะห์โพรลีน ซึ่งมีรายงานกล่าวไว้ว่า *OsP5CS1* เป็นยีนในการสังเคราะห์โพรลีนในข้าวภายใต้ภาวะเครียดจากความเค็ม การให้กรดแอบไซซิคจากภายนอกสามารถชักนำการต้านทานความเครียดจากความเค็มและเพิ่มการสะสมโพรลีน ซึ่งเป็นที่เข้าใจว่าเป็นผลจากการแสดงออกของยีน *OsP5CS1* ด้วยการให้กรดแอบไซซิคจากภายนอก อย่างไรก็ตาม การตรวจสอบการแสดงออกของยีน *OsP5CS1* ที่ระยะเวลาต่าง ๆ ภายหลังจากการให้กรดแอบไซซิค และถูกชักนำภายใต้สภาวะเครียดจากความเค็ม แสดงให้เห็นว่าการเพิ่มระดับการแสดงออกของ *OsP5CS1* ไม่สัมพันธ์กับรูปแบบการเพิ่มขึ้นของการสะสมโพรลีน ในทางตรงกันข้ามรูปแบบการสะสมโพรลีนสัมพันธ์กับรูปแบบการแสดงออกของ *OsP5CR* ซึ่งเป็นอีกยีนหนึ่งในกระบวนการสังเคราะห์โพรลีน ดังนั้นการเพิ่มการต้านทานความเครียดจากความเค็มผ่านทางการให้กรดแอบไซซิคจากภายนอก สัมพันธ์กับการทำงานของ *OsP5CR* มากกว่า *OsP5CS1* นอกจากนี้ กลไกการส่งสัญญาณผ่าน *CaM1-1* ยังเกี่ยวข้องกับการสะสมโพรลีนภายใต้ภาวะเครียดจากความเค็มด้วย

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SIRIPORN SRIPINYOWANICH: CHARACTERIZATION OF THE NOVEL SALT STRESS RESPONSIVE GENE IN RICE *Oryza sativa* L. THESIS ADVISOR: ASSOC. PROF. SUPACHITRA CHADCHAWAN, Ph.D., THESIS CO-ADVISOR: PROF. HONGYA GU, Ph.D., ASSOC. PROF. TEERAPONG BUABOOCHA, Ph.D., 204 pp.

The goals of this research have two main points; the first is to characterize the novel salt-responsive gene, *OsNUC1*, in rice, in order to indicate its function as a salt-resistant gene. The other is to elucidate of salt-tolerant mechanism of *OsNUC1* and *OsP5CS1* (the key regulator gene in proline biosynthesis) in relation with abscisic acid (ABA) signaling pathway.

RiceNucleolin genes, *OsNUC* genes are the members of a small gene family in rice, consisting of two genes, *OsNUC1* and *OsNUC2*, which are located on chromosome 4 and 8 respectively. *OsNUC1*encodes the protein with three conserved domains of Nucleolins found in other species, which are the acidic glycine rich domain with nuclear localization signal (NLS) at amino-terminal, two RNA recognition motifs (RRM) at the central domain, and glycine-and arginine-rich (GAR) domain at carboxyl terminal. The cDNA clones encoding the polypeptide consisting of all three domains and the one encoding the polypeptide consisting of the central and carboxyl-terminal domains exist in rice genome database. The localization experiment using GFP-Nucleolin fusion protein, performed in the onion epidermal peel, showing that GFP-three-domain Nucleolin1 fusion protein was localized in nucleus only, while the GFP-two-domain Nucleolin1 fusion protein, which was the fusion protein between GFP and central and carboxyl terminal domains of Nuclelin1, was localized in both nucleus and cytoplasm. This suggested that the two-domain Nucleolin1 could be transported to the nucleus without NLS at N-terminal. The *OsNUC1* transcripts were found in leaf, root, seed, and flower tissues. The highest expression level was found in flowers and roots. Based on quantitative real-time PCR, salt stress can induce *OsNUC1* gene expression up to 9 days after treatment. The salt resistant lines, LPT123-TC171 and FL530-IL showed the higher *OsNUC1* gene expression, when compared to their salt-susceptible isogenic lines, LPT123, and KDML105, respectively. This suggests that *OsNUC1* may function as a salt resistant gene in rice. The cDNA clone encoding the central and carboxyl terminal domains was over-expressed in transgenic Arabidopsis, and three independent transgenic lines with the different gene expression levels were used to investigate the effect of *OsNUC1* gene expression. It was shown that *OsNUC1* gene expression can enhance root growth, increase leaf number in both normal and salt-stress conditions. These data confirm the hypothesis that *OsNUC1* is involved in salt resistant mechanism in plants. Moreover, it was found that *OsNUC1* could increase at least two salt resistant genes, *AtSOS1* and *AtP5CS1*, which supports the role of *OsNUC1* in salt resistant function. Exogenous ABA can induce *OsNUC1* gene expression in rice, which is consistent with the existence of ABRE *cis*-element in the promoter region. However, during salt stress, the application of abamineSG, ABA synthesis inhibitor, did not affect *OsNUC1* expression, suggesting that ABA may not required for *OsNUC1* gene expression during salt stress.

The signaling response via ABA was also studied in proline synthesis mechanism. *OsP5CS1*was reported to be responsible for proline synthesis in rice during salt stress. The topical application of ABA can induce salt resistance and proline accumulation, assuming that exogenous ABA induces *OsP5CS1* gene expression. However, the quantitative real-time PCR detecting *OsP5CS1* gene expression at various timing after ABA application and salt stress, showed that the up-regulation of *OsP5CS1* was not well correlated with the increase in proline accumulation pattern. On the contrary, the proline accumulation pattern was consistent with the gene expression pattern of *OsP5CR*, another gene in proline synthesis pathway. This suggested that the increase in salt resistance via the topical ABA application was due to *OsP5CR* function rather than *OsP5CS1* function. Moreover, the experiment showing the involvement of *CaM1-1* signaling pathway in proline accumulation during salt stress was also performed.

Field of Study : Biological Science Student's Signature .....

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LIST OF ABBREVIATIONS

ABA	abscisic acid
ABRE	ABA responsive element
Amp^+	amplicilin resistance
AtEF-1α	Arabidopsis elongation factor-1α
AtNUC1	Arabidopsis nucleolin1
AtP5CS1	Arabidopsis pyrroline 5-carboxylate synthetase1
AtSOS1	Arabidopsis salt overly sensitive1
Arg	arginine
^oC	degree Celsius
CaM	calmodulin
CAMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
C-terminus	carboxyl terminus
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside 5'-triphosphate
DRE	dehydration-responsive element
DW	dry weight
EDTA	ethylene diamine tetraacetic acid
FL530-IL	KDML105 introgression line
FW	fresh weight
g	gram

GAR	glycine- and arginine- rich domain
Gen^{+}	gentamycin
GFP	green fluorescent protein
Gly	glycine
HCl	hydrochloric acid
HPLC	high performance liquild chromatography
Kan^{+}	kanamycin resistance
kb	kilobase pairs in duplex nucleic acid
	kilobase in single-stranded nucleic acid
KCl	potassium chloride
kDa	kiloDalton
KDML105	Khao Dawk Mali 105
KOH	potassium hydroxide
l	liter
IPTG	Iso-1-thio-β -D-thiogalactopyranoside
Mg^{2+}	magnesium ion
µg	microgram
µl	microliter
µM	micromolar
M	mole per liter (Molar)
mg	milligram
min	minute
ml	milliliter
mM	milimolar
MW	molecular weight

N	normal
NaCl	sodium chloride
NaClO	Sodium hypochlorite
NaOAc	sodium acetate
NCED	9-*cis*-epoxycarotenoid dioxygenase
ng	nanogram
NLS	nuclear localize signal sequence
nm	nanometer
nmol	nanomole
no.	number
N-terminus	amino terminus
OsCam1-1	rice calmodulin1-1
OsCIPK15	rice calcium independent protein kinase
OD	optical density
OsNUC1	rice nucleolin1
OsNUC2	rice nucleolin2
OsP5CS1	rice pyrroline5-carboxylate synthetase1
OsP5CR	rice pyrroline5-carboxylate reductase
PCR	polymerase chain reaction
RBP	RNA-binding protein
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
Rif^+	rifampicilin resistance
RRM	RNA-recognition motif

Tris	tris (hydroxyl methyl) aminomethane
v/v	volume by volume
w/v	weight by volume
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide
X-gal	5-Bromo-4-chloro-3-indole-beta-D-galactopyranoside

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is arguably the world's most important food. Almost two billion people - one third of the world's population - depend primarily on rice for basic nourishment (IRRI, 2002; Coats, 2003). Rice has always been the staple food of the Thai people, and plays a crucial role as the essence of Thai life. Unfortunately, various abiotic stresses as the climate changes and water scarcity increases and salinity soil exposure limit rice production in rainfed environments, which comprise about 45% of the global rice area (Lafitte *et al*., 2004). In the fact of global scarcity of the increased salinization of soil, salt stress is already a major limiting factor for plant growth and will soon become even more severe desertification, which will cover more and more of the world's terrestrial area. No toxic substances restrict plant growth more than salt stress exposure on a world scale. In coastal areas, periodic invasions of sea water directly added salts to the soil. Soils in other semi-arid or arid regions, especially those with ineffective drainage, accumulate salts as irrigation water evaporates, leaving deposits of soluble salts. Interestingly, the salt-stress exposure not only effects on plant growth, but also increases salinization of arable land which resulting in 30% land loss within 25 years and up to 50% by the year 2050 (Wang *et al*., 2003; Ashraf, 1994). Widespread salinity leads to huge losses in terms of productivity and agricultural land especially in rice production.

Because of their sessile nature, plants have evolved sophisticated mechanisms to cope with environmental challenges (Zhu, 2002). The regulation of gene expression at the post-transcriptional as well as the transcriptional level is of crucial importance for growth, development, and stress responses in eukaryotes. Post-transcriptional mechanisms based on alternative splicing and RNA processing define the actual transcriptome supporting the stress response.

The pre-mRNA splicing mechanisms in post-transcriptional level are emerging as key regulators for tuning the plant's response to environmental and developmental processes (Hugouvieux *et al*., 2001; Xiong *et al*., 2001; Li *et al*., 2002; Xiong *et al*., 2002). The regulation is mainly achieved either directly or indirectly by many RNA-binding proteins (RBPs) which contain one or more RNA-recognition motifs (RRMs) at the N-terminus and a variety of auxiliary motifs at the C-terminus, such as glycine-rich domain, arginine-rich domain, SR-repeats, and RD-repeats (Burd and Dreyfuss, 1994; Albà and Pagès, 1998; Lorkovic and Barta, 2002). The main component of proteins in pre-mRNA splicing has been demonstrated to consist of RNA recognition motif (RRM) (Amrein *et al*., 1988); furthermore, RRM also found to be involved in pre-ribosomes (Bourbon *et al*., 1983), in poly(A) tail synthesis and maturation (Adam *et al*., 1986), in translational control (Naranda *et al*., 1994), and in mRNA stability (Zhang *et al*., 1993).

RNA recognition motif (RRM) also called consensus RNA-binding domain (CS-RBD) which is referred to be ribonucleoprotein (RNP) motif and RNP consensus sequence (Kenan *et al*., 1991; Burd and Dreyfuss, 1994a). A number of proteins with RRM have been found in organisms ranging from *Escherichia coli* to human (Fukami-Kobayashi *et al*., 1993) that are conserved among different species (Serin *et al*., 1997) and involved in the posttranscriptional regulation of gene expression (Burd and Dreyfuss, 1994a). For example, poly(A)-binding proteins have one RRM and a proline-rich domain and regulate mRNA stability and translation. Some hnRNPs, such as hnRNP A1, have two RRMs and a glycine-rich domain and play important roles in mRNA biogenesis, such as splicing and RNA export (Burd and Dreyfuss, 1994b).

Recently, a family of proteins consisting of one amino-terminal consensus sequence RNA-binding domain (CS-RBD) and one carboxyl-terminal glycine-rich domain is referred to as a glycine-rich RNA-binding protein (GRP) family and some of them have been demonstrated to be induced by a number of external stimuli including cold, osmotic stress, high salinity, wounding, and viral infection (Bergeron *et al*., 1993; Carpenter *et al*., 1994; Heintzen *et al*., 1994; Sachetto-Martins *et al*.,

2000). They act as chaperones that modulate RNA-RNA interactions or shuttle the mRNA for efficient processing. Under stress conditions, GR-RBPs may stabilize mRNA either during the transfer from the nucleus to cytoplasm or directly in the cytoplasm, allowing efficient mRNA processing. Support this idea by report of Kim and colleagues (2005) that GR-RBPs in Arabidopsis, one of which is *atRZ1a*, play roles in post-transcriptional regulation of gene expression in plants under various stress conditions. The overexpression of *atRZ1a* in Arabidopsis plants resulted in the increasing in freezing tolerance under cold stress. Moreover, the knockout of a small RBP in rice, *OsDEG10*, with RNAi technique caused the transgenic lines to be more sensitive to high light and cold stresses (Park *et al*., 2009).

Interestingly, RNA-recognition motif is one of the unique features of nucleolin and highly conserved during evolution (Ginisty, 1999). Nucleolin is known to be the multifunctional important protein that has been implicated to be involved directly or indirectly in many metabolic processes. It is one of the most abundant non-ribosomal proteins of nucleolus (Bugler, 1982). Some evidences reported that plant nucleolin-like protein was developmentally and cell cycle regulation in alfafa (Bogre *et al*., 1996), light regulated in pea (Tong *et al*., 1997), and involved in splicing and/or processing of chloroplast RNAs in Arabidopsis (Didier and Klee, 1992). The *AtNUC-L1*disruption affects on plant growth and development in Arabidopsis (Pontvianne *et al*., 2007). Three main domains are found in nucleolin; N-terminal domain rich in acidic residues, which is the site of numerous phosphorylations by casein kinase 2 (CK2) and cyclin dependent kinase 1 (CDK1); a central domain containing RRM motifs and a C-terminal domain rich in glycine and arginine residues (the GAR domain) (Mongelard and Bouvet, 2006). Nucleolin is found in organisms ranging from yeast (*Saccharomyces cerevisiae*) to plants to mammals. The RNA recognition motif was found in high concentration of nucleolin in the dense fibrillar region of the nucleolus (Escande *et al*., 1985; Lischwe *et al.*, 1981). Moreover, the function of Glycine-Arginine rich repeat segments (GAR) of nucleolin is involved in pre-RNA recognition, condensing, and packaging (Ghizolfi *et al*., 1992; Creancier *et al*., 1993) that acts via possesses binding affinity for some ribosomal proteins and interacts with a subset through its carboxyl-terminal domain (Bouvet *et al*., 1998). Therefore, the

role of GAR domain was proposed to be a protein-protein interaction domain. The RRMs and GAR domain in central and carboxyl-terminal domains of nucleolin may play a role in the assembly of ribosomal proteins and RNA which are the important components in post-transcriptional mechanism. Even though the role of these distinguish domains of nucleolin-like protein seems to be a lot of support in stress tolerance function, it still was not clearly identified. So, we would like to show the regulation of *OsNUC1* on salt stress to enhance salt resistant mechanism in plants and the role of RRMs and GAR domain of OsNUC1 in salt resistance.

Furthermore, the salt tolerant mechanism in plants also involved in perceive and respond adaptively to stress and importantly that the adaptive process is controlled mainly by ABA which is a crucial phytohormone and plays a critical role in response to various stress signals. ABA is an important signal for triggering plant responses to adverse environmental conditions during vegetative growth (Leung and Giraudat, 1998; Nambara and Marion-Poll, 2005). ABA coordinates many of these stress responses, including the immediate stomatal closure, osmolyte accumulation and the induction of the synthesis of stress-related proteins, such as late embryogenesis abundant and heat shock proteins, reactive oxygen scavengers, etc. and thus ABA is aptly called a stress hormone. ABA levels are induced in response to various stress signals and generally accompanied by an increase in endogenous ABA levels prior to activation of a number of water-and salt-stress-induced genes (Chandler and Robertson, 1994), the products of which are thought to be involved in protection of the cell or in recovery from the stress-mediated physiological insult. Stress responsive genes can be expressed either through an ABA-dependent or ABA-independent pathway (Shinozaki and Yamaguchi-Shinozaki, 1997).

To determine how the gene responds to salt stress, the gene expression should be analyzed in relation to abscisic acid (ABA) function. Several ABA deficient mutants were used for determining gene function as they were reported in *Arabidopsis* (Koornneff *et al*., 1998), tobacco, tomato, and maize (Liotenberg *et al*., 1999). They showed the requirement of the induction of these ABA-inducible genes in response to salt stress (Xiong *et al*., 2001). Besides, ABA-inducible genes are predicted to play

an important role in the signal transduction of the salt tolerance mechanism in rice (Gupta *et al*., 1998). In support of this, there are observations that application of the exogenous ABA to both whole plants, and in tissue culture explants or protoplasts, facilitated the adaptation to subsequent increased salinity in several phylogenetically diverse plants. It has been well documented that while exogenous ABA application enhances the tolerance of plants or plant cells to cold (Chen *et al*., 1983), heat (Robertson *et al*., 1994), drought (Lu *et al*., 2009), anoxia (Kato-Noguchi, 2000) and heavy metal stresses (Hsu and Kao, 2000). Several studies also should that the exogenous ABA treatment enhances plant tolerance to salinity. Larosa *et al*. first reported that ABA enhances the adaptation of cultured tobacco cells to salinity (1985). Later, this was also found to be true for cultured rice cells (Kishor, 1989). The exogenous ABA treatment has great agronomic potential for improving in the growth of japonica rice (*Oryza sativa* L.) (Khomsakul, 2004) and the common bean (*Phaseolus vulgaris*) (Khadri *et al*., 2007), to reduce leaf abscission and increase salt tolerance in citrus plants (Gómez Cardenas *et al*., 2003), and to induce salt adaptation in jojoba (*Simmondsia chinensis*) shoots grown *in vitro* (Mills *et al*., 2001). As all above supporting an idea that application of exogenous ABA could enhance salt tolerant ability and considered to be involved in the induction of salt-inducible genes. Therefore, it is interesting to determine the salt responsive gene in relation to ABA function by using the exogenous ABA application.

Beside, among these adapted mechanisms (osmotic adjustment), ion and proline concentrations are considered to be involved in the protection of enzymes (Solomon *et al*., 1994) and cellular structures (Van Rensburg *et al*., 1993) and to act as a free radical scavenger (Alia *et al*., 1995). Proline accumulation is one of the most frequently reported modifications induced by water and salt stresses in plants and often considered to be involved in stress resistance mechanisms. A number of reports indicated the positive correlation between the accumulation of this compound and the adaptation to osmotic stress (Stewart and Lee, 1974; Greenway and Setter, 1972; Goas *et al*., 1982; Weimberg *et al*., 1982; Torello and Rice, 1986).

Proline biosynthesis from glutamic acid via P5C constitutes a main route under stress conditions (Stewart, 1997; Delauney and Verma, 1993). In first step of this proline synthesis pathway, glutamic acid is catalyzed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and is rate-limiting (Delauney and Verma, 1993; Hu *et al*., 1992; Kavi Kishor *et al*., 1995; Yoshiba *et al*., 1995; Igarashi *et al*., 1997; Hong *et al*., 2000). Homologous *P5CS* genes have been isolated from various plant species, including two *P5CS* gene homologues in rice, *OsP5CS1* (Igarashi *et al*., 1997) and *OsP5CS2* (Hien *et al*., 2003). However, their partial characterization to date reveals different transcript expression patterns between them. *OsP5CS1* transcripts are upregulated by NaCl, osmotic, dehydration and cold shock (Igarashi *et al*., 1997), whilst *OsP5CS2* transcripts are additionally up-regulated by mannitol (Hien *et al*., 2003). In addition, both genes are up-regulated by exogenous ABA under normal conditions, the kinetics differ with a faster and stronger up-regulation of *OsP5CS1* transcripts compared to that for *OsP5CS2* in whole indica rice seedlings, and differential expression levels in different tissues were reported (Hur *et al*., 2004). The data summarized above raise the issue of whether *OsP5CS1* gene expression is related directly to salt-stress and ABA function in *Oryza sativa* L., since it remains unknown if *OsP5CS1* acts via an ABA-dependent pathway of salt-stress response. Thus, this study was predicted the *OsP5CS1* in ABA signaling and also established the relationship between *OsP5CS1* gene expression levels and proline accumulation. Based on previous reports, where exogenous ABA treatment increased *P5CS* transcript levels in *A. thaliana* (Strizhov *et al*., 1997; Yoshiba *et al*., 1999; Abraham *et al*., 2003), we considered the possibility that *OsP5CS* might be regulated by ABA in rice. Khomsakul (2004) presented the 100µM ABA is an appropriate concentration of exogenous ABA application that could induce rice salt tolerance and also trigger proline accumulation. If that is the case, we speculate that exogenous ABA application at a dose that induces salt tolerance and proline accumulation should induce *OsP5CS1* transcript levels. The proline synthesis pathway with glutamate as a precursor requires two enzymes, pyrroline-5-carboxylate synthase (P5CS), and pyrroline-5-carboxylate reductase (P5CR). Therefore, if exogenous ABA application does not act via *OsP5CS1* gene expression, other genes, such as *OsP5CR*, in the proline biosynthesis pathway should be investigated.

Under osmotic stress, only *AtP5CS*, but not *AtP5CR*, gene expression was well correlated with proline content (Yoshiba *et al*., 1995; Savouré *et al*., 1997). In contrast, in maize and wheat the activity of P5CR during water stress was increased in correlation with the accumulation of proline, whilst increased Ca^{2+} levels also led to the elevated accumulation of proline in both plant species (Nayyar, 2003). The calmodulin antagonist, trifluoperazine, decreased P5CR activity and proline content, suggesting the involvement of Ca^{2+} and calmodulin in P5CR function (Nayyar, 2003).

To determine if ABA regulates the gene expression, an anti-ABA biosynthesis substance (abamineSG) application as well as exogenous ABA can be used to clarify the role of ABA in salt-tolerant gene expression. AbamineSG is a competitive inhibitor of the 9-*cis*-epoxycarotenoid dioxygenase enzyme (NCED) (Kitahata *et al*., 2006). The application of abamineSG during osmotic stress was reported to scale down the level of ABA accumulation, inhibit the ABA-responsive genes and ABA-catabolic genes in abamineSG-treated Arabidopsis and also induce plant growth retardation (Kitahata *et al*., 2006). Thus, it is possible to think that abamineSG should enable us to elucidate the functions of the interested genes, *OsNUC1* and *OsP5CS1*, on salt tolerance mechanism involved in ABA signaling pathway. The result of gene expression analysis when rice seedlings were treated with exogenous ABA and anti-ABA biosynthesis, abamineSG, substances during normal and salt stress will show if these genes is induced via ABA dependent or ABA independent pathway under salt stress condition.

In this research, two pairs of indica rice cultivars were used: LPT123 and its isogenic line (LPT123-TC171) (Vajrabhaya and Vajrabhaya, 1991; Thikart *et al*., 2005), and the other is KDML105 and FL530-IL (the recombinant inbred line of KDML105) (Suriya-arunroj *et al*., 2004). Each pair of two rice lines with a similar genetic background, but that differ in their salt-tolerant ability, the wild type rice cultivars: LPT123 and KDML105 (the salt-sensitive cultivar), and their mutant lines: LPT123-TC171 and FL530-IL (the salt-tolerant cultivar), will be used as a model to test the pathway responsible for salt tolerance in rice.

Leung Pra Tew 123 (LPT123) is the indica rice cultivar and its isogenic line, LPT123-TC171 that was obtained from somaclonal variation and already tested for salt tolerant ability and has been selected under salt-stressed conditions for seven generations (Vajrabhaya and Vajrabhaya, 1991; Khomsakul, 2004). KDML105, commonly known as "Jasmine rice" or "Thai Hom Mali rice", is a photoperiod-sensitive cultivar and is well adapted to the rainfed environment in which the fields are normally low fertility, frequently experiencing drought and salt stresses and also often attacked by diseases and insect pests. 'KDML 105' was grouped in salt-susceptible cultivar that had high Na^+ uptake but low K^+ uptake resulting in imbalance Na^+ and K^+ in their cells (Suriya-arunroj *et al*., 2005) whereas, FL530-IL (salt-resistant cultivar) which is the recombinant inbred line of KDML105 had low Na^+/K^+ ratio (Suriya-arunroj *et al*., 2005). A salt-resistant rice line was developed by the Rice Gene Discovery Unit. To obtain FL530, the hybrid line between 'Pokkali' and 'IR29' was crossed with 'KDML105' and the resulting progeny was backcrossed with KDML105 for three more generations. The salt-tolerant phenotype is believed to derive from the first chromosome of 'Pokkali' rice. Therefore, 'LPT123-TC171' and 'FL530-IL' contain the genetic background similar to their wild type varieties, but they have higher salt tolerance, which is suitable for comparative studies. This research consists of two main parts; the first one is characterization of the novel salt responsive gene or *OsNUC1* and the second are study on the role of *OsP5CS1* in proline biosynthesis when the plants were applied with the exogenous ABA. The gene expressions were detected in comparative level of salt-susceptible and -resistant rice lines.

The differential expression of *OsNUC1* gene between normal and salt-stress conditions in rice was clarified. Characterization of *OsNUC1* gene contains nine parts which are (i) Structure and database information, (ii) Southern blot analysis, (iii) Subcellular localization that imply the locate of each domain of this gene, (iv) *OsNUC1* gene expression profile in different organs and under salt-stress exposure, (v) The *OsNUC1* gene function on salt stress in correlation with ABA function, (vi) The effect of exogenous ABA application on this gene expression, (vii) Induction of overexpressed *OsNUC1* transgenic Arabidopsis, (viii) *OsNUC1* enhances plant

growth, and (ix) *OsNUC1* could promote the other salt-responsive genes regulation. This is the first report to reveal the function of nucleolin in salt-stress resistance, which suggests the posttranscriptional regulation and promotion of the other salt-resistant genes.

The evaluation of how ABA involves in the salt tolerant ability via proline accumulation was performed. Moreover, the role of *OsCam1-1* in proline biosynthesis was also proposed. The function of calmodulin in proline accumulation during salt stress was observed in Cam antagonist-treated rice seedlings to imply the role of *OsCam1-1* on proline accumulation.

Objectives:

1. To characterize *OsNUC1*, a novel salt-stress responsive gene, including gene organization and gene expression during the normal and salt-stress conditions.
2. To determine the gene effects on salt tolerance by overexpression of the novel gene and test for salt-tolerant ability of the transgenic plants.
3. To determine if the *OsNUC1* and *OsP5CS1* genes act via ABA dependent or ABA independent pathway using exogenous ABA and anti-ABA substance application.
4. To determine if the *OsNUC1* is induced in the same signal transduction pathway as *OsP5CS1* (the gene responsible for proline synthesis) gene induction during salt stress.
5. To determine if the *OsP5CS1* or *OsP5CR* is the rate-limiting gene on proline biosynthesis when the plants get the exogenous ABA together with salt stress.
6. To determine if the calmodulin involves in proline biosynthesis during salt stress.

Contents of the thesis:

The organization of this dissertation is started with an introduction of the research including rational, problems, and objectives. Then, chapter II presents the literature reviews about the toxicity of salt exposure, the role of ABA, osmotic adjustment and plant nucleolin-like protein and background of rice and Arabidopsis that were used for this study. Materials and methods obtained in this study are also mentioned in the following chapter. Chapter IV, results and discussion, is divided into 3 parts corresponding to: (i) characterization of *OsNUC1*, a novel salt-responsive gene, in rice, (ii) determination of *OsNUC1* and *OsP5CS1* function on ABA regulation under normal and salt-stress conditions, (iii) study in the effect of exogenous ABA on the changes of *OsP5CS1* and *OsP5CR* gene regulation in proline biosynthesis pathway. After that, all results are summarized in chapter V and future perspectives are also described. Appendices indicated other information that did not included in the previous chapters.

CHAPTER II

LITERATURE REVIEWS

1. Salinity effects on plants

Salinity, a major environmental stress, is a condition where excessive salts in soil solution cause inhibition of plant growth or plant death. On a world scale, no toxic substances restrict plant growth more than high dose of salts (Zhu, 2007). It is a substantial constraint to crop production (Boyer, 1982) and limits agriculture all over the world, particularly on irrigated farmlands (Rausch, 1996). High concentrations of salts in soil in many arid and semi-arid regions are causing large decreases in yields for a wide variety of crops all over the world, which affect plants through osmotic effects and ion specific effects (Munns, 2002; Pitman and Lauchli, 2002; Sekmen *et al*., 2007). Currently, 20% of the world's cultivated land is affected by salinity, which results in the loss of 50% of agricultural yield (Zhu, 2001; Bartels and Sunker, 2005). Many crop plants such as barley, maize and rice, are often subject to salinity stress (Sairam and Tyagi, 2004). Salt stress causes alterations in plant metabolism, including reduced water potential, ion imbalances and toxicity and sometimes severe salt stress may even threaten survival. Such plasmolysis affects the metabolism of the cells and the functions of macromolecules and, ultimately, results in the cessation of growth (Le Rudulier, 2005).

In extreme cases, salt exposure not only imposes limitation on crop productivity but also limits land available for farming. Agricultural land could no longer sustain agricultural production and had to be abandoned. One-third of the land being irrigated worldwide is affected by salinity, but salinity also occurs in non-irrigated land (Allen *et al*., 1994). Moreover, salinity can be detected within one hundred years of settlement on of land (Estes, 1997). The loss of farmable land is directly in conflict with the needs of the world population which is projected to increase by 1.5 billion in the next 20 years. Therefore, soil salinity also becomes a serious problem in many coastal, arid and irrigated production systems.

In area of low rainfall (such as Northeast area of Thailand), salts accumulate because percolating moisture is insufficient to wash out salts added by irrigation. Huge losses of valuable crop plant production, such as rice productivity, in Thailand were faced by salinity exposure, thus highlighting a greater need for understanding how plants respond to adverse conditions with the hope of improving tolerance of plants to environmental stress (Joseph et al., 2010). An increase in salinity resistance in crop plants is necessary for further expansion for growing area because good agricultural land is limited (Toenniessen, 1984).

Salt response follows an early similarity with drought, whereas in the long term plants are responding to ion toxicity. There are species-specific responses to salt. High salinity causes both ionic stress and osmotic stress that, in turn, affect virtually every aspect of plant physiology and metabolism (Hasegawa *et al*., 2000a; Zhu, 2001; Chinnusamy *et al*., 2006). A combination of ionic and hyperosmotic imbalance mainly inhibit growth and can affect development or cause cell death (Hasegawa *et al*., 2000b; Zhu, 2001, 2002). For ion toxicity, sodium ions are toxic to most plants, and some plants are also inhibited by high concentrations of chloride ions. For the latter, high salt represents a water deficit or osmotic stress because of decreased osmotic potential in the soil solution. Some plants are able to prevent salt entry (salt exclusion at the whole plant or the cellular level) or to minimize its concentration in the cytoplasm (by compartmentalizing salt in the vacuoles), thus avoiding toxic effects on the key metabolic processes such as photosynthesis.

1.1 Ionic stress and ion homeostasis

Excessive sodium ions at root surface disrupt plant potassium nutrition. Because of a common proteins transport Na^+ and K^+, Na^+ competes with K^+ for intracellular influx (Niu *et al*., 1995; Amtmann and Sanders, 1999; Blumwald *et al*., 2000). Many K^+ transport systems have some affinity for Na^+, i.e., Na^+/K^+ symporters. Thus external Na^+ negatively impacts intracellular K^+ influx. Potassium deficiency inevitably leads to growth inhibition because potassium, as the most abundant cellular cation, plays a critical role in maintaining cell turgor, membrane

potential and enzyme activities. Once sodium gets into the cytoplasm, it inhibits the activities of many enzymes.

Most cells maintain relatively high K^+ and low concentrations of Na^+ in the cytosol. This is achieved through a coordinated regulation of transporters for H^+, K^+, Ca^{2+} and Na^+. The absorption and the extrusion of the Na^+ through the plasma membrane and the vacuole regroup a multiplicity of research that addresses the role of K^+ channels as well as Na^+/H^+ antiporters associated to with H^+-ATPase pump (Sussman, 1994; Munns, 2005) that generates a proton motive force driving the transport of Na^+. For example, *SOS1* functions as a Na^+/H^+ antiporter on the plasma membrane and plays a crucial role in sodium efflux from root cells and the long distance Na^+ transport from root to shoot (Shi *et al*., 2000). Increased ATPase-mediated H^+ translocation across the plasma membrane is a component of the plant cell response to salt imposition (Braun *et al*., 1986; Warad *et al*., 1991). In addition, the compartmentalization of Na^+ (and Cl^-) into the vacuole allows the plants to use NaCl as an osmoticum, maintaining an osmotic potential that drives water into the cells, which is necessary for cell volume regulation and development (Hasegawa *et al*., 2000a). Even in the case of halophytes that accumulate large quantities of sodium inside the cell, their cytosolic enzymes are just as sensitive to sodium as enzymes of glycophytes. This implies that halophytes have to compartmentalize the sodium into the vacuole, away from cytosolic enzymes.

1.2 Osmotic stress and regulation of osmotic potential

When plants are challenged with hyperosmolarity, accumulation of ions such as Na^+ in the vacuoles can serve as a means to lower osmotic potential of the cells, thereby the water potential imbalance between the apoplast and symplast leads to turgor decrease (Bohnert *et al*., 1995; Taiz and Zeiger, 1998). This process is perhaps cost-effective with regard to the amount of energy and resources spent (Yeo, 1985), which if severe enough can cause growth reduction. Growth cessation occurs when turgor is reduced below the yield threshold of the cell wall.

However, to maintain turgor may contribute by osmotic adjustment in order to despite low water potentials and proceed to the uptake of K^+, compartmentalization of Na^+ and Cl^- into the vacuole or synthesis of compatible solutes such as proline, glycinebetaine, polyol, sugar etc. (Asraf, 1994). For synthesis of compatible solutes, they are called compatible because they are non-toxic at high concentration; they have low weight, are highly soluble and protect plants from stress by turgor maintenance, detoxification of radical oxygen species (ROS), and by stabilization of quaternary structure of proteins (Yancey *et al.*, 1982; Smirnoff and Cumbes, 1989; Bohnert and Jensen, 1996; Nuccio e*t al*., 1998; Hong *et al*., 2000). In salt-acclimated plants, it was also shown that primary metabolites linked to amino acid and nitrogen or carbohydrate and polyol metabolism do increase; these compatible solutes play a role in osmotic adjustment, membrane and protein protection or scavenging of reactive oxygen species (ROS) and of excess accumulated ammonium ions. Different compatible solutes are synthesized according to the species. In cereal species (wheat, barley, rice and maize), Ayliffe *et al*. (2005) noted the presence of proline and demonstrated the importance of accumulation and degradation of proline in the mechanisms of tolerance to abiotic stress namely drought and salt.

2. Salt-stress signal transduction for plant adaptation

Because of their sessile nature, plants have evolved sophisticated mechanisms to cope with environmental challenges (Zhu, 2002). Plants respond and adapt to environmental stresses through not only physiological and biochemical processes but also molecular and cellular processes. Plant adaptation under salt exposure has been regulated by salt stress signaling, in which, can be divided into three functional categories: ionic and osmotic stress signaling for their establishment of cellular homeostasis under stress conditions, detoxification signaling to control and repair stress damages, and signaling to coordinate cell division and expansion to levels suitable for the particular stress conditions (Figure 2.1). Homeostasis signaling negatively regulates detoxification responses because, once cellular homeostasis is reestablished, stress injury would be reduced, and failure to reestablish homeostasis would aggravate stress injury (Zhu, 2001, 2002). When the ionic and osmotic change,

in turn, lead to the other changes for the detoxification pathways, e.g. phospholipid hydrolysis, changes in the expression of dehydrin-type genes and proteinases that remove denatured proteins, and activation of enzymes involved in the generation and removal of reactive oxygen species and other detoxification proteins. Homeostasis and detoxification signaling lead to stress tolerance and are expected to negatively regulate the growth inhibition response, i.e., to relieve growth inhibition.

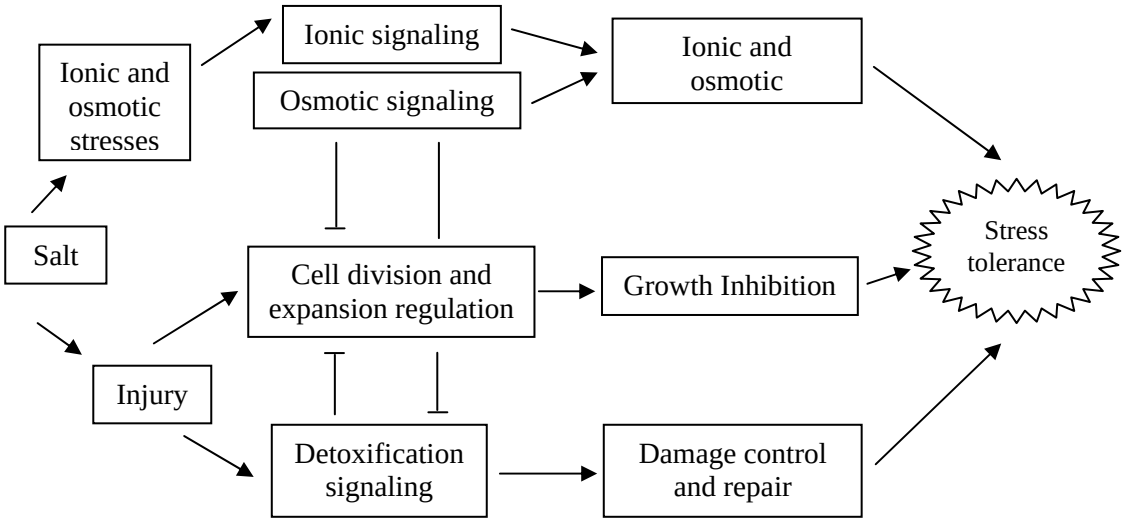


Figure 2.1 Functional demarcation of salt and drought stress signaling pathways. The inputs for ionic and osmotic signaling pathways are ionic (excess NaCl) and osmotic (e.g., turgor) changes. The output of ionic and osmotic signaling is cellular and plant homeostasis. Direct input signals for detoxification signaling are derived stresses (i.e., injury), and the signaling output is damage control and repair (e.g., activation of dehydration tolerance genes) (Zhu, 2002).

Stress signals are sensed and decoded by all plants via distinct and interconnecting signal pathways that are response relays for the control of unique and stress-specific programs. These response relays that control genetic programs and coordinate determinants and processes are required for adaptation. Stress signaling are dependent upon the activation of cascades of molecular networks involving in stress perception, transcription, and the expression of specific stress-related genes. A cascade of complex events is called signal transduction. The extracellular stress signal is first perceived at membrane level by the membrane receptors, ion channel and then activates large and complex signaling cascade intracellular including the generation of secondary signal molecules such as Ca^{2+}, ROS and IP_3. The stress signal then transduces inside the nucleus induce multiple stress responsive genes, the products of which ultimately lead to plant adaptation to stress tolerance directly or indirectly (Trewavas and Malho, 1997; Mahajan and Tuteja, 2005). Overall, the stress response could be a coordinated action of many specific genes, which may cross-talk with each others. These specific genes could encode for proteins implicated in Na^+ sequestration, in synthesis of compatible osmolytes, in the detoxification of toxic compounds, in signal perception and regulating factors and other unknown functions. These activated stress response mechanisms reestablish ion homeostasis, protect and repair damaged proteins and membranes (Ashraf, 1994).

Many stress-responsive genes are regulated by different signaling pathways in response to stress. Stress responses primarily include transcriptional regulation of gene expression and this depends on the interaction of transcription factors with *cis*-regulatory sequences (Shinozaki and Shinozaki, 2005). *cis*-acting elements function in response to environmental stress in stress-responsive promoters. Interactions among different types of *cis*-acting elements function in cross-talk between different signals. For example, the interactions between DRE/CRT and ABRE in the *rd29a* promoter. The DRE/CRTs functions as a coupling element of ABRE (Narusaka *et al*., 2003) and constitutes an ABA response complex in ABA-inducible gene expression (Shinozaki and Shinozaki, 2005). ABRE and DRE are major *cis*-acting elements in the abiotic stress-inducible gene expression to control the molecular procresses of stress responses and stress tolerance. DRE functions in early stress signaling, whereas

ABRE functions after the accumulation of ABA during drought and high salinity stress response. Furthermore, in several in instances, the quantity and availability of regulatory proteins may depend on their own expression patterns. Such autocatalytic controls may be exerted on the transcriptional, post-transcriptional or translational level.

3. Abscisic acid (ABA)

ABA is defined as a stress hormone and plant growth regulator (PGR). Plant perceive and respond adaptively to stress imposed by salt and the adaptive process are controlled mainly by the abscisic acid (ABA) (Swamy and Smith, 1999). Its rapid accumulation in response to stresses and its mediation of many stress responses helps plant to survive under stresses (Zhang *et al*., 2006). Under salt exposure, the production of ABA was triggered which are responsible for the induction of many genes (some are fast response and others slow response genes) since they were required for protein synthesis (Mahajan and Tuteja, 2005; Swamy and Smith, 1999; Xiong *et al*., 2002; Chinnusamy *et al*., 2004). It is believed that a large group of these genes are regulated by the stress-induced ABA (Bray, 1997; Chandler and Robertson, 1994; Leung and Giraudat, 1998; Finkelstein *et al*., 1990; Zhu, 2002).

The main function of ABA is to regulate plant water balance and osmotic stress tolerance. Several ABA-deficient mutants have been reported for Arabidopsis, namely *aba1*, *aba2*, and *aba3* (Koornneef *et al*., 1998). There are also ABA-deficient mutants for tobacco, tomato, and maize (Liotenberg *et al*., 1999). The mutants, such as the Arabidopsis *aba1*, *aba2*, and *aba3*, have slightly smaller statures, which may be caused by unavoidable stress even under the best growth conditions (Koornneef *et al*., 1998). Additionally, the smaller stature of the *aba* mutants may also be due to ABA regulation of the cell cycle and other cellular activities. Under drought stress, ABA-deficient mutants readily wilt and die if the stress persists. Under salt stress, ABA-deficient mutants also perform poorly (Xiong *et al*., 2001). The role of ABA in drought and salt stress is at least two fold: water balance and cellular dehydration tolerance. Whereas the role in water balance is mainly through guard cell regulation,

the latter role has to do with induction of genes that encode dehydration tolerance proteins in nearly all cells.

The action of ABA can target specifically guard cells for induction of stomatal closure and inhibit stomatal opening but may also signal systemically for adjustment towards severe water shortage. ABA promotes the efflux of K^+ ions from the guard cells, which results in the loss of turgor pressure leading to stomata closure. ABA also triggers cytosolic calcium ($[Ca^{2+}]_{cyt}$) increases (McAinsh *et al*., 1990). $[Ca^{2+}]_{cyt}$ elevations activate two different types of anion channels: slow-activating sustained (S-type; Schroeder and Hagiwara, 1989) and rapid transient (R-type; Hedrich *et al*., 1990) anion channels. Both mediate anion release from guard cells, causing depolarization. This change in membrane potential deactivates inward-rectifying K^+ (K^+_{in}) channels and activates outward-rectifying K^+ (K^+_{out}) channels (Schroeder *et al*., 1987), resulting in K^+ efflux from guard cells. The sustained efflux of both anions and K^+ from guard cells via anion and K^+_{out} channels contributes to loss of guard cell turgor, which leads to stomatal closing.

As a result of stomatal closing, photosynthesis declines (Kawasaki *et al*., 2001) and photoinhibition and oxidative stress occur. Another factor that contributes to decreased photosynthesis is the inhibitory effect of salt stress on the efficiency of translocation and assimilation of photosynthetic products. Therefore, a perturbed growth, evidenced particularly by inhibition of cell expansion under salt stress, maybe controlled to a large extent by hormone homeostasis.

ABA is synthesized in both roots and shoots (Thompson *et al*., 2000). ABA content in roots is well correlated with both soil moisture and the relative water content of roots in many plant species (Zhang and Davies, 1989, Liang, 1997). In addition, ABA can act as a long distance communication signal between osmotic stress roots and leafs (Zeevaart and Creelman, 1988, Miborrow, 2001, Hartung *et al*., 2002), thereby the xylem ABA came from two sources, which are roots and the older leaves that wilted earlier than the younger leaves. Since ABA synthesized in the root under salt stress maybe an endogenous signal that ascends with transpirational flow to

regulate shoot growth, stomatal aperture and hence stress tolerance (reviewed in Davies and Zhang, 1991). ABA transport from root to shoot is modified by the ionic conditions and pH in the xylem (Wilkinson, 1998; Bacon, 1998). ABA is a weak acid it may be come protonated or deprotonated in the pH range found in the apoplast of leaves. This was clearly shown in the early 1980s in experiments on mesophyll that documented high rates of ABA uptake at acidic pH and almost no uptake at the more alkaline pH of 7.5 (Kaiser and Hartung, 1981). In certain plant species, the pH of the leaf apoplast increases as the osmotic stress because of the delivery of xylem saps that is of a more alkaline pH. The ABA that arrives from roots via the xylem will remain deprotonated under the more alkaline conditions and will not be taken up passively by mesophyll cells. The result is that less ABA is transported into the mesophyll cells and a build-up of ABA in the apoplast leads to enhanced stomatal closure (Hartung, 1998).

ABA not only promotes the closure of stomata to minimize transpirational water loss, it also mitigates stress damage through the activation of many stress-responsive genes that encode enzymes for the biosynthesis of compatible osmolytes and LEA-like proteins, which collectively increase plant stress tolerance (Hasegawa *et al*., 2000, Bray, 2002, Finkelstein *et al*., 2002). ABA function also involves in complex regulatory mechanisms that control signal perception and transduction. In addition, ABA has been shown to offset the inhibitory effect of stress-induced ethylene on plant growth (Sharp, 2002). Despite the ease with which ABA can enter cells, there is evidence for extracellular as well as intracellular perception of ABA (reviewed by Leung and Giraudat, 1998, Rock, 2000). Genetic studies have identified many loci involved in ABA synthesis and response and analyzed their functional roles in ABA physiology (reviewed in Leung and Giraudat, 1998, Rock, 2000). Many likely signaling intermediates correlated with ABA response (e.g. ABA-activated or induced kinases and DNA-binding proteins that specifically bind ABA-responsive promoter elements) have also been identified. Cell biological studies have identified secondary messengers involved in ABA response (Finkelstein and Rock, 2002).

Many genes that are induced under environmental stresses such as low temperature, high salt and drought stresses are also induced by exogenous ABA (reviewed by Shinozaki and Yamaguchi-Shinozaki, 1997). Higher plants under such stresses produce ABA endogenously and this ABA induces the expression of various genes allowing higher plants to tolerate these stresses (Mansfield, 1987). Many ABA-responsive genes have been identified successfully (Skriver and Mundy, 1990). It has been demonstrated that transgenic plants overexpressing some of these ABA-responsive genes show significantly improved tolerance to stresses (Xiang *et al*., 2008; Godoy *et al*., 1990).

Many approaches, especially the use of ABA-deficient and ABA-insensitive mutants (Koornneef *et al*., 1998), have shown that signaling of salt stress may be understood in two major pathways: the ABA-dependent and ABA-independent gene expression pathways (Shinozaki and Yamaguchi-Shinozaki, 1997). Some osmotic stress responsive genes are induced completely independent of ABA, some are fully dependent on ABA, and others are only partially ABA dependent (Shinozaki and Yamaguchi-Shinozaki, 1996). For example, the *rd29a* gene has served as an excellent paradigm of ABA-dependent and-independent gene regulation. Osmotic stress induction of *rd29a* transcript accumulation is only partially blocked by *aba1* or *abi1* mutations, thus suggesting this gene does not require ABA for their expression under drought and salt conditions but do respond to exogenous ABA (Thomasshow, 1994; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1996).

Exogenous application of ABA has been documented that enhances the tolerance of plants or plant cells to high salt (Li *et al*., 2010), cold (Chen *et al*., 1983), heat (Robertson *et al*., 1994), drought (Lu *et al*., 2009), anoxia (Kato-Noguchi, 2000) and heavy metal stresses (Hsu and Kao, 2003). The application of exogenous ABA has been shown to improve the salinity tolerance of several plant species, such as rice (Kishor, 1989, Bohra *et al*., 1995), tobacco (Larosa *et al*., 1985), finger millet (Aarati *et al*., 2003) and common bean (Khadri *et al*., 2007). Applications of ABA are known to affect plant growth and development, mimicking the effects of ionic and osmotic stresses, thereby helping plants to better survive stress conditions (Davies and Jones,

1991, Taiz and Zeiger, 2006). Treatment of exogenous 100µM ABA application on indica rice cultivars during salt stress resulted in early accumulation of proline for the enhancement in salt tolerance (Klomsakul, 2004). This phenomenon is generally known as induced resistance, and it suggests that ABA has great agronomic potential for improving the stress tolerance of agriculturally important crops.

ABA is a sesquiterpenoid ($C_{15}H_{20}O_4$). The naturally occurring form is S-(+)-ABA; the side chain of ABA is by definition 2-cis, 4-trans (Figure 2.2). Trans, trans-ABA is biologically inactive (Zeevaraart and Creelman, 1988, Toorop *et al*., 1999). ABA is involved in many aspects of plant responses to salt stress, including both plant growth and development (Rock, 2000; Bray, 1997; Skriver and Mundy, 1990; Ingram and Bartel, 1996; Zhu *et al*., 1997; Chandler and Robertson, 1994). It plays a critical role in regulating plant water status through guard cells and growth as well as by induction of genes that encode enzymes and other proteins involved in cellular dehydration tolerance (Luan, 2002; Zhu, 2002).

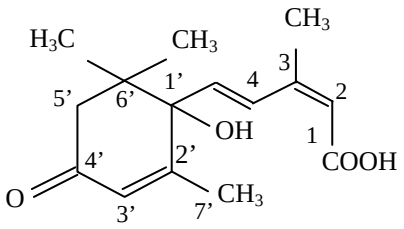


Figure 2.2 Structure of S-(+)-abscisic acid

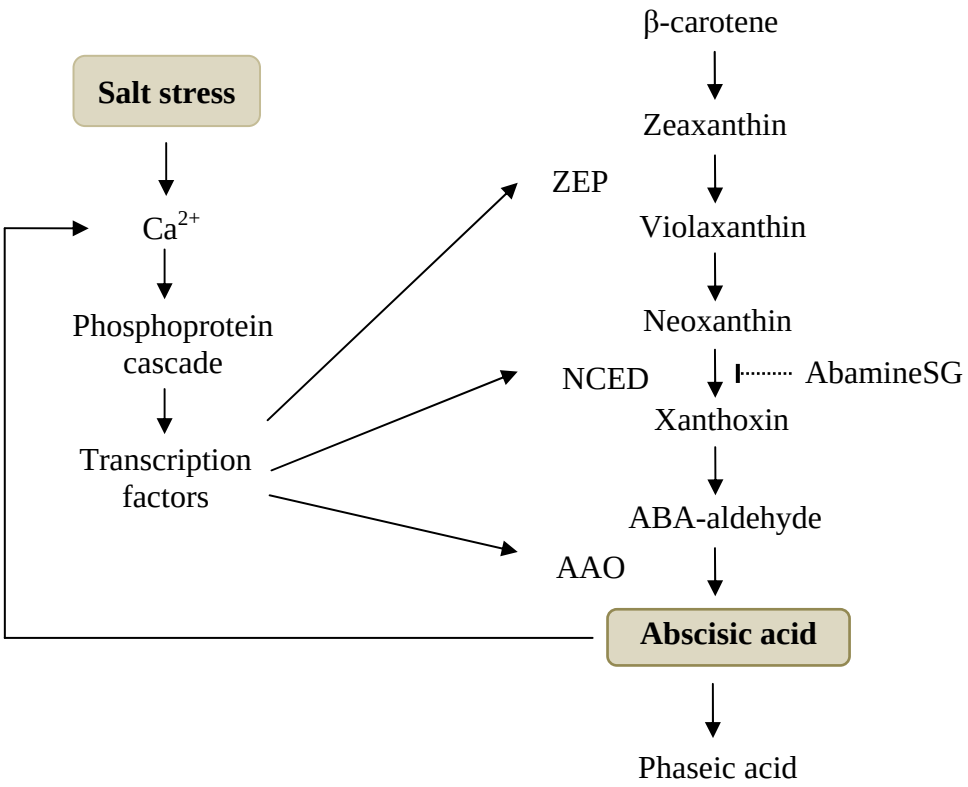
3.1 ABA biosynthesis

Salt stress and osmotic stress induced ABA accumulation is a result of both activation of synthesis and inhibition of degradation. The induction of genes for enzymes responsible for ABA biosynthesis has been identified. ABA biosynthesis primarily occurs in the leaves as ABA is synthesized from β-carotene through several enzymatic steps. The first step that is more specific to the ABA biosynthesis pathway is the epoxidation of zeaxanthin to violaxanthin, which occurs in plastids (Duckham *et al*., 1991; Rock and Zeevaart, 1991; Marin *et al*., 1996). This step is catalyzed by a

zeaxanthin epoxidase (ZEP), whose molecular identity was first revealed in tobacco (Marin *et al*., 1996). After a series of structural modifications, violaxanthin is converted to 9-cis-epoxycarotenoid. The 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes the oxidative cleavage of 9'-*cis*-neoxanthin to xanthoxin (Schwartz *et al*., 1997). This step was considered the first committed step in the ABA biosynthesis pathway. The *ZmNCED* gene was isolated using the maize *vp14* mutant (Tan et al., 1997). The product xanthoxin is then exported to the cytosol, where it is converted to ABA through a two-step reaction via ABA-aldehyde. ABA aldehyde oxidase catalyzes the last step (Figure 2.3). The rate-limiting step in ABA biosynthesis is NCED that is xanthophylls precursors (Tan *et al*., 1997; Liotenberg *et al*., 1999; Qin and Zeevaart, 1999; Thompson *et al*., 2000). Expressions studies with *ZEP*, *NCED*, and *AAO* indicated that these genes are all upregulated by drought and salt stress (Audran *et al*., 1998; Seo *et al*., 2000; Iuchi *et al*., 2001; Xiong *et al*., 2001). The expression of *NCED* gene is a rapid and transient process, e.g., in maize leaves dehydration will lead to an accumulation of *ZmN* mRNA to the highest level within 5h, following which the *ZmNCED* mRNA will rapidly decrease (Qin and Zeevaart, 1999, 2002). Importantly, overexpression of a 9'-*cis*-epoxycarotenoid dioxygenase gene led to a substantial accumulation of ABA and enhanced drought tolerance in tobacco (Qin and Zeevaart, 2002).

3.2 ABA biosynthesis inhibitor (abamineSG)

AbamineSG (Figure 2.4) is a competitive inhibitor of the enzyme NCED. An important structural feature of abamineSG is a three-carbon linker between the methyl ester and the nitrogen atom. Treatment of osmotically stressed plants with 100 µM abamineSG inhibited ABA accumulation by 77% as compared to the control. Moreover, the expression of ABA-responsive genes, *RD29B* and *RAB18*, and ABA catabolic gene, CYP707, was strongly inhibited in abamineSG-treated plants under osmotic stress. The growth of Arabidopsis seedlings also was inhibited by abamineSG (Kitahata *et al*., 2006). Thus, it is possible to think that abamine should enable us to elucidate the functions of ABA in cells or plants and to find new mutants involved in ABA signaling.



(Zhu, 2002)

Figure 2.3 ABA biosynthesis pathway and its regulation. ABA is synthesized from β-carotene via the oxidative cleavage of neoxanthin and conversion of xanthoxin to ABA via ABA-aldehyde (Xiong *et al*., 2001; Zhu, 2002). AbamineSG could inhibit enzyme NCED function. ZEP, zeaxanthin epoxidase; NCED, 9'-*cis*-epoxycarotenoid dioxygenase; AAO, ABA-aldehyde oxidase (Kitahata *et al*., 2006).

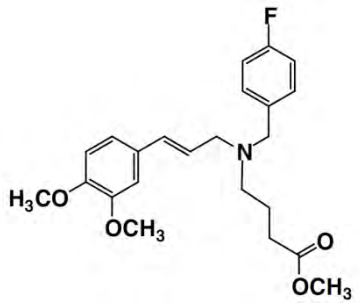


Figure 2.4 Structures of abamineSG. The length of the linker between the methyl ester and the nitrogen atom was crucial for the inhibition of ABA biosynthesis.

4. Compatible solute and osmotic adjustment

Under osmotic stress, an important consideration is to accumulate osmotically active compounds called osmolytes or compatible solutes in order to lower the osmotic potential in the cells. A common feature of compatible solutes is that these compounds can accumulate to high levels without disturbing intracellular biochemistry (Bohnert *et al*., 1995). Compatible solutes have the capacity to persevere the activity of enzymes that are in saline solutions. In addition, the osmolytes are considered to stabilized proteins and cellular structures (Yancey *et al*., 1982). This response is homeostatic for cell water status and protein integrity, which is perturbed in the face of soil solutions containing higher amounts of NaCl and the consequent loss of water from the cell.

The accumulation of these osmolytes is believed to facilitate "osmotic adjustment", by which the internal osmotic potential is lowered and may then contribute to tolerance (Delauney and Verma, 1993; McCue and Hanson, 1990). With accumulation proportional to the change of external osmolarity within species-specific limits, protection of structures and osmotic balance supporting continued water influx (or reduced efflux) are accepted functions of osmolytes. Accumulation of compatible solutes results in an increase in cellular osmolarity that can drive influx of water or reduce the efflux. This provides the turgor that is necessary for cell expansion (Kishor *et al*., 2005). Moreover, the accumulation of osmotically active

compounds in the cytosol increases the osmotic potential to provide a balance between the apoplastic solution, which itself becomes more concentrated with Na^+ and Cl^- ions, and the vacuolar lumen, which in halophytes can accumulate up to 1M Na^+(and Cl^-). For a short-term stress, this may provide the cells with the ability to prevent water loss. However, for continued growth under salinity stress, an osmotic gradient (towards the cytosol) must be kept in order to maintain turgor, water uptake and facilitate cell expansion (Greenway and Munns, 1980).

Some compatible osmolytes are essential elemental ions; such as K^+, but the majority is organic solutes, consist of proline, mannitol, and glycine betaine. Compatible solute accumulation as a response to osmotic stress is an ubiquintous process in organism as diverse as bacteria to plants species. Many organic osmolytes, such as proline, glycine betaine, are presumed to be osmoprotectants, as their levels of accumulation are insufficient to facilitate osmotic adjustment. Not only are the organic solutes nor harmful, they may also have a protective effect against damage by toxic ions of dehydration. Many of the osmoprotectants enhance stress tolerance of plants when expressed as transgene products (Bohnert and Jensen, 1996; Zhu, 2001). An adaptive biochemical function of osmoprotectants is the scavenging of reactive oxygen species that are by-products of hyperosmotic and ionic stresses and cause membrane dysfunction and cell death (Bohnert and Jensen, 1996).

In this study, we interested in proline accumulation and biosynthesis under salt stress exposure. Proline is one of the compatible osmolyte, is not charged at neutral pH and is highly soluble in water (Hare and Cress, 1997). In plants, proline accumulation has been reported to occur after salt, drought, high temperature, low temperature, heavy metal, pathogen infection, nutrient deficiency, atmospheric pollution and UV irradiation (Hare and Cress, 1997; Saradhi *et al*., 1995; Siripornadulsil *et al*., 2002). The level of proline accumulation in plants varies from species to species and can be 100 times greater than in control situation. Proline metabolism in plants has mainly been studied in response to osmotic stress. Proline accumulation is believed to play adaptive roles in plant stress tolerance and maybe part of the stress signal influencing adaptive responses (Maggio *et al*., 2002).

5. Role of proline in salt stress condition

Under osmotic stress, membrane integrity must be maintained to prevent protein denaturation. Proline may interact with enzymes to preserve protein structure and activities. Proline has a property of forming hydrophilic colloids in aqueous media with a hydrophobic backbone interacting with the protein in order to protect protein structure (Chadalavada *et al*., 1994). On the other hand, effects of proline might be involved in the hydration layer surrounding phospholipids and possibly its interaction between phospholipid head groups (Bellinger and Larher, 1987). Indeed, it has been shown in vitro to reduce enzyme denaturations caused due to NaCl stress (Hamilton and Heckathorn, 2001). Sodium chloride curtailed carboxylase activity of Rubisco and enhanced the oxygenase activity, but not quite surprisingly, salt stress induced oxygenase activity was suppressed by proline even at a concentration of 50 mM NaCl (Sivakumar *et al*., 2000). It showed that proline plays a critical role in protecting photosynthetic activity under salt stress. Hamilton and Heckathorn (2001) found that while complex II is protected by proline under NaCl stress condition and it also prevents the photoinhibitory loss of photochemical activity by producing a reduction in lipid peroxidation (Sivakumar *et al*., 2000, Alia *et al*., 1991). Proline also acts as a reserve source of carbon, nitrogen and energy during recovery from stress (Zhang *et al*., 1997) and be ROS scavenger (Smirnoff and Cumbes, 1989). In organisms ranging from bacteria to higher plants, there is a strong correlation between increased cellular proline levels and the capacity to survive both water deficit and the effects of high environmental salinity.

5.1 Proline biosynthesis

In plants, there are two different precursors for proline that can be synthesized from either glutamate or ornithine, however, glutamate pathway is the primary route for proline biosynthesis during osmotic stressed. The glutamate pathway (Figure 2.5) consists of two important enzymes, via pyrroline carboxylic acid synthetase (P5CS) and pyrroline carboxylic acid reductase (P5CR). Transcripts corresponding to both cDNAs accumulate in response to NaCl treatment. Proline biosynthetic pathway starts with the phosphorylation of glutamate, which gets converted to γ-glutamyl phosphate and then to glutamic-γ-semialdehyde (GSA) by the

enzymes γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase, respectively. GSA gets converted to pyrroline 5-carboxylate (P5C) by spontaneous cyclization. On the other hand, glutamate is directly catalysed to GSA by pyrroline 5-carboxylate synthetase (P5CS) in plants and other eukaryotes (Hu *et al*., 1992). P5C is then reduced to proline by P5C reductase (P5CR) in both prokaryotes and eukaryotes.

In rice, Roy *et al*. (1992) estimated that proline accumulation in response to NaCl could be attributed both to an increase in P5CR activity and to a decrease in PDH activity. Some authors suggest that proline accumulation maybe related to the degree of salt tolerance in rice (Igarashi *et al*., 1997). Levels of proline in the transgenic Arabidopsis were twice that of control plants grown in the absence of stress, and three times higher than in control plants grown under stress. The high levels of proline were correlated with an improvement in tolerance to salinity (Yosiba, 1995).

Many approaches aimed at engineering higher concentrations of proline began with the over-expression of genes encoding the biosynthetic enzymes pyrroline-5-carboxylate (P5C) synthase (P5CS) and P5C reductase (P5CR) that catalyze the two steps between the substrate, glutamic acid and the product, proline. *P5CS* over-expression in tobacco dramatically elevated free proline in transgenic tobacco (Kishor *et al*., 1995). Further, a two-fold increase in free proline was achieved in tobacco plants transformed with a *P5CS* modified by site-directed mutagenesis (Hong *et al*., 2000). The capacity of proline degradation is also decreased under prolonged hyperosmotic for proline accumulated during stresses (Hare and Cress, 1997). Proline catabolism, via *proline dehydrogenase* (*ProDH*), is upregulated by free proline and there exists a strong evidence that free proline inhibits *P5CS* (Roosens *et al*., 1999). Free cellular proline levels are also transcriptionally and translationally controlled. An alternative approach to attain significant free proline levels, where antisense cDNA transformation was used to decrease *ProDH* expression, was utilized (Nanjo. 1999).

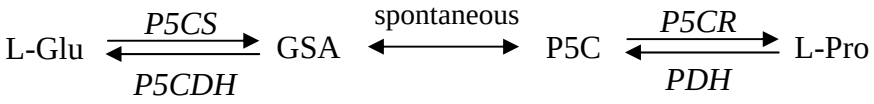


Figure 2.5 Proline metabolic pathways via L-glutamic acid in plants. The main precursor of Pro synthesis is L-glutamic acid (L-Glu). L-Glu is first reduced to glutamate semialdehyde (GSA), by P5C synthase (P5CS). The second reduction, of P5C to Pro, is catalyzed by P5C reductase (P5CR). This pathway is found in the cytosol and in plastids. Pro is catabolized to Glu in mitochondria by Pro dehydrogenase (PDH) and P5C dehydrogenase (P5CDH).

5.2 Δ^1-pyrroline-5-carboxylate synthase (P5CS)

P5CS is a bifunctional enzyme that catalyses the first two steps of proline biosynthesis in plants and consequently the increase in osmotolerance (Kishor *et al*., 1995; Hong *et al*., 2000; Szoke *et al*., 1992). It is a rate-limiting enzyme in the pathway and exhibits both γ-glutamyl kinase as well as glutamic-γ-semialdehyde dehydrogenase activities. This essential role is probably due to two levels of control exerted at those steps: proline feedback inhibition on the P5CS protein (Zhang *et al*., 1995; Fujita *et al*., 1998) and regulation of the *P5CS* transcript level in response to osmotic stress conditions (Igarashi *et al*., 1997; Fujita *et al*., 1998; Savoure *et al*., 1995).

In plants, numerous studies suggest that P5CS is feedback regulated by proline and there lease of feedback inhibition of P5CS enzyme can lead to an increase in proline accumulation in response to stress conferring better osmotolerance (Fujita *et al*., 1998; Boggess *et al*., 1976). Transgenic tobacco overexpressing a feedback insensitive form of the enzyme, accumulates more proline and shows more resistance to osmotic stress than the plant expressing the wild type *P5CS* (Hong *et al*., 2000). Indeed, the *P5CS* gene is transcriptionally induced by osmotic stress and its induction precedes the accumulation of proline (Yoshiba *et al*., 1995). Accumulation of proline and expression of the *P5CS* gene were strongly induced after 48 h in salt-tolerant rice under high-salt conditions (250mM) when compared with a sensitive one (Igarashi *et*

al., 1997). This strongly supports that proline accumulation and the induction of *P5CS* shortly after the start of salt stress maybe related to the degree of salt tolerance in *Oryza sativa*. Moreover, expression of this *P5CS* transgene under the control of a stress-inducible promoter led to stress-induced overproduction of the P5CS enzyme and proline accumulation in transgenic rice plants (Igarashi *et al*., 1997). Second generation transgenic plants showed an increase in biomass under salt and water stress conditions (Zhu *et al*., 1998).

Overwhelming evidence supports the conclusion that P5CS is the rate-limiting enzyme in proline biosynthesis (Kavi Kishor *et al*., 1995). This suggests that *P5CS* has a key role in the biosynthesis of proline under osmotic stress (Yoshiba *et al*., 1995). Although levels of transcript encoding *P5CR* have been reported to increase under stress in at least two legumes (Hare and Cress, 1997), and P5CR enzyme activity has been shown to increase under stress in several plant species (Mattioni *et al*., 1997), controversy has arisen concerning whether or not Arabidopsis *P5CR* transcript abundance is sensitive to salinization. Whereas one group has reported induction of *AtP5CR* mRNA levels in response to osmotic stress (Verbruggen *et al*., 1993, Savoure´ *et al*., 1997), others have contested a significant accumulation of transcripts encoding P5CR in osmotically-stressed Arabidopsis (Yoshiba *et al*., 1995). Using an Arabidopsis *P5CR* cDNA as a probe, Mattioni *et al.* (1997) concluded that an increase in P5CR activity in *Triticum durum* seedlings during dehydration and salt stresses is unrelated to levels of the corresponding transcript.

Homologous *P5CS* genes have been isolated from various plant species, including two *P5CS* gene homologues in rice (*Oryza sativa* L.), *OsP5CS1* and *OsP5CS2* (Igarashi *et al*., 1997; Hien *et al*., 2003). However, their partial characterization to date reveals different transcript expression patterns between them. Thus, *OsP5CS1* transcripts are upregulated by NaCl, osmotic, dehydration and cold shock (Igarashi *et al*., 1997), whilst *OsP5CS2* transcripts are additionally up-regulated by mannitol (Hien et al., 2003). In addition, although both genes are up-regulated by exogenous ABA under normal conditions, the kinetics differ with a faster and

stronger up-regulation of *OsP5CS1* transcripts compared to that for *OsP5CS2* in whole indica rice seedlings, and differential expression levels in different tissues (Hur *et al*., 2004). This is in good agreement with the differential expression patterns of the two homologous genes in *Arabidopsis*, *AtP5CS1* and *AtP5CS2*, in terms of tissue tropism and the kinetics and levels of transcript expression (Strizhov *et al*., 1997; Yoshiba *et al*., 1999; Abraham *et al*., 2003).

6. Nucleolin

Nucleolin is one of the most abundant non-ribosomal proteins that ubiquitously expressed in various cell compartments, especially in the nucleolus, of which is a major component. Intact Nucleolin is the major species and represents 5% of nucleolar protein in actively dividing cells. In non-dividing cells, degraded forms of various molecular sizes are predominantly expressed due to autodegradation (Lapeyre *et al*., 1987; Chen *et al*., 1991; Fang and Yeh, 1993; Olson *et al*., 1990). Nucleolin is a multifunctional protein primarily involved in ribosome synthesis; however, it has been implicated in many other metabolic processes including cell proliferation and growth, ribosomal components, transcriptional repression, replication, signal transduction and chromatin decondensation (reviewed in Ginisty, 1999). Nucleolin also potentially stabilizes mRNA in response to UVA radiation (Zhang *et al*., 2008). Nucleolin have been reported in multiple species, including hamsters (Bouche *et al*., 1984; Lapeyre *et al*., 1987), human (Srivastava *et al*., 1989; Tuteja *et al*., 1995), mouse (Bourbon *et al*., 1988), rat (Ohmori *et al*., 1990), chicken (Maridor and Nigg, 1990), *Xenopus laevis* (Caizergues-Ferrer *et al*., 1989), insects (Olson, 1990), yeast (Lee *et al*., 1991, 1992; Kondo and Inouye, 1992; Gulli *et al*., 1995; Leger-Silvestre *et al*., 1997) and in plants (Didier and Klee, 1992; Martine *et al*., 1992; Bogre *et al*., 1996; Tong *et al*., 1997; deCarcer *et al*., 1997). The proteins from yeast and plants, such as pea, alfafa and Arabidopsis, which are only structurally related to Nucleolin without having a very similar sequence to animal Nucleolin are called Nucleolin-like proteins. However, the studies on plant Nucleolin-like protein function have been few and far between and had no report on Nucleolin-like protein in rice. Even though Nucleolin structure in several species was clearly identified, the

structure of rice Nucleolin-like protein and the role of this gene on stress response remain unidentified.

6.1 Plant Nucleolin-like protein

In plant, the presence of Nucleolin-like protein was first reported from onion root meristematic cells where it is associated with chromatin and helps in decondensation of chromatin, in rDNA transcription, and in the early steps of pre-rRNA processing (Martin *et al*., 1992). In onion root cells, it has also been shown that Nucleolin together with other components of the nucleolar processing complex co-localize during mitosis and later segregated to daughter cell nucleoli (Medina *et al*., 1995). It is also suggested that ribosome biogenesis restarts not only after mitosis at the level of transcription but also at the intermediate levels of pre-rRNA processing. The Arabidopsis Nucleolin-like protein has the same gene organization as three ribonucleoproteins of tobacco chloroplast (Li and Sugiura, 1990), which are suggested to be involved in splicing and/or processing of chloroplast RNAs (Didier and Klee, 1992).

On the other hand, plant Nucleolin-like protein is involved in developmentally, cell-cycle, and light regulation (Tuteja and Tuteja, 1998). The transcript and protein levels of Nucleolin-like protein in alfafa correlate with cell proliferation, and *Nucleolin* gene expression is induced in the G1 phase of cell cycle after mitogenic stimulation of Go-arrested leaf cells, similar to the D-type cyclin gene (Bogre *et al*., 1996). In proliferating cells of alfafa, *Nucleolin*'s transcript level is not changed in a cell cycle phase-specific manner but disappears when cells exit the cell cycle and undergo differentiation or polar growth, indicating the role of Nucleolin in cell proliferation (Bogre *et al*., 1996). *Nucleolin* gene expression is also known as a marker for proliferation events during flower development. In pea, *Nucleolin* is shown to be light regulated (Tong *et al*., 1997). Light is also known to increase the rate of nuclear rRNA gene transcription in several plants and the light receptor for this response is the photoreversible pigment phytochrome (Thien and Schopfer, 1982). Moreover, rRNA transcription is a crucial step in ribosome assembly (Olson, 1990).

Moreover, the genome of the model plant *Arabidopsis thaliana* encodes two Nucleolin-like proteins, AtNUC-L1 and AtNUC-L2. Only the *AtNUC-L1* gene is ubiquitously expressed in normal growth conditions. Pontvianne and colleagues (2007) reported that the *AtNUC-L1*disruption affects plant growth and development. *AtNUC-L1* is localized in the nucleolus mainly in the dense fibrillar component. *Atnuc-L1* plants grew lower than WT plants and the newly emerged leaves were smaller, pointed, irregular shaped, and scrunched. The major phenotypic changes in flower, sepals, anthers and siliques of *Atnuc-L1* were reported. The reduction of seeds was also found (Pontvianne *et al*., 2007).

6.2 Structure of Nucleolin

Nucleolin has three domains of different structure and function, namely the N-terminal, central and C-terminal domain (Ginisty *et al*., 2000; Ginisty *et al*., 1999). The different nucleolar functions of Nucleolin and Nucleolin-like proteins must rely on the presence of several distinct structural domains. It is likely that the N- and C- terminal domains of Nucleolin are involved in protein-protein interaction whereas the central domain, which contains the RBD motifs, is involved in specific interactions with nucleic acids. The different functions of Nucleolin are also likely to be the result of the assemblies of Nucleolin with other factors to form large complexes specialized in a specific function.

6.2.1 N-terminal Domain

The amino-terminal domain contains several highly charged acidic sequence repeats interspersed with basic segments. The number of acidic stretches differ in different species, mouse, human, and chicken Nucleolin contain four, whereas the Nucleolin-like protein from pea and Arabidopsis contain seven and alfafa contains nine (Bourbon *et al*., 1988; Srivastava *et al*., 1989; Maridor and Nigg, 1990; Bogre *et al*., 1996; Tong *et al*., 1997). The plant Nucleolin-like proteins contain considerably more but shorter acidic repeats as compared with others. The acidic sequence repeats is the site of numerous phosphorylations by casein kinase 2 (CK2) and cyclin dependent kinase1 (CDK1) (Mongelard and Bouvet, 2006). Downstream part of the N-terminal domain contain bipartite NLS (NLS: nuclear localize signal)

(Xue *et al*., 1993). The functional bipartite NLS in the pea Nucleolin-like protein KKGKRQAEEEIKKVSAKKQK (Tong *et al*., 1997) was shown to be responsible for targeting Nucleolin to the nucleus. However, there is no consensus signal sequence for targeting Nucleolin to the nucleolus. Instead, it is proposed that the accumulation of Nucleolin in the nucleolus results from specific binding of Nucleolin to other nucleolar components, particularly rDNA, rRNA, and also protein constituents of nucleolar matrix structure (Schmidt-Zachmann and Nigg, 1993). Of the several structural domains present in Nucleolin, only the N-terminal was found to be dispensable for nucleolar accumulation (Tuteja and Tuteja, 1998).

6.2.2 Central Domain

This domain of Nucleolin is globular and contains four RNA recognition motifs (RRM) also called consensus RNA-binding domain (CS-RBD) that are conserved among different species (Serin *et al*., 1997). However, Nucleolin-like proteins from yeast, pea, alfafa, and Arabidopsis contain only two CS-RBDs. The CS-RBD is found in proteins implicated in heterogeneous RNA packaging (Dreyfuss *et al*., 1993), pre-mRNA splicing (Amrein *et al*., 1993), as components of pre-ribosomes (Bourbon *et al*., 1983), in poly (A) tail synthesis and maturation (Adam *et al*., 1986), in translational control (Naranda *et al*., 1994), and in mRNA stability (Zhang *et al*., 1993). The central domain interacts and binds specifically with short RNA stem-loop structures of 18S and 28S ribosomal RNA (Bugler *et al*., 1987; Ghisolfi *et al*., 1992; Serin *et al*., 1997; Bouvet *et al*., 1998).

6.2.3 C-terminal Domain

The COOH-terminal proximal portion of Nucleolin consists of Glycine- and Arginine-rich (GAR) repeat segments, as Arg-Gly-Gly (RGG) repeats interspersed, thereby it also called RGG domain (Bouvet *et al*., 1998). The length of the GAR/RGG domain is variable among Nucleolins, with its sequence and arrangement of the repeats not well conserved (Bogre *et al*., 1996). This domain is in an extended conformation and has no hydrophobic regions (Jordan, 1987; Lapeyre *et al*., 1987). The presence of this domain in a protein is associated with the presence of an RNA-binding domain (RBD or others) (Burd and Dreyfuss, 1994) and it’s essential

for efficient binding of Nucleolin to RNA but does not itself contribute to the specificity of the interaction (Ghisolfi *et al*., 1992b, Heine *et al*., 1993). One function of this domain could be to facilitate the interaction of Nucleolin RBD domains with targets located within large and complex RNA, such as rRNA (Ghisolfi *et al*., 1992b; Heine *et al*., 1993). The GAR domain appears likely to function primarily in protein-protein interactions (Bandziulis *et al*., 1989). Bouvet *et al*., (1998) showed that Nucleolin interacts with several ribosomal proteins through its GAR domain. However, this GAR domain may also influence the polynucleotide binding properties of CS-RBD. This domain is known to destabilize rRNA/rRNA helical regions so that rRNA regions can be recognized by the central domain of the Nucleolin (Ghisolfi *et al*., 1992a). However, GAR domain could be an important signal for the regulation of these interactions, the stability of the protein, or its localization.

6.3 The complex role of Nucleolin in ribosome biogenesis

Nucleolin might play a key role in ribosome biogenesis that includes transcription and processing of rRNA as well as ribosome assembly and maturation. Ribosome biogenesis in eukaryotes is a complex process that involves the coordinate expression of a large number of genes. The main function of Nucleolin is participation in the rRNA processing from rDNA transcription to assembly of preribosomal particles. It induces changes in chromatin structure, elongation of primary rRNA transcript and ribosome maturation. The main steps of ribosome biogenesis occur in the nucleolus (Sommerville, 1986) where ribosomal genes are actively transcribed by RNA polymerase I. The intranuclear location of the nucleolus around the chromosomal regions that code for ribosomal regions that code for ribosomal RNAs (5.8S, 18S, and 28S) facilitates the active transcription of these genes by RNA polymerase I (Hadjiolov, 1985; Shaw and Jordan, 1995; Shaw, 1996). The nascent transcripts are associated with two types of proteins: ribosomal proteins, found in the mature cytoplasmic ribosomes and a group of proteins that are transiently bound to pre-ribosomes in the nucleolus. These latter proteins play a role in the transcription process in the transcription process, in the packaging of pre-RNA or in its maturation. Nucleolin is among one of them that is highly conserved during evolution and was originally called C23 (Orrick *et al*., 1973). The specific interaction

of Nucleolin with the pre-rRNA substrate is required for the processing reaction *in vitro*. The N-terminal domain together with the RBD region is required for Nucleolin activity. It is a well-characterized major nucleolar phosphorylation that represents up to 5% of the nucleolar proteins in exponentially growing cells (Sapp *et al*., 1986; Lapeyre *et al*., 1987). It is a highly phosphorylated multifunctional non-ribosomal acidic protein and is present at the heart of the nucleolus (Jordan, 1987). Because of its specific nucleolar localization it is called Nucleolin.

6.4 The other role of Nucleolin-like protein

Tuteja *et al*. (1995) have discovered the Nucleolin as a nuclelic acid helicase. RNA helicases are not only essential for most processes of RNA metabolism including ribosome biogenesis, pre-mRNA splicing and translation initiation (Jankowsky, 2010, Jankowsky *et al*., 2010, Fairman-Williams *et al*., 2010), but also for sensing viral RNAs in the context of the innate immune system and for the biogenesis and function of miRNAs (Linder and Owttrim, 2009, Yoneyama and Fujita, 2010). The nucleic acid unwindings are needed transiently and are known to be essential for DNA replication, repair, recombination, transcription, translation initiation, RNA splicing, ribosome assembly, and mRNA stabilization, turnover, and export (Figure 2.6) (Dalbadie-McFarland and Abelson, 1990; Pause and Sonenberg, 1992; Lavoie *et al*., 1993; Matson *et al*., 1994; Tuteja and Tuteja, 1996; Tuteja, 1997; Venema *et al*., 1997). Nucleolin is also known to be up-regulated during times of increased cell proliferation (Bogre *et al*., 1996). Since in cell proliferation, the DNA helicases play an important role whether the GAR domain of pea Nucleolin, similarly to the GAR domain human Nucleolin (Tuteja *et al*., 1995), also functions as DNA helicase. Mostly helicases are either DNA helicase or RNA helicase and play important roles in the processing of DNA and/or RNA (Matson *et al*., 1994). Additionally, Nucleolin has been shown to be involved, either directly or indirectly, in the regulation of transcription by Pol I and Pol II (Figure 2.6); it suggests a role in both the activation and the inhibition of transcription (Roger, 2002; Ying, 2000; Hanakahi, 1997; Schulz, 2001; Grinstein *et al*., 2002). A positive correlation has been demonstrated between the quantity of Nucleolin, the rate of Pol I transcription and the proliferation rate of a cell (Derenzini, 2000).

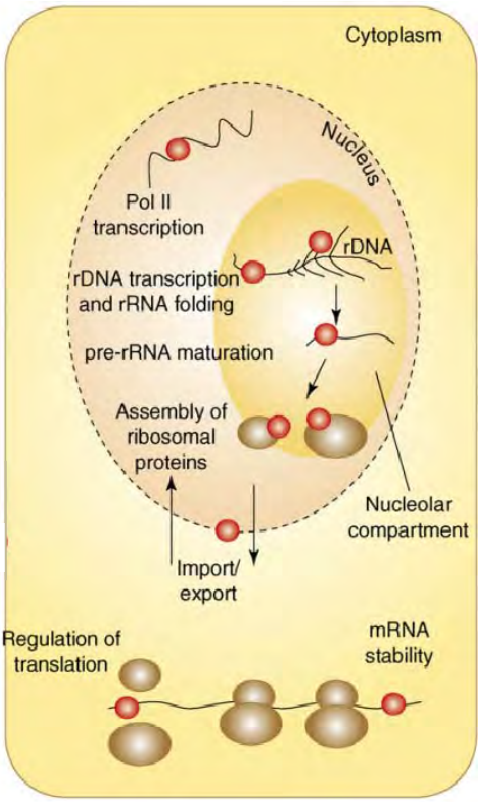


Figure 2.6 The cellular compartment locations of Nucleolin. The wide localization, and the large number of Nucleolin-associated factors, is compatible with the multiple functions of Nucleolin. Red dots, the different possible localizations and functions of Nucleolin. (i) In the nucleolus, association of Nucleolin with nucleolar chromatin (rDNA) could be involved in the regulation of Pol I transcription. (ii) Nucleolin has been seen on nascent pre-rRNA transcripts and is believed to participate in co-transcriptional pre-rRNA folding, (iii) maturation at the first processing site and (iv) assembly of pre-rRNA with ribosomal proteins. (v) The shuttling of Nucleolin between the nucleus and the cytoplasm might participate in the import and/or export of several nucleolar components or proteins, such as ribosomal proteins. (vi) In the nucleoplasm, Nucleolin has been also found associated with several genes transcribed by Pol II and with mRNAs, with functions from (vii) the regulation of translation to (viii) mRNA stability (Mongelard and Bouvet, 2006).

7. Rice is the important crop food of the world and the model plant of monocots.

Rice is a staple food for a large part of the world's human population, especially in East and Southeast Asia, making it the most consumed cereal grain (FAO, 2004; Coats, 2003). Due to population increases, the demand for rice is estimated to be 2,000 million metric tons by 2030 (FAO, 2004), thus, the improvement of rice production is required. The most important factor to reduce rice production is salt stress affects in worldwide (Suriya-arunroj *et al*., 2005). In Asia alone, 21.5 million ha are affected by salt stress, it limits crop yield and restricts use of land previously uncultivated (FAO, 2005). Rice is the most important agricultural crop and assesses high valuable crop food not only in Thailand, but also including multiple countries in Asia. Thailand is the largest exporting country of the world (26% of world exports) and the second exporter is Vietnam (15%), whereas, China (31% of world production) and India (20%) could produced rice in the first two highest countries of the world's rice production, many countries in Asia were reported to be the considerable rice producer and exporter of the world (FAO, 2004).

Unfortunately, rice is constantly bombarded with environmental signals both biotic and abiotic, some of which cause stress and limit growth and development and affect the yield and quality. Rice thrives in waterlogged soil and can tolerate submergence at levels that would kill other crops, is moderately tolerant of salinity and soil acidity, but is highly sensitive to drought and cold. According to salinity effects approximately 20% of world's arable land and approximately 40% of irrigated land to various degrees, thereby significant proportion of cultivated land is salt-affected (Rhoades and Loveday, 1990). Therefore, advances in physiology, molecular biology, and genetics have greatly improved the understanding of how rice responds to salt stress and the basic of varietal differences in tolerance.

Rice plant offers various advantages as an experimental plant compared with other monocot species, such as small genome size, a known genome sequence (Sasaki and Burr, 2000) and self-fertilization. Moreover, it is an annual plant that is nevertheless able to survive for several years. For those reasons, rice is considered a

model plant of monocots. Rice belongs to the grass family (*Poaceae*) together with barley, wheat, maize and sorghum, which are important cereal crops that support the global food supply. In addition, these grasses are inferred to have a monophyletic origin (Clark *et al*., 1995), indicating that information obtained from rice is helpful for studying other cereal crops. In fact, several reports have elucidated genome synteny between rice and other grasses (Bennetzen and Ma, 2003). Salt sensitivity of rice varies not only among genotypes but also among developmental stages of the plant (Akbar and Yabuno, 1974). However rice is very tolerant to salt during germination, but very sensitive during the early seedling stages (Pearson and Ayers, 1960).

Oryza sativa is an annual grass, which grows best when submerged in water. It grows in upland areas, rainfed lowland areas, and flood-prone areas. Rice is highly adaptable and can be grown in diverse environments. It resembles a weed, 2 to 5 feet tall, depending on the variety and depth of submersion. Among the Asia rice cultivars, *Oryza sativa*, three sub-species are commonly distinguished based on geographic conditions, japonica, indica, and javanica, all of them which include glutinous and non-glutinous varities.

Rice was the cereal selected to be sequence as a priority and has gained the status of the "model organism". Rice with its relatively small genome size (~430Mb), relatively easy transformation process, well developed genetics, availability of a dense physical map and molecular markers (Chen *et al*., 2002; Wu *et al*., 2002a), high degree of chromosomal co-linearity with other major cereal such as maize, wheat, barley, and sorghum (Ohyanagi *et al*., 2006) and together with its complete genome sequence (Sasaki *et al*., 2005) is considered a model monocot system. It is being used to understand several fundamental problems of plant physiology, growth, and developmental processes ranging from elucidation of a single gene function to whole metabolic pathway engineering. In addition, rice shares extensive synteny among other cereals thereby, increasing the utility of this system (Devos and Gale, 2000). These, together with availability of rice information databases (Hirochika *et al*., 2004) have made rice a worthy forerunner among the plants especially among the cereals.

8. Arabidopsis as a model plant to study salt tolerance.

Arabidopsis is a glycophytic plant that is sensitive to growth inhibition and damage by salt stress. The Arabidopsis genome has been fully sequenced; the comparison between ecotypes may localize salt tolerance QTLs and potentially lead to the molecular identification of some major loci, if relatively large differences in salt tolerance exist between ecotypes. Research with other glycophytic plant species has shown that upon exposure to high salinity, plants may exhibit a reduced growth rate, accelerated development and senescence or death if the stress is severe or prolonged (reviewed by Lazof and Bernstein, 1999). Like many other glycophytes, the sensitivity of Arabidopsis to salt stress is exhibited at all stages of development. A brief (8hr) treatment with 150mM NaCl during seed development stage resulted in callose deposition and abnormal changes in ovule and embryo structures indicative if cell death (Sun and Hauser, 2001). However, in Arabidopsis salt sensitivity is most evident at the seed germination and seedling stages. Even after cold stratification of imbibed seeds to break dominancy, Arabidopsis seed germination was greatly impaired at salt concentration at or above 75mM. After germination, seedling growth is also very sensitive to NaCl. Although lower concentrations of NaCl (<50mM NaCl) may have a slightly stimulating effect on fresh weight gain in culture media, higher than 50mM of NaCl clearly inhibits plant growth and eventually kills the plants (Xiong and Zhu, 2002).

With the complete sequencing of the Arabidopsis genome, Arabidopsis should find even more use as a model system for studying plant osmotic stress responses. Genetic analysis of salt stress signaling has demonstrated the promise offered by this model system, despite of its glycophytic nature (Zhu, 2000). As Arabidopsis is a glycophyte and is very sensitive to salt, one might assume that this plant is not suitable for studying the mechanisms of salt tolerance. However, previous studies with cultured glycophytic plant cells indicated that these cells could be adapted to tolerance high concentrations of salt that would kill unadapted cells. Hasegawa *et al.* (1994) described about the physiological studies with adaptation of glycophytic plants as well as cell cultures, one consistent that emerged from their studies was that salt-sensitive plants do have salt tolerance genes. Cell cultures derived from many

different glycophytes were made salt tolerant with relative ease by gradual adaptation to higher levels of NaCl. For example, a salt-sensitive tobacco cell culture was adapted to grow over tens of thousands of generations in medium containing near seawater-level NaCl (Hasegawa *et al.*, 1994). Salt-sensitive plants were similarly adapted to grow in the presence of high salinity. New crop varieties have not been obtained by salt adaptation, mainly because adapted plants and cells, although they survive well under high salinity, grow very slowly even without salt stress. Nevertheless, these studies illustrate that all plants have in their genomes genes for salt tolerance. Without adaptation, the salt tolerance genes may not be properly expressed to confer salt tolerance.

8.1 Strategies for finding salt tolerance determinants in Arabidopsis

These studies are typically supported by transgenic studies where the genes of interest are overexpressed and/or under expressed and the resultant transgenic plant phenotypes analyzed. The gene expression profiling approach has been the most popular one for the last decade and a half. Eventually, functional analysis through transgenic overexpression or underexpression, or through reverse genetics to identify knockout mutants is needed to establish whether an induced gene is functionally important for plant salt tolerance. The genetic approach utilizes natural or induced variations in stress tolerance or stress gene regulation. Gene mutations in Arabidopsis plants can be easily created by using one of several mutagens such as ethylmethane sulfonate (EMS), fast neutrons, transposons or T-DNA. Mutations in salt tolerance genes are expected to cause decreased salt tolerance in plants, unless they are functionally redundant. Therefore, one can search for plant mutants that are hypersensitive to salt stress, and these mutants will lead to the identification of salt tolerance genes. Of course, screening for the opposite phenotype (i.e. increased salt tolerance) would identify negative regulators of stress tolerance or gain-of-function mutations.

The promoter-reporter approach is an efficient and especially powerful in identifying gene mutations that have only subtle visible or tolerance phenotypes (Ishitani *et al*, 1997; Xiong *et al*., 1999a). Transgenic plants expressing candidate genes under the control of a constitutive promoter (such as *35SCaMV*) have been widely used to examine gene function in salt and other stress tolerance (Chuang and Meyerowitz, 2000; Smith *et al*., 2000). The use of inducible promoters or tissue-specific promoters may avoid the adverse side effects of constitutive or ubiquitous expression. Kasuga *et al* (1999) found that plants expressing CBF/DREB under the stress-responsive *rd29a* promoter were more stress-tolerant but were not compromised in growth, unlike *35SCaMV* promoter-driven expression. These studies are exciting in providing leads for the genetic engineering of stress tolerance in crop plants. However, caution should be taken when the overexpression results are used to infer the native function of the endogenous plant genes. For example, overexpression of a transcription factor in a tissue or condition where it is normally not expressed may activate other target genes and thus lead to phenotypes unrelated to its normal function.

9. A systematic validation in real-time PCR

Real-time RT-PCR (also known as quantitative RT-PCR) is a powerful tool for quantifying gene expression, combining both high sensitivity and specificity with efficient signal detection. It has relatively recently begun to be used to monitor gene expression in plants (reviewed in Gachon *et al*., 2004) and remains under used, considering its ability to discriminate between the expression of closely related genes and to quantify transcript levels of very weakly expressed genes (Czechowski *et al*., 2004). The main advantages of real-time PCR are its higher sensitivity, specificity.

Real-Time PCR detects the accumulation of amplicon during the reaction. The data is then measured at the exponential phase of the PCR reaction. Traditional PCR methods use agarose gels or other post PCR detection methods, which are not as precise, whereas real-time PCR makes quantitation of DNA and RNA easier and more precise than the past methods. To account for between-sample variations in the amounts of starting material and the efficiency of the quantification process,

determinations of mRNA species in real-time RT-PCR analysis should be normalized according to the total amounts of mRNA present in the samples. The expression levels of target genes are described in terms of the ratios of target mRNA levels to the level of a reference mRNA species, which should be the product of a stably expressed gene whose abundance is strongly correlated to the total amounts of mRNA present in each sample (Huggett *et al*., 2005). The availability of non-specific double-stranded DNA (dsDNA) binding fluorophors, such as SYBR Green, and 384-well-plate real-time PCR machines that can measure fluorescence at the end of each PCR cycle make it possible to perform qRT-PCR on hundreds of genes or treatments in parallel. This has facilitated the comparative analysis of all members of large gene families, such as transcription factor genes (Czechowski *et al*., 2004).

PCR has three phases, exponential phase, linear phase and plateau phase as shown in Figure 2.6. The exponential phase is the earliest segment in the PCR, in which product increases exponentially since the reagents are not limited. The linear phase is characterized by a linear increase in product as PCR reagents become limited. The PCR will eventually reach the plateau phase during later cycles and the amount of product will not change because some reagents become depleted. Real-time PCR exploits the fact that the quantity of PCR products in exponential phase is in proportion to the quantity of initial template under ideal conditions (Heid *et al*., 1996, Gibson *et al.*, 1996). During the exponential phase PCR product will ideally double during each cycle if efficiency is perfect, i.e. 100%. It is possible to make the PCR amplification efficiency close to 100% in the exponential phases if the PCR conditions, primer characteristics, template purity, and amplicon lengths are optimal.

The basis of real-time PCR is a direct positive association between a dye with the number of amplicons. As shown in Figure 2.27 B and C, the plot of logarithm 2 based transformed fluorescence signal versus cycle number will yield a linear range at which logarithm of fluorescence signal correlates with the original template amount. A baseline and a threshold can then be set for further analysis. The cycle number at the threshold level of log-based fluorescence is defined as Ct number, which is the

observed value in most real-time PCR experiments, and therefore the primary statistical metric of interest.

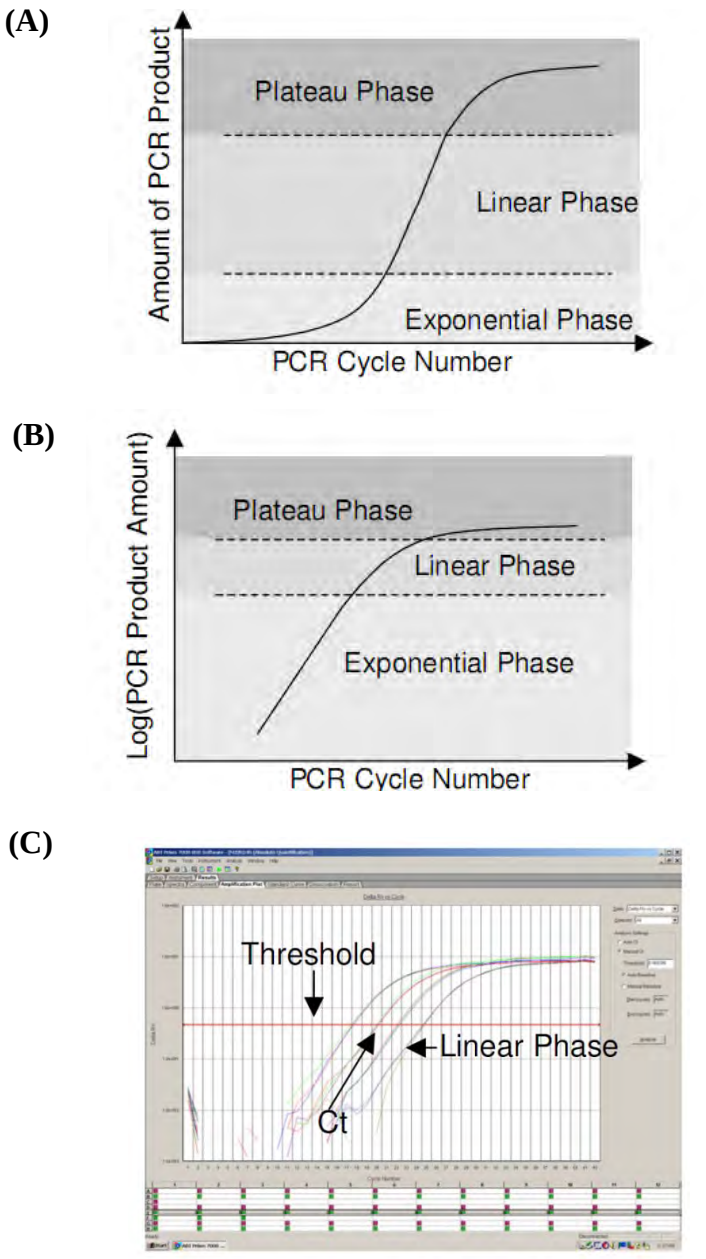


Figure 2.27 Real-time PCR. (A) Theoretical plot of PCR cycle number against PCR product amount is depicted. Three phases can be observed for PCRs: exponential phase, linear phase and plateau phase. (B) shows a theoretical plot of PCR cycle number against logarithm PCR product amount. Panel (C) is the output of a serial dilution experiment from an ABI 7000 real-time PCR instrument (Yuan *et al*., 2006).

Since relative quantification is the goal for most for real-time PCR experiments, several data analysis procedures have been developed. Two mathematical models are very widely applied: the efficiency calibrated model (see in appendix B-9) (Pfaffl, 2001, Pfaffl *et al*., 2002) and the ΔΔCt model (Livak and Schmittgen, 2001). The experimental systems for both models are similar. The experiment will involve a control sample and a treatment sample.

CHAPTER III

MATERIALS AND METHODS

I. MATERIALS

1. Plant materials

1.1 Rice (*Oryza sativa* L.) seeds

1.1.1 Rice cultivar Khao Dok Ma Li 105 (KDML105)
Rice cultivar FL530-IL (a recombinant inbred line of KDML105)

Seeds of the rice cultivar KDML105 (salt-susceptible) and FL530-IL (salt-resistant) (Suriya-arunroj *et al*., 2004) were kindly provided by the Rice Gene Discovery Unit in collaboration with the National Center for Genetic Engineering and Biotechnology (BIOTEC) and the DNA Technology Laboratory (DNATEC) of Kasetsart University, Khamphang-sean, Thailand.

1.1.2 Rice cultivar Leung Pra Tew 123 (LPT123)
Rice cultivar Leung Pra Tew 123-TC171 (LPT123-TC171) (a salt-resistant isogenic line of LPT123)

The Thai indica rice cultivar Leung Pra Tew 123 (LPT123) was obtained from the Agriculture Department, Ministry of Agriculture and Cooperation, Thailand, and was used in comparison with the salinity-resistant line, LPT123-TC171 (Vajrabhaya and Vajrabhaya, 1991; Khomsakul, 2004; Maneeprasopsuk, 2004; Thikart *et al*., 2005; Udomchalothon *et al*, 2008; Sripinyowanich *et al*, 2010), which has been selected under salt-stressed conditions for seven generations.

1.2 Arabidopsis (*Arabidopsis thaliana* L.) seeds

1.2.1 Arabidopsis, ecotype Columbia, served as wild-type

1.2.2 The independent lines of *OsNUC1* overexpression transgenic Arabidopsis under the regulation of constitutively expressed promoter (*35SCaMV*)

1.2.3 The independent lines of *OsNUC1* overexpression transgenic Arabidopsis under the regulation of salt-inducible promoter (*rd29a*)

1.3 Onion epidermal cells

The onion epidermal cells were prepared by using a pointed forceps removed the adaxial epidermis from the underlying tissue. Then, place the strip of epidermis on MS agar plate added 3% sucrose with the adaxial surface of the epidermis facing down.

2. Bacterial strains

2.1 *Eschericia coli* strain DH5α

2.2 *Agrobacterium tumefaciens* strain GV3101 harboring the activation-tagging plasmid pSKI015 (Weigel *et al*., 2000)

3. Instruments

3.1 Equipment for plant growing

- Glass bottle 50 ml
- Glass bottle 100 ml
- Plastic tray 8 x 12 x 4 $inch^2$
- Sand

3.2 Equipment for DNA and RNA extraction

- Deep freezer -80^oC
- Mortars and pestles
- Spectula

- Microcentrifuge tube (Labcon North American, USA)
- Waterbath
- Refrigerated centrifuge (Universal16, Hettich, Germany)
- Eppendorf tubes 1.5 ml
- Spectrophotometer (Agilent Technology, USA)
- Vortex mixture

3.3 Equipment for bacterial growth

- Incubator
- Incubator shaker
- Glass plate
- Lamina flow (Astec Microflow, Bioquell Medical Limited, UK)
- 0.22 um Millipore membrane filter (Millipore, USA)

3.4 Equipment for plasmid DNA extraction

- Deep freezer -80^{o}C
- Microcentrifuge
- Eppendorf tubes 1.5 ml
- Vortex mixer

3.5 Equipment for electrophoresis

- Horizontal gel electrophoresis (MiniRun GE-100, Hangzhou BIOER Technology Co., Ltd., China)
- Gel Doc^{TM} 2000 and UV transsilluminator (Bio-Rad, USA)
- Microwave oven

3.6 Equipment for southern blot analysis

- Film cassette with intensifying screen: 35x43 cm (Amersham Pharmacia Biotech UK limited, UK)
- Hybond N^{+} (Amersham Pharmacia Biotech UK limited, UK)
- Filter paper: Whatman No.1 (Whatman International Ltd.)
- Bio-Rad GS Gene Linker^{TM} UV Chamber

- Hybridization oven (Techne Hybridiser HB-1D, Lab Extreme, Inc., USA)
- Waterbath
- Orbital shaker
- X-ray film (Kodak (Australia) PTY, LYD., Australia)
- Hot air oven
- Hot plate
- Microcentrifuge
- Eppendorf tubes 1.5 ml
- Forceps

3.7 Equipment for cloning

- PCR thin wall microcentrifuge tube 0.2 ml (Axygen Scientific, Inc., USA)
- PTC-100TM programmable thermal controller (MJ research, USA)
- Microcentrifuge
- Eppendorf tubes 1.5 ml
- Waterbath
- Spectrophotometer
- Cuvette
- Incubator
- Incubator shaker
- Lamina flow

3.8 Equipment for particle bombardment

- Particle bombardment (Bio-rad, USA)
- Glass plate
- Lamina flow
- Forceps
- Macrocarrier
- Vortex mixer

3.9 Equipment for subcellular localization

- Leica laser-scanning confocal microscope (Leica Microsystem, Germany)
- Forceps
- Glass slide
- Cover slip

3.10 Equipment for real-time PCR and RT-PCR

- Microcentrifuge
- 96-well plate
- Opticon2 Continuous Fluorescence Detector System (MJ research, USA)
- PCR thin wall microcentrifuge tube 0.2 ml (Extragene, USA)
- PTC-100TM programmable thermal controller (MJ research, USA)
- Vortex mixture

3.11 Equipment for ABA extraction

- Deep freezer -80^{o}C
- Mortars and pestles
- Microcentrifuge tube
- Centrifuge (Universal16, Hettich, Germany)
- Refrigerated centrifuge (Universal16, Hettich, Germany)
- Refrigerator
- 0.22 um Millipore membrane filter (Millipore, USA)
- Centrifuge tubes 50 ml
- Eppendorf tubes 1.5 ml
- Spatula
- Syringe

3.12 Equipment for ABA determination by HPLC

- Round flask
- Beaker
- Microcentrifuge tube

- Rotary evaporator
- Vial 5 ml
- Vacuum pump
- Nipro disposable synringe 5 ml
- HPLC (Agilent Technologies Series 1100, USA)
- 250 nm x 4 mm column packed with 5µm ODS Hypersil (Shandon Runcorn, UK)

3.13 Equipment for collecting plant growth

- Aluminium foil
- Balance: Sartorius CP423s (Scientific Promotion Co. USA)
- Forceps
- Hot-air oven
- Scissor

3.14 Equipment for proline content analysis

- Centrifuge tube
- Spectrophotometer (Agilent Technology, USA)
- Cuvette
- Autopipette (Gibson, Inc., USA)
- Mortars and pestles
- Filter paper: Advantec No.1 (Toyo Roshi Kaisha, Ltd., Japan)
- Hot plate
- Vortex mixer
- Glass funnel

4. Chemicals and reagents

4.1 Chemicals for rice seedlings preparation

- Modified WP no.2 nutrient solution (see in Appendix A)
- Sodium chloride (Merck, Germany)

4.2 Chemicals for DNA and RNA extraction

- CTAB buffer (see in Appendix A)
- RNA extraction buffer (see in Appendix A)
- Phenol: Chloroform: Isoamyl alcohol (25:24:1) (v/v)
- Absolute ethanol (Liquid Distillery Organization Excise Dept, Thailand)
- Lithium chloride (Sigma Chemical Company, USA)
- TE buffer (see in Appendix A)
- Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich Co., USA)
- Hydrogen peroxide
- Chloroform (Merck, Germany)
- 80% ethanol
- Liquid nitrogen
- Sodium acetate (CH_3COONa) (Sigma-Aldrich Co., USA)
- 2-mercaptoethanol (Merck, Germany)

4.3 Chemicals for southern blot analysis

- Restriction endonuclease: *BamHI* (New England Biolabs, USA)
- Depurination solution (see in Appendix A)
- Denaturation solution (see in Appendix A)
- Neutralization solution (see in Appendix A)
- Developer and fixer solution (Kodax) (Australia PTY. LTD., Australia)
- ECL direct nucleic acid labeling and detection system (Amersham International, Amersham, UK)
- 20x SSC (see in Appendix A)

4.4 Chemicals for plasmid DNA extraction

- Solution I (see in Appendix A)
- Solution II (see in Appendix A)
- Solution III (see in Appendix A)
- Isopropanol (Merck, Germany)

- Phenol:Chloroform (1:1)(v/v) (Merck, Germany)
- TE buffer (see in Appendix A)
- RNase A (Sigma-Aldrich Co., USA)
- 80% ethanol
- Liquid nitrogen (from TIG, Thailand)
- Sodium acetate (CH_3COONa) (Sigma-Aldrich Co., USA)

4.5 Chemicals for electrophoresis

- Agarose (Research Organics, USA)
- TBE buffer (Tris Borate EDTA) (see in Appendix A)
- Ethidium bromide (Gibco BRL, USA)
- RNA loading dye (see in Appendix A)
- DNA loading dye (see in Appendix A)
- DNA marker (1KB DNA ladder, New England Biolabs, USA)

4.6 Chemicals for subcellular localization

- Restriction endonucleases: *Bgl*II and *Xba*I (New England Biolabs, USA)
- Gold particle
- pRTL vector
- Murashige and Skoog (MS) (1962) medium (see in Appendix A) with 3% sucrose

4.7 Chemicals for bacterial transformation

- LB medium (see in Appendix A)
- 5-Bromo-4-chloro-3-indole-beta-D-galactopyranoside: X-gal (Sigma Chemical Company, USA)
- Iso-1-thio-β-D-thiogalactopyranoside: IPTG (BioPoint Ltd., UK)
- Antibiotic: Ampicillin, Kanamycin, Rifampicin, Gentamycin
- Glycerol (Ajax Finechem Pty Ltd., Australia)

4.7 Chemicals for gene cloning

- Calcium chloride
- pGEM®-T vector system I (Promega), a vector for cloning (see in Appendix C for a map)
- *pJim19* (see in Appendix C for a map)
- Restriction endonucleases: *Xba*I, *Sac*I, *Hind*III (New England Biolabs, USA)
- *pfu* polymerase reaction mixture (Invitrogen, La Jolla, CA, USA)
- T4 DNA ligase (Takara Bio Inc., Japan)
- StarPerp Gel Extraction Kit (GenStar Biosolutions Co., Ltd., China)
- Sodium acetate (CH_3COONa) (Sigma-Aldrich Co., USA)
- 80% ethanol
- Isopropanol (Merck, Germany)

4.8 Chemicals for the exogenous ABA application and the abamineSG treatment

- ± Abscisic acid (Sigma, USA)
- AbamineSG (kindly provided by Prof. Dr. Tadao Asami, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan)
- Triton X-100 (Sigma-Aldrich Co., USA)
- Ethanol (Liquid Distillery Organization Excise Dept, Thailand)
- Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich Co., USA)

4.9 Chemicals for Real-time PCR and RT-PCR

- 100mM dATP, dCTP, dGTP, dTTP (Promega)
- oligo$(dT)_{15}$ (Merck, Germany)
- Sodium acetate (Sigma-Aldrich Co., USA)
- Absolute ethanol
- RQ1 RNase-free *DNase*I (Takara Bio Inc., Japan)
- MMLV reverse Transcriptase (Promega, USA)
- Sensicript Reverse Transcriptase (QIAGEN)
- iQ™ SYBR® Green Super Mix (Bio-Rad, USA)

- SYBR Green Master Mix Reagent (Toyobo)

4.10 Chemicals for seed sterilization

- Sodium hypochlorite (Avantor™ Performance Materials, Inc., USA)
- Tween20 (Promega, USA)

4.11 Chemicals for Arabidopsis seedlings preparation and growth analysis

- Murashige and Skoog (MS) (1962) medium (see in Appendix A)
- sucrose
- 1.5% agar
- Sodium chloride (Ajax Finechem Pty Ltd., Australia)

4.12 Chemicals for calmodulin antagonist treatment

- N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (Sigma-Aldrich Co., USA)
- Triton X-100 (Sigma-Aldrich Co., USA)

4.13 Chemicals for proline content analysis

- Acid ninhydrin (see in Appendix A)
- Glacial acetic acid (Merck, Germany)
- Chloroform (BDH, UK)
- Toluene (Panreac Química S.A.U., Spain)
- Sulfosalicylic acid (Avantor™ Performance Materials, Inc., USA)
- Phosphoric acid (Panreac Química S.A.U., Spain)
- L-Proline (Sigma-Aldrich Co., USA)

II. METHODS

1. Amino acid identity and motif analyses of rice Nucleolin1-like protein

The Basic Local Alignment Search Tool (BLAST) algorithms (BlastP) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) using the protein sequences of the novel salt-responsive gene (accession number: DQ012289) as query sequences against the rice genome was conducted. Nucleotide and amino acid sequences as well as information regarding gene of interest were obtained, and the protein comparison of homology and similarity was done with ClustalW by comparing full-length rice Nucleolin1-like protein of indica and japonica cultivars and full-length Arabidopsis Nucleolin1 with the corresponding Nucleolin amino acid sequences obtained from GenBank. Gene annotation at the Rice Annotation Project (RAP-DB) was used to confirm the existence and sequences of this gene.

2. Southern blot analysis

Leaf tissues of three weeks old rice seedlings were collected for genomic DNA extraction (appendix B-2). Two microlitres of 10mg/ml RNaseA and *Bam*HI endonuclease were added into 100µg of genomic DNA and the mixture was incubated at 37^{o}C for 3 hours. After that, DNA was extracted with one volume of phenol: chloroform: isoamylalcohol (25:24:1). The sample was mixed thoroughly and immediately centrifuged for 1 minute. The upper aqueous phase was transferred to a fresh tube. One volume of chloroform was added and mixed gently. The mixture was centrifuged at 14,000rpm for 1 minute at 4^{o}C and the top aqueous phase was collected. Then, 0.1 volumes of 3M NaOAc and 2.5 volumes of iced-cold absolute ethanol were added and the suspension was mixed gently and allowed to stand for 10-30 minutes on ice (or at -20^{o}C). The DNA pellet was obtained after centrifugation at 13,000rpm for 10 minutes. The genomic DNA was resuspended with 50 µl of sterilized water. The digested genomic DNA concentration was estimated by spectrophotometer as indicated in appendix B-2. The digested genomic DNA were fractionated in an agarose gel (see in appendix B-3). The gel was run until the tracking dye reached ¼ from the bottom of the gel. Before blotting, the resolved DNA bands were visualized on an UV transilluminator and photograph was taken with the ruler, in corresponding to a marker. The DNA molecular weight fractionated

genomic DNA samples were transferred to a charged nylon membrane (see in appendix B-4). The *OsNUC1* cDNA (accession no. AK063918) in p*ME18* vector labeled with ECL^{TM} direct nucleic acid labeling system was used as probe for hybridization. The probe labeling and detection of the hybridization signal were performed according to the manufacturer's protocol (see in appendix B-4).

3. Subcellular localization of *OsNUC1* protein

3.1 primer design

Gene-specific primers were designed in order to amplify the specific cDNA fragment of each construct, the full-length *OsNUC1* (FL-*OsNUC1*), the 5' and of *OsNUC1* (5'-*OsNUC1*) and the 3' end of *OsNUC1* (3'-*OsNUC1*). The position of the primers and the sequence of the primers are indicated in Table 3.1 and Figure 3.1. *Bgl*II recognition site and *Xba*I recognition site were added to the 5'end of forward (F) and reverse (R) primers, respectively, for cloning into pRTL expression vector.

Table 3.1 Primer pairs and PCR parameter for cloning full-length *OsNUC1*, amino-terminal *OsNUC1* (5'-*OsNUC1*), central and carboxyl-terminal *OsNUC1* (3'-*OsNUC1*), of which *Bgl*II restriction site was added to the 5'end of the forward primer (-F) and *Xba*I restriction site was added to the 5'end of the reverse primer (-R), respectively.

Primer Name	Sequence	Tm	Amplified region
FL-*OsNUC1*-F	GGCAGATCTCTTAATATGGGCAAGTCAAGC	64.8	-6 to 15
FL-*OsNUC1*-R	GCATCTAGACGGCTCACTAGAATCGCATA	64.9	2183 to 2202
5'-*OsNUC1*-F	GGCAGATCTCTTAATATGGGCAAGTCAAGC	64.8	-6 to 15
5'-*OsNUC1*-R	AATTCTAGACGAACCAACTCACACAGCAG	63.4	1250 to 1269
3'-*OsNUC1*-F	GGCAGATCTAGCTCCGATGAATCTTCT	63.5	1174 to 1191
3'-*OsNUC1*-R	GCATCTAGACGGCTCACTAGAATCGCATA	64.9	2183 to 2202

Figure 3.1 Schematic diagram of oligonucleotide sequence primers (forward and reverse strands) of full-length *OsNUC1*, amino-terminal *OsNUC1* (5'-*OsNUC1*), central and carboxyl-terminal *OsNUC1* (3'-*OsNUC1*) as shown in *OsNUC1* cDNA (accession no.AK103446).

GATTACCTCCCCGCCCGCGCACCTCGAGCCCCCAGGTCCGCCTCGCCTCTCCGTCGCTTGCCGCCGCCC

GCAGTGGTCGCCGGCCGCGCGGGGCTTCGCCGGTGTCGCTTCCTCGTTTGGAATTAATTAAGCAATACT

FL-*OsNUC1*-F, 5'-*OsNUC1*-F

TAAT**ATG**GGCAAGTCAAGCAAGAAATCTGCTGTTGAAGTTGCACCTACCTCTGTCTCAGTCTCAGAGGG

GAAATCTGGGAAGAAGGGAAAGAGAAATGCAGAAGATGAGATTGAGAAAGCTGTGAGTGCCAAGAAACA

AAAGACTGTACGTGAGAAGGTTGTGCCCTCAAAGGAGGAAGCCAAAAAAGTGAAGAAGCAGCCCCCACC

GAAGAAGGTTGAGAGCAGCAGTTCTGAGGAGGATTCTTCAGAATCTGAAGAGGAGGTAAAGGCCCAACC

AAAGAAGACTGTCCAACCGAAGAAGGCTGCACAACCTGCTAAAGAGGAGTCAAGTGATGATAGCAGTGA

TGATAGCTCCTCAGATGATGAGCCTGCAAAAAAACCTGTTGCTCGTCCAAATAAGGCTGCACTTTCTAC

CAACAGTAGCAGCAGTGATGATAGCAGTGATGAGAGTTTATCAGATGATGAACCTGTGAAAAAGCCTGC

TGCCCCTTTGAAGAAGCCAGTTGCACTTGCTACCAATGGATCAAAAAAGGTTGAGACAGACAGCAGCAG

CTCTGATAGCAGCTCTGATGAGGAGTCTGATGAGGATGATAAAAAAACTGCTGCTCCAGTGAAGAAACC

TTCAGTTGCTGCTATACAAAAGAAGACCCAGGAGTCTGACAGTTCTGATAGTGACTCTGATTCTGAATC

AGATGAGGATGTGCCGACTAAAGCACCAGCAGTAGCCAAGAAAAAGGAAGAATCCAGTGAAAGCTCTGA

TTCTGAAAGTGATTCAGACTCTGATGATGAGGCTGCTGCTGTTAAAAAGGAAGAAGAATCCAGTGATAG

CTCAGACAGTGACTCTGAATCTGAGTCTGATTCTGATGAACCAGCAAAACCTACTATTCCTGCAAAAAG

GCCACTGACAAAAGACACAAAGAAGGGACAATCCAAGGATGAATCTGAAGATAGTTCTGATGAGAGTTC

TGAGGAAAGTGGTGATGAACCTCCGCAAAAGAAGATTAAGGATTCTACAACTTCTGGTACTACCAAGCC

TTCCCCTAAGGCTACCAAGAAAGAAATCAGCAGTGATGACGAAAGTGATGAAGATGACAGTTCTGATGA

AAGCTCTGATGAGGATGTTAAGCAAAAACAAACTCAAGCTAAGAAGCAAGCACCAGTAGCACAAGAGAG

3'-*OsNUC1*-F

TAGCAGCTCCGATGAATCTTCTGAAGAAGATAGTGACATGGAAAGTGATGAACCAGCAAAAACTCCCCA

5'-*OsNUC1*-R

AAAGAAGGAAACTGCTGTGTCTGTTGGTTCGAATAAGTCTGCGACAAAACCGGGACAAGAGGAACCAAA

AACGCCTGCCAGCAACCAAAATCAAGCTACCGGGTCAAAGACTCTTTTTGTTGGAAATTTACCATACAA

TGTGGAGCAAGAACAAGTGAAGCAATTTTTCCAGGAGGCAGGTGAAGTTGTTGATATTCGTTTCAGTAC

CTTTGAAGATGGGAACTTCAGGGGCTTTGGACATGTTGAATTTGCCACAGCGGAAGCTGCTAAGAAGGC

ACTTGAACTTGCTGGTCATGACCTGATGGGACGGCCGGTCAGGCTTGACCTGGCTCGTGAGAGAGGCGC

GTATACTCCTGGCAGCGGGAGGGACAATAGTTCTTTCAAGAAGCCTGCTCAAAGCTCAGGAAACACTAT

ATTTATTAAAGGCTTTGATACTTCTCTTGACATACACCAGATCCGGAATTCACTTGAAGAACATTTTGG

CTCGTGTGGAGAGATTACACGGGTTTCAATTCCAAAGGATTATGAAACCGGTGCAAGCAAAGGGATGGC

GTACATGGATTTTGCGGACAACGGTTCCTTGTCGAAAGCATACGAACTGAATGGATCTGACCTCGGTGG

ATACAGCTTGTATGTTGATGAAGCGAGGCCTAGGCCCGATAACAACAGAGAGGGTGGCTTCAGCGGTGG

AAGAGACTTTAACAGCAGTGGGAGAGGAGGAAGACGTGGTGGACGTGGTGATGGTAGCCGTGGACGCGG

TGACCGTGGACGTGGTAGAGGCTTTGGTAGGGGTGACAGGGGCCATGGTGGACGGGGTACACCGTTCAA

GCAGAGCGCTGGTACACCTAGTGCAGGAAAGAAGACAACATTCGGTGACGACGACTAGACGAACAATTT

FL-*OsNUC1*-R, 3'-*OsNUC1*-R

GAGCCGATTTTGTTCTAGTACTGTAGTAATCTCCATCCCCCCTAATATATGCGATTCTAGTGAGCCGTG

AATTGGCTACCTCCGGACTGTTACATTCTAGCCGTGTTATGTACTTTGTTGGCAGTTGGGACCGTGTTG

GTAAATTTGCCCGACAGATTTGGCAATTTTGG

Figure 3.2 Schematic diagram of amino acid sequence of full-length OsNUC1, amino-terminal OsNUC1 (5'-OsNUC1), central and carboxyl-terminal OsNUC1 (3'-OsNUC1)

(A) Full-length OsNUC1

```
1    MGLSSLLSAVGVAPTSVSVSGGLSGLLGLAAAGAGIGLAVSALLGLTVAGLVVPSLGGAL    60
61   LVLLGPPPLLVGSSSSGGASSGSGGGVLAGPLLTVGPLLAAGPALGGSSAASSAASSSAA    120
121  GPALLPVAAPALAALSTASSSSAASSAGSLSAAGPVLLPAAPLLLPVALATAGSLLVGTA    180
181  SSSSASSSAGGSAGAALLTAAPVLLPSVAAIGLLTGGSASSASASASGSAGAVPTLAPAV    240
241  ALLLGGSSGSSASGSASASAAGAAAVLLGGGSSASSASASGSGSASAGPALPTIPALAPL    300
301  TLATLLGGSLAGSGASSAGSSGGSGAGPPGLLILASTTSGTTLPSPLATLLGISSAAGSA    360
361  GAASSAGSSAGAVLGLGTGALLGAPVAGGSSSSAGSSGGASAMGSAGPALTPGLLGTAVS    420
421  VGSALSATLPGGGGPLTPASAGAGATGSLTLPVGALPTAVGGGGVLGPPGGAGGVVAIAP    480
481  STPGAGAPAGPGHVGPATAGAALLALGLAGHALMGAPVALALAAGAGATTPGSGAAASSP    540
541  LLPAGSSGATIPILGPATSLAIHGIAASLGGHPGSCGGITAVSIPLATGTGASLGMATMA    600
601  PAAAGSLSLATGLAGSALGGTSLTVAGAAPAPAAAAGGGPSGGAAPASSGAGGAAGGAGA    660
661  GSAGAGAAGAGAGPGAGAAGHGGAGTPPLGSAGTPSAGLLTTPGAAAEnd              708
```

(B) Amino-terminal OsNUC1 (5'-OsNUC1)

```
1    MGLSSLLSAVGVAPTSVSVSGGLSGLLGLAAAGAGIGLAVSALLGLTVAGLVVPSLGGAL    60
61   LVLLGPPPLLVGSSSSGGASSGSGGGVLAGPLLTVGPLLAAGPALGGSSAASSAASSSAA    120
121  GPALLPVAAPALAALSTASSSSAASSAGSLSAAGPVLLPAAPLLLPVALATAGSLLVGTA    180
181  SSSSASSSAGGSAGAALLTAAPVLLPSVAAIGLLTGGSASSASASASGSAGAVPTLAPAV    240
241  ALLLGGSSGSSASGSASASAAGAAAVLLGGGSSASSASASGSGSASAGPALPTIPALAPL    300
301  TLATLLGGSLAGSGASSAGSSGGSGAGPPGLLILASTTSGTTLPSPLATLLGISSAAGSA    360
361  GAASSAGSSAGAVLGLGTGALLGAPVAGGSSSSAGSSGGASA                      402
```

(C) Central and carboxyl-terminal OsNUC1 (3'-OsNUC1)

```
402  MGSAGPALTPGLLGTAVSVGSALSATLPGGGGPLTPASAGAGATGSLTLPVGALPTAVGG   462
463  GGVLGPPGGAGGVVAIAPSTPGAGAPAGPGHVGPATAGAALLALGLAGHALMGAPVALAL   522
523  AAGAGATTPGSGAAASSPLLPAGSSGATIPILGPATSLAIHGIAASLGGHPGSCGGITAV   582
583  SIPLATGTGASLGMATMAPAAAGSLSLATGLAGSALGGTSLTVAGAAPAPAAAAGGGPSG   642
643  GAAPASSGAGGAAGGAGAGSAGAGAAGAGAGPGAGAAGHGGAGTPPLGSAGTPSAGLLTT   702
703  PGAAAEnd                                                       708
```

3.2 Construction of the subcellular localization vector

The DNA specific regions, as indicated in 3.1, were amplified with *pfu* polymerase (Invitrogen, USA) according to manufacturer's protocol. The thermal cycle used was as follows: 94^{o}C for 5min, then 40 cycles of 94^{o}C for 45sec, 61^{o}C for 1min and 72^{o}C for 3min, then 72^{o}C for 10min. Each amplified fragment was fused in frame with the coding region of GFP in the pRTL vector at *Bgl*II-*Xba*I cloning sites which is constitutively expressed under the control of *35SCaMV* promoter. Gene cloning was performed as indicated in appendix B-5. Transformant cells were selected on the LB agar plate which contains 100µg/ml amplicilin. After restriction map confirmation of the position clones, the sequences of the GFP-full length/ partial OsNUC1 gene were determined using the service of ...Plasmids in each construct; (1) *35SCaMV*:*GFP*-full-length *OsNUC1*, (2) *35SCaMV*:*GFP*-N-terminus *OsNUC1*, and (3) *35SCaMV*:*GFP*-C-terminus *OsNUC1* were isolated by BioDev Plasmid Minipreps Purification kit (BioDev-Tech, China) and prepared in final concentration of 1µg/µl. The plasmids in each construct were bombarded on onion epidermal cells by particle bombardment (Bio-Rad) as performed in appendix B-6. The fusion protein localization was observed using a Leica laser-scanning confocal microscope (Leica Microsystem).

4. Expression profile of *OsNUC1* gene

Expression pattern of *OsNUC1* gene was determined in various rice organs, roots, leaf sheath, leaf blade, flowers and seeds, of 3.5 month-old, soil-grown rice seedlings. For the expression pattern under salt-stress, three week-old, hydroponic grown (three-leaf stage) rice seedlings of four rice cultivars, LPT123, LPT123-TC171, KDML105, and FL530-IL were grown in the WP No.2 nutrient solution (appendix B-1) supplemented with 0.5% (w/v) NaCl. Leaf tissues were collected at

the different time points, 0, 1, 2, 3, 6, and 9 days, after treatment. RNAs extracted from those tissues were used as the templates to first strand cDNA synthesis as shown in appendix B-8. The primers for quantitative real-time PCR of *OsNUC1* gene expression were designed manually, and then confirmed with the analysis with Oligoanalyzer 3.1 software (www.idtdna.com). The forward primer and reverse primer are as followed: 5'-ATGGATCTGACCTCGGTGGA-3' and 5'GTCTTCCT CCTCTCTCAGTG-3', respectively. To normalize level of total cDNA loading, a parallel amplification of *OsActin* (accession number: AK101613), the housekeeping control gene, was performed using *OsActin* specific primers; forward primer: 5'-AGCTATCGTCCACAGGAA-3' and reverse primer: 5'-ACCGGAGCTAATCAG AGT-3'. The quantitative real-time PCR was conducted on the Opticon2 Continuous Fluorescence Detector System (MJ research, USA). At least 3 independent real-time PCR reactions were performed on the same cDNA preparation. Each reaction contained 10µl of 2x SYBR Green Master Mix Reagent (Toyobo), 1µl of cDNA samples, and 200nM gene-specific primers in a final volume of 20µl. The thermal cycle used was as follows: 94^{o}C for 20 min, then 40 cycles of 94^{o}C for 20 sec, 53^{o}C for 20 sec and 72^{o}C for 20 sec, followed by a final extension at 72^{o}C for 10 min.

5. The effect of exogenous ABA and abamineSG application on *OsNUC1* gene expression during normal and salt-stress conditions

5.1 ABA and abamineSG treatment

Four rice cultivars; LPT123, LPT123-TC171, KDML105, and FL530-IL were germinated and grown under normal condition until three-leaf stage as described in appendix B-1 then they were subjected to salt stress condition by addition of 0.5% NaCl into nutrient solution. The normal grown plants were used as control. ABA treatment was performed by spraying (±) ABA (Sigma) solution (appendix A), at a concentration of 0µM (Control) and 100µM onto leaves of rice seedlings until fully soaked in the early morning of every day during the experimental period. (±) ABA solution was treated onto the three week-old rice seedlings grown in normal or salt stress condition. Rice leaf tissues were collected at different time points; 0, 1, 2, 3, 6, and 9 days after treatment. They were immediately frozen in liquid nitrogen and kept at -80^{o}C, until the RNA was isolated.

For abamineSG treatment, 50µM of abamineSG, ABA biosynthesis inhibitor (Kitahata *et al*., 2006), was soakly sprayed onto the rice leaves in the morning every two days. The control treatment was spraying with abamineSG buffer (DMSO solution). The experiment was performed with both normal (WP nutrient solution) and salt-stress (WP containing 0.5% (w/v) NaCl) grown plants. Rice leaf tissues were harvested at 0, 1 and 2 days of treatment and were immediately frozen in liquid nitrogen and then were stored at -80^{o}C. Longer experimental periods than two days led to severe damage of salt-stress seedlings, therefore, the experiment had to be determined.

5.2 RNA extraction and detection of *OsNUC1* gene expression

Total RNA samples were extracted by the standard hot-phenol method (Thikart *et al*., 2006), then they were treated with *DNase*I and used as templates for cDNA synthesis by using reverse transcriptase enzyme as shown in appendix B-8. The *OsNUC1* transcript expression level was quantified by real-time PCR technique (see in appendix B-9) in the similar way as indicated previously.

5.3 The effect of abamineSG application on some ABA-inducible genes

To study the effect of abamineSG on the transcript expression of the other ABA-inducible genes, *OsCIPK15* and *OsCYP707A5*, were chosen as they were previously reported that their function involved in ABA signaling (Xiang *et al*., 2007) and ABA catabolism (Kushiro *et al*., 2004, Saito *et al*., 2004, Kitahata *et al*., 2006), respectively. LPT123 (salt-sensitive line) and LPT123-TC171 (salt-resistant line) were used in this study, thereby, they were grown in modified WP No.2 nutrient solution. Three-leaf stage of rice seedlings (three week old seedlings) grown in normal and salt stress conditions were treated with 50µM abamineSG in the same way as describe previously. Rice leaf tissues were harvested on day 0, 1 and 2 after treatment and immediately frozen in liquid nitrogen, and then they were kept at -80^{o}C until the RNA extraction step. Total RNA was extracted by the standard hot-phenol procedure (Thikart *et al*., 2006) as described in appendix B-7. The *DNase*I treatment and the first strand cDNA synthesis were done as described in appendix B-8. RT-PCR was performed in a 20µl solution containing a 1µl aliquot of the first strand

cDNA reaction, 0.5µM of each of the gene-specific primers, 0.5U *Taq* polymerase (FastStart Taq DNA polymerase, Roche Applied Science), and 1% reaction buffer containing 2mM $MgCl_2$. The reaction included an initial 2 min denaturation at 95^{o}C, followed by 28 cycles of PCR (95^{o}C for 1 min; annealing temperature as indicated later for 1 min, 72^{o}C for 2 min) and 72^{o}C for 10 min. The primers for RT-PCR analysis of genes were designed from the *OsCIPK15* cDNA (accession no. NM_001072106) and *OsCYP707A5* cDNA (accession no. DQ887714). *OsACTIN* (accession no. AK101613) gene was used as internal control. The primers for the detection of each gene and the annealing temperature used are as followed:

Table 3.2 Primer pairs and PCR parameter for gene expression determination of ABA-inducible genes; *OsCIPK15* (accession no. NM_001072106), *OsCYP707A5* (accession no. DQ887714) via RT-PCR technique. *OsActin* (accession no. AK101613) was used as the internal control.

Gene	Primer	Sequence	Annealing temperature (^{0}C)	Amplified region
OsCIPK15	F	5'-CTTGGTGTGAGACGCAAGAA-3'	54^{o}C	1147 to 1166
	R	5'-GATGCCCTCCTCCAGTAGC-3'		1521 to 1539
OsCYP707A5	F	5'-TGTGCCATTGCTGTCCATCT-3'	55^{o}C	543 to 562
	R	5'-CGGCTCGTCATCTTCATCCG-3'		985 to 1004
OsACTIN	F	5'-CTGCCGAGCGGGAAATTGTC-3'	55^{o}C	573 to 592
	R	5'-CTGGCGGAGCGACAACCTTG-3'		946 to 965

After amplification, the DNA fragments were separated on 0.8% agarose gel (see in appendix B-3). Then it was strained with ethidium bromide. The visualization of DNA fragments was done using UV transllliluminator and the photograph of the gel was taken.

5.4 The effect of abamineSG application on ABA accumulation

The preparation of rice seedlings and abamineSG treatment were done in the same way as indicated in 5.1, and the leaf tissues were collected of the same period of time as they were collected for gene expression analysis. ABA extraction and determination via HPLC technique were performed as described in appendix B-10.

6. Characterization of the transgenic Arabidopsis with the expression of the partial *OsNUC1* overexpression cassette and the Arabidopsis transformation.

6.1 Partial *OsNUC1* gene construction

For cloning of the *OsNUC1* gene, the primer pairs *OsNUC1* were designed according to the cDNA sequence from the National Center for Biotechnology Information (NCBI) (accession no AK063918). A fragment of central and carboxyl-terminal *OsNUC1* ORF that contains RRMs and GAR domains was PCR-amplified from *OsNUC1* cDNA (RIKEN) by use of the primer cOsNUC1-F, 5'-AATCTAGACAGTAGCAGCAGTGATGATAGC-3' and cOsNUC1-R, 5'AAGAGCTCACCAACACGGTCCCAACT-3', which contained the addition *Xba*I and *Sac*I recogntion sites (underlines), respectively. Polymerase chain reaction (PCR) was performed with *pfu* polymerase (Invitrogen, USA). The PCR condition was as follows: 94^{o}C for 5min, then denaturation at 40 cycles of 94^{o}C for 45 sec, 59^{o}C for 1 min and 72^{o}C for 3 min, then a final extension at 72^{o}C for 10 min. Then, dATP was added to the 3' end of the fragment by *Taq* polymerase reaction (Appendix B-5.3) before cloning into pGEM-T vector (Appendix C). The sequence of the cloned AK063918 fragment was confirmed by sequence analysis using Invitrogen service.

To construct the expression vector for plants, the targeted gene was inserted into *pJim19*, the binary vector for Agrobacterium transformation, containing the *npt*II gene as the plant selectable marker. AK063918 fragment with the correct sequence in pGEM-T vector was digested with *Xba*I and *Sac*I and the fragment was purified before cloning into the cloning sites, *Xba*I and *Sac*I, of *pJim19* (Figure 3.3) to generate pJim19_ccOsNUC1 for Arabidopsis transformation.

To create the gene construct containing partial *OsNUC1* gene expression with the salt stress-inducible promoter, *35SCaMV* promoter was substituted with Arabidopsis *rd29a* promoter (accession no. EF090409). The *rd29a* promoter was amplified with the forward primer: 5'-AAGCTTCCCGACC GACTACTAA-3' and reverse primer: 5'-TCTAGAGGCGTCTTCCATG-3', which contained additional *Hind*III and *Xba*I recognition sites (underlines). PCR reaction was performed as followed: 94^{o}C for 5 min, then 40 cycles of 94^{o}C for 45 sec, 53^{o}C for 30 sec and 72^{o}C for 40 sec, and 72^{o}C for 10 min.

The additional adenine deoxyribonucleotide was inserted to the 3' end of the amplified fragment using *Taq* polymerase reaction (Appendix B-5.3) before cloning into pGEM-T vector (Appendix C). The sequence of the cloned fragment was confirmed for the correct one, before use for *35SCaMV* promoter substitution in pJim19_ccOsNUC1 vector to create pJim19_scOsNUC1 vector.

The promoter substitution was done by digesting pGEM-T vector containing *rd29a* fragment and pJim19_ccOsNUC1 with *Hind*III and *Xba*I to release *rd29a* fragment and pJim19_ccOsNUC1 fragment without *35SCaMV* promoter (Figure 3.4). Then, after the target fragment purification, both fragments were ligated together to generate pJim19_scOsNUC1 (Figure 3.4) to use for *Agrobacterium* transformation in the next step.

6.2 Agrobacterium transformation

Both pJim19_ccOsNUC1 and pJim19_scOsNUC1 were purified and transferred into *Agrobacterium tumefaciens* L. strain GV3101 by heat-shock transformation method. The LB medium (Appendix A) containing 50µg/ml kanamycin (transformation selection), 25µg/ml rifampicin and 50µg/ml gentamycin (*A. tumefaciens* GV3101 selection) was used for transformant selection. The details are described in Appendix B-11).

Figure 3.3 Strategy for construction of partial *OsNUC1* constitutive expression vector

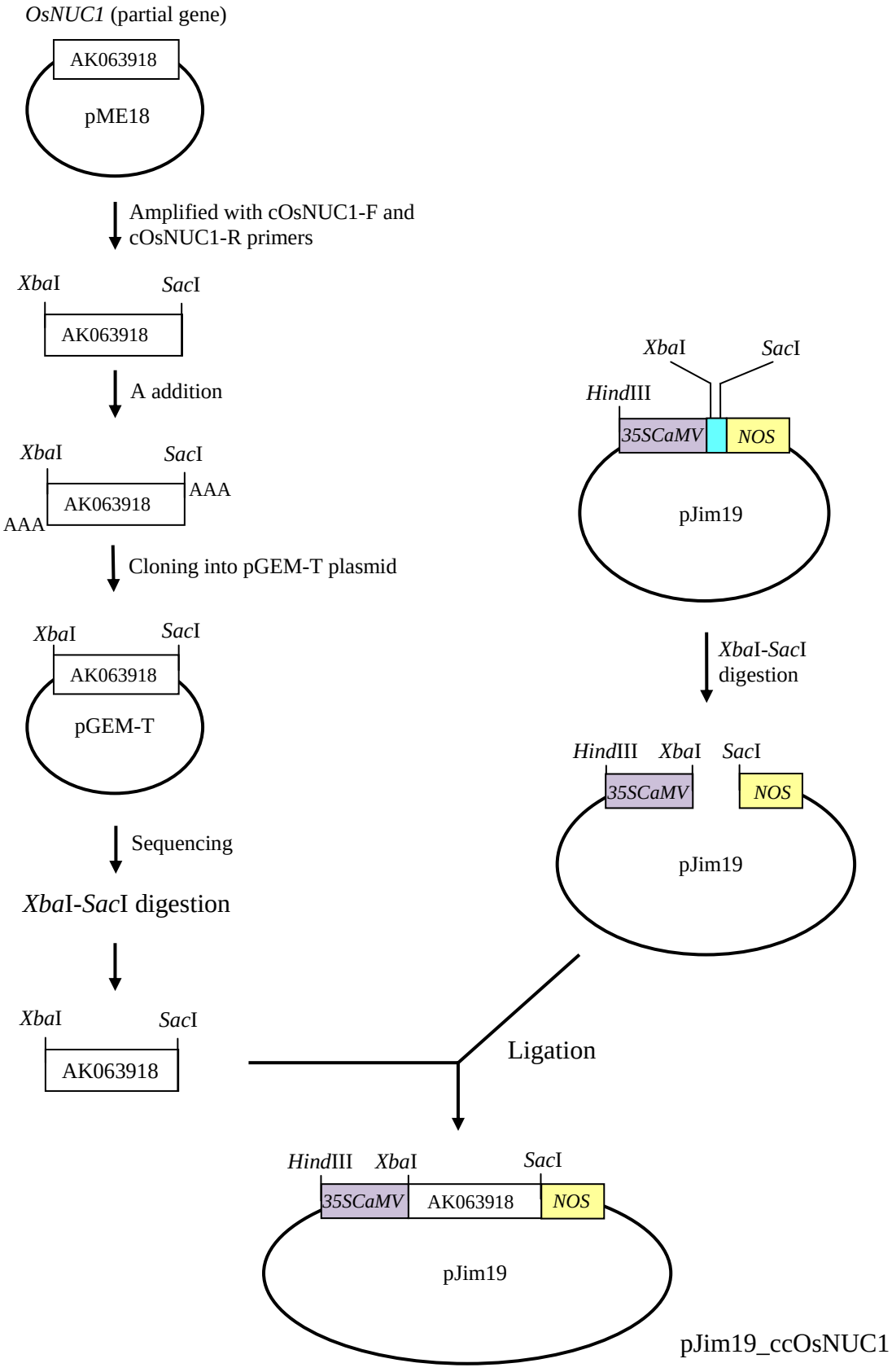
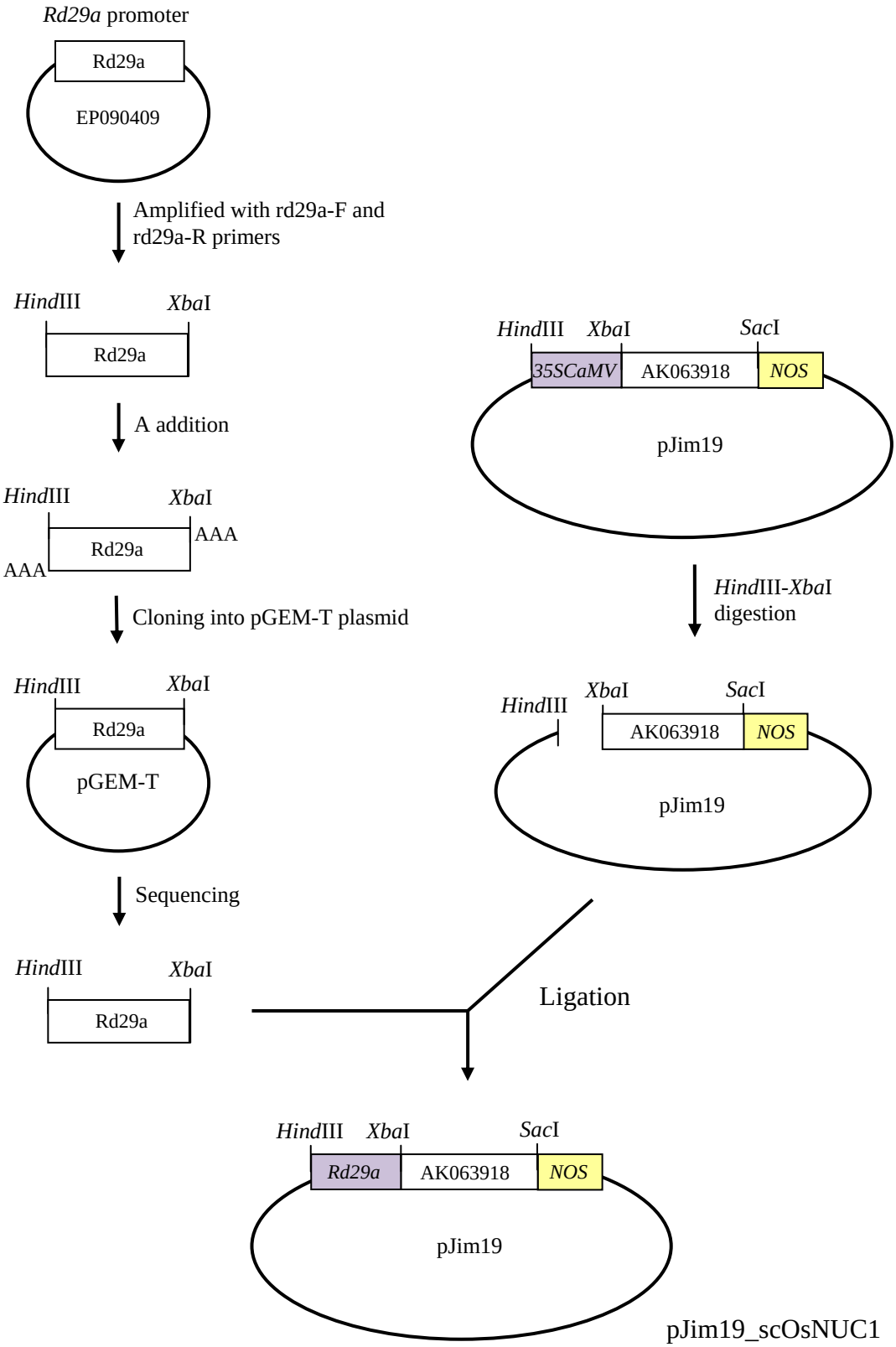


Figure 3.4 Strategy for construction of partial *OsNUC1* salt-inducible expression vector



6.3 Arabidopsis transformation and transgenic plant selection

T-DNA in pJim19_ccOsNUC1 and pJim19_scOsNUC1 were introduced into *Arabidopsis thaliana* L., ecotype Columbia by the floral dip method (Bechtold and Pelletier, 1998). T_o seeds and progenies were plated on half-strength MS medium containing 50µg/ml kanamycin resistance for transgenic plant selection. Lines, showing T_1 seedlings segregation in kanamycin resistance, were collected for the selection of homozygous transgenic lines in the next generation.

6.4 Detection of *OsNUC1* gene expression in transgenic Arabidopsis

OsNUC1 gene expression was detected in ten independent T_1 transgenic lines of Arabidopsis, six of which were the transgenic plants containing 35SCaMV_AK063918_nos-ter construct (constitutive *OsNUC1* over-expressing lines) and four of which were the transgenic plants containing rd29a_AK063918_nos-ter construct (stress-inducible *OsNUC1* over-expressing lines). Transgenic Arabidopsis and wild type were grown on MS medium containing 1% sucrose at 22^{o}C under a 16: 8 hour of light: dark photoperiod for a week. Then, they were treated with salt stress by growing on 100mM NaCl containing MS medium, thereby, the gene transcript expression was determined in comparison to that of the ones grown in normal condition.

After a week of treatment leaf tissues were harvested for RNA extraction. Total RNA was isolated from leaf tissue with TRIzol reagent (Invitrogen, USA), according to the manufacturer's protocol to determine the expression level of *OsNUC1*. The *Dnase*I treatment and reverse transcription were performed in appendix B-8. After reverse transcription reaction, the quantitative real-time PCR was carried out on the Opticon2 Continuous Fluorescence Detector System (MJ research) according to the SYBR gene detection protocol (SYBR Premix Ex Taq system, Toyobo). Each reaction contained 10µl of 2x SYBR Green Master Mix Reagent, 1µl of cDNA samples, and 200nM gene-specific primers in a final volume of 20µl. Differences in cycles during the linear amplification phases were compared with the transcript of Arabidopsis *EF-1α* as an internal control. The specific primers for *OsNUC1* and Arabidopsis *EF-1α* were designed manually, and then confirmed

with the analysis with Oligoanalyzer 3.1 software (www.idtdna.com) from *OsNUC1* cDNA (accession number: AK063918) and *AtEF-1α* cDNA (At5g60390) as followed:

OsNUC1-F	5'-ATGGATCTGACCTCGGTGGA-3'
OsNUC1-R	5'GTCTTCCTCCTCTCTCAGTG-3'
AtEF-1α-F	5'-TTCTCCGAGTACCCACCTTT-3'
AtEF-1α-R	5'-ATTTGGCACCCTTCTTCACT-3'

The thermal cycle used was as follows: 94^{o}C for 20min, than 40 cycles of 94^{o}C for 20sec, 56^{o}C for 20sec and 72^{o}C for 20sec, then a final extension at 72^{o}C was done for 10 min.

After screening with *OsNUC1* gene expression level, three of constitutive *OsNUC1* over-expressing line and two of stress-inducible *OsNUC1* expressing lines were chosen for further characterization.

6.5 Plant growth and salt resistant ability analyses of the transgenic Arabidopsis

6.5.1 Preliminary test for salt-resistant ability

T_1 Independent lines of constitutive *OsNUC1* over-expressing and stress-inducible *OsNUC1* expressing transgenic Arabidopsis were tested for salt-resistant ability in comparison with wild-type by growing the one-week old seedlings on the MS medium supplemented with NaCl at various concentrations, 0, 50, 100 and 150mM for a week. The photographs for seedling phenotypes were taken for comparison.

6.5.2 Plant growth analysis

Arabidopsis lines selected in 6.4 were used for growth analysis experiments in both normal and salt stress condition. The experiments were performed with complete randomized design (CRD) with 4 replicates. Each replicate represented at least 20 plants. The comparison of means in each parameter was performed with Duncan Multiple Range Test (DMRT).

For growth analysis in normal condition,plants were germinated on ½ MS medium containing 50µg/ml kanamycin for 2 weeks, then root and leaf number, root length, leaf area, fresh weight, and dry weight were determined. The Image Pro-Plus 5.1 computer program was used to facilitate the determination of root length. For fresh weight determination, pool of 10 Arabidopsis plants was used for each measurement. Then, they were dried at 60^{o}C for at least 48 hours or until all water was removed from seedlings to determine plant dry weight.

For growth analysis during salt-stress condition, the transgenic plants were germinated on MS medium for a week before transferred to the MS medium containing 100mM NaCl. The same experimental design and statistical analysis were done as the experiment in normal condition. The growth determination on the same parameters was performed after a week of the treatment.

6.6 Detection of *AtNUC1*, *AtSOS1*, and *AtP5CS1* gene expression in transgenic Arabidopsis

Gene expression of *AtNUC1*, *AtSOS1*, and *AtP5CS1* was detected in the transgenic plant lines chosen in 6.4. Plant tissue preparation, RNA extraction, and cDNA synthesis were done in the same way as indicated in 6.4. Then, cDNA templates were used for quantitative real-time PCR reaction in order to detect gene expression level of *AtNUC1*, *AtSOS1*, and *AtP5CS1* using the specific primers as indicated in table 3.3. The real-time PCR condition was similar to what shown in 6.4. AtEF-1α gene expression was used as the internal control.

Table 3.3 Primer pairs of *AtNUC1*, *AtSOS1,* and *AtP5CS1* for gene expression determination via quantitative real-time PCR. *AtEF-1α* was used as the internal control.

Gene	Primer	Sequence	Amplified region
AtNUC1	F	5'-GCAGCCGAAGACACCTACTA-3'	1110 to 1129
	R	5'- ACATCGACAACTTCACCAGC-3'	1219 to 1238
AtSOS1	F	5'-CGGCAGCATGGTTAATGTGT-3'	3126 to 3145
	R	5'-TTGGCTGAAACGAGACCTTG-3'	3232 to 3251
AtP5CS1	F	5'-CGCCAGCACACGATTCTCAG -3'	1953 to 1972
	R	5'-TTCCATTGTCTCCGTCG -3'	2099 to 2116

7. The effect of exogenous ABA and abamineSG application on *OsP5CS1* gene expression during normal and salt-stress conditions

Four rice cultivars; LPT123, LPT123-TC171, KDML105, and FL530-IL were germinated and grown under normal condition until three-leaf stage as described in appendix B-1. Treatments with ABA or ABA biosynthesis inhibitor (AbamineSG) were done in the same way as indicated in 5. Under the exogenous ABA application treatment, rice leaf tissues were collected at different time points; 0, 1, 2, 3, 6, and 9 days after treatment. Whereas, the abamineSG substance treatment, leaf tissues were harvested at 0, 1 and 2 days of treatment. Because longer experimental periods than two days led to too severe damage of seedlings to be assayed for gene expression. At the indicated time point, leaf tissue were immediately frozen in liquid nitrogen and kept at -80^{o}C until needed for RNA extraction. RNA was extracted as indicated by Thikart *et al*. (2008) (Appendix B). Then, total RNA was treated with *DNase*I and the reverse transcription as described in appendix B-7. The *OsP5CS1* transcript expression level was performed by real-time PCR technique (see in appendix B-9).

The quantitative real-time PCR was performed in a final volume of 20µl containing a 1µl aliquot of the first strand cDNA reaction, 0.05µM of each of the gene-specific primers, 1x iQ^{TM} SYBR$^{®}$ Green Super Mix (Bio-Rad, USA), and 3 mM $MgCl_2$. The reaction included an initial 8 min denaturation at 95^{o}C, followed by 40 cycles of PCR (95^{o}C for 30 sec; 57^{o}C for 30 sec; 72^{o}C for 45 sec). The specific primer for *OsP5CS1* were designed manually, and then confirmed with the analysis with Oligoanalyzer 3.1 software (www.idtdna.com) from the 3'UTR region of the *OsP5CS1* cDNA (accession no AK102633) as follows: *OsP5CS1*-F forward primer 5'- TCTGCTCAGTGATGTGGATG-3' and *OsP5CS1*-R reverse primer: 5'-CCTACACGAGATTTGTCTCC- 3' to yield an expected amplicon of 123bp. The *OsACTIN* gene was used as internal control via the specific primers *OsActin*–F 5'-AGCTATCGTCCACAGGAA-3' and *OsActin*–R 5'-ACCGGAGCTAATCAGA GT- 3' yielding an expected amplicon of 100bp. The PCR reactions were performed as above except the annealing temperature was reduced to 49^{o}C. The level of *OsP5CS1* gene expression was determined in comparison with *OsActin* gene expression in reference to the expression on Day0 of the treatment (Czechowski *et al*., 2005). At least 3 independent real-time PCR reactions were performed on the same cDNA preparation. The calculation of the level of gene expression was done as demonstrate in the appendix B-9.

8. The effect of exogenous ABA application on *OsP5CR* transcript expression during salt-stress condition

LPT123 (salt-sensitive line) and LPT123-TC171 (salt-resistant line) were used in this study, thereby, they grown in modified WP No.2 nutrient solution. The nutrient solution was changed once a week during the experimental period. Three-leaf stage of rice seedlings were treated with 100µM ABA during salt-stress conditions in the same way as indicated in 5. The gene expression of *OsP5CR* in co-treatment of ABA and salt stress was compared with that in normal and salt-stress conditions. Rice leaf tissues were harvested at 0, 3, 6 and 9 days after treatment. And RNA was extracted, followed by cDNA synthesis as indicated in 8. The *OsP5CR* gene expression was determined by RT-PCR, which was performed in 20µl solution

containing a 1µl aliquot of the first strand cDNA reaction, 0.5µM of each of the gene-specific primers, 0.5U Taq polymerase (FastStart Taq DNA polymerase, Roche Applied Science), and 2mM $MgCl_2$. The reaction included an initial 2 min denaturation at 95°C, followed by 28 cycles of PCR (95°C for 1 min; annealing temperature as indicated later for 1 min, 72°C for 2 min) and 72°C for 10 min. The *OsP5CR* specific primers were designed manually, and then confirmed with the analysis with Oligoanalyzer 3.1 software from the *OsP5CR* cDNA *(*accession no AK070184*)* as follows: *OsP5CR*-F forward primer 5'-AATAGAGGCCATGGC TGATG-3' and *OsP5CR*-R forward primer R 5'-AATGCACCCTTCTCAAGCTC-3', annealing temperature 60°C. *OsActin* (accession no AK101613) gene was used as internal control with primers as follows: *OsActin*-F 5'-CTGCCGAGCGGGA AATTGTC-3' and *OsACTIN*-R 5'-CTGGCGGAGCGACAACCTTG-3', annealing temperature 55°C. The expression level was analyzed by agarose gel electrophoresis then, it was strained with ethidium bromide. The visualization of DNA fragment was done using UV transllliluminator and photographed.

9. The effect of calmodulin antagonist on proline accumulation during salt-stress condition

LPT123 and LPT123-TC171 were grown hydroponically with WP modified nutrient solution (Vajrabhaya and Vajrabhaya, 1991) for 3 weeks until they reached the three-leaf stage. Then three treatments were started. The normal grown condition was the transfer of the seedlings to the WP nutrient solution, while the salt-stress treatment, they were transferred to the WP solution containing 0.5% (w/v) NaCl.

To detect if calmodulin plays a role in proline accumulation during salt stress in rice, 100mM calmodulin antagonist, W-7, was added to the WP nutrient solution (Vajrabhaya and Vajrabhaya, 1991) containing 0.5% (w/v) NaCl. Then, the difference in proline content in leaves at three treatments were compared using CRD and DMRT on day0, 3, 6, and 9 after treatment. The proline content analysis was performed according to Bates and colleagues (1972) (see in Appendix B-12).

CHAPTER IV

RESULTS AND DISCUSSION

1. Amino acid identify and motif analyses of rice nucleotide-like protein

Firstly, Somporn Maneeprasopsuk (2004) discovered the novel salt-responsive gene (accession no.DQ012289) in indica rice cultivar Leung Pra Tew123 (LPT123) by the differential display method. This salt-responsive gene was observed to differentially express in the salt-sensitive (LPT123) and the salt-resistant (LPT123-TC171) rice lines of being grown under normal and salt-stress condition. The short sequence of DQ012289, 67 amino acids, is a part of Os04g0620700 protein coding sequence (accession no.NP_001053909) (http://www.ncbi.nlm.nih.gov/UniGene/). The 706 amino acids sequence of Os04g0620700 was used as a query for tblastn algorithm. It is identical to the japonica rice *nucleolin1* gene. Based on RAP-DB (Rice Annotation Project Database), two full-length cDNA clones from this locus was reported, AK103446 with 2,447bp long and AK063918 with 1,943bp long. AK103446 showed 85.8% homology with the *nucleolin1* gene of indica rice (accession no.HO714H04.9). The alignment of nucleolin1-like protein of the japonica rice cultivar, the indica rice cultivar and *Arabidopsis thaliana* (Petricka and Nelson, 2007), was shown in Figure 4.1. Furthermore, analysis of the japonica rice genome databases (RAP-DB) revealed two genes with homology to the Arabidopsis *AtNUC*-L1, called *OsNUC1* (accession no.AK103446) and *OsNUC2* (accession no.AK103422). *OsNUC1* is located on chromosome 4, while *OsNUC2* is on chromosome 8. *OsNUC1* was found to have the 52.9% identity with *OsNUC2* at the nucleotide level, and 46.4 % identity at amino acid level. These rice nucleolin-like proteins are conserved with nucleolin-like proteins from other plant species, such as Arabidopsis (*Arabidopsis thaliana* L.), sorghum (*Sorghum bicolor*), pea (*Pisum sativum* L.), tobacco (*Nicotina tabacum*) and alfalfa (*Medicago sativa* L.), in the part of the consensus RNA-binding domain (CS-RBD).

```
OsNUC1japonica      MGKSSKKS-AVEVAPTSVSVSEGKSGKKGKRNAEDEIEKAVSAKKQKTVR---EKVVPS- 55
OsNUC1indica        MGKASKKSVAVAVAPAAVPAK-GKGGKK--REAEDEIEKAVSAKKQKAAAAPPAKAVPAP 57
AtNUC-L1            MGKSKSATKVVAEIKATKPL------KKGKREPEDDIDTKVSLKKQK-------KDVIAA 47

OsNUC1japonica      KEEAKKVKKQPPPKKVESSSS----EEDSSESEEEVKAQPKKTVQPKKAAQPAKEESSDD 111
OsNUC1indica        KADAKKAKKQPPPKKAASSSSGSSSEEDSSESEEEVKVQVKKTTKP------VKQESSSD 111
AtNUC-L1            VQKEKAVKKVP--KKVESSDD----SDSESEEEEKAKKVPAKKAAS----------SSDE 91

OsNUC1japonica      SSDDSSSDDEPAKKPVARPNKAALSTNSSSSDDSSDESLSDDEPVKKPAAPLKKPVALAT 171
OsNUC1indica        ESSDESSDDEDAKP--ADP-------------------------------------VAN 131
AtNUC-L1            SSDDSSSDDEPAPKKAVAA-------------------------------------TNG 113

OsNUC1japonica      NGSKKVETDSSSSDSSSDEESDEDDKKTAAPVKKPSVAAIQKKTQESDSSDSDSD-SESD 230
OsNUC1indica        NGLKKGKPASSDSESDSDDEMDEDEK-PAAPVKKTSVTAQKKKD-DSDSSESESDESDSD 189
AtNUC-L1            TVAKKSKDDSSSSDDDSSDEEVAVTKKPAAAAKNGSVKAKKESSSEDDSS---------S 164

OsNUC1japonica      EDVPTK--APAVAKKKEESSESSDSESDSDSDDEAAAVKKEEESSDSSDSDSESESDSDE 288
OsNUC1indica        EDVPTKSKAPAVAAKNDDSTDGS--ESESDSEDEDAAPKG------------------- 227
AtNUC-L1            EDEPAKKPAAKIAKPAAKDSSSSDDDSDEDSEDEKPATKK-------------------- 204

OsNUC1japonica      PAKPTIPAKRPLTKDTKKGQSKDESEDSSDESSEESGDEPPQKKIKDSTTSGTTKPSPKA 348
OsNUC1indica        ------------------------------------------------------------A 228
AtNUC-L1            ------------------------------------------------------AAPAA 209

OsNUC1japonica      TKKEISSDDESDEDDSSDESSDEDVKQKQTQAKKQAPVAQESSSSDESSEEDSDMESDEP 408
OsNUC1indica        AKKESSSDEE---DDSSEESSDDEPKQPQQKK------AQEESS-EESSEEDSDEEDEKL 278
AtNUC-L1            AKAASSSDSS--DEDSDEESEDEKPAQKKADTK-----ASKKSSSDESSESEED-ESEDE 261

OsNUC1japonica      AKTPQKKETAVSVGSNKSATKPGQEEPKTPASNQNQATGSKTLFVGNLPYNVEQEQVKQF 468
OsNUC1indica        AKTPKKK--------TPAATKSQNDEPKTPASNQSQGTESATLFMGNLSFNLNQDQVKEF 330
AtNUC-L1            EETPKKKSSD---VEMVDAEKSSAKQPKTPSTP--AAGGSKTLFAANLSFNIERADVENF 316

OsNUC1japonica      FQEAGEVVDIRFST-FEDGNFRGFGHVEFATAEAAKKALELAGHDLMGRPVRLDLARERG 527
OsNUC1indica        FQEVGEVISVRLAT-HEDGSSRGFGHVQFASSEEAKKALELHGCDLDGRPVRLDLAHERG 389
AtNUC-L1            FKEAGEVVDVRFSTNRDDGSFRGFGHVEFASSEEAQKALEFHGRPLLGREIRLDIAQERG 376

OsNUC1japonica      --AYTPGSGRDNSSFKKPAQSSG-NTIFIKGFDTSLDIHQIRNSLEEHFGSCGEITRVSI 584
OsNUC1indica        --AYTPHSRNDTGSFQKQNRGSS-QSIFVKGFDSSLEESKIRESLEGHFADCGEITRVSV 446
AtNUC-L1            ERGERPAFTPQSGNFRSGGDGGDEKKIFVKGFDASLSEDDIKNTLREHFSSCGEIKNVSV 436

OsNUC1japonica      PKDYETGASKGMAYMDFADNGSLSKAYELNGSDLGG-YSLYVDEARPRPDNNREGGFSGG 643
OsNUC1indica        PMDRETGASKGIAYIDFKDQASFSKALELSGSDLGG-YNLYVDEAKPKGDSRDGGGRRGG 505
AtNUC-L1            PIDRDTGNSKGIAYLEFSEGK--EKALELNGSDMGGGFYLVVDEPRPRGDSSGGGGFGRG 494

OsNUC1japonica      RDFNSSG--RGGRRGGRGDGSRGRGDRGRGRGFGRGDRG-HGGRG-TPFKQSAGTPSAGK 699
OsNUC1indica        RSGDRFGGRSGDRFGGRSGGRFGGRDGGRRGGRGGRDGGRRGGRGGFQSRQSAGTASTGK 565
AtNUC-L1            NGRFGSG---GGRGRDGGRGRFGSG-GGRGRDGGRGRFGSGGGRGSDRGRGRPSFTPQGK 550

OsNUC1japonica      KTTFGDDD 707
OsNUC1indica        KTTFGDE- 572
AtNUC-L1            KTTFGDE- 557
```

Figure 4.1 Comparison of OsNUC1 amino acid sequences in japonica (accession no. BAD05605) and indica (accession no. AAU01907) rice cultivars and AtNUC-L1 amino acid sequence (accession no. NP_175322). A putative bipartite NLS, and acidic/Ser-rich region, two RNA recognition motifs (RRM) and a carboxyl-terminal Gly- and Arg-rich (GAR) domain are highlighted with orange, purple, pink, and yellow, respectively.

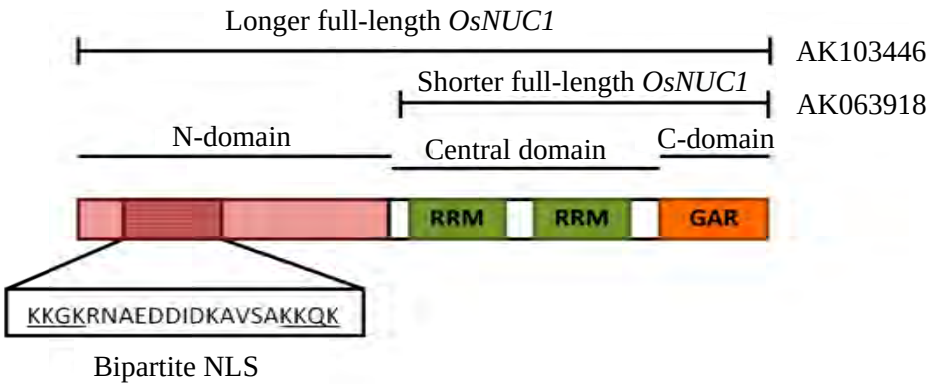


Figure 4.2 Schematic representation of domain diagram of structure features of rice nucleolin-like protein.

The rice nucleolin protein consists of three distinguished domains with a specific function; (1) an amino-terminal domain rich in acidic residues and bipartite nuclear-localized signal sequence (NLS), (2) a central domain containing two RNA-binding domains (RBDs) also called RNA-recognition motifs (RRMs) and (3) a carboxyl-terminal domain rich in Glycine and Arginine residues (GAR domain) (Ginisty *et al*., 1999). The longer full-length cDNA, AK103446, encodes the polypeptide with complete sets of the three domains while the shorter cDNA, AK063918, encodes the polypeptide containing only the central domain and the carboxyl-terminal domain (Figure 4.2). The unique tripartite feature as described for nucleolin was found in various eukaryotic cells, animals, plants and yeast (Srivastava *et al*., 1989, Bourbon *et al*., 1988, Maridor and Nigg, 1990, Caizergues-Ferrer *et al*., 1989, Rankin *et al*., 1993). In contrast to animals and yeast, which have four RRMs, the rice and Arabidopsis sequences contain only two RRM motifs (Figure 4.1).

The N-terminal domain is made up of highly acidic regions interspersed with the basic sequences and contains multiple phosphorylation sites. The potential bipartite nuclear-localized signal sequence (NLS) of rice nucleolin-like protein, KKGKRNAEDEIEKAVSAKKQK, affirms a consensus motif for nuclear targeting (Srivastava *et al*., 1989, Lee *et al*., 1991, Tong *et al*., 1997). However, the N-terminal domain was found to be dispensable for nucleolar accumulation (Tuteja and Tuteja, 1998). The central domain contains two RNA-recognition motifs (RRMs), which are

conserved with nucleolin-like proteins from other species (Serin *et al*., 1997). The RRM is found in proteins implicated in many metabolic processes, such as pre-ribosomes (Bourbon *et al*., 1983), in poly(A) tail synthesis and maturation (Adam *et al*., 1986), in translational control (Naranda *et al*., 1994), RNA packaging (Dreyfuss *et al*., 2002), translational control (Naranda *et al*., 1994), and mRNA stability (Zhang *et al*., 1993). Moreover, the main component of proteins in pre-mRNA splicing has been demonstrated to consist of RNA recognition motif (RRM) (Amrein *et al*., 1988). The pre-mRNA splicing mechanisms in post-transcriptional level are emerging as key regulators for tuning the plant's response to environmental and developmental processes (Hugouvieux *et al*., 2001; Xiong *et al*., 2001; Li *et al*., 2002; Xiong *et al*., 2002). Whilst, Gly-and Arg-rich domain in the carboxyl-terminal domain appears likely to function primarily in protein-protein interactions (Bandziulis *et al*., 1989) that could be to facilitate the interaction of nucleolin RRMs domains with targets located within large and complex RNA, such as rRNA (Ghisolfi *et al*., 1992b; Heine *et al*., 1993). The presence of this domain in a protein is associated with the presence of an RNA-binding domain (Burd and Dreyfuss, 1994).

2. Southern blot analysis

Two pairs of rice cultivars/lines, which are different in salt-tolerant ability, were used in these studies. Leung Pra Tew 123 (LPT123) and Khao Dok Mali 105 (KDML105) rice cultivars are considered salt-sensitive line while the isogenic line of LPT123 (LPT123-TC171) and the recombinant inbred line of KDML105 (FL530-IL), are salt-resistant ones. To estimate the number of rice *Nucleolin* genes present in the indica rice genome, leaf tissues of three-leaf stage rice seedlings were used for genomic DNA extraction and digested with *BamH*I. Ten micrograms of the digested genomic DNA were fractionated in an agarose gel and transferred to a charged nylon membrane. Southern blot analysis was performed by using the shorter *OsNUC1*-japonica cDNA (assession no.AK063918) as a probe.

Based on the genomic DNA hybridization (Figure 4.3), at least seven positive bands were detected. According to the *OsNUC1* and *OsNUC2* genomic sequences retrieved from the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp) one of *BamH*I restriction sequences exist in *OsNUC2* genomic DNA, while genomic *OsNUC1* contains no *BamH*I restriction sites. Therefore, the large positive band which was longer than 7 kb should be the signal of *OsNUC1* gene, while the fragment at the size of 6.4 and 7.3 kb may represent *OsNUC2*. All other detected bands, may be resulted from the cross hybridization with other genes containing RRM and GAR domains.

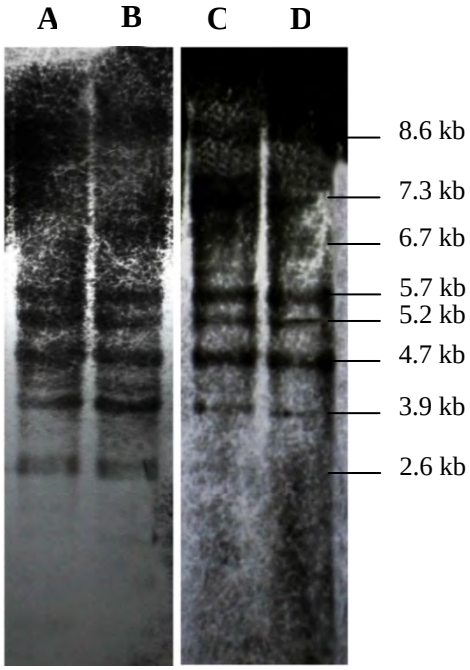


Figure 4.3 Southern analysis of genomic DNA from indica rice. Total DNA of KDML105 (A), FL530-IL (B), LPT123 (C), and LPT123-TC171 (D), was digested with *Bam*HI and hybridized with *OsNUC1* probe.

3. Subcellular localization of OsNUC1 proteins

In order to investigate the subcellular localization of OsNUC1 that reflecting role of acidic-rich N-terminal domain and the RRM-Gly-Arg rich domain at the C-terminus, the expression vector of GFP-full-length OsNUC1 protein, GFP-partial N-terminus (containing acidic domain) protein, and GFP-partial C-terminus of OsNUC1 (containing RRM – GAR domains) protein were constructed (Figure 4.4). And then each construct was bombarded on the epidermal peel of onion. After 18-30 hours of incubation on MS medium, the expression of the GFP was visualized with Leica laser-scanning confocal microscope (Leica Microsystem) and the GFP fluorescence was induced by excitation at 450 nm with an argon laser.

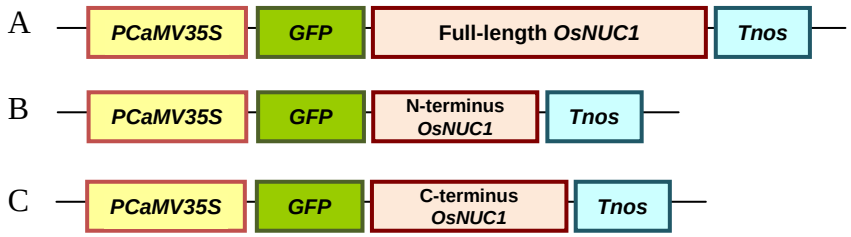


Figure 4.4 The *GFP*-fusion constructs used for subcellular localization,
(A) *35SCaMV*: *GFP*-full-length *OsNUC1*
(B) *35SCaMV*:*GFP*-N-terminus *OsNUC1*
(C) *35SCaMV*:*GFP*-C-terminus *OsNUC1*

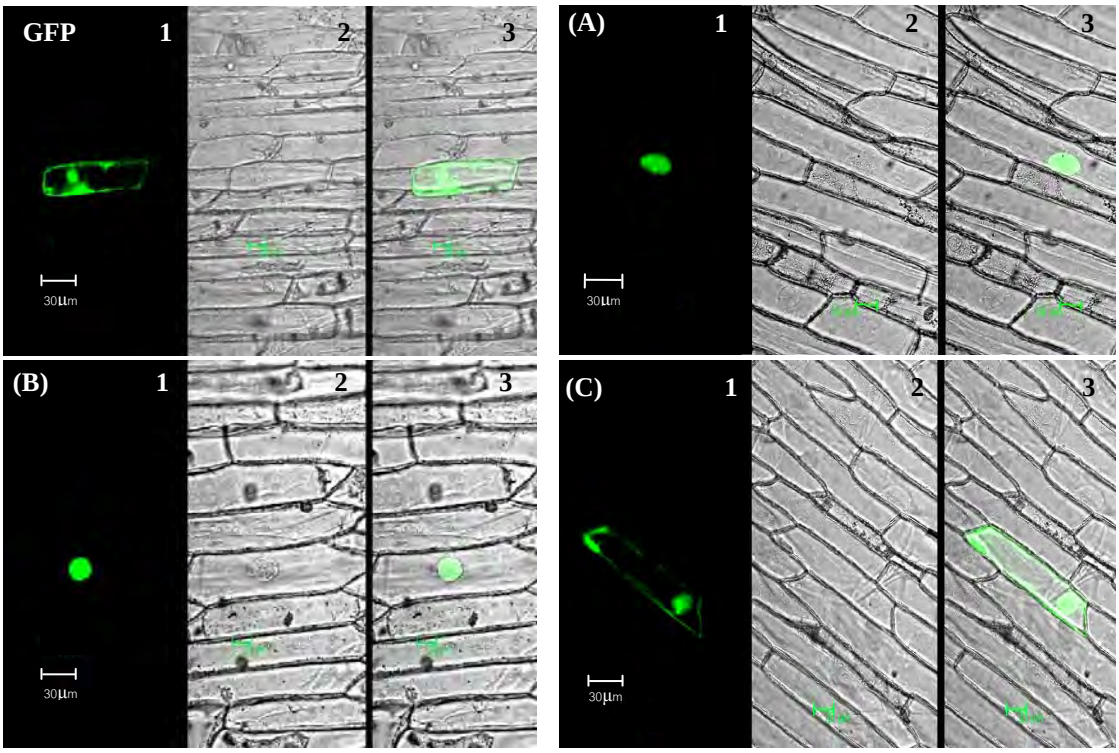


Figure 4.5 Subcellular localization of the *GFP-OsNUC1*. *OsNUC1-GFP* fusion proteins (followed by A, B and C constructs as shown in Figure 4.4) and *GFP* alone were each expressed transiently in onion epidermal cells and observed under a confocal microscope (1) fluorescence image; (2) bright field image; (3) overlay of both. The scale bar is indicated in each photo.

The GFP-full-length OsNUC1 and GFP-N-terminal OsNUC1 fusion proteins which contain the bipartite NLS domain resulted in the localization of green florescence only in the nucleus (Figure 4.5, A and B). To make a comparison between these two expressed proteins, some special bright spots were found in the GFP-full-length OsNUC1 protein that it may be apparent from the interaction between the RNA binding domains (RRM) of OsNUC1 with RNA molecules in the nucleus. Additionally, the fluorescent protein product of the GFP - central and C - terminus OsNUC1, containing RRM motifs and GAR domain, showed the localization of the fluorescence both in the nucleus and the cytoplasm of cells (Figure 4.5C). This expressed protein was also found as the special bright spots in the

nucleus suggesting the function of RRM motif in RNA binding activity, whereas the control GFP protein distributed over the cytoplasm and nucleus without any bright spot. Our finding that the nuclear fluorescence was strongly localized to a subnuclear region, presumably the nucleolus (Figure 4.5, A and C), is consistent with the localization of nucleolin reported in the other systems (Bugler *et al*., 1982, Shaw and Jordan, 1995, Petricka and Nelson, 2007). The bright spots were found within the tissues bombarded with the construct of the GFP - central and C - terminus OsNUC1 implies that only the RRMs and GAR domains are enough for nuclear localization.

4. Expression profile of *OsNUC1*

LPT123 rice seedlings were hydroponically grown in WP No.2 solution. Three-leaf stage LPT123 rice seedlings were transferred to soil and grown in the greenhouse. To speculate which organs the *OsNUC1* function, the expression of *OsNUC1* was investigated in various organs of rice; roots, leaf sheath, leaf blade, flowers and seeds.

4.1 Expression pattern of *OsNUC1*

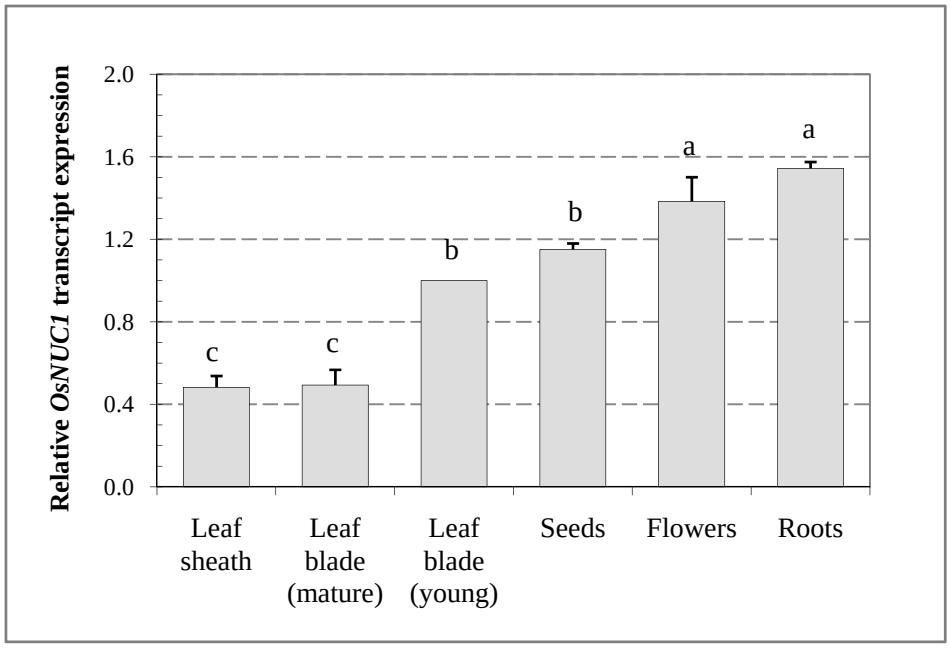


Figure 4.6 Real-time quantitative PCR analysis of *OsNUC1* expression in various rice organs.

* Means with a different letter are significantly different from each other (P<0.05; Duncan's multiple means test).

Expression of *OsNUC1* was detected in almost all plant tissues. It was strongly expressed in roots and flowers which fully contain dividing cells. Among leaf tissues, younger leaf blade (first fully expanded leaf of two week-old rice seedlings) showed the higher level of *OsNUC1* gene expression than that of mature leaf (first fully expanded leaf of 3.5 month-old rice), which was similar to the expression level found in leaf sheath tissue (Figure 4.6). Moreover, the high level of *OsNUC1* expression was found in seeds, flowers and roots, which are tissues containing developing cells. It suggested the potential role of *OsNUC1* in association with cell growth and/or differentiation. Supporting this idea, alfafa *nucleolin* is absent in stationary cells, but highly expressed before the onset of DNA replication at both the transcriptional and protein levels in logarithmically dividing cells (Bögre *et al*., 1996). Furthermore, the highest level of *nucleolin* transcript was observed in the floral tissue of Arabidopsis (Didier and Klee, 1992) and in the root meristematic cells of alfalfa (Bögre *et al.*, 1996). These results support a link between rice nucleolin-like protein and cell division that could explain the growth enhancement in transgenic Arabidopsis with *OsNUC1* overexpression (see in experiment 7).

4.2 Comparison of *OsNUC1* gene expression when plants were grown in normal and salt stress condition

Leaf blade tissue of rice seedlings was the target model to investigate the effect of salt stress on *OsNUC1* gene expression, as the leaf blades of seedlings have shown the certain level of *OsNUC1* gene expression and RNA extraction from this tissue is relatively easy when compared to the RNA yield obtained from other tissue sources. The salt-stress induced *OsNUC1* gene expression was detected in four rice lines, LPT123, LPT123-TC171, KDML105, and FL530-IL. After growing three-leaf stage rice seedlings (three weeks old) to induce *OsNUC1* gene expression in all rice cultivars/ lines tested in WP nutrient solution, containing 0.5% (w/v) NaCl. The highest level of *OsNUC1* gene expression in the salt-resistant lines, LPT123-TC171 and FL530-IL was higher than the expression level found in salt-sensitive cultivar with the same genetic background (Figure 4.7).

OsNUC1 expression was significantly induced by salt stress after 1-2 days of treatment. The salt-resistant lines, LPT123-TC171 and FL530-IL, showed the higher level of gene expression when compared to that of LPT123 and KDML105, respectively. In this assay, the maximum levels attain for both LPT123-TC171 and FL530-IL are approximately 9.0 and 2.5 folds higher than that of the seedlings grown in normal condition (Figure 4.7). The up-regulation *OsNUC1* gene expression by salt stress and the higher level in the salt resistant lines suggest the salt stress resistant role of OsNUC1 gene. These data reveal that *OsNUC1* expresses in the different tissues, at specific developmental stages, and in response to salt-stress condition.

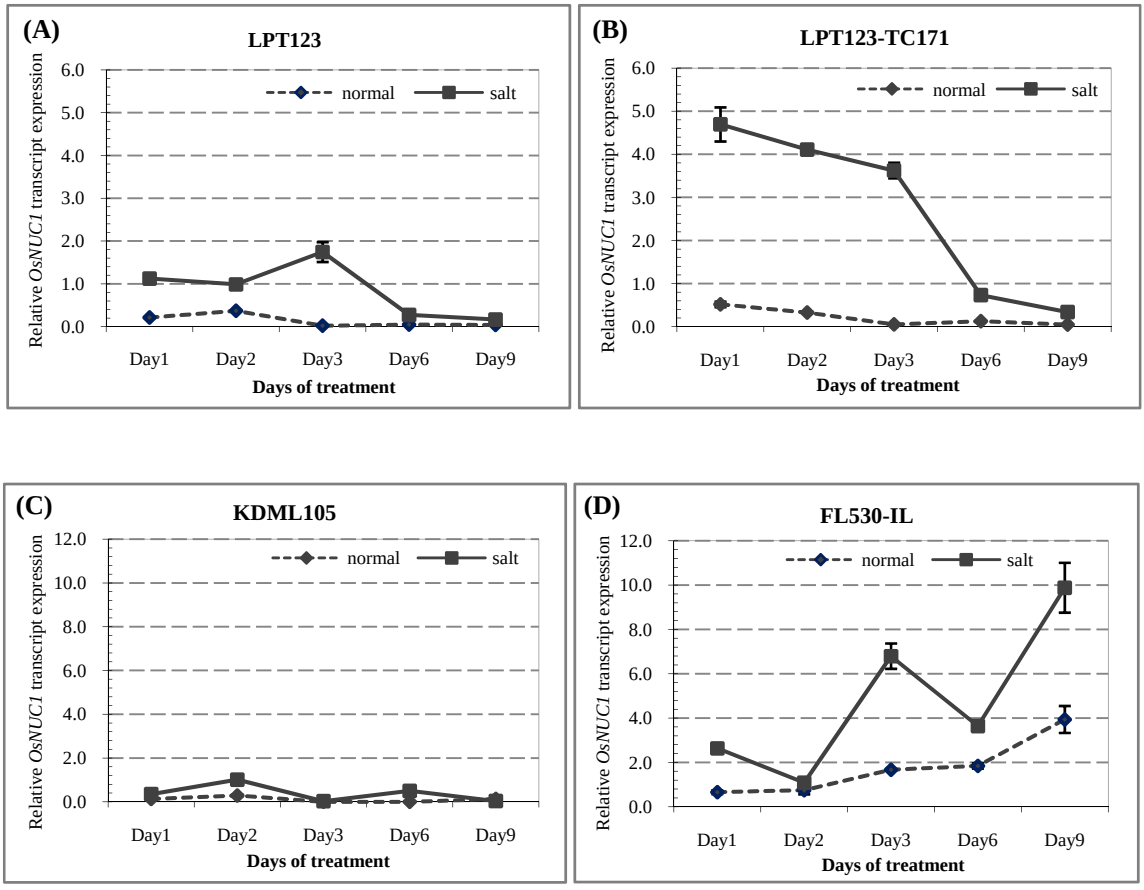


Figure 4.7 The up-regulation of *OsNUC1* in rice seedlings by salt stress exposure. Two salt sensitive cultivars, LPT123 (A) and KDML105 (C) and their isogenic salt resistant lines, LPT123-TC171 (B) and FL530-IL (D), respectively were used for the salt-responsive experiment.

Salt-stress induced *OsNUC1* gene expression is supported with the presence of several putative stress-responsive *cis*-elements, such as MYB and MYC binding sites (Abe *et al*., 2003) and TC-rich repeats and abscisic acid responsive element (ABRE) (Shinozaki and Yamaguchi-Shinozaki, 1997) found in the 1500bp sequence upstream of the 5' end of the start codon of the gene. All of these *cis*-elements were reported to show the response to salt stress (Zhu, 2002).

Moreover, the function of *OsNUC1* in salt-tolerant ability was attained for the function of RNA-binding domain which is the specificity of *nucleolin*. RNA-recognition motifs (RRMs) are incorporated into RNA-binding proteins (RBPs) (Burd and Dreyfuss, 1994, Albà and Pagès, 1998, Lorkovic and Barta, 2002). RBPs in eukaryotes have crucial roles in all aspects of post-transcriptional gene regulation, while the regulation of gene expression at the post-transcriptional as well as the transcriptional level that based on the alternative splicing and RNA processing is important for growth, development, and stress responses. RRMs are the main components of proteins in pre-mRNA splicing (Amrein *et al*., 1988) and act as regulators of RNA processing (Bourbon *et al*., 1983; Adam *et al*., 1986) and/or mRNA stability (Zhang *et al*., 1993). This in turn would lead to the accumulation of stress-related proteins and the secondary metabolites having protective function. This evidence was reported in the radiation exposure showing the binding of nucleolin to the mRNAs of peroxiredoxin1 and glutathione peroxidase which function in antioxidative peocess to protect the cells from over exposure to radiation (Park *et al*., 2000). Many Arabidopsis RBPs are involved in the regulation of plant-specific processes (e.g. flowering) or are involved in plant responses to changing environmental conditions (Lorkovic, 2008).

According to NCBI database, the *OsNUC1* gene is located on the position of 31.94 - 31.98 Mbp on chromosome 4. Many QTL loci (available in http://grameme.org/rice), for example, drought tolerance (Champoux *et al*., 1995), rooting depth (Kamoshita *et al*., 2002), root pulling force (Zheng *et al*., 2000), and penetrated root thickness (Zheng *et al*., 2000), were shown to be associated in that region. However, the QTL involving in salt resistance have not been identified. At

OsNUC2 location on chromosome 8, the marker associated with relative root growth character (Nguyen *et al*., 2002) was determined. This suggests that *OsNUC1* and *OsNUC2* genes may have the indirect function(s) on salt-resistant ability.

5. The effect of exogenous ABA and abamineSG application on *OsNUC1* gene expression during normal and salt stress condition.

By analysis of the application of 100µM ABA could enhance growth of rice seedlings during salt stress condition by increasing of shoot and root fresh and dry weights and reducing sodium accumulation in cells (Bohra *et al*., 1995; Khomsakul, 2004), besides, the salt-tolerant improvement by exogenous ABA spraying also depended on the concentration of abscisic acid (Khomsakul, 2004). This phenomenon is generally known as ABA induced salt resistance, and it suggests that the exogenous ABA has great agronomic potential for improving and enhancing the stress tolerance in rice.

This experiment was designed in order to determine if the regulation of *OsNUC1* gene expression is under ABA function during normal and salt-stress condition. Rice seedlings of four rice lines, LPT123, LPT123-TC171, KDML105, and FL530-IL, were hydroponically grown in WP No.2 solution. To test the effect of exogenous ABA application on *OsNUC1* transcript expression, 100µM ABA was sprayed onto leaves of three-leaf stage rice seedlings with/ without salt stress. 0µM ABA spraying was used as control buffer treatment. Rice leaf tissues were harvested at different time points (0, 1, 2, 3, 6 and 9 days). The relative *OsNUC1* expression level of was determined by using quantitative real-time PCR. *OsActin* was used for the internal control.

The similar design was also performed using the spraying of abamineSG, the ABA synthesis inhibitor instead of ABA solution in order to determine the dependence of *OsNUC1* gene expression on ABA molecules. The effect of abamineSG on ABA synthesis resulted in the limited ABA content and the reduction of ABA responsive gene expression, *OsCIPK15* and *OsCYP707A5* was also demonstrated to show the inhibition of ABA synthesis.

5.1 The effect of exogenous ABA on *OsNUC1* transcript expression in LPT123 and LPT123-TC171 under normal condition.

A significant induction of *OsNUC1* transcript levels by exogenous ABA was detected in both rice lines after a day of treatment. Up to four - fold induction was detected in salt-resistant line, LPT123-TC171, while only two - fold induction was found in the salt sensitive one, LPT123. The expression level of this gene in LPT123-TC171 rapidly decreased back towards basal levels within 6 days after treatment, while the expression level observed in the LPT123 cultivar were down to the normal level after two days of treatment. This indicated that *OsNUC1* induction by ABA was prolonged in the salt-resistant rice line (Figure 4.8). Therefore, given the proposed involvement of ABA as an inducer of *OsNUC1* transcript, the exogenous 100µM ABA spraying markedly induced the *OsNUC1* gene expression. This is in agreement with *OsNUC1* promoter sequence analysis that showed one ABA responsive element (ABRE; ACGTGG/TC).

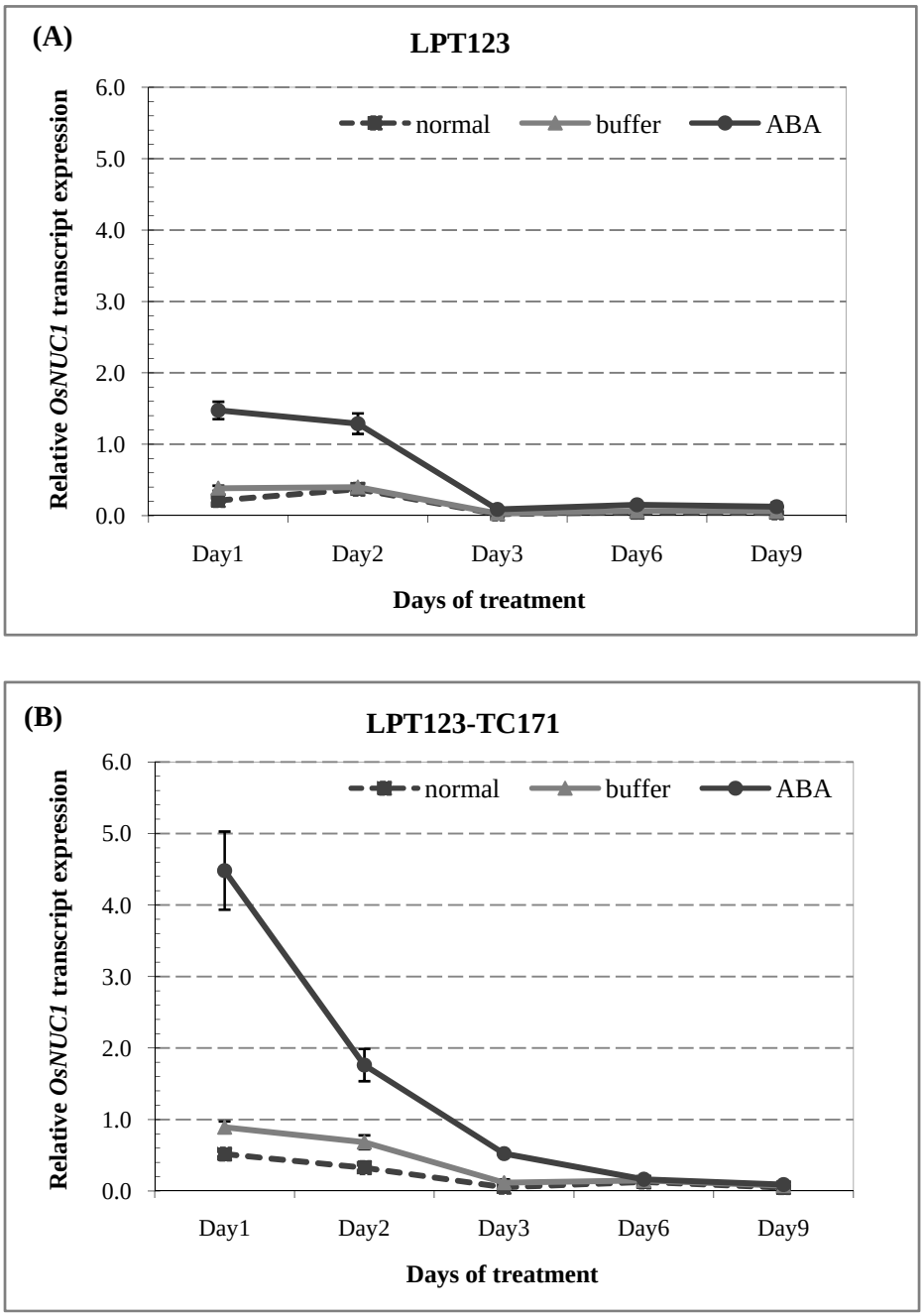
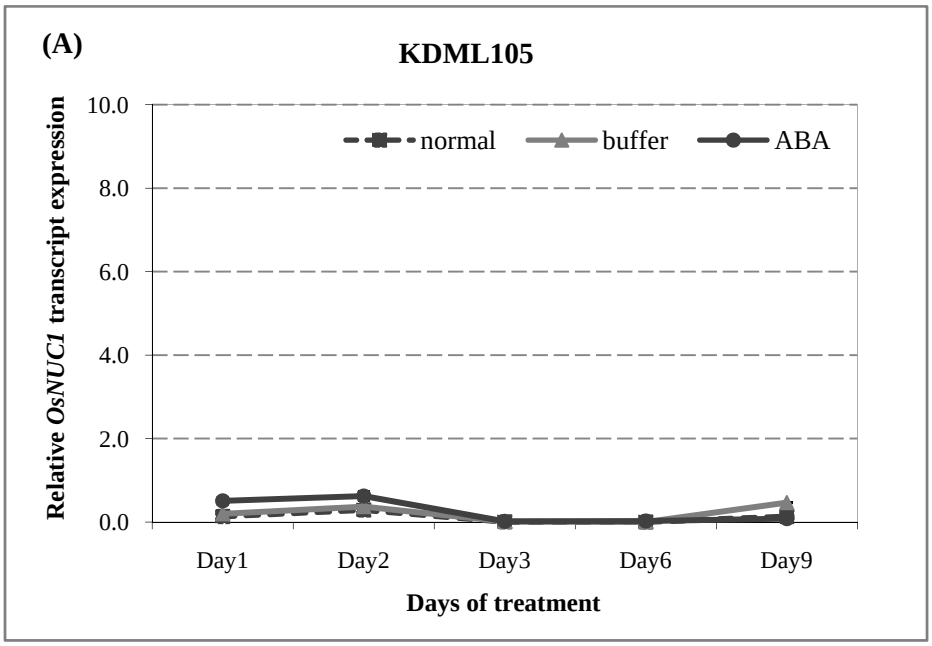


Figure 4.8 The effects of exogenous 100µM ABA on *OsNUC1* transcript expression levels in the salt-sensitive (LPT123) (A) and salt-resistant (LPT123-TC171) (B) indica rice lines grown under normal condition.

5.2 The effect of exogenous ABA on *OsNUC1* transcript expression in KDML105 and FL530-IL under normal condition.

The up-regulation of *OsNUC1* by 100μM ABA treatment was detected only in salt-resistant line (FL530-IL) (Figure 4.9). The exogenous ABA application induced the *OsNUC1* expression higher up to 2.51 folds after nine days of the treatment. For salt-sensitive line (KDML105), *OsNUC1* expression was slightly increased (but did not show significant difference statistically) after 1-2 days of treatment. The *OsNUC1* gene expression in the salt-resistant was higher than that of the salt-sensitive rice under both normal and ABA-treated conditions. The buffer spraying on leaf tissues showed no significant affect on *OsNUC1* transcript level when compared with normal condition (Figure 4.9). With the consistency in the up-regulation of *OsNUC1* in FL530-IL line and LPT123-TC171 line, it is suggested that ABA is involved in *OsNUC1* induction in rice under normal condition.



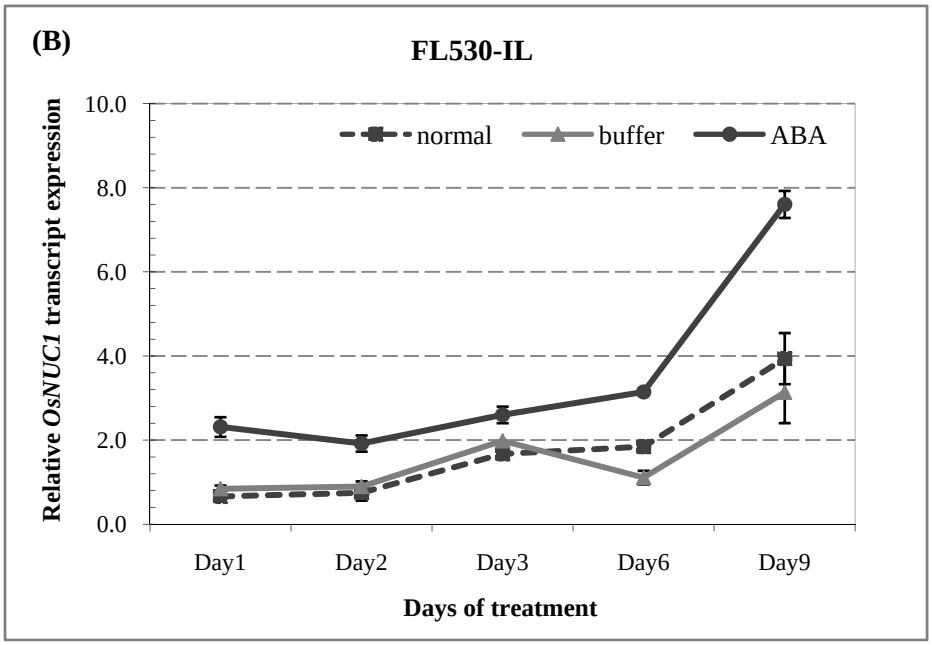


Figure 4.9 The effects of exogenous 100µM ABA on *OsNUC1* transcript expression levels in the salt-sensitive (KDML105) (A) and salt-resistant (FL530-IL) (B) indica rice lines grown under normal condition.

5.3 The effect of exogenous ABA on *OsNUC1* transcript expression in LPT123 and LPT123-TC171 under salt-stress condition.

Due to previous experiment showing the exogenous ABA could elevate *OsNUC1* transcript levels under normal condition, we would like to investigate if the similar phenomenon could be detected during salt stress condition. Interestingly, the exogenous ABA application was significantly enhanced *OsNUC1* transcript level only in day1 of treatment in both rice lines when compared with the ones treated with salt stress only (Figure 4.10). Then the *OsNUC1* expressions were decreased to the same level as the expression in the salt-treated plants with the buffer spray, which showed the lower level of *OsNUC1* gene expression than the level in salt-treated plants, while is normally seen, because the buffer spray reduces the salt-stress effect due to the increase in humidity during the buffer spray treatment.

It should be pointed out that the highest level of *OsNUC1* gene expression due to the ABA application was not higher than the highest level of *OsNUC1* gene expression induced by salt-stress treatment. Therefore, it implies that exogenous ABA application during salt stress causes the faster response in *OsNUC1* gene expression, but does not show the addition effect on *OsNUC1* gene expression during salt stress. However, the up-regulation of *OsNUC1* gene expression via ABA or salt stress is temporary. The level of gene expression was down to the normal level with 6-9 days (Figure 4.10).

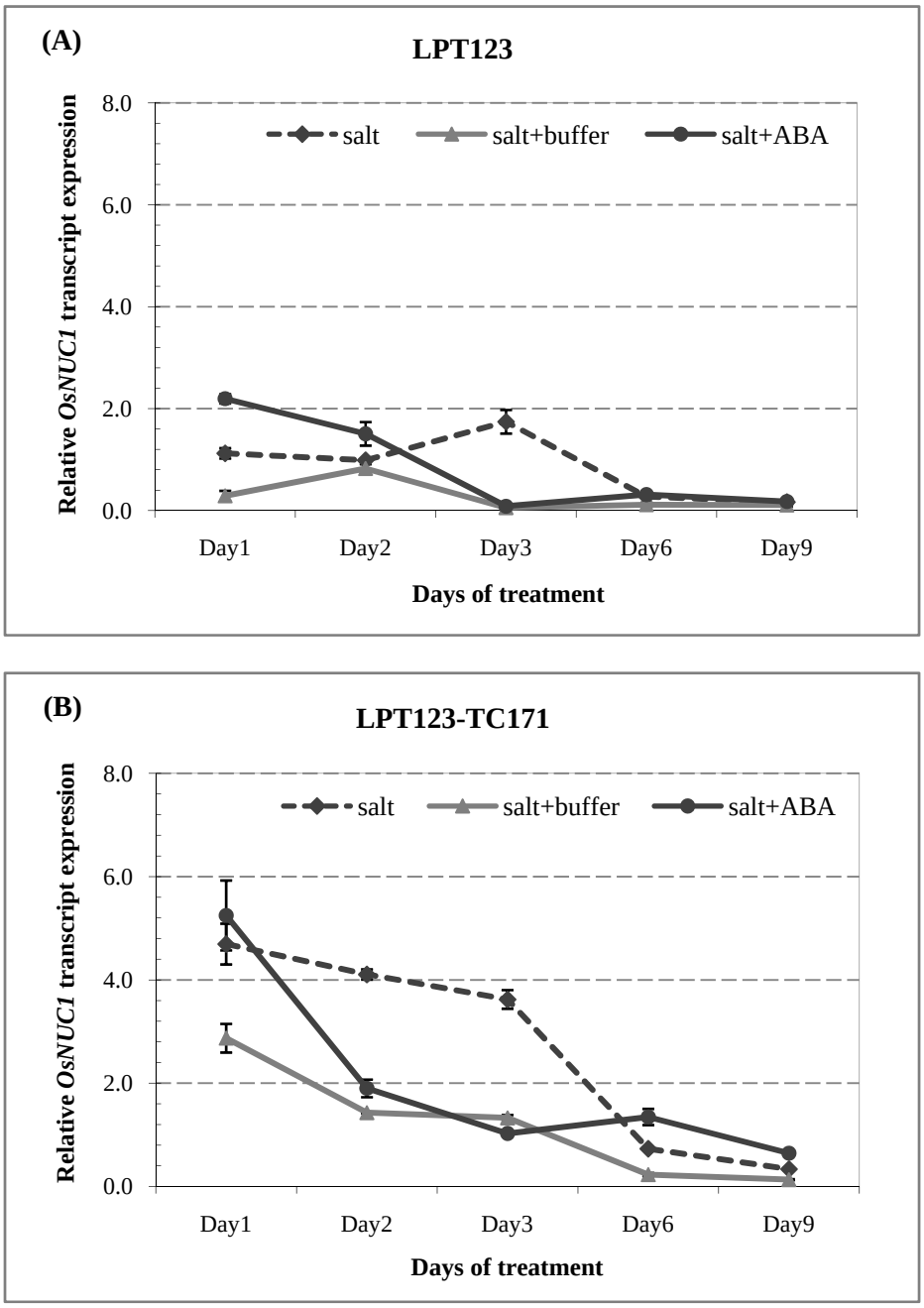


Figure 4.10 The effects of exogenous 100µM ABA on *OsNUC1* transcript expression levels in the salt-sensitive (LPT123) (A) and salt-resistant (LPT123-TC171) (B) indica rice lines grown under salt stress (0.5% NaCl) condition.

5.4 The effect of exogenous ABA on *OsNUC1* transcript expression in KDML105 and FL530-IL under salt-stress condition.

The similar experiment was performed with rice seedlings with KDML105 genetic background. The exogenous ABA application obviously induced the *OsNUC1* expression after 1 day of treatment. This indicated that exogenous ABA could promote the *OsNUC1* transcript level in salt stress. However, it was clearly seen only in the salt resistant line, FL530-IL. Additionally, the *OsNUC1* expression level also decreased on buffer spraying with 0µM ABA in both rice lines when compared with the salt stress treatment. It is consistent with the lower level of *OsNUC1* in experiment 5.2 that may results from salt-stress toxicity reduction.

In FL530-IL line, the biphasic induction of *OsNUC1* gene expression due to salt stress was clearly seen (Figure 4.11B). The ectopic application of buffer or ABA did not affect this pattern. We would like to point out that this biphasic response may be due to the genetic background of KDML105 as we do not see this pattern in the response of rice with LPT123 genetic background.

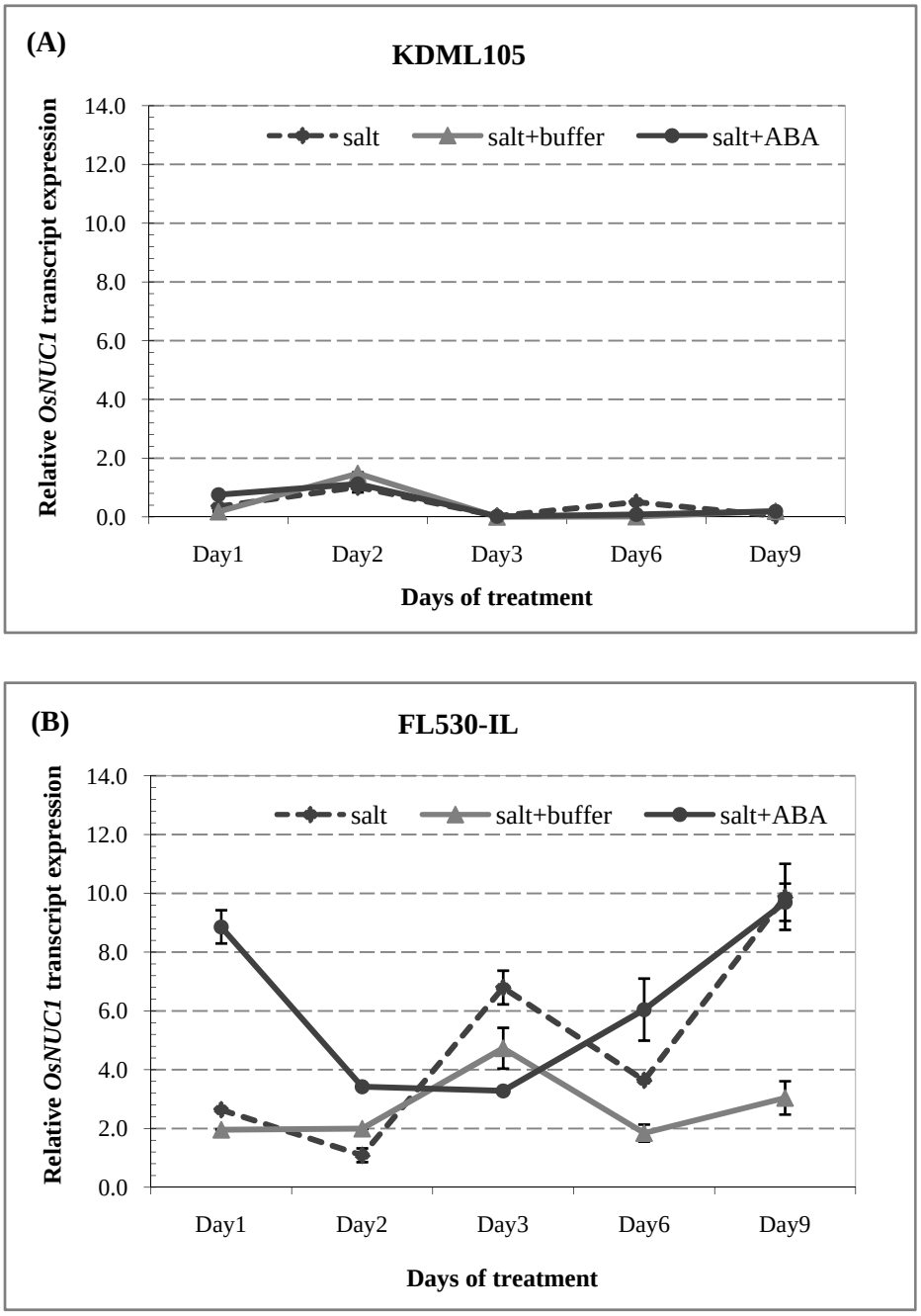


Figure 4.11 The effects of exogenous 100µM ABA on *OsNUC1* transcript expression levels in the salt-sensitive (KDML105) (A) and salt-resistant (FL530-IL) (B) indica rice lines grown under salt stress (0.5% NaCl) condition.

5.5 AbamineSG, a specific ABA biosynthesis inhibitor, causes a reduction in the ABA content and inhibits other ABA-inducible genes.

In order to confirm the action of abamineSG as an ABA biosynthesis inhibitor, the decreasing of ABA accumulation and down-regulation of other ABA-inducible genes were determined. Rice seedlings of LPT123 and LPT123-TC171 were hydroponically grown in WP No.2 solution. After that, three-leaf stage rice seedlings were treated with 50μM abamineSG under both normal and salt-stress conditions, whereas buffer spraying with 0μM abamineSG was used for control treatment. Determinations of ABA content and expression of other ABA-inducible genes were shown at different time points (0, 1 and 2 days of treatment). The measurement of the ABA content and expression level had to be determined within this short period because rice seedlings subjected to both salt stress and abamineSG application did not survive for a longer period. The blocking in ABA biosynthesis should be noted to lead to severe damage of seedlings.

5.5.1 The effect of abamineSG on ABA accumulation

As abamineSG is a competitive inhibitor of NCED, the key enzyme in ABA biosynthesis pathway, the application of abamineSG led to a decrease in ABA accumulation in plant cells. ABA content in rice seedling leave was quantitated by HPLC measurement (Agilent Technologies Series 1100, USA). The application of abamineSG caused an approximately 10- to 12-folds reduction in the ABA content after two days of treatment (Figure 4.12) during salt stress condition. The statistically different reduction was seen in both two rice cultivars. The ABA content reduction was also detected in the normally grown plants. The increasing of ABA content in salt resistant rice line under both normal and salt stress conditions can be used to explain the relation of ABA content and salt-resistant ability and confirm the action of ABA in plant stress hormone.

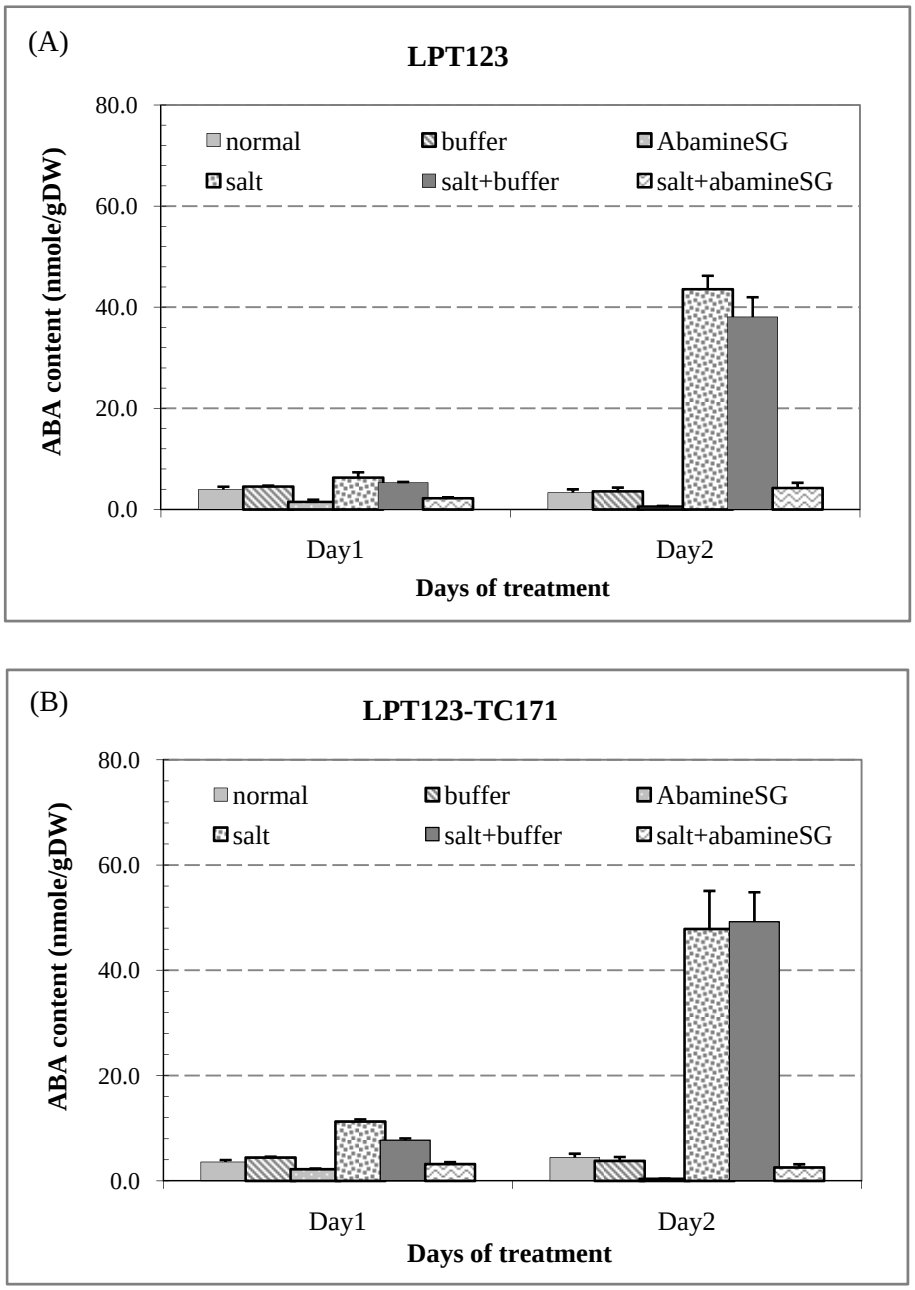


Figure 4.12 The effects of 50μM abamineSG on the ABA accumulation in two rice lines, LPT123 (A) and LPT123-TC171 (B), grown under normal (WP) and salt-stress (0.5% NaCl) conditions.

5.5.2 The effect of abamineSG on expression of other ABA-inducible genes.

To examine whether inhibition of ABA biosynthesis influenced the transcription levels of ABA-inducible genes, RT-PCR analysis was performed in this study. Two ABA-inducible genes, *CIPK15 and CYP707A5*, are potent candidate genes for this study. *OsCIPK15* gene was reported to be participating in the cross talks of signaling pathway for drought, salt stresses and ABA treatment (Xiang *et al*., 2007). *CYP707A5* gene encodes ABA 8'-hydeoxylase and that 8'-hydroxylation by CYP707A proteins is the key step in the conversion of ABA to PA (Yang and Choi, 2006). Induction of the *CYP707A* genes in response to osmotic stress was strongly inhibited in abamineSG-treated Arabidopsis (Kitahata *et al*., 2006). As the information of these ABA-inducible genes described on above, we expected to find the down-regulation of these two genes in abamineSG-treated plants.

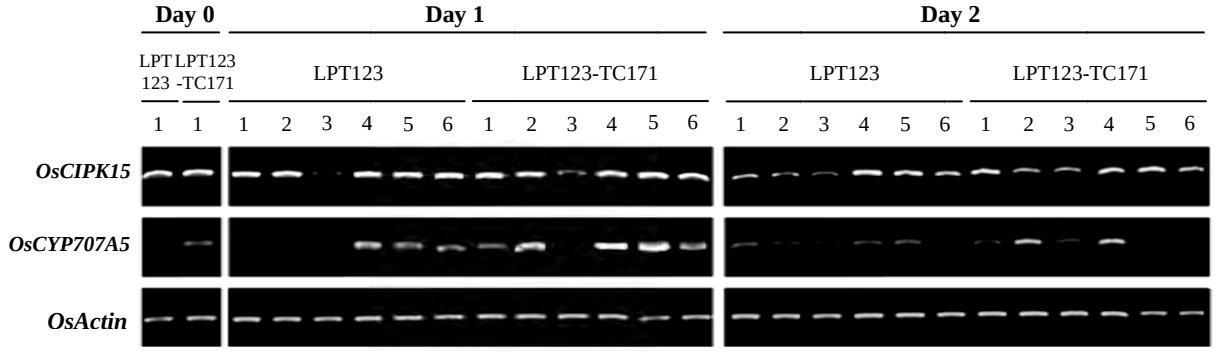


Figure 4.13 The effects of 50µM abamineSG on *OsCIPK15* and *OsCYP707A5* gene expression levels during normal (1-3) and salt stress (4-6) conditions. The controls were performed using untreated plants grown under either normal (1) or salt stress (4) conditions. The treated plants were sprayed with 0µM (2, 5) or 50µM (3, 6) abamineSG.

With respect to *OsCIPK15* gene expression, under normal conditions a reduction in the *OsCIPK15* expression level was clearly seen on the second day of the treatment and this reduction was comparable in both rice lines. Under the salt-stress condition, the reduced *OsCIPK15* transcript levels were only detected after two days of combined salt stress and abamineSG treatment. However, abamineSG treatment

caused a further significant reduction in the transcript levels in both the LPT123 and LPT123-TC171 rice lines grown under both normal and salt stress conditions, with the exception of the salt-resistant LPT123-TC171 line grown under salt stress, in which only a slightly reduced transcript level was found (Figure 4.13).

With respect to the expression of *OsCYP707A5*, abamineSG treatment clearly resulted in a reduced transcript level in both rice lines, when grown under normal conditions. Salt-stress alone induced a significant up-regulation of *OsCYP707A5* transcript levels in both rice lines, but the magnitude of regulation was reduced with abamineSG treatment (Figure 4.13). Moreover, the expression of *OsCYP707A5* was different between these two tested rice lines, with the salt-sensitive LPT123 cultivar showing a lower transcript level than the salt-resistant LPT123-TC171 rice line. With abamineSG treatment under normal growth conditions, a reduction in the *OsCYP707A5* transcript level was clearly detected after one day of treatment in both rice lines, but under the salt-stress conditions, transcript levels only decreased towards normal levels after two days of treatment. Induction of the *OsCIPK15* and *OsCYP707A5* genes in response to salt stress was strongly inhibited in abamineSG-treated plants. These results could confirm the administration of abamineSG strongly attains ABA biosynthesis inhibition.

5.6 The effect of abamineSG on OsNUC1 gene expression in rice.

Rice seedlings of four rice lines, LPT123, LPT123-TC171, KDML105, and FL530-IL, were hydroponically grown in WP No.2 solution for 3 weeks. AbamineSG, the competitive inhibition of NCED, the key enzymes in ABA biosynthesis pathway (Kitahata *et al.*, 2006), was sprayed onto leaves of rice seedlings. The changes of *OsNUC1* transcript level were determined under six conditions, normal condition (WP No.2 nutrient solution), spraying of 0µM abamineSG and 50µM abamineSG under normal condition, salt stress (WP added 0.5% NaCl), salt stress with spraying of 0µM abamineSG and 50µM abamineSG. Leaf tissues were harvested at different time points (0, 1 and 2 days). Longer experimental periods than two days led to severe damage of seedlings and so were not assayed. The relative *OsNUC1* expression level of was determined by using quantitative real-time PCR. *OsActin* was used for the internal control.

5.6.1 The effect of abamineSG on *OsNUC1* gene expression in LPT123 and LPT123-TC171.

OsNUC1 transcript level under normal condition was clearly observed (Figure 4.14). When ABA biosynthesis pathway was blocked by abamineSG, the *OsNUC1* transcript level did not show statically significant changes when compared with that of the buffer treatment. This indicates the blocking of ABA biosynthesis by abamineSG did not affect on *OsNUC1* gene expression under normal condition, suggesting that ABA may not be required for *OsNUC1* gene expression in normal condition.

Under salt stress condition, the spraying of buffer or abamineSG, resulted in the down-regulation of *OsNUC1* gene expression. In LPT123, the decrease in *OsNUC1* gene expression was detected only in the first day after treatment, while in LPT123-TC171, the reduction of *OsNUC1* gene expression due to buffer spraying may be caused by the less salt stress toxicity occurred. However, abamineSG application during salt stress did not clearly decrease *OsNUC1* gene expression in LPT123 and LPT123-TC171. This suggested that ABA was not the required element for *OsNUC1* gene expression during salt stress.

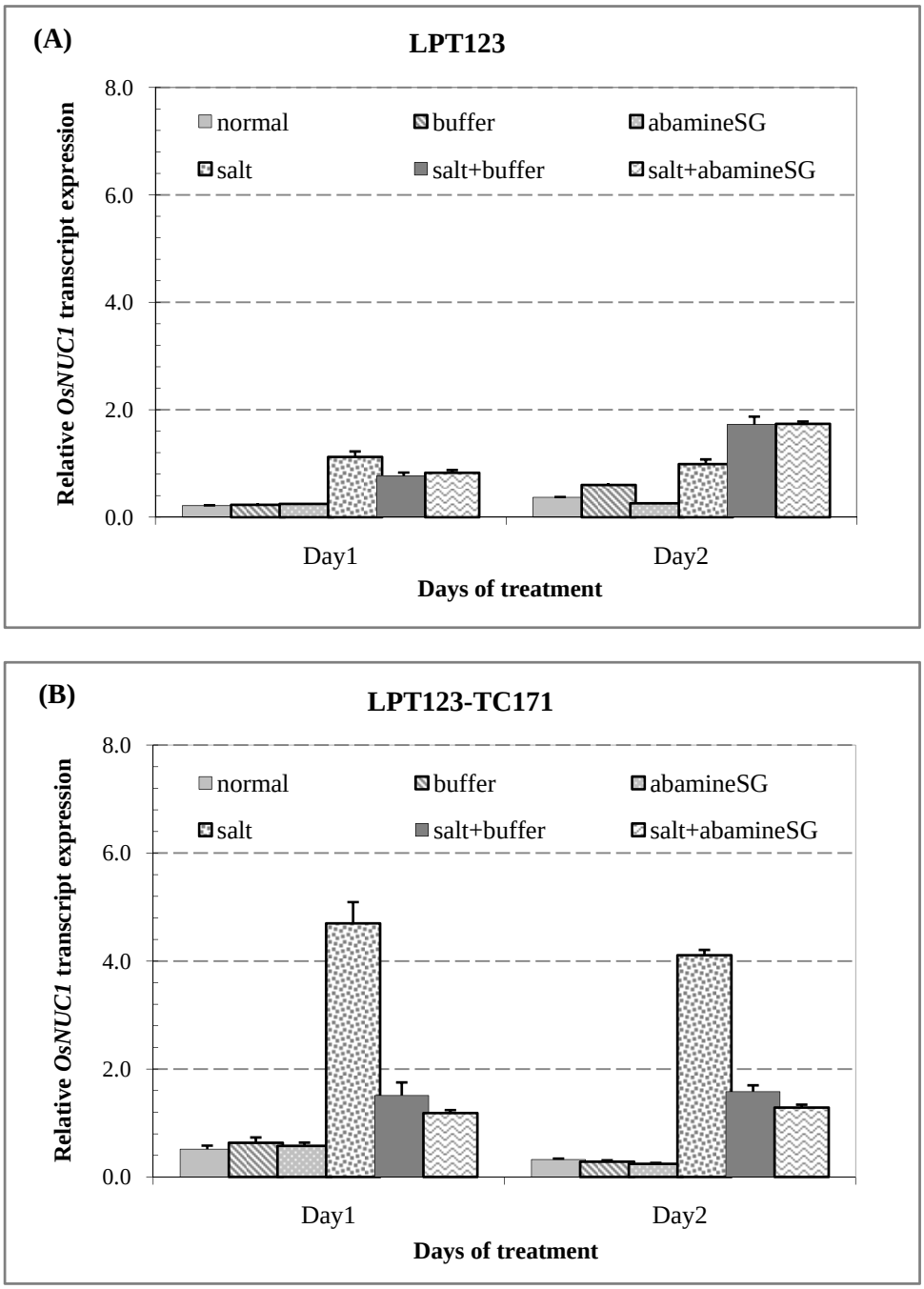


Figure 4.14 The effects of abamineSG application on *OsNUC1* transcript expression levels in salt-sensitive (LPT123) (A) and salt-resistant (LPT123-TC171) (B) lines during grown under normal and salt stress (0.5% NaCl) conditions.

5.6.2 The effect of ABA biosynthesis inhibitor (abamineSG) on *OsNUC1* transcript expression in KDML105 and FL530-IL.

The similar experiment to the experiment in 5.6.1 was performed with rice seedlings with KDML105 genetic background, the salt sensitive one, KDML105 and the salt-resistant line, FL530-IL. The result was shown in figure 4.15, in which the similar response for *OsNUC1* gene expression to abamineSG was detected. Therefore, these data confirm that ABA may not be required for *OsNUC1* induction by salt stress.

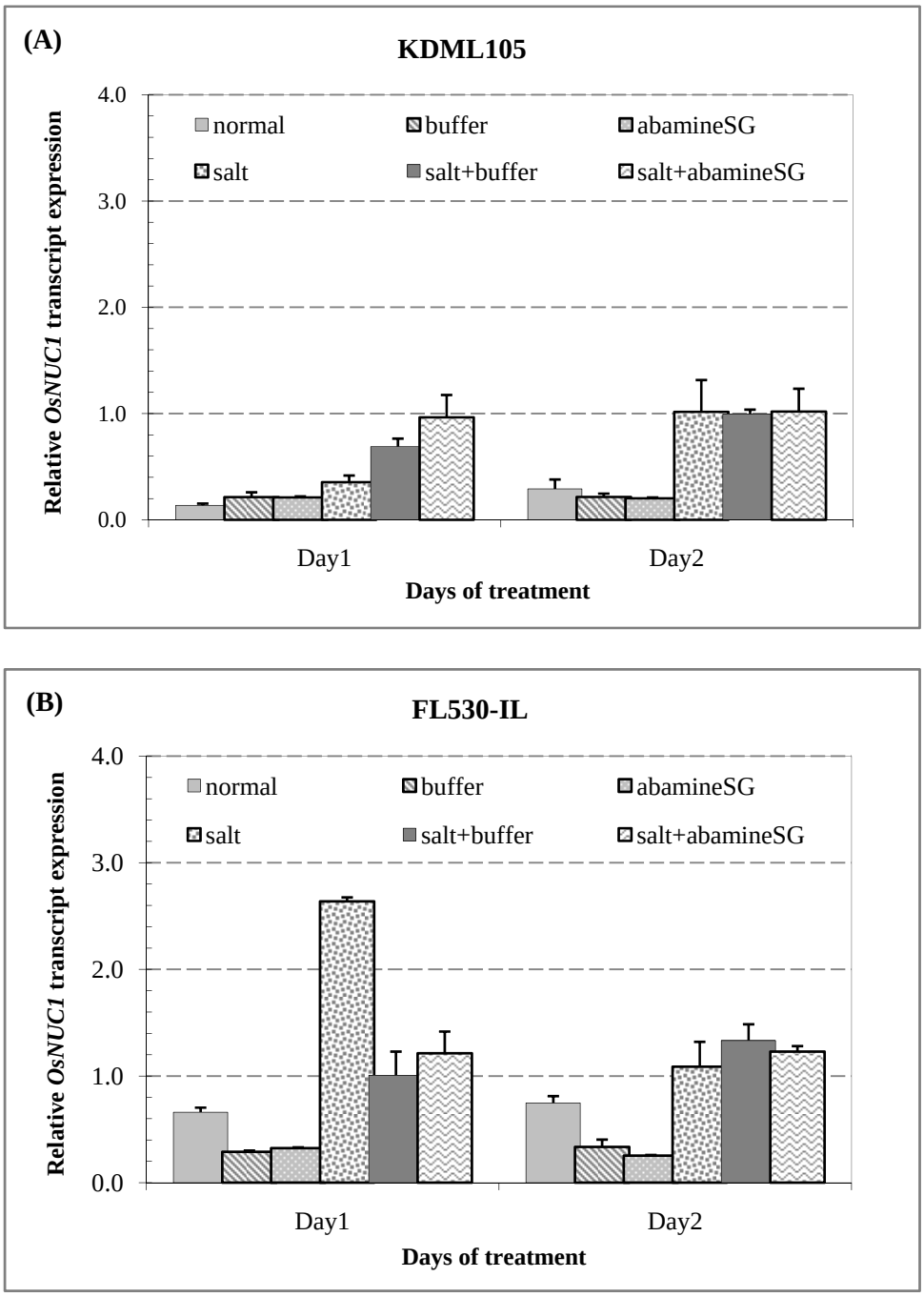


Figure 4.15 The effects of abamineSG application on *OsNUC1* transcript expression levels in salt-sensitive (KDML105) (A) and salt-resistant (FL530-IL) (B) lines during grown under normal and salt stress (NaCl) conditions.

6. Characterization of the transgenic Arabidopsis with the expression of partial *OsNUC1* gene.

A number of intriguing reports on RRMs and GAR domain suggest that they mainly function in post-transcription mechanism such as pre-mRNA splicing and mRNA stability (Amrein *et al*., 1988; Zhang *et al*., 1993; Burd and Dreyfuss, 1994; Albà and Pagès, 1998; Lorkovic and Barta, 2002). The regulation of gene expression at the post-transcriptional is of crucial for stress responses in eukaryotes (Hugouvieux *et al*., 2001; Xiong *et al*., 2001; Zhu, 2002). The over-expression of *OsNUC1* using AK063918 cDNA, which encodes polypeptide containing two RRM motifs and GAR domain in the model plants as Arabidopsis can be used to test for the function of these domains in salt resistance. Two types of promoter, *35SCaMV* promoter and *rd29a* promoter were used for regulation at transcriptional level (Figure 4.17) in order to regulate the constitutive expression and stress-inducible expression respectively. These constructs were transferred into *Arabidopsis thaliana*, ecotype Columbia via flora dip method (Bechtold and Pelletier, 1998).

Sometimes the defective phenotypes, like small figure, low growth rate etc. (Zhao *et al*., 2000) were detected in overexpression of exogenous gene transgenic plants. These problems have been solved by using organ specific or inducible promoter to driven gene transcription. Use of the stress-inducible *rd29a* promoter instead of the constitutive *35SCaMV* promoter for the overexpression of *OsNUC1* in transgenic Arabidopsis minimizes the negative effects on plant growth (Kasuga *et al*., 1999). *Rd29a* promoter induced by salinity, osmotic, and cold stresses. *Rd29a* promoter has been found to contain two major *cis*-acting elements, the dehydration-responsive element (DRE)/C-repeat (CRT) which is involved in high-salt, dehydration and low-temperature-induced gene expression (Liu *et al*., 2000), and the ABA-responsive element (ABRE) (Shinozaki and Yamaguchi-Shinozaki, 1997), both are involved in stress-inducible gene expression. In this study, the *rd29a* promoter was cloned from Arabidopsis *rd29a* cDNA (At5g52310) (accession no.EF090409) as shown in Figure 4.16.

DRE/CRT
CCCGACCGACTACTAATAATAGTAAGTTACATTTTAGGATGGAATAAATATCATACCGACAT
DRE/CRT
CAGTTTGAAAGAAAAGGGAAAAAAAGAAAAAATAAATAAAAGATATACTACCGACATGAGTT
CCAAAAAGCAAAAAAAAAAGATCAAGCCGACACAGACACGCGTAGAGAGCAAAATGACTTTGA
ABRE
CGTCACACCACGAAAACAGACGCTTCATACGTGTCCCTTTATCTCTCTCTCAGTCTCTCTAT
AAACTTAGTGAGACCCTCCTCTGTTTTACTCACAAATATGCAAACTAGAAAACAATCATCAG
GAATAAAGGGTTTGATTACTTCTATTGGAAAGAAAAAAATCTTTGGACC

Figure 4.16 *Rd29a* promoter containing two dehydration-responsive/C-repeat elements (DRE/CRT) and the ABA-responsive element (ABRE).

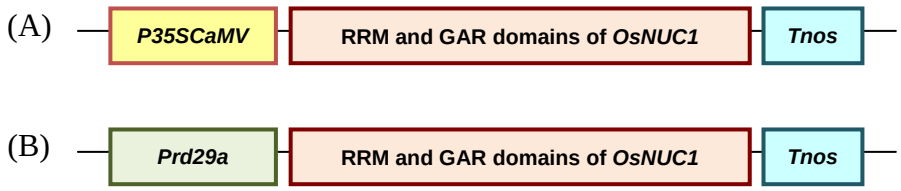


Figure 4.17 The constructs for the overexpression of truncated *OsNUC1* in Arabidopsis. (A) *35SCaMV*:*OsNUC1*:*pJim19* and (B) *rd29a*:*OsNUC1*:*pJim19*

Two constructs, *35SCaMV*:*OsNUC1*:*pJim19* and *rd29a*:*OsNUC1*:*pJim19* (Figure 4.17) were transferred into *Arabidopsis thaliana*, ecotype Columbia. Molecular and genetic studies provided the evidence that the dicot Arabidopsis and the monocot rice share common regulatory mechanisms of gene expression. The *cis*-acting elements play important roles in the regulation of gene expression in response to abiotic stresses and most of *cis*-acting elements are common between rice and Arabidopsis (Nakashima *et al*., 2009).

Total of 80 putative transgenic seeds (T_0) were obtained. Each T_0 seedling was screened on MS plate, containing 50µM kanamycin. The resistant seedlings were transferred to soil and grown to get T_1 seeds.

T_1 seeds of each line were germinated on MS plates containing 50µM kanamycin. The lines whose T_1 seedlings showing the resistant: sensitive kanamycin segregation at the 3:1 were selected for further studies, as it indicates the single locus insertion of the transgene, which will be appropriate for production of homozygous transgenic lines. The plants were germinated on MS medium (1962) at 22^{o}C for 1 week, and then they were treated under salt stress condition for a week.

6.1 Preliminary test for salt resistance in putative transgenic Arabidopsis

Some of T_1 seedlings showing the kanamycin resistance were collected to test for salt resistance. By evaluate salt tolerance of the overexpressed *OsNUC1* transgenic Arabidopsis, plants were germinated on MS medium (1962) at 22^{o}C for 1 week, and then they were treated under salt stress conditions by adding various concentrations of NaCl which are 0mM, 50mM, 100mM, and 150mM into MS agar medium. Then, agar plates were placed on vertical direction and plant grown for a week.

In normal condition (0mM NaCl), the putative transgenic Arabidopsis containing *35SCaMV-OsNUC1* construct showed the higher growth rate. Larger leaves of the transgenic seedlings were also found. It was clearly seen that the independent T_1 transgenic lines showed the higher level of salt resistance than the wild type plant (Figure 4.19). The transgenic Arabidopsis seem to have more leaves and roots when grew on the medium supplemented with NaCl. This phenomenon was clearly shown, when the seedlings were treated with 100mM NaCl. The similar phenotypes were detected with the transgenic lines possessing partial *OsNUC1* regulated by salt-inducible *rd29a* promoter. Additionally, as the partial OsNUC1 lacks of nucleus localized signal sequence (NLS) and based on the localization data which showed that the protein might locate in both the nucleus and cytoplasm, it can be proposed that the mechanism of OsNUC1 on salt resistance can function either in the nucleus or in the cytoplasm or both. One may speculate that *OsNUC1* promotes the other salt-responsive gene transcription in the nucleus. A recent report indicated that Nucleolin has histone chaperone activity and helps promote transcription through nucleosome (Mongelard and Bouvet, 2006). In addition, it may involve in

stabilization the mRNA of other salt-resistant gene in the cytoplasm and/or the involvement of RRMs and GAR domain in posttranscriptional regulation under salt stress exposure. For example, glycine-rich RNA-binding proteins (GR-PBRs) were reported to be involved in the plant response to various stress conditions (Kim and Kang, 2006, Kim *et al*., 2005, Kim *et al*., 2007; 2008, Kwak *et al*., 2005). It is likely that they act as regulators of RNA processing and/or stability for mRNAs that are highly expressed during stress conditions.

Surprisingly, this experiment also found the early flowering in T_2 *35SCaMV-OsNUC1* transgenic Arabidopsis under normal condition. Two weeks-old transgenic plants can induce flowering (Figure 4.18). Many Arabidopsis RRMs are involved in the regulation of plant-specific processes (e.g. flowering) (Quesada *et al*., 2005). In higher plants, RNA-binding proteins perform a crucial role in key developmental processes such as floral transition and flower development or stress tolerance (Cheng and Chen, 2004, Terzi and Simpson, 2008, Simpson, 2004, Quesada *et al*., 2005). Furthermore, constitutive expression of *LFY* and *AP1* genes which are the most famous of the meristem identity genes cause early flowering of transgenic plants (Shulga *et al*., 2009).

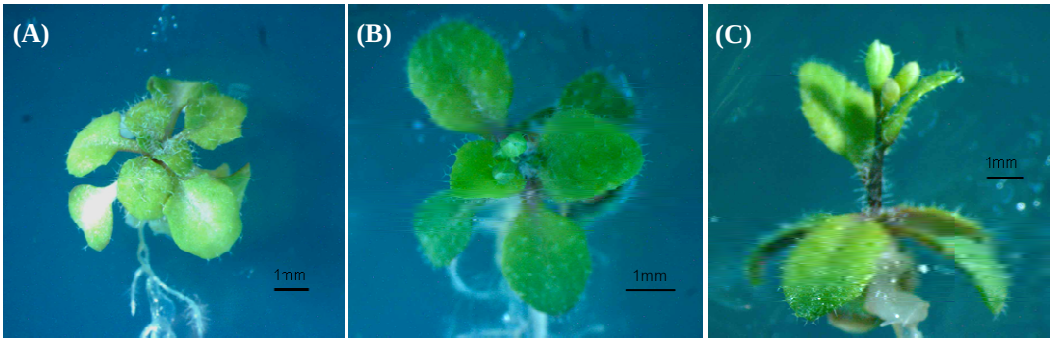


Figure 4.18 Early flowering of transgenic *35SCaMV-OsNUC1* Arabidopsis. (A) 2 week-old wild-type Arabidopsis, (B-C) 2 week-old *OsNUC1*-overexpressing Arabidopsis (B: Top view and C: Side view)

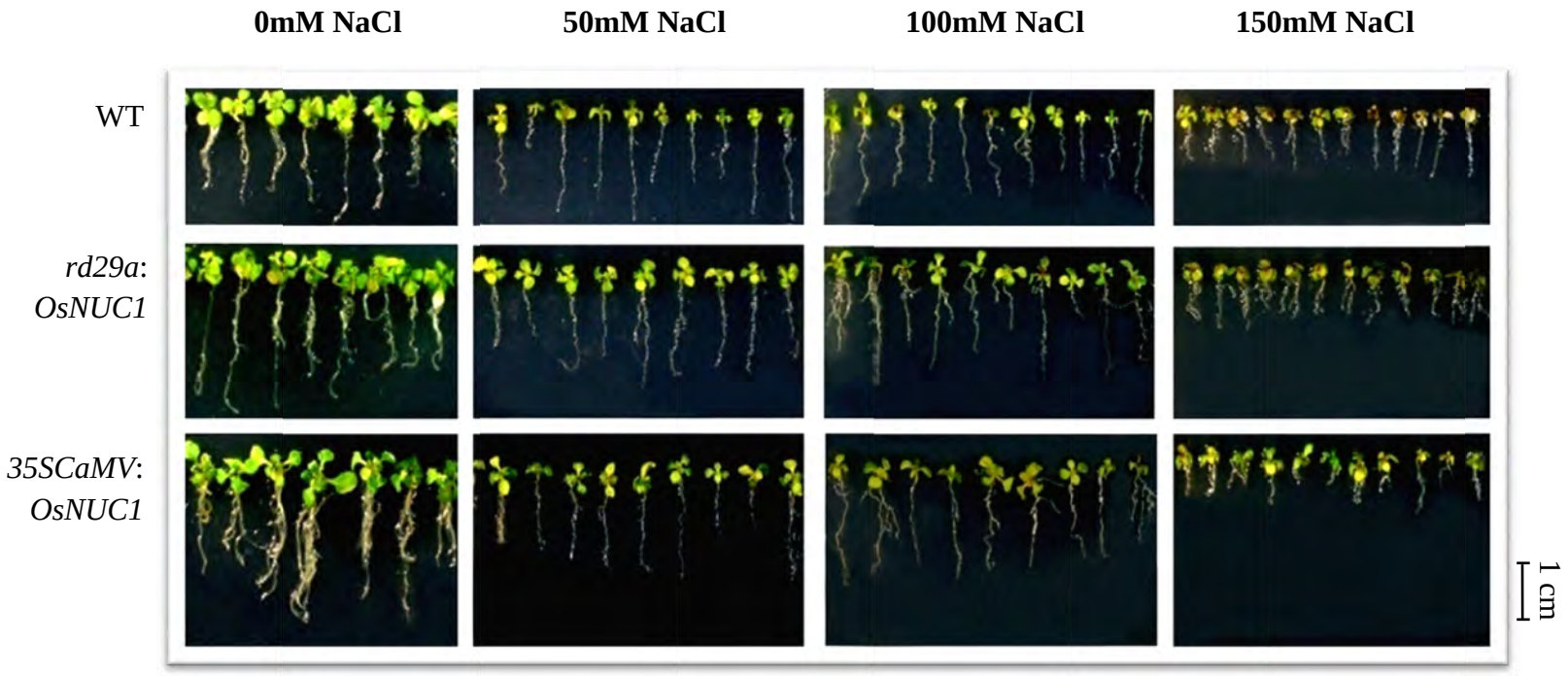


Figure 4.19 Seedlings morphology of the wild type (WT) and T_1 Arabidopsis after a week in normal (0mM NaCl) or salt stress (50, 100 or 150mM NaCl) condition.

6.2 Detection of *OsNUC1* gene expression in transgenic Arabidopsis.

After obtaining the homozygous transgenic lines (T_2) six independent lines of the constitutive expression, 35S-1, 35S-2, 35S-3, 35S-4, 35S-5, 35S-7, and four lines of the inducible expression, rd-9, rd-10, rd-13 and rd-14, were analyzed for *OsNUC1*gene expression.

They were germinated on MS medium for a week, then transferred to MS containing 100mM NaCl and grown for another week. *OsNUC1* transcript expression levels of the transgenic plants were estimated by real-time PCR. The *OsNUC1* primers were designed for gene expression analysis. Arabidopsis *EF-1α* was used as internal control. The *OsNUC1* expression level of each line was represented in comparison with the lowest level of *OsNUC1* gene expression.

OsNUC1 gene expression was shown in Figure 4.20. Among the *OsNUC1* constitutive expressing lines, 35S-7 showed the highest *OsNUC1* gene expression, while 35S-1 showed the lowest level of the gene expression. Therefore, three independent lines with various levels of *OsNUC1* gene expression were selected for further characterization.

Among the salt-inducible *OsNUC1* expressing lines, rd-10 showed the highest level while rd-9 showed the lowest level. Rd-13 and rd-14 were selected for further studies.

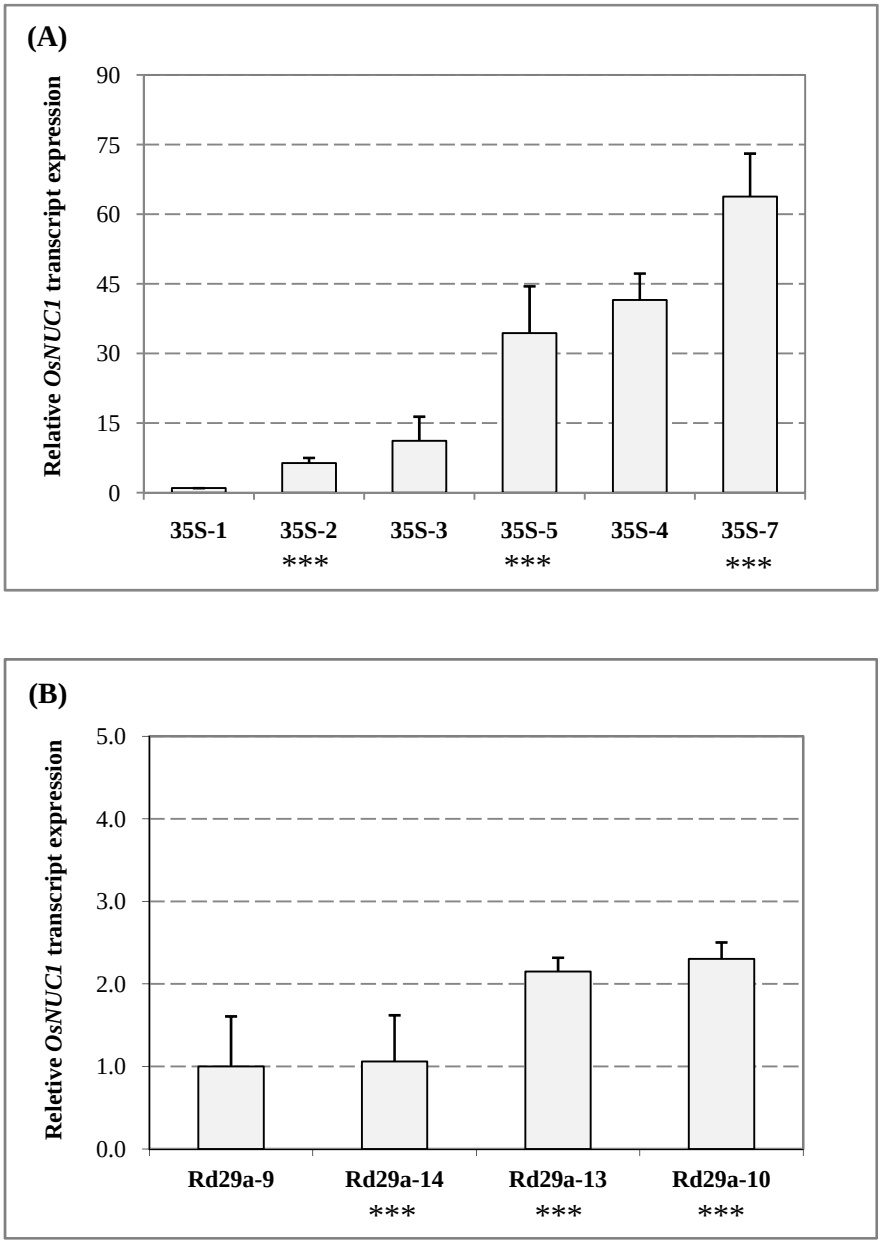


Figure 4.20 Expression levels of *OsNUC1* in the six *35SCaMV:OsNUC1* (A) and four *rd29a:OsNUC1* (B) transgenic Arabidopsis. The lines chosen for further characterization were marked with three asterisks.

Three independent transgenic Arabidopsis lines with different level of *OsNUC1* gene expression, 35S-2, 35S-5, and 35S-7 (Figure 4.20A), were chosen for further analysis. The truncated *OsNUC1* fragment was amplified from the genomic DNA of transgenic Arabidopsis lines to confirm the gene insertion in plant model genome (Figure 4.21).

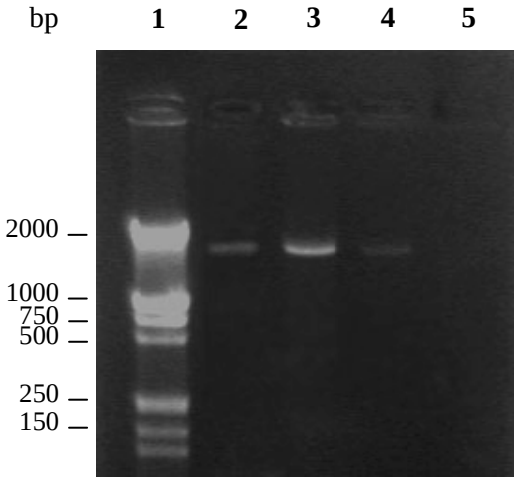


Figure 4.21 PCR analysis of partial *OsNUC1* gene in transgenic Arabidopsis genome (Lane 2-4) and wild type (Lane5). Lane1, DL2000 DNA marker; Lane2, transgenic 35S-2; Lane3, transgenic 35S-5; Lane4, transgenic 35S-7; Lane5, wild-type Arabidopsis

6.3 Plant growth and salt resistant ability analyses of the transgenic Arabidopsis

Significantly difference in fresh weight and dry weight under normal condition was detected in all transgenic lines (Figure 4.22 A). The ratio of fresh weight and dry weight of transgenic plants under normal condition also showed the a significant increase (Figure 4.22 E). The higher ratio of fresh weight per dry weight is probably a result of larger and more intercellular spaces holding more water. However, fresh weight and dry weight of transgenic *OsNUC1ox* plants under stress condition showed significant increase only in 35S-7, rd-13 and rd-14. The increasing of dry weight of 35S-7 under both normal and stress conditions and of rd-13 and rd-14 under stress exposure may be due to the enhancement of plant cell number.

The transgenic Arabidopsis overexpressing *OsNUC1* gene were a strongly increase in root elongation, root number, and leaf number at the seedling stage. It was clearly shown that in normal condition the *35SCaMV*:*OsNUC1* transgenic plants had more leaves and roots (Figure 4.23, C and E). By contrast, in the salt-stress condition, no significantly difference in root number could be detected between the transgenic lines with constitutive expression of *OsNUC1* and the wild type control. However, the transgenic lines show the higher root number than the wild type. Whereas, root number and elongation was significantly increased in the transgenic lines with expression construct *rd29a-OsNUC1* (Figure 4.23 D). Leaf number and root length of the transgenic lines were also higher than that of wild type (Figure 4.23, B and F). Arabidopsis *Nucleolin* mutants (*parl1*) were also characterized by a slight slow growth phenotype and by an increase in unprocessed 35S pre-rRNA in seedlings (Petricka and Nelson, 2007). It is feasible that *OsNUC1* could enhance plant growth. Such growth enhancement was also reported in tobacco overexpressing *OsARP* (a new rice vacuolar antiporter regulating gene) (Uddin *et al*., 2008) and *AtZEP* (Zeaxanthin epoxidase) (Park *et al*., 2008) which is an important enzyme in ABA biosynthesis.

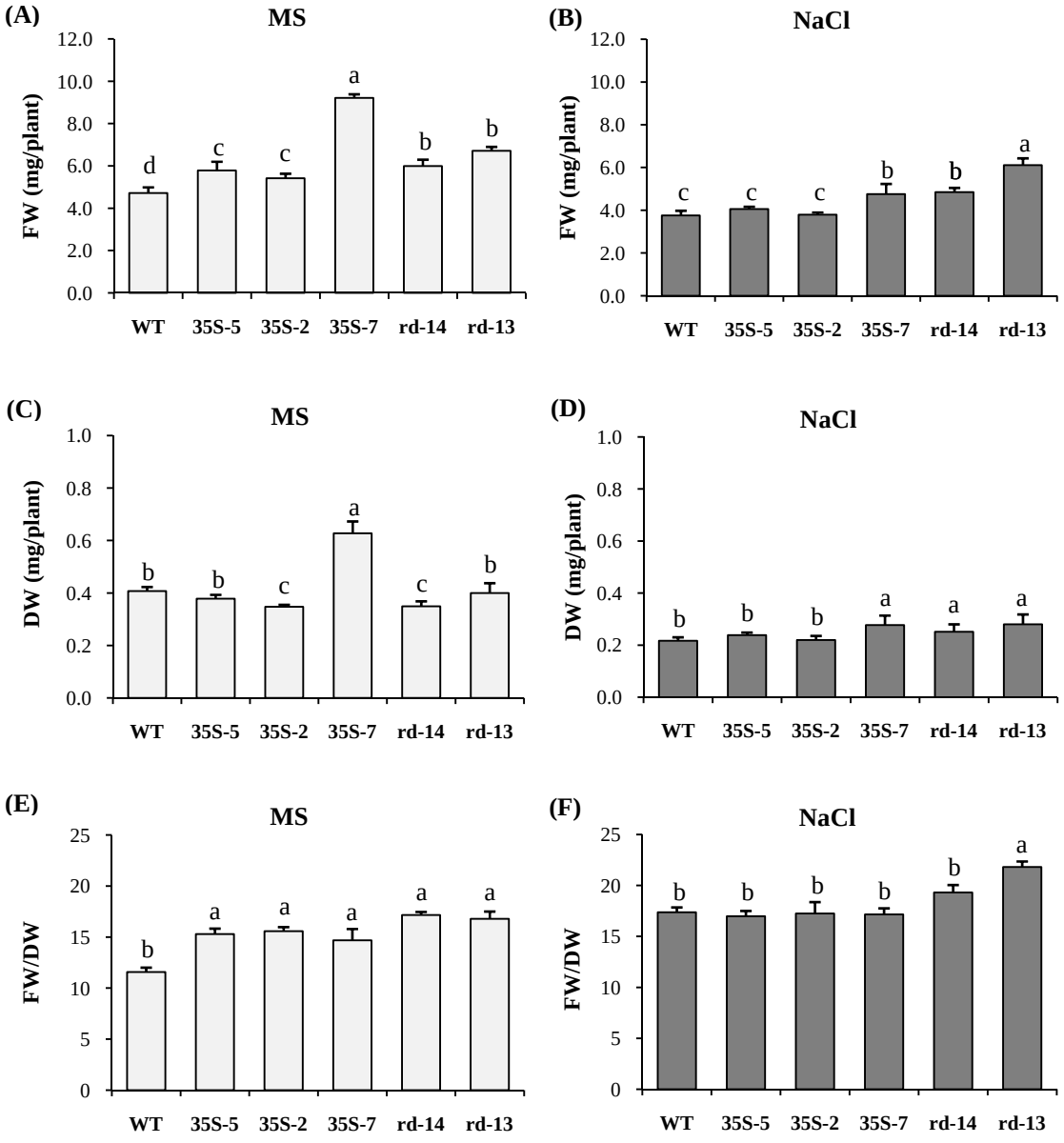


Figure 4.22 The overexpression of truncated *OsNUC1* gene in Arabidopsis effects on plant growth indicated by fresh weight (A, B), dry weight (C, D), and fresh weight per dry weight (E, F) when grown in normal condition or NaCl containing medium for 3 weeks. Five independent transgenic Arabidopsis with different *OsNUC1* gene expression and WT were used in the experiment.

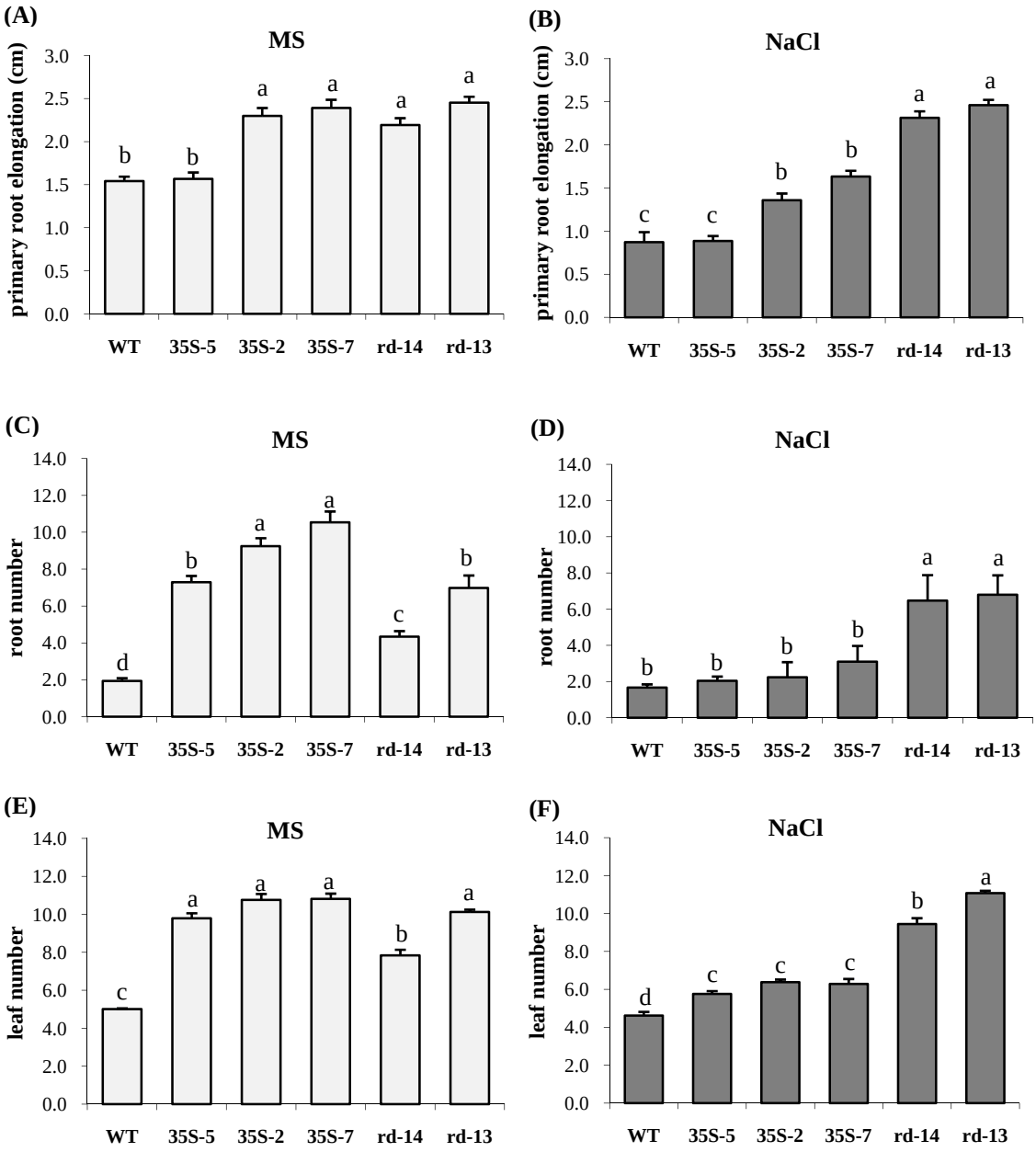


Figure 4.23 The overexpression of truncated *OsNUC1* gene in Arabidopsis effects on plant growth indicated by root length (A, B), root number (C, D) and leaf number (E, F) when grown in normal condition or NaCl containing medium for 3 weeks. Five independent transgenic Arabidopsis with different *OsNUC1* gene expression and WT were used in the experiment.

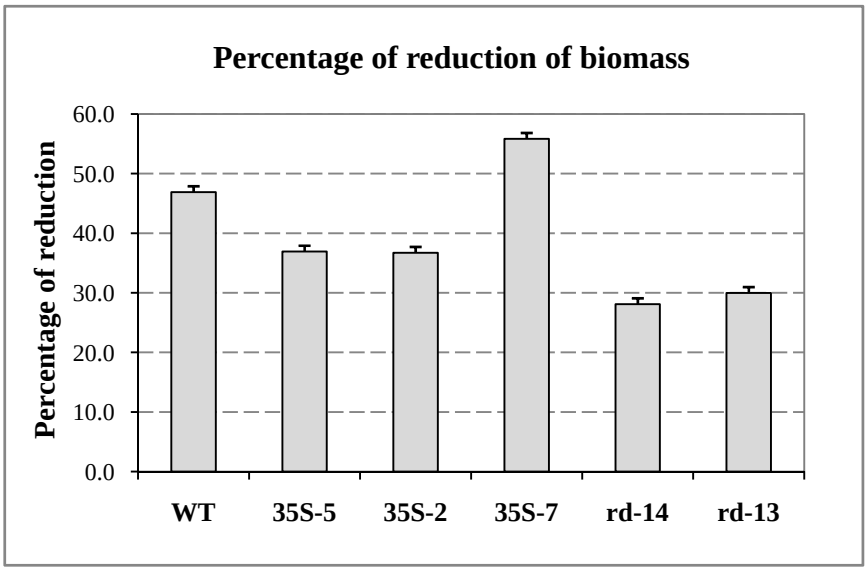


Figure 4.24 Percentage of reduction of biomass.

6.4 Detection of *OsNUC1*, *AtNUC1*, *AtSOS1* and *AtP5CS* gene expression in transgenic Arabidopsis

Transcript expression level of the endogenous Arabidopsis *NUC1* and the other salt-responsive genes in transgenic *OsNUC1*-overexpressed Arabidopsis was determined by real-time PCR. Three and two independent transgenic Arabidopsis lines of *35SCaMV-OsNUC1* and *rd29a-OsNUC1*, respectively, with different level of *OsNUC1* gene expression, 35S-2, 35S-5, 35S-7, rd-13 and rd-14 were germinated and grown on MS medium containing kanamycin (50mg/L) for a week, and then they were treated under salt stress (by adding 100mM NaCl). Whereas, normal condition, 7-days old seedlings were transferred to new MS medium and grown for a week. After that, leaf tissues were harvested at different time points (0, 2, 5, 12 and 24 hours). The specific primers for each gene were designed and used for detected the gene expression level. Arabidopsis *EF-1α* was used as internal control.

6.4.1 The *OsNUC1* transcript level in *35SCaMV*:*OsNUC1* and *rd29a*:*OsNUC1* plants

Expression pattern of the *OsNUC1* gene in *rd29a-OsNUC1* is different from that of *35SCaMV-OsNUC1* plants. The *OsNUC1* transcript levels in *35SCaMV-OsNUC1* plants rapidly elevated after they were treated on salt stress until five hours of treatment, then they were immediately decreased (Figure 4.25). Whereas, the *OsNUC1* expression levels in *rd29a-OsNUC1* plants have peaked after twenty-four hours of treatment. The stress-inducible *rd29a* probably show late induction of *OsNUC1* under salt stress. On the other hand, the highest expression of the *OsNUC1* gene in rd-13 is still lower than that in 35S-7 Arabidopsis. These results revealed the different kind of promoters, which are constitutively expressed (*35SCaMV*) and stress-inducible promoter (*rd29a*), in the regulation of *OsNUC1*.

Three individual lines of *35SCaMV-OsNUC1* have the different level of *OsNUC1* expressions, which 35S-7, 35S-2, and 35S-5 were arranged from highest to lowest level, respectively (Figure 4.25 A). This result is consistent with the level of gene expression in subsequent study. The strongly expressed of *OsNUC1* by the induction of constitutively expressed *35SCaMV* promoter after 5 hours of NaCl treatment led to down regulation of this gene expression on the next detected timings. Moreover, the similar *OsNUC1* expression pattern of these three transgenic Arabidopsis also was performed. For the *rd29a-OsNUC1* plants, the expression of *OsNUC1* gene is higher in the rd-13 than that in the rd-14 and rd-10 (Figure 4.25 B). Surprisingly, one of these three *rd29a-OsNUC1*, rd-10 showed the distinctive expression pattern of *OsNUC1*. It wasn't up-regulated after 24 hours of treatment which was different for the other two transgenic lines with the inducible with the inducible promoter (Figure 4.26). Therefore, we decided to examine salt tolerance ability of *rd29a-OsNUC1* only in rd-13 and rd-14 transgene lines.

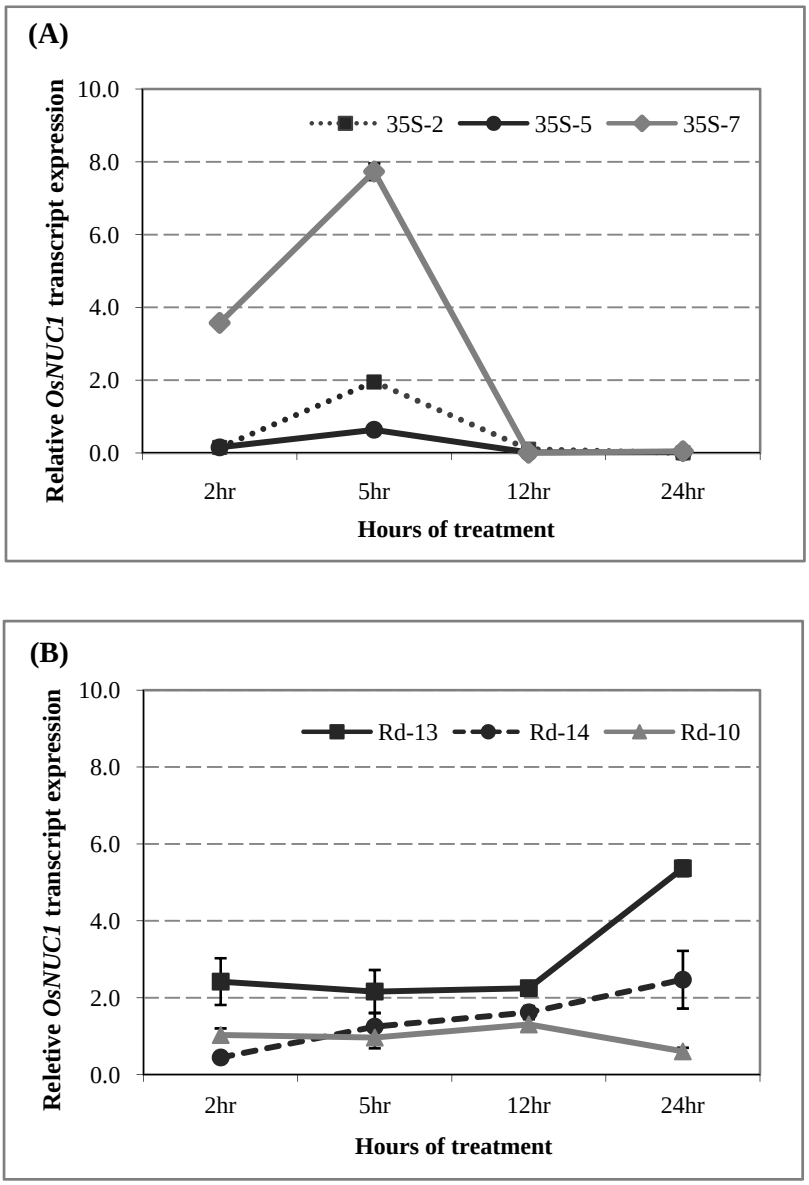


Figure 4.25 Expression pattern of *OsNUC1* in *35SCaMV-OsNUC1* (A) and *rd29a-OsNUC1* (B) transgenic Arabidopsis under salt stress. Three independent transgenic Arabidopsis of each construct were used in the experiment.

6.4.2 The *AtNUC1* gene expression in *35SCaMV-OsNUC1* and *rd29a:OsNUC1* plants

6.4.2.1 *35SCaMV-OsNUC1*

Endogenous *AtNUC1* gene in wild-type under salt stress showed higher expression than that in normal condition, the strongly up-regulation of *AtNUC1* by salt stress was detected after 12 hours of treatment (Figure 4.26, C and D). This result could support the function of *NUC1* in salt-stress response. When compared the transcript level of *AtNUC1* in transgenic plants and wild type under salt stress, the *AtNUC1* in *35SCaMV-OsNUC1* was continuously decreased until 12 hours of salt-stress treatment, then up-regulated on 24 hours (Figure 4.26, C and D). Therefore, the up-regulation of *OsNUC1* (Figure 4.26, A and B) that peak on 5 hours of treatment, did not represent any effect on the expression of endogenous *AtNUC1*. This implies that the potent function of *OsNUC1* was impact on salt stress instead of the endogenous *AtNUC1* function in transgenic plants.

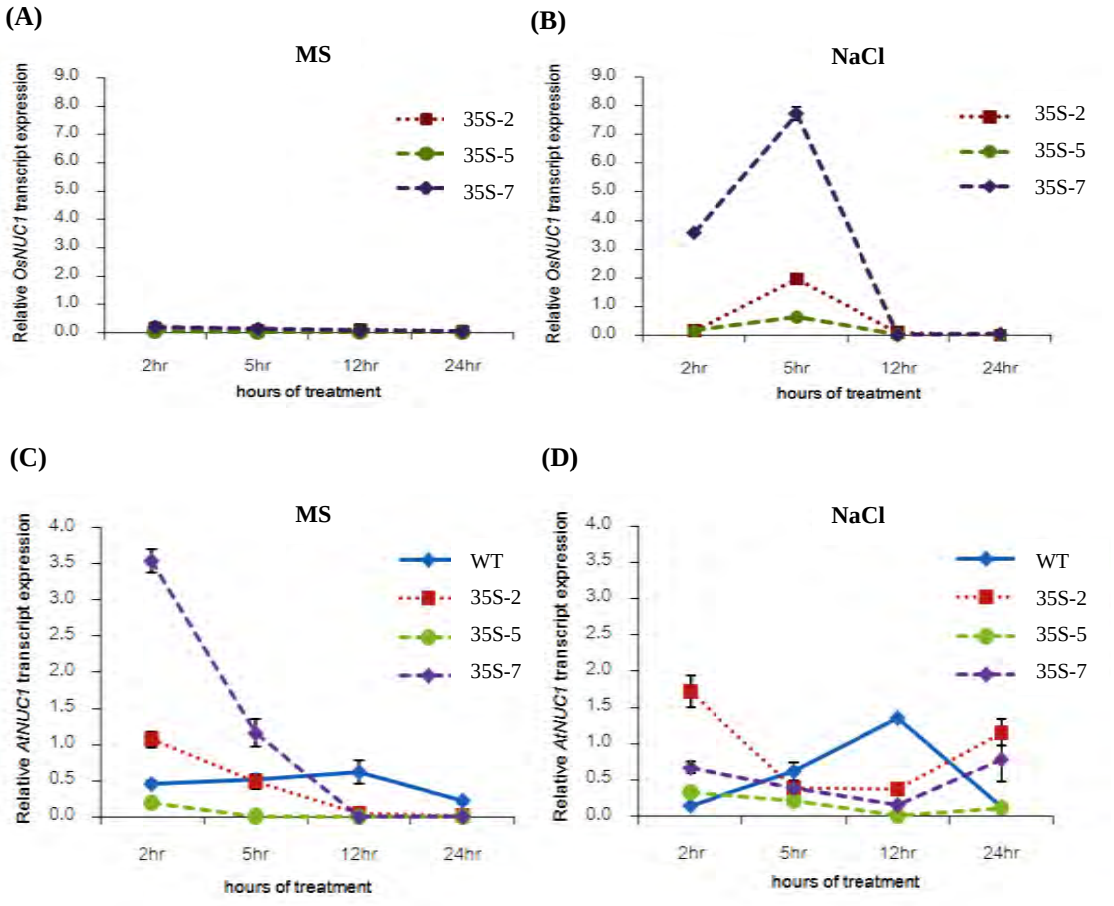


Figure 4.26 Expression pattern of *OsNUC1* (A and B) and endogenous *AtNUC1* (C and D) in the *35SCaMV-OsNUC1* overexpressors during normal (A and C) and salt-stress (B and D) conditions. Three independent transgenic Arabidopsis and wild type were used in the experiment.

6.4.2.2 *rd29a-OsNUC1*

When compared the transcript level of *AtNUC1* in transgenic plants and wild type under salt stress, the *AtNUC1* in *rd29a-OsNUC1* was immediately induced to high level after 5 hours of salt-stress treatment, and then down-regulated to low level (Figure 4.27, A and B). However, the *OsNUC1* in *rd29a-OsNUC1* has peak at 24 hour (Figure 4.25 B), which is not consistent with the *AtNUC1* expression pattern. Therefore, this result confirmed the up-regulation of *OsNUC1* did not represent any effect on the expression of endogenous *AtNUC1*.

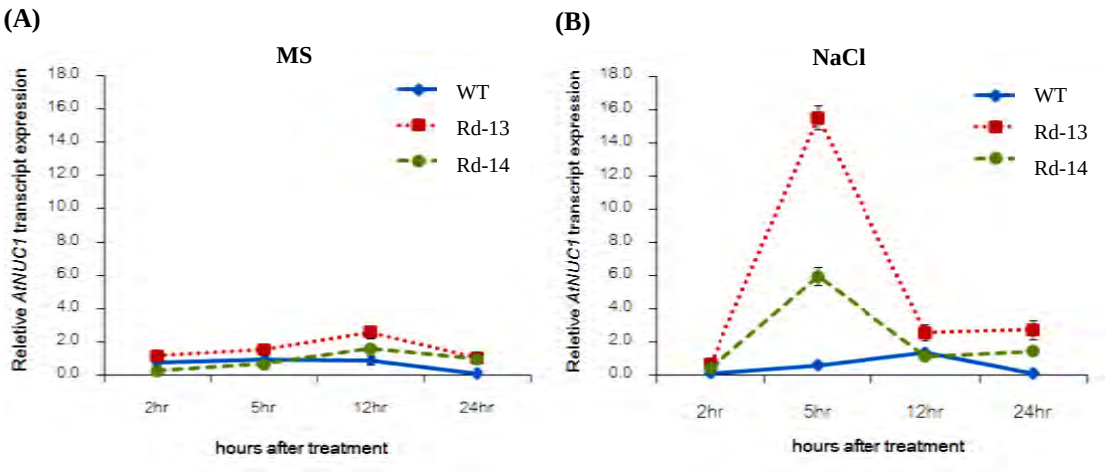


Figure 4.27 Expression pattern of endogenous *AtNUC1* in the *rd29a-OsNUC1* overexpressors during normal (A) and salt-stress (B) conditions. Three independent transgenic Arabidopsis and wild type were used in the experiment.

6.4.3 The other salt-resistant gene expression, *AtSOS1* and *AtP5CS1*, in *35SCaMV-OsNUC1* and *rd29a-OsNUC1* plants

The *AtSOS1* and *AtP5CS* are known and well characterized (Kishor *et al*., 1995; Lui and Zhu, 1997; Strizhov *et al*., 1997; Liu and Zhu, 1998; Halfter *et al*., 2000; Liu *et al*., 2000; Shi *et al*, 2000; 2002a; 2002b; Qiu *et al*., 2002; Quintero *et al*., 2002; Szekely *et al*., 2008). In Arabidopsis, ion homeostasis is mediated mainly by the SOS signal pathway. *AtSOS1* encodes a plasma membrane Na^+/H^+ antiporter that play a critical role in sodium extrusion and in controlling long-distance Na^+ transport from the root to shoot (Shi *et al*., 2000; 2002a). On the other hand, *P5CS1* gene encodes Δ^1-pyrroline-5-carboxylate synthetase1, which known as the rate-limiting enzyme in the proline biosynthesis in *Arabidopsis thaliana* (Delauney and Verma, 1993; Hu *et al*., 1992; Kavi Kishor *et al*., 1995; Yoshiba *et al*., 1995; Igarashi *et al*., 1997; Hong *et al*., 2000). In any event, higher expression of stress responsive genes was an advantage to the plants under stress to improve plant tolerance (Shinozaki and Yamaguchi-Shinozaki, 1997; Strizhov *et al*., 1997; Zhu, 2002). As the involvement in salt resistant function of these two genes, they were chosen to use in this study in order to investigate the effects of the overexpression of *OsNUC1* on the other salt-resistant gene transcripts.

6.4.3.1 *35SCaMV-OsNUC1*

The *AtSOS1* expression level in all *35SCaMV-OsNUC1* transgenic plants was strongly increased 5 hrs of treatment of assay in both normal and salt-stress conditions (Figure 4.28, C and D). The *AtSOS1* gene expression pattern under both conditions was consistent with the *OsNUC1* expression (Figure 4.28, A and B) in overexpressed *OsNUC1* Arabidopsis. The up-regulation of *AtSOS1* may result in the higher salt resistant in the transgenic line. The highest to lowest *OsNUC1* gene expression are as followed 35S-7, 35S-2, and 35S-5, respectively. This is consistent with the level of the transcription detected earlier. This indicated that the increasing of *OsNUC1* gene expression increase *AtSOS1* gene expression under salt stress.

Surprisingly, the *AtP5CS1* gene expression pattern during salt stress in transgenic *35SCaMV*:*OsNUC1* was similar to the expression pattern of *AtSOS1*. Under salt stress, the expression of *AtP5CS1* gene in transgenic was approximately 30 folds higher than that in wild type (Figure 4.28 F). It was obviously shown that the increasing of *OsNUC1* in transgenic Arabidopsis was strongly affected on the up-regulation of endogenous *AtSOS1* and *AtP5CS1* in both conditions. With respect to the function of RRM motif and GAR domain in *OsNUC1* on salt tolerance, this revealed that the up-regulation of *OsNUC1* could promote other salt-resistant genes.

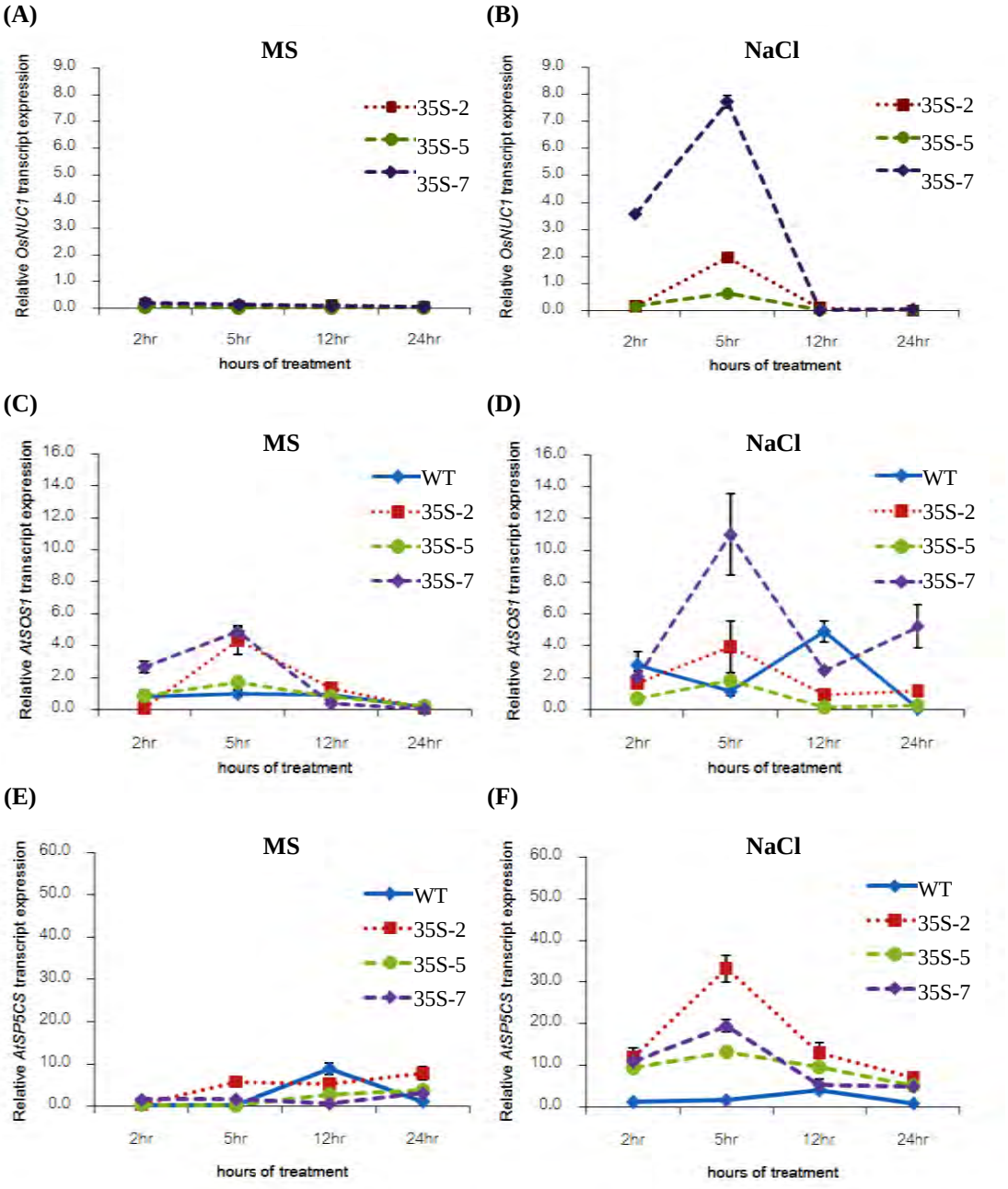


Figure 4.28 Expression pattern of *OsNUC1* (A and B), *AtSOS1* (C and D) and *AtP5CS* (E and F) in the *35SCaMV-OsNUC1* overexpressors during normal (A, C and E) and salt-stress (B, D and F) conditions. Three independent transgenic Arabidopsis and wild type were used in the experiment.

6.4.3.2 *rd29a-OsNUC1*

The stress-inducible *rd29a* promoter for the regulation of *OsNUC1* optimized the effects on the gene expression of the other salt responsive genes. The expression of *OsNUC1* regulated by stress-inducible *rd29a* promoter show the up-regulation of these two stress-responsive genes on the second time point of salt-stress treatment (Figure 4.29, B and D). Stronger *AtP5CS* expression was detected in the *rd29a-OsNUC1* plants under stress condition (Figure 4.29 D). However, in *rd29a-OsNUC1* Arabidopsis plants, both genes were weakly expressed under the normal condition. The expression level of these induced genes in the transgenics was higher than that in the control plants. As the peaked of *OsNUC1* transcript level in *rd29a-OsNUC1* was shown at twenty-four hour of treatment (Figure 4.25 B).

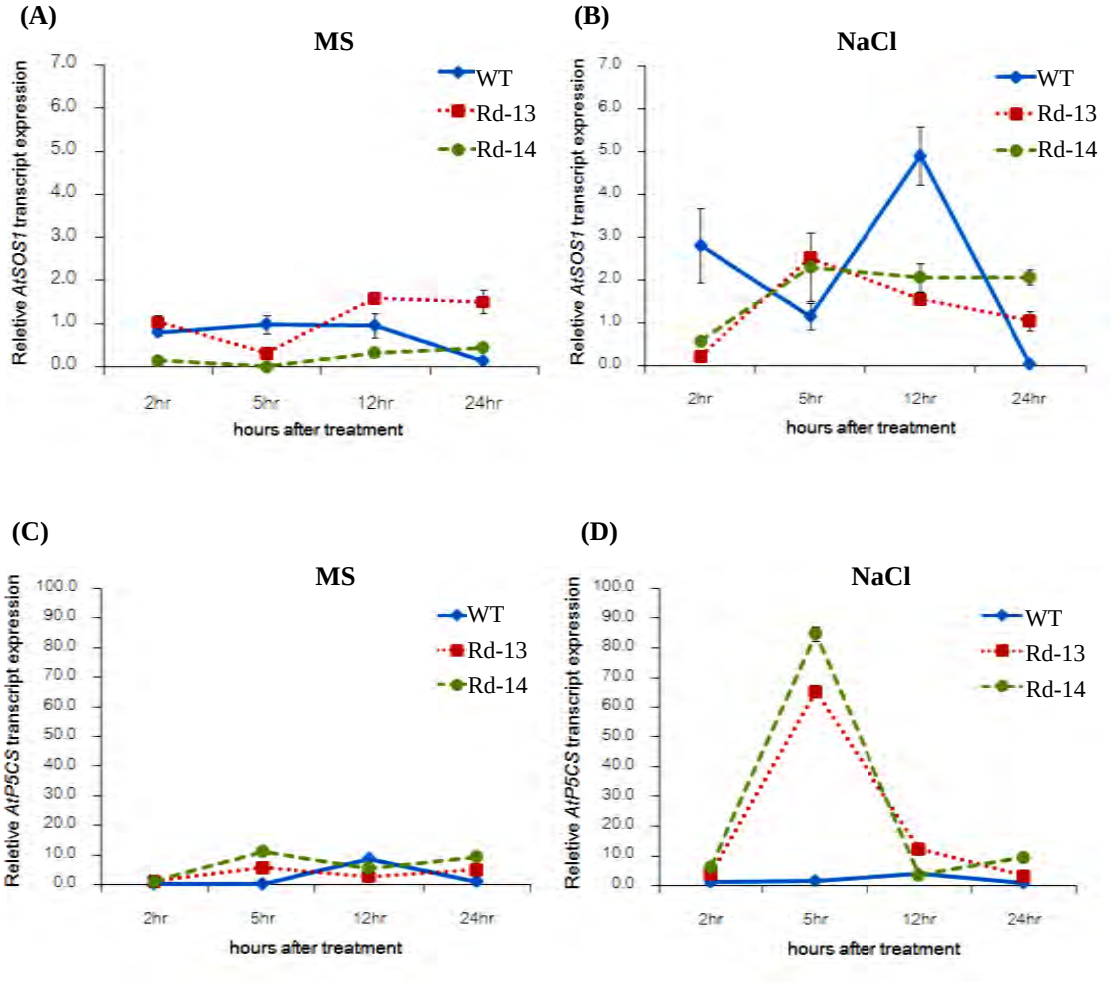


Figure 4.29 Expression pattern of *AtSOS1* (A and B) and *AtP5CS* (C and D) in the *rd29a-OsNUC1* overexpressors during normal (A-C) and salt-stress (B-D) conditions. Two independent transgenic Arabidopsis and wild type were used in the experiment.

7. The effect of exogenous ABA and abamineSG application on *OsP5CS1* gene expression during normal and salt-stress conditions.

Under salinity stress, the plants will produce a series of reactions and synthesize important metabolic materials to resist stress. Because *OsP5CS1* is required in proline biosynthesis pathway to synthesize proline which is an important osmoprotectant to protect against the damage caused by stresses (Hong *et al*., 2000), thereby contributing to the *OsP5CS1* gene function in proline biosynthesis has held much interest. Based on previous reports, where exogenous ABA treatment increased *P5CS* transcript levels in *Arabidopsis thaliana* (Strizhov *et al*., 1997; Yoshiba *et al*., 1999; Abraham *et al*., 2003), a similar differential expression pattern between the two *P5CS* homologues has been reported (Strizhov *et al.*, 1997; Yoshiba *et al*., 1999; Abrahan *et al*., 2003) and ABA-independent. ABA-dependent regulations of proline biosynthesis following cold and osmotic stress have been characterized (Savoure *et al*., 1997). The transcript levels of rice *P5CS* genes, *OsP5CS1* and *OsP5CS2* were also shown to strongly correlate with proline accumulation during salt stress in three rice cultivars that differed in their salt and drought tolerance levels (Hien *et al*., 2003); however, drought, cold and salt stress, in addition to exogenous ABA application, induce *OsP5CS1* gene expression in rice (Hur *et al*., 2004; Igarashi *et al*., 1997). The two homologues show different tissue localization and kinetic responses. Rice treated with NaCl or ABA showed a higher level of *OsP5CS1* transcript expression than that of *OsP5CS2* (Hur *et al*., 2004). Thus we considered the possibility that *OsP5CS1* might be regulated by ABA in rice, as a required prelude to evaluate such, the objective of the present investigation was to establish the relationship between *OsP5CS1* gene expression and ABA function.

To test the effect of exogenous ABA application, three-leaf stage rice seedlings were treated with 100μM ABA spraying and grown on the WP No.2 medium or WP No.2 medium containing 0.5% NaCl. The 0μM ABA or buffer spraying was used as control. The expression level of this gene was determined by using quantitative real-time PCR at different time points of treatment (0, 3, 6 and 9 days). *OsActin* was used for the internal control.

7.1 The effect of exogenous ABA on *OsP5CS1* transcript expression in LPT123 and LPT123-TC171 under normal condition.

After three days of exogenous ABA application, significantly increases in the *OsP5CS1* transcript levels were clearly seen in the two week-old seedlings of both the salt-sensitive and the salt-tolerant rice lines compared to those grown under normal conditions (Figure 4.30). However, the numerically higher induced *OsP5CS1* transcript level seen in the LPT123 cultivar than those in the LPT123-TC171 line was not statistically significant. After six days the transcript levels of the LPT123 cultivar decreased to the same level as those seen from the plants without ABA application (Figure 4.30 A), whereas in the salt-tolerant LPT123-TC171 line the transcript levels were further and significantly increased up to two folds higher than those on day 3 (Figure 4.30 B). In both of these indica rice lines the *OsP5CS1* transcript levels decreased close the basal levels after 9 days of treatment. However, the gene expression level was still significantly higher than the normal level. Thus, *OsP5CS1* appears to be the ABA-inducible gene.

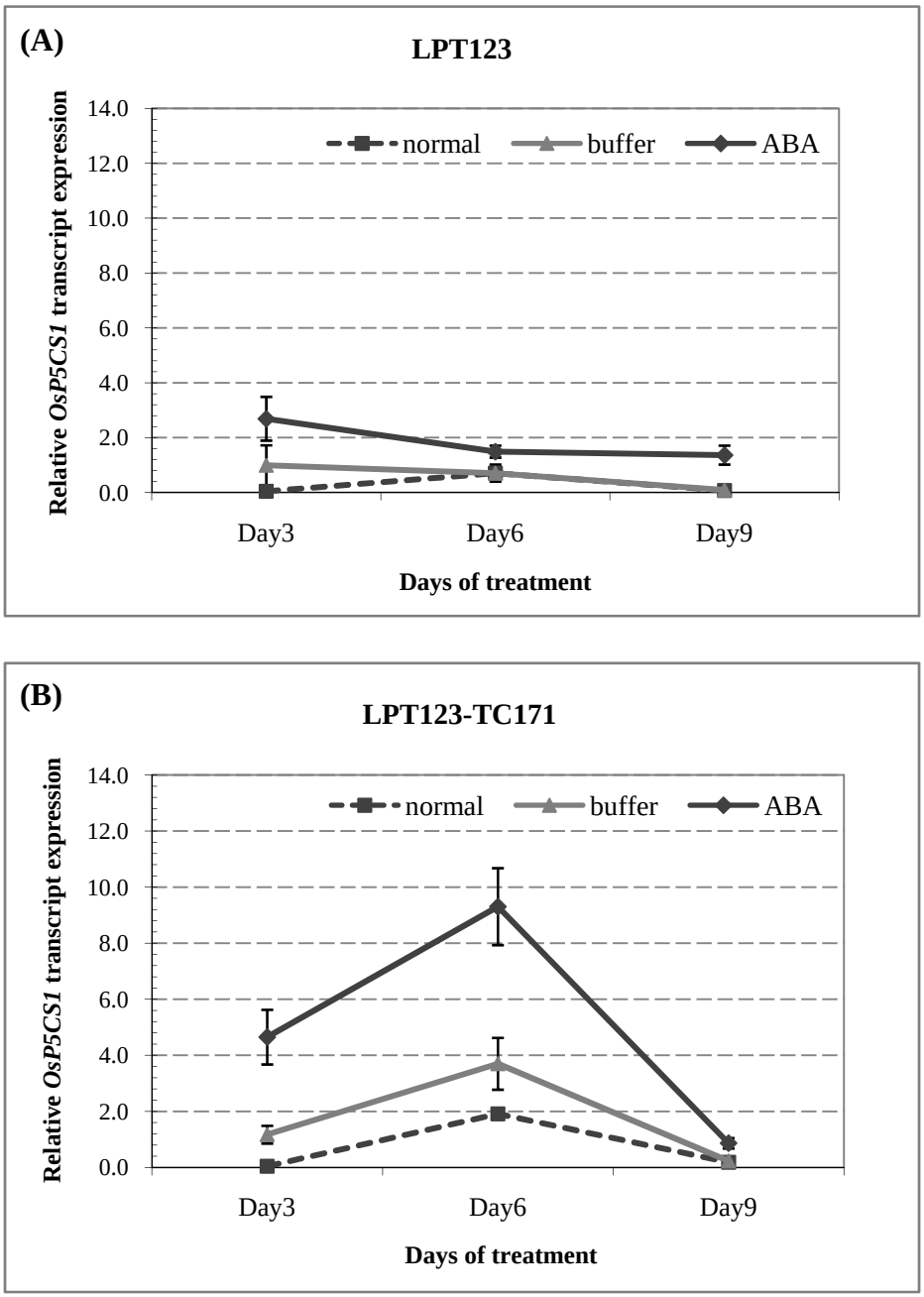


Figure 4.30 The effects of exogenous 100µM ABA on *OsP5CS1* transcript expression levels in the salt-sensitive (LPT123) (A) and salt-resistant (LPT123-TC171) (B) indica rice lines grown under normal condition.

7.2 The effect of exogenous ABA on *OsP5CS1* transcript expression in LPT123 and LPT123-TC171 under salt stress.

Although salt stress was found to induce the *OsP5CS1* transcript expression in both rice lines, the magnitude and kinetics markedly differed (Figure 4.31) from that induced by topical ABA application (Figure 4.30). A significant induction of *OsP5CS1* transcript levels by salt stress was clearly detected in both rice lines after three days of salt-stress treatment, with an almost two fold higher level being observed in the LPT123-TC171 line compared to that of the salt-sensitive cultivar (Figure 4.31). The expression levels declined thereafter, but the LPT123-TC171 line still showed highly elevated transcript levels after six days compared to the more rapid decrease back towards basal levels that was observed in the LPT123 cultivar. Interestingly, given the proposed involvement of ABA as an inducer of *OsP5CS1* transcripts, the application of 100µM ABA onto seedlings markedly decreased the level of *OsP5CS1* transcripts induced by salt stress in both rice lines after three days of treatment, and numerically slightly (but not statistically significantly) thereafter at days six and nine for the LPT123 cultivar. However, after six days of exogenous ABA application together with salt stress, the *OsP5CS1* transcript levels appeared to be up-regulated in the salt-tolerant LPT123-TC171 line. After nine days of treatment, no significant effect of exogenous ABA application upon the *OsP5CS1* transcript levels was observed in both lines (Figure 4.31). These results indicate that the exogenous ABA application during salt stress in the LPT123 and LPT123-TC171 rice lines reduced and delayed, respectively, the salt stress-mediated induction of *OsP5CS1* gene expression.

The higher *OsP5CS1* transcript levels were detected in the salt-tolerant line during salt stress and the observed proline accumulation levels in leaves broadly followed this pattern showing a good correlation with the *OsP5CS1* transcript levels. This is consistent with that reported by Hien *et al*. (2003), who showed that a salt-tolerant rice cultivar also accumulated a higher level of proline. In contrast, they reported that comparable levels of *OsP5CS1* and *OsP5CS2* transcripts were found in their salt-sensitive and salt-tolerant rice cultivars. The different genetic backgrounds of the rice plants under investigation between, as well as within the Hien *et al*. study,

are likely to affect the pattern of salt stress responses. The two indica rice cultivars used in this study, LPT123 and LPT123-TC171, have a much greater similarity in their genetic background since the LPT123-TC171 is a salt-tolerant mutated line isolated from the LPT123 cultivar (Vajrabhaya and Vajrabhaya, 1991; Thikart *et al*., 2005), whereas the rice cultivars used by Hein *et al*. (2003) likely had different genetic backgrounds between the salt-resistant and salt-tolerant lines used. If we accept this hypothesis for now, then the level of acquired proline may result from the overall outcome of different pathways in plants. The different sensitivity of the *OsP5CS1* transcript expression in response to salt stress, and the different sensitivity to ABA induction of *OsP5CS1* during salt stress, in the two rice lines used in this study (LPT123-TC171 and LPT123), together suggest that the salt tolerance of the LPT123-TC171 rice line may be caused by a mutation in the ABA signaling pathway, which includes the receptors and the signal transduction pathways that function to perceive ABA (Hirayama and Shinozaki, 2007). If that is so, then these rice lines may be useful for analysis of such receptors and pathways in the future. It is possibly that the synthesis of macromolecules likes proline for cell growth was inhibited by salinity; the exogenous ABA treatment could partially overcome this inhibition. The improved biosynthesis of amino acids and other organic solutes might be an efficient way for osmotic adjustment, leading to the better water status in seedlings that undergo exogenous ABA treatment.

However, surprisingly, the *OsP5CS1* expression pattern in the cotreatment of exogenous ABA treatment and salt stress was not comparable with proline accumulation in rice seedlings as reported by Klomsakul (2004). Proline content was considerably increased from six days of treatment onwards reaching, within the 12-day period assayed, a potential maximum after 9 (LPT123) or 12 (LPT123-TC171) days. In contrast, the *OsP5CS1* transcript reached the maximum on 6-day of assay and back nearly to the basal level on day 9 of treatment. These results would indicate that the *OsP5CS1* gene was not the key regulator gene in proline biosynthesis when exogenous ABA was applied under salt stress, thereby, the determination to find out another gene in proline biosynthesis pathway had held much interest.

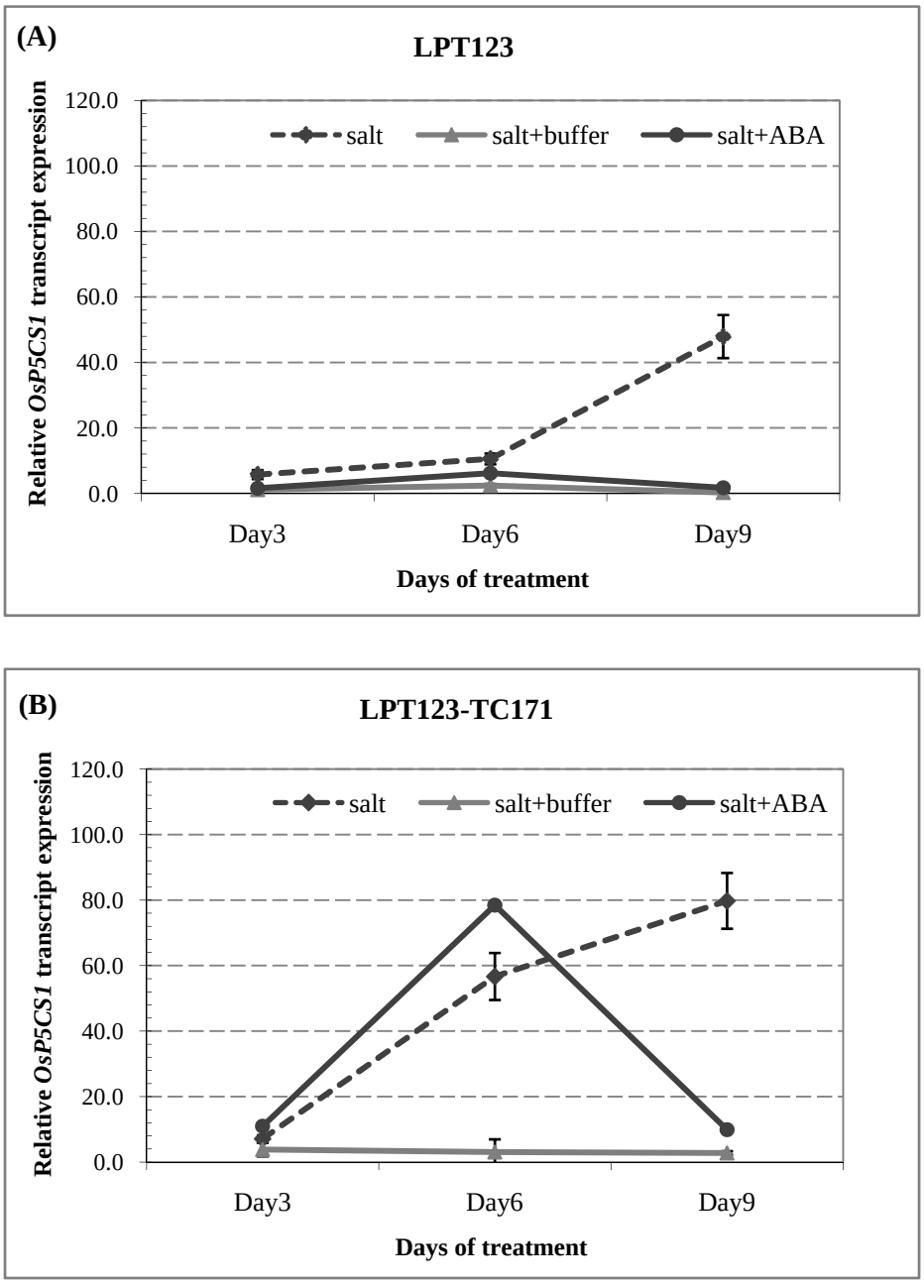


Figure 4.31 The effects of salt stress and exogenous ABA application during salt stress on *OsP5CS1* transcript expression levels in the salt-sensitive LPT123 and salt-resistant LPT123-TC171 indica rice cultivars.

7.3 The effect of exogenous ABA on *OsP5CS1* transcript expression in KDML105 and FL530-IL under normal condition.

After six days of exogenous ABA application, slightly decreased in the *OsP5CS1* transcript levels were clearly seen in both the salt-sensitive and the salt-tolerant rice lines compared to those grown under normal conditions. When after nine days the transcript levels had strongly induced in the salt-tolerant line by exogenous ABA (Figure 4.32). Therefore, relative to the salt-sensitive line, the salt-tolerant line appears to have a higher induction level, and a more induced response, of elevated *OsP5CS1* transcript levels. This data could support the notion that *OsP5CS1* is an ABA-inducible gene.

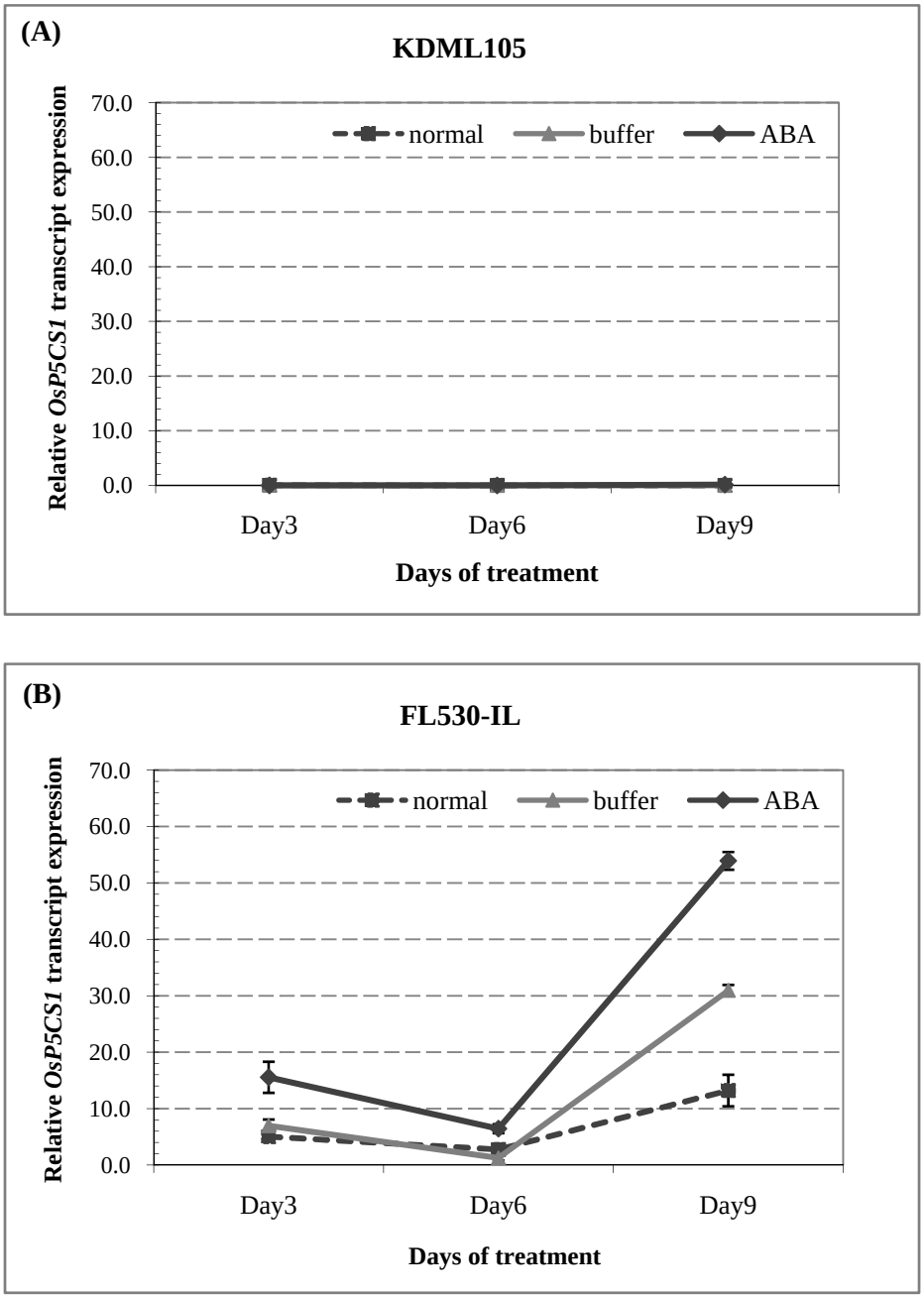


Figure 4.32 The effects of exogenous 100µM ABA on *OsP5CS1* transcript expression levels in the salt-sensitive (KDML105) (A) and salt-resistant (FL530-IL) (B) indica rice lines grown under normal condition.

7.4 The effect of exogenous ABA on *OsP5CS1* transcript expression in KDML105 and FL530-IL under salt stress.

A significant induction of *OsP5CS1* transcript levels by salt stress was clearly detected in both rice lines after six days of salt-stress treatment, with a strongly increased in the salt-resistant line on day9 of salt-stress treatment, about six fold higher level being observed in comparing with that of the salt-sensitive cultivar (Figure 4.33). Interestingly, in salt-resistant cultivar the kinetic of *OsP5CS1* transcript level was observed in co-treatment of salt-stress and exogenous ABA application, and they also induced the highest elevation of *OsP5CS1* transcript expression after nine days of treatment (Figure 4.33). This result confirmed the function of this gene on salt stress in related to the ABA signaling.

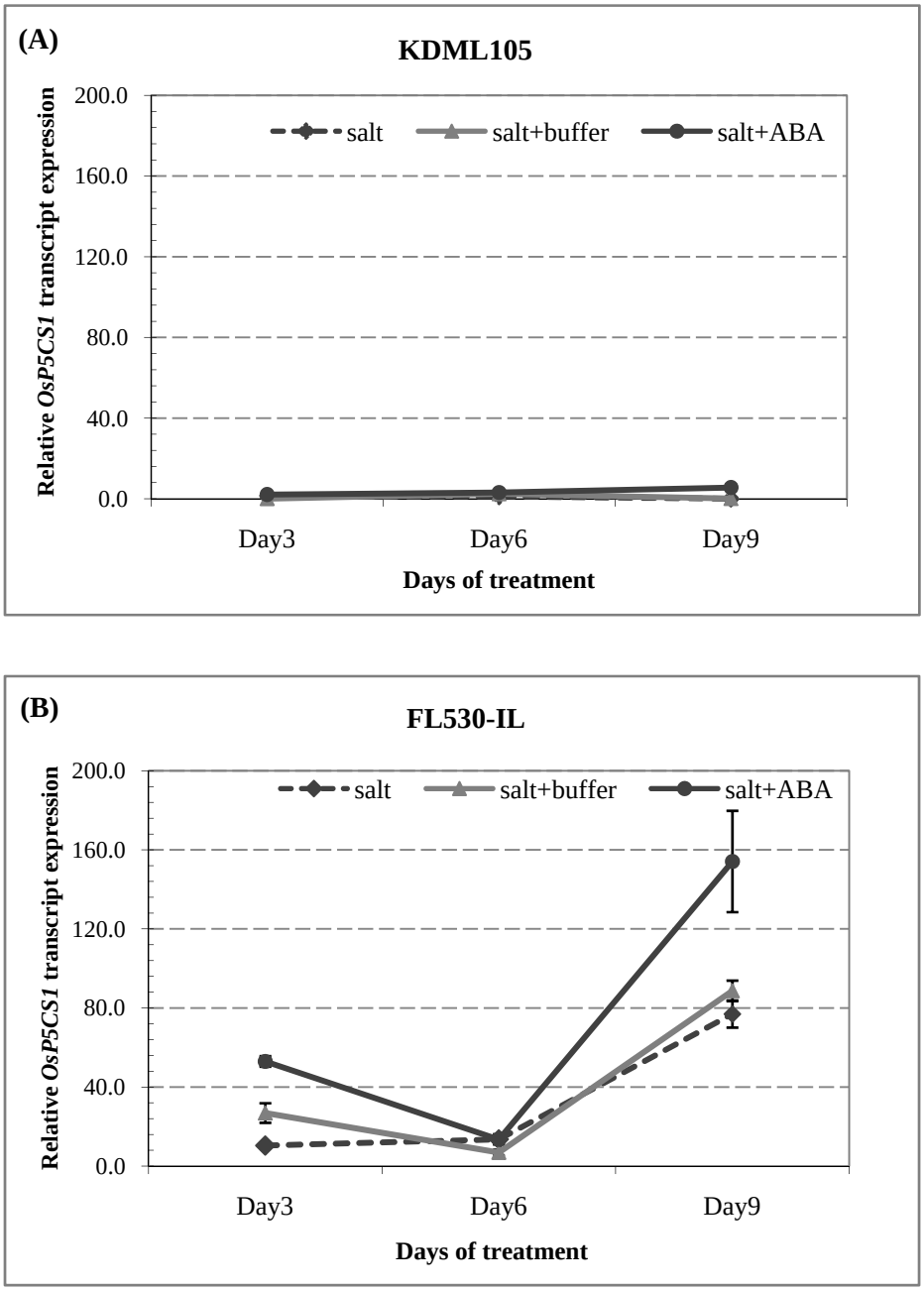


Figure 4.33 The effects of salt stress and exogenous ABA application during salt stress on *OsP5CS1* transcript expression levels in the salt-sensitive KDML105 and salt-resistant FL530-IL indica rice cultivars.

7.5 The effect of ABA biosynthesis inhibitor (abamineSG) on *OsP5CS1* transcript expression in LPT123 and LPT123-TC171

OsP5CS1 gene expression was reported to be directly related to salt-stress (Igarashi *et al*., 1997; Zhu *et al*., 1998; Choudhary *et al*., 2005) and ABA function (Hur *et al*., 2004) in *Oryza sativa* L., since it remains unknown for the ABA signaling pathway that *OsP5CS1* acts under salt-stress. The potential involvement of ABA in *OsP5CS1* gene expression was also investigated by the same method with the study in *OsNUC1* transcript expression as performed in the experiment 5, which is the study on the effect of ABA biosynthesis inhibitor application on the gene expression level.

After only two days of salt stress, the *OsP5CS1* transcript levels were increased up to eight fold higher in both rice lines, compared to the level in normal condition (Figure 4.34). Interestingly, the salt-stress-induced elevation (e.g. two days after treatment) of the *OsP5CS1* transcript expression was reduced by the administration of abamineSG (Figure 4.34). Moreover, this inhibition was more marked in the salt-resistant line. Taken together, these data support the involvement of ABA in the control of *OsP5CS1* gene expression in both rice lines, even though the two lines exhibited slightly different responses to ABA. Additionally, this result is the analysis of the putative promoter region of the *OsP5CS1* gene in Indica rice. The ABA responsive element (ABRE) (Marcotte *et al*., 1989) was found in the promoter region and it likely binds transcription factors involving in the ABA-regulated gene during salt stress. Therefore, taken all together, we suggest that the salt-stress-induced *OsP5CS1* transcript expression in rice is controlled, at least in part, by an ABA-dependent pathway (Bray *et al*., 2000; Zhu, 2002).

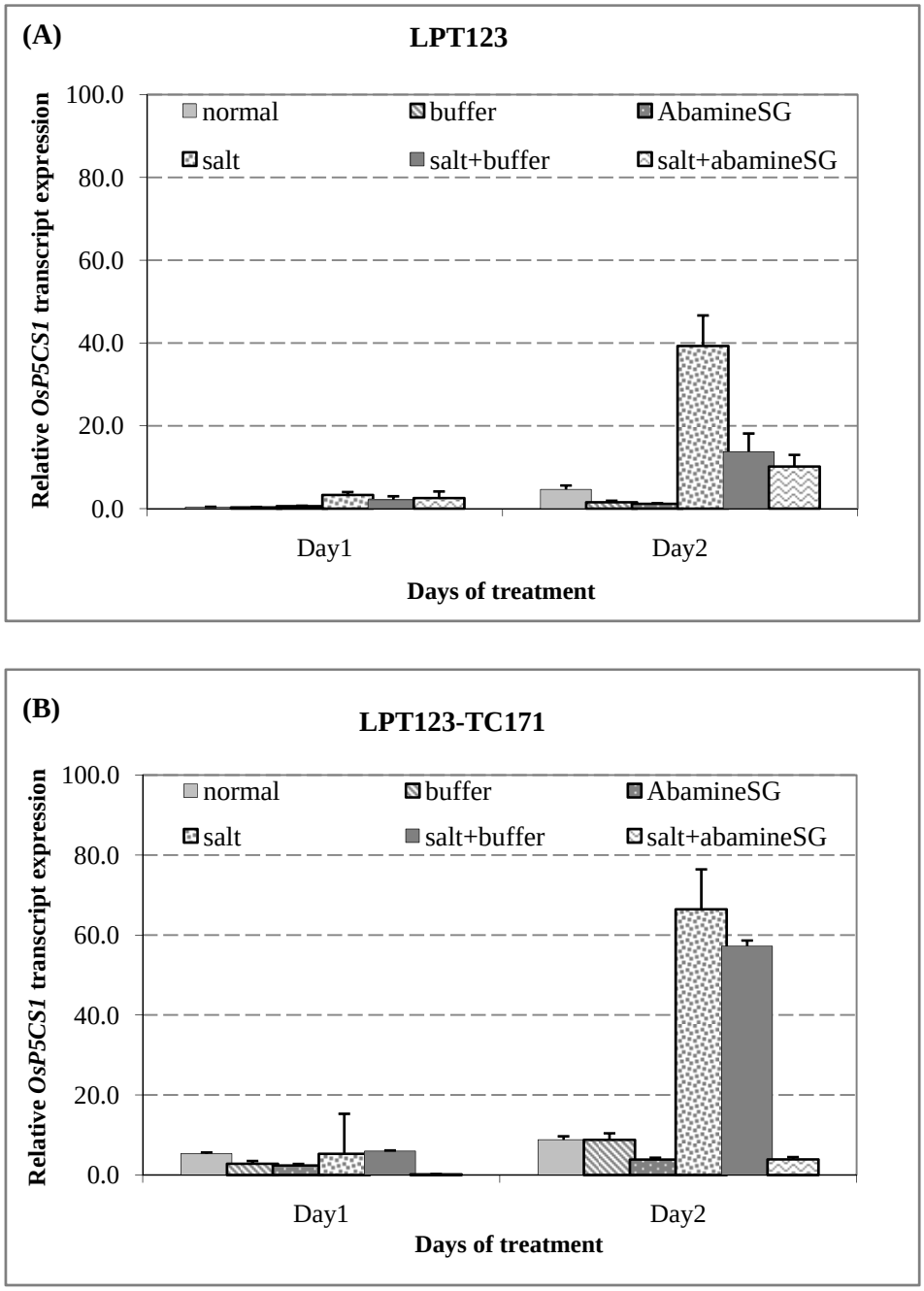


Figure 4.34 The effects of abamineSG application on *OsP5CS1* transcript expression levels in salt-sensitive (LPT123) (A) and salt-resistant (LPT123-TC171) (B) lines during grown under normal and salt stress (NaCl) conditions.

7.6 The effect of ABA biosynthesis inhibitor (abamineSG) on *OsP5CS1* transcript expression in KDML105 and FL530-IL.

Salt stress was also found to induce the *OsP5CS1* gene expression in both these two rice lines. After two days of salt stress, the *OsP5CS1* transcript levels were increase to two folds higher than the level of the transcript in normal condition (Figure 4.35). The salt-stress-induced *OsP5CS1* gene expression was significantly reduced by the application of abamineSG under both normal and salt-stress conditions. The decreasing in *OsP5CS1* expression levels by ABA biosynthetic inhibitor was consistent with that of Leung Pra Tew rice cultivars, which supports the involvement of ABA in the control of *OsP5CS1* gene expression in salt-sensitive and salt-resistant rice lines.

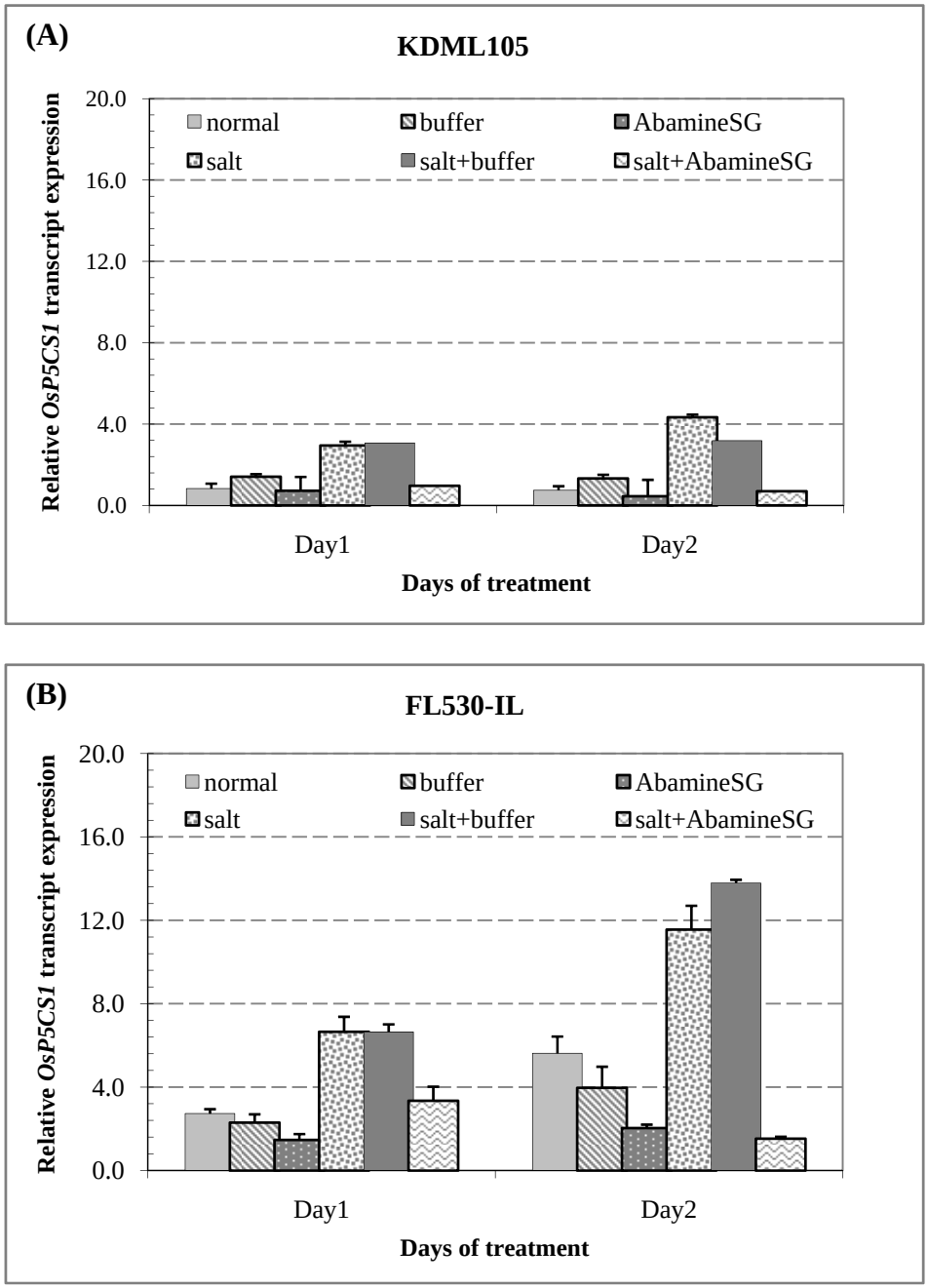


Figure 4.35 The effects of abamineSG application on *OsP5CS1* transcript expression levels in salt-sensitive (KDML105) (A) and salt-resistant (FL530-IL) (B) lines during grown under normal and salt stress (NaCl) conditions.

8. The effect of exogenous ABA on *OsP5CR* gene expression during salt-stress condition.

The exogenous ABA application had an effect on proline accumulation in salt-treated rice seedlings resulting in salt tolerant enhancement. When 100µM ABA was topically applied to the salt-treated seedlings, the kinetics and magnitude of the increased proline content was changed with a significant increase in proline levels, which was detected within three days after treatment in both rice lines. The proline content in the LPT123-TC171 line was, however, 100% higher than that in the LPT123 cultivar (Khomsakul, 2004). Whereas, the exogenous ABA did not clearly up-regulate *OsP5CS1* transcript levels in both rice lines examined, in contrast to the proline accumulation pattern found, we further investigated if any other genes in the proline synthesis pathway were up-regulated by topical ABA application. Under osmotic stress, only Arabidopsis *P5CS*, but not *AtP5CR*, gene expression was well correlated with proline content (Yoshiba *et al*., 1995; Savouré *et al*., 1997). In contrast, in maize and wheat the activity of P5CR during water stress was increased in correlation with the accumulation of proline, whilst increased Ca^{2+} levels also led to the elevated accumulation of proline in both plant species (Nayyar, 2003). So, this is a great idea to search for the *OsP5CR* regulation if it acts as the key regulator gene in proline biosynthesis when the exogenous ABA applied.

Here, the *OsP5CR* expression pattern when rice seedlings get the exogenous ABA together with salt stress was determined. Two rice cultivars, salt-sensitive (LPT123) and salt-resistant (LPT123-TC171) line, were germinated on normal WP No.2 solution and then treated with salt stress and together with 100µM exogenous ABA application. Leaf tissues were harvested at different time points (0, 3, 6 and 9 days after treatment) to be used for gene expression analysis via RT-PCR technique.

Salt stress induced *OsP5CR* gene expression and exogenous ABA application additionally further up-regulated the *OsP5CR* transcript levels in both the LPT123 and LPT123-TC171 rice lines after day 3 and 6 after treatment (Figure 4.36). However, after nine days, the levels of the *OsP5CR* transcripts in both rice lines were comparable to those of the controls. The up-regulation of this gene on three and six

days of exogenous ABA treatment suggesting that this gene may be responsible for the higher level of proline accumulation when the exogenous ABA was applied. This suggests that proline accumulation in the LPT123 rice cultivar does not directly result from the up-regulation of *OsP5CS1* expression, but rather is due to the induction of *OsP5CR* gene expression.

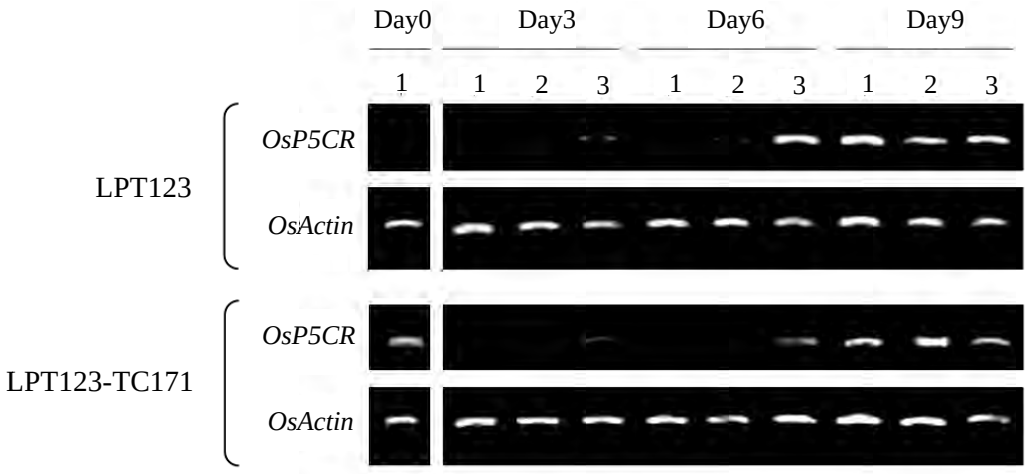


Figure 4.36 The effects of exogenous ABA treatment prior to salt-stress condition (3) on the *OsP5CR* gene expression levels, in comparison of the expression of the gene during normal (1) and salt-stress (0.5% NaCl) (2) conditions. The *OsActin* gene was used as the internal control of equal loading of the mRNA.

9. The effect of calmodulin antagonist on proline accumulation during salt-stress condition.

Recently, the exogenous ABA application was reported to be inducer of *OsCam1-1* gene expression in both rice lines during salt stress, and ABA can modify the perception and/or signal transduction involving *OsCam1-1* that may trigger salt-stress response in rice (Boonburapong, 2008). This evidence showed the possible study in the investigation if *OsCam1-1* plays a role in the proline accumulation during salt stress in rice. 100μM of W-7, the calmodulin inhibitor, was used as the strategy of this experiment to determine the change in proline accumulation level, when W-7 was applied during salt stress. Furthermore, in maize and wheat, the calmodulin

inhibitor (TFP) was shown to decrease both the P5CR activity and the proline content during water stress (Nayyar, 2003), which is consistent with our experiment that the calmodulin inhibitor (W-7) could inhibit proline accumulation in response to salt stress in both rice lines (Figure 4.38). It was clearly shown that a three- and 8.6-fold reduction in the proline content was found in the LPT123 and LPT123-TC171 rice lines, respectively, when W-7 was applied to the rice seedlings during salt stress (Figure 4.37).

An important factor in the battle between sodium and potassium ions is calcium. Increased calcium supply has a protective effect on plants under sodium stress. Calcium sustains potassium transport and potassium/ sodium selectivity in sodium-challenged plants. This beneficial effect of calcium is mediated through an intracellular signaling pathway that regulates the expression and activity of potassium and sodium transporters. Calcium may also directly suppress sodium import mediated by nonselective cation channels.

With regard to the *OsCam1-1* gene expression, the salt-susceptible LPT123 cultivar failed to induce *OsCam1-1* gene expression after salt stress, whilst in the resistant LPT123-TC171 line it was significantly induced after 0.5 hour of the treatment. However, the *OsCam1-1* transcript levels reported in the LPT123 cultivar were different from those found in the Khoa Dawk Mali105 (KDML105) rice cultivar, where *OsCam1-1* was the major calmodulin transcript that was induced by salt stress (Phean-o-pas *et al*., 2005). Perhaps then the salt susceptibility of the LPT123 rice strain is due to the deficiency in salt-stress perception via the Ca^{2+} signal transduction cascade, as it had a lower level of *OsCam1-1* gene expression than the salt-resistant one.

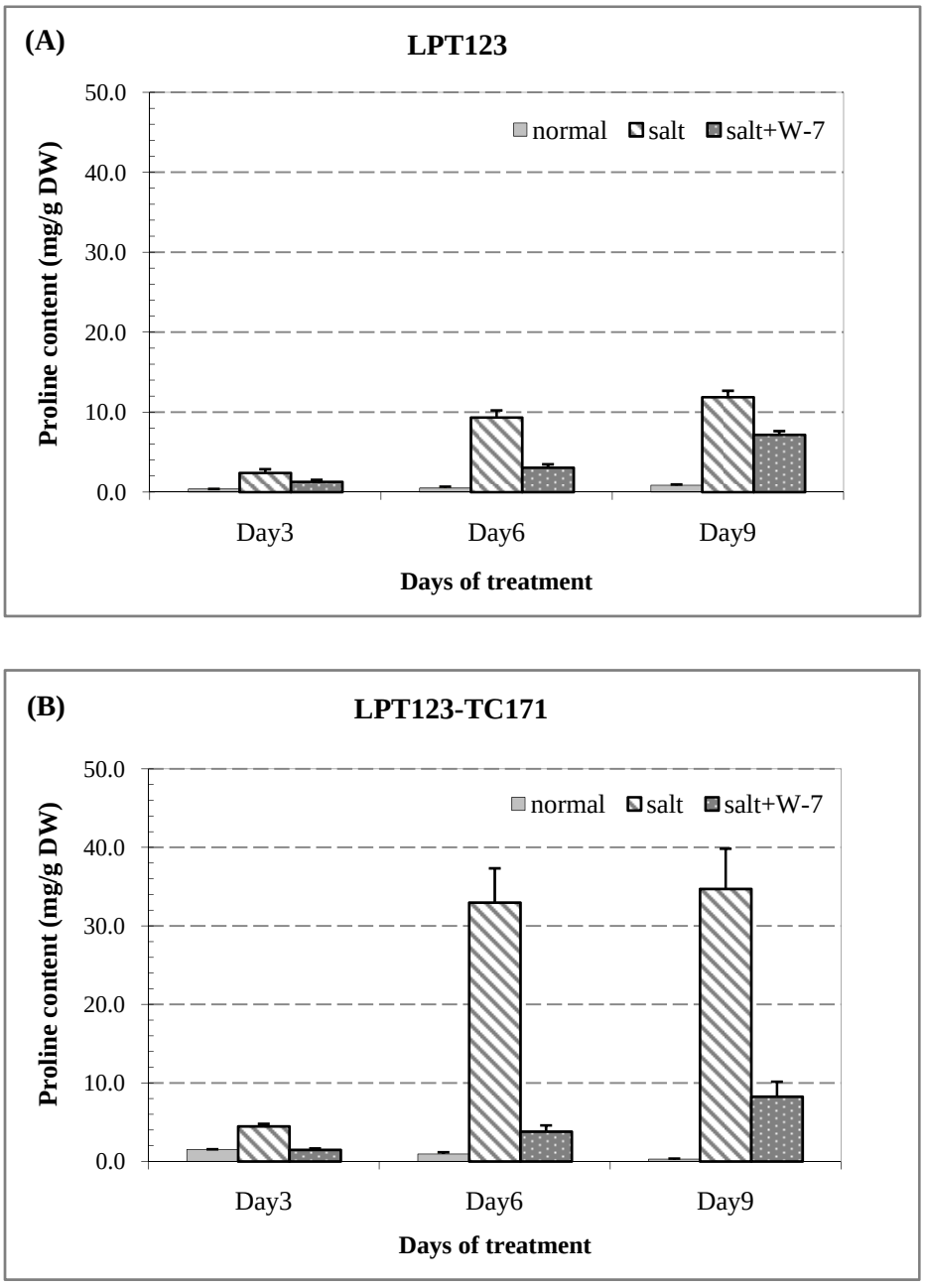


Figure 4.37 The effects of the calmodulin inhibitor, W-7, on proline accumulation in LPT123 (A) and LPT123-TC171 (B) rice cultivars.

CHAPTER V

CONCLUSION

1. Two homologues of rice *nucleolin* (*OsNUC*) genes, *OsNUC1* and *OsNUC2*, are located in different chromosomes based on RAP-DB. The result from blastP analysis showed that the novel salt-responsive gene (accession no. DQ012289) in rice has the highest similarity with shorter full-length cDNA of *OsNUC1* gene (accession no. AK063918) which encodes two RNA recognition motifs (RRMs) and Glycine- and Arginine-rich (GAR) repeat segment as a unique feature in the central and carboxyl-terminal domains. Whereas, the full-length *OsNUC1* contains three main domains: an amino-terminal domain rich in acidic residues and bipartite NLS, in addition to RRM and GAR at central and C-terminal domains.

2. The fluorescent product of the full-length OsNUC1 showed the localization only in the nucleus with some special bright spots, this resulted from the function of the bipartite NLS in the amino-terminal domain that is responsible for targeting nucleolin to the nucleus. Whereas, the fluorescent protein product of the partial OsNUC1 consisting of RRM and GAR domain, showed the localization in the nucleus and the cytoplasm. Moreover, some special bright spots were also detected in the nucleus. This suggests that only the RRMs and GAR domain of *OsNUC1* may be enough for nuclear localization. Additionally, some bright spots suggest the binding of RRM domain to RNA in the nucleus as no bright spots in nucleus was found for N-terminal OsNUC1 lacking RRM and GAR domain. However, the fluorescent protein was localized only in the nucleus.

3. *OsNUC1* is expressed in leaf blade, leaf sheath, roots, seeds, and flowers. It is abundant in roots and flowers, which suggests that the role of *OsNUC1* may involve cell growth and differentiation. Salt stress induces the up-regulation of *OsNUC1* in all four rice lines/cultivars tested, LPT123, LPT123-TC171, KDML105, and FL530-IL, and the

salt resistant lines show the higher level of gene expression than the salt sensitive lines with the similar genetic background.

4. ABA signaling is not required for *OsNUC1* regulation. However, exogenous ABA can induce *OsNUC1* gene expression.

5. The RRMs and GAR domains of *OsNUC1* play an important role on plant growth, and contribute to salt-stress resistant character in plants.

6. The over-expression of RRMs and GAR domains of *OsNUC1* in transgenic Arabidopsis promotes the elevation of endogenous salt-responsive genes, such as *SOS1* and *P5CS1*. One may speculate that nucleolin promotes the other salt stress-resistant gene expression in the nucleus. It may also involve in stabilization of mRNA of the other salt-resistant genes and regulate RNA processing in the cytoplasm.

7. The high expression of *OsP5CS1* during salt stress was clearly observed in all four indica rice cultivars, supporting the role of *OsP5CS1* on salt-tolerant mechanism.

8. ABA is involved in *OsP5CS1* transcript induction, as exogenous ABA application induced *OsP5CS1* expression under normal growth condition, whilst application of the ABA biosynthetic inhibitor, abamineSG, decreased *OsP5CS1* levels during salt stress. These indicate that the *OsP5CS1* regulation in salt-stress response depends on ABA signaling.

9. When the exogenous ABA is used to induce salt resistance, the expression pattern of *OsP5CS1* was not consistent with proline accumulation levels as reported by Khomsakul (2004). We hypothesized that under exogenous ABA treatment, *OsP5CS1* is not the key regulator gene on proline biosynthesis. However, *OsP5CR* is responsible for the higher level of proline accumulation in plant when topical ABA is applied to salt-stressed plants.

10. Under salt exposure, the inhibition on calmodulin leads to lower level of proline accumulation.

Suggestions for further studies

1. The genomic DNA sequence and/ cDNA sequence of *OsNUC1* in both salt-sensitive (LPT123) and salt-resistant (LPT123-TC171) rice lines should be analyzed to compare the promoter and coding sequences.

2. The complementation test of *OsNUC1* gene in the *NUC1* mutant should be performed to confirm nucleolin function of *OsNUC1*.

3. The overexpression of *OsNUCI* in the salt sensitive rice lines to confirm the salt resistant function of *OsNUC1*.

4. The location of each OsNUC1domain at subcellular level should be investigated to interpret the appropriate function (s) of the gene.

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APPENDICES

APPENDIX A

Chemical solution

1. MS complete medium (Murashige and Skoog, 1962)

Inorganic salts

NH_4NO_3	Ammonium nitrate	1650.0 mg
KNO_3	Potassium nitrate	1900.0 mg
KH_2PO_4	Potassium Phosphate dibasic anhydrous	180.0 mg
$MgSO_4.7H_2O$	Magnesium sulfate	380.0 mg
$CaCl_2.2H_2O$	Calcium chloride dihydrate	440.0 mg
$MnSO_4$-$4H_2O$	Magnesium sulfate	22.3 mg
$FeSO_4.7H_2O$	Ferrous sulfate	27.8 mg
Na_2.EDTA	Disodium ethylenediaminetetraacetate	37.3 mg
$Zn_4.7H_2O$	Zinc sulfate	8.6 mg
H_2BO_3	Boric acid	6.2 mg
KI	Potassium iodide	0.83 mg
$CuSO_4.5H_2O$	Cupric sulfate	0.0025 mg
$Na_2MoO_4.2H_2O$	Sodium molybdate	0.25 mg
$CoCl_2.6H_2O$	Cobalt chloride	0.025 mg

Organic constituents

Sucrose	30.0 mg
Glycine	2.0 mg
Myo-Inositol	100.0 mg
Nicotinine acid	0.5 mg
Pyridoxine	0.5 mg
Thiamine.HCl	0.1 mg
Agar	8.0 gm

2. Southern blotting

2.1 Depurination solution:

HCl	40 ml (0.2N)
dd H_2O	960 ml

2.2 Denaturation solution:

NaCl	21.9 g (1.5 M)
NaOH	5 g (0.5 N)
ddH_2O	200 ml

Adjust the volume to 250 ml with distilled water.

2.3 Neutralization solution:

Tris base	30.3 g (0.5 M)
NaCl	87.6 g (3 M)
ddH_2O	400 ml

Adjust pH to 7.2 ($\pm$0.3) with HCl and adjust the volume to 500 ml with distilled water.

2.4 20X SSC:

NaCl	3 M
sodium acetate	0.3 M

2.5 Primary wash buffer with Urea

Urea	360 g
SDS	4 g
20x SSC	25 ml

Adjust the volume to 1 liter. This can be kept for up to 3 months in a refrigerator at 2-8^{o}C. Stringency may be increased by using a lower final SSC concentration, e.g., 0.1x SSC instead of 0.5x SSC.

2.6 Secondary wash buffer

SSC	2x

This can be kept for up to 3 months in a refrigerator at 2-8^{o}C.

3. Modified WP No.2 nutrient solution

3.1 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

3.2 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
H_2O	800 ml

Stir with a magnetic stirrer, add 2 ml of $FeSO_4.7H_2O$ and adjust the volume to 1 L with water.

[a] **Preparation of 30 g/L $FeSO_4$ stock**

Na_2EDTA	40 g
$FeSO_4.7H_2O$	30 g

Stir each chemical solution with a magnetic stirrer and adjust the volume to 1 L with water

4. LB Medium (Luria-Bactani Medium)

: Per litre

To 950ml of deionized H_2O add:

bacto-tryptone	10 g
bacto-yeast extract	5 g
NaCl	10 g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5N NaOH. Adjust the volume of the solution to 1 litre with deionized H_2O.

LB Agar medium

- LB medium
- 1.5% LB Agar

5. Plasmid DNA extraction

8.1 Solution I:

Glucose	50 mM
Tris-HCl	25 mM
EDTA	10 mM

8.2 Solution II:

NaOH	0.2 N
SDS	1%

8.3 Solution III:

Potassium acetate	5 M
Glacial acetic acid	

6. Electrophoresis

6.1 5x TBE:

Tris base	54 g
Boric acid	27.5 g
EDTA pH 8.0	20 ml (0.5 M)

6.2 DNA loading dye and RNA loading dye for agarose gel:

Glycerol in water	30% (v/v)
Bromophenol blue	0.25% (w/v)
Xylene cyanol	0.25% (w/v)

7. 1mM Abscisic acid (ABA) stock

± Abscisic acid	5.27 mg
Ethanol	500 µl

Dissolve in a final volume of 20 ml ddH_2O

8. 0µM Abscisic acid (ABA) stock

Ethanol	500 µl

Dissolve in a final volume of 20 ml ddH_2O

9. RNA extraction buffer

Tris pH 9.0	100 mM
NaCl	100 mM
EDTA	20 mM
lauryl sarcosinate	1% (w/v)
2-mercaptoethanol	0.1% (v/v)
DEPC (diethyl pyrrocarbonate)	0.1% (v/v)

10. 80mM Calmodulin antagonist (W-7) stock

W-7	30 mg

Dissolve in a final volume of 1 ml ddH_2O

11. Acid ninhydrin

Ninhydrin	0.625 g
Glacial acetic acid	30 ml
6M Phosphoric acid	20 ml

The solution was heated on hot plate by setting of warm temperature with agitation, until dissolved. The reagent is prepared fresh each time it is required.

APPENDIX B

Protocol

1. Plant materials and growing conditions

1.1 Normal condition

The wild type rice (LPT123 and KDML105) and its derived salt-resistant lines, LPT123-TC171 (Thikart *et al*., 2005) and FL530-IL (Suriya-arunroj *et al*., 2004) (kindly provided by the utilization of rice gene laboratory, Kasetsart University, Khumpangsan, Thailand) were germinated on sand for a week. Then, they were grown in modified WP No.2 nutrient solution (Vajrabhaya and Vajrabhaya, 1991) in the greenhouse with natural light (93–99 μmol photon.m^{-2}.s^{-1}) and a relative humidity of between 74% and 81%. The nutrient solution was changed once a week during the experimental period.

1.2 Salt-stress condition

Rice seedlings at three-leaf stage were treated with 0.5% (w/v) NaCl by adding into the WP No.2 nutrient solution. The nutrient solution was changed once a week during the experimental period. The planting and salt-stress treatment in the WP nutrient solution and WP containing 0.5% NaCl was essentially the same as described previously (Udomchalothorn *et al*., 2009).

1.3 Tissue collection

Rice tissue was immediately frozen in liquid nitrogen after collection and kept at -80^{o}C.

2. Genomic DNA preparation

The plant materials were kept frozen at -80^{o}C. Rice leaf tissues were ground to a fine powder in liquid nitrogen using chilled mortars and pestles. Then, the ground tissues were added into 800μl of hot CTAB buffer (60^{o}C) and 3μl of β-mercaptoethanol. After that, the mixture was inverted several

times to mix and incubated at 60^{o}C for 30 minutes, and inverted gently every 10 minutes. Afterward, the mixture was left at room temperature for 5 minutes. Then, 500µl of chloroform: isoamylalcohol (24:1) was added and mixed gently for 5 minutes. The mixture was centrifuged at 14,000rpm for 10 minutes at 4^{o}C. The upper aqueous phase was transferred to a fresh microcentrifuge tube. Genomic DNA was precipitated by the addition of 0.1 volumes of 3M NaOAc and 0.6 volumes of isopropanol. The suspension was mixed gently and allowed to stand for 30 minutes on ice (or at -20^{o}C). Then, the pellet was collected by centrifugation at 14,000rpm for 10 minutes at 4^{o}C. The pellet was allowed to dry in room temperature for 10-15 minutes. After that, the genomic DNA was resuspended with 100µl of TE buffer. The concentration was estimated by measuring the absorbance at 260 nm, and calculated in µg/ml unit, using the following equation:

$$[DNA] = A_{260} \times \text{dilution factor} \times 50^{*}$$

*The absorbance at 260nm (A_{260}) of 1.0 corresponds to the DNA of approximately 50µg/ml (Sambrook *et al*., 1989).

3. Agarose gel electrophoresis

DNA samples were mixed with 1x DNA loading buffer and loaded into the TBE agarose gel which the concentration depends on size of the DNA fragment to be prepared, generally use 0.8-1.5% agarose. Electrophoresis was carried out at constant 100 volts/cm until the tracking dye reached ¼ from the bottom of the gel. After electrophoresis, the gel was stained with ethidium bromide solution (5-10µg/ml in distilled water) for 3-5 minutes and destained with distilled water by gently shaking for 10 minutes to remove unbound ethidium bromide from agarose gel. The resolved DNA bands were visualized on an UV transilluminator and photographed.

4. Southern blot analysis

4.1 Southern blotting

After electrophoresis, the gel to be blotted was removed from the electrophoresis chamber and soaked in 5-10 gel volumes of denaturation solution for 20 minutes with gently agitation, then transferred to 5-10 gel volumes of depurination solution for 20 minutes. After depurination, the gel was submerged in 5-10 gel volumes of neutralization solution for 15 minutes with gentle agitation. Genomic DNA in the soaked gel was transferred to positively charged Hybond^{TM} nylon membrane. When transfer is complete, the nylon membrane was carefully removed from the gel by flat-tipped forceps. The DNA was immobilized by UV cross-linking in Bio-Rad GS Gene Linker^{TM} UV Chamber. The blot was stored at room temperature until hybridization.

4.2 Oligolabeling

ECL^{TM} direct nucleic acid labeling system was used for probe labeling, according to the manufacturer's protocol.

4.3 Hybridization and detection

Membrane was hybridized for 16 hours at 42^{o}C in gold hybridized buffer, containing 5% blocking reagent and 0.5M NaCl. Then, membrane was washed twice in primary wash buffer at 42^{o}C for 20 minutes and twice in secondary wash buffer (Appendix A) at room temperature for 5 minutes each times. Membrane was exposed on X-ray film for half an hour. After exposure, the X-ray film was developed and fixed with developer and fixer solution (Appendix A) respectively, to obtain the signal.

5. Gene cloning

5.1 PCR Amplification

Gene-specific primers were designed in order to amplify the specific cDNA fragments. Polymerase chain reaction (PCR) was performed in a 25µl *pfu* polymerase reaction mixture (Invitrogen, La Jolla, CA, USA) and carried out by using a PTC-100TM programmable thermal controller (MJ research, USA) in a 25µl *pfu* polymerase reaction mixture (Invitrogen, Lo Jolla, CA, USA). The thermal cycle used was as follows: 94^{o}C for 5min, than 40 cycles of 94^{o}C for 45sec, 61^{o}C for 1min and 72^{o}C for 3min, then 72^{o}C for 10min. PCR products were separated on agarose gel electrophoresis and visualized by ethidium bromide staining.

5.2 Extraction of DNA fragments from agarose gel

Extraction of DNA fragment from agarose gel was performed using StarPerp Gel Extraction Kit (GenStar Biosolutions Co., Ltd., China). After electrophoresis, the gel slide containing the desired DNA fragment was excised as gel slice from an agarose gel using a scalpel and transferred to a microcentrifuge tube. Three volumes of melting buffer (supplied by manufacturer) were added and incubated for 10 minutes at 65^{o}C or until the gel slice has completely dissolved. The gel mixture was vortexed every 2 to 3 minutes during the incubation period. The mixture was transferred into column and centrifuged at 12,000rpm for 1 minute. The flow through solution was discarded. Another 550µl of washing buffer (supplied by manufacturer) was added and centrifuged at 12,000rpm for 30 seconds. Washing step was repeated twice. The flow through solution was discarded. The column was centrifuged to remove a trace element of the washing solution. The column was placed into a new sterilized 1.5ml microcentrifuge tube. DNA was eluted by addition of 30µl of sterilized water to the center of the column and the column was let

stand for 1 minute, and then it was centrifuged at 12,000rpm for 1 minute to obtain the DNA solution for cloning.

5.3 Ligation

Before ligation, polyA was added at the ends of each DNA fragment, in order to ligate to the pGEM®-T vector (see in Appendix C). A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of insert used in ligation reaction, the following equation was used:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

The 10µl of ligation reaction was composed of 5µl of 2x ligase buffer, 3 weiss units of ligase, and the appropriate amount of the inserted fragment and water. The reaction was incubated at 16^{o}C overnight.

5.4 Bacterial Transformation

5.4.1 Preparation of competent *E. coli* cells

Competent *E. coli*, strain DH5α, was prepared according to the method of Sambrook *et al.* (1989). A single colony of *E.coli* strain DH5α was inoculated in 15ml LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) and incubated at 37^{o}C with shaking at 250rpm overnight. Then, 1ml of the starting culture was inoculated into 14.4 ml of LB broth and continued culture at 37^{o}C with shaking 250 rpm for 1.5 hours. Ten milliliters of the cells from previous step were inoculated into 90ml LB and it was incubated at 37^{o}C with shaking at 250 rpm for 2 hours until the optical density of the culture at the wavelength of 600 nm (OD600) reached 0.4-

0.6. The cells were chilled on ice for 10 minutes and harvested by centrifugation at 8,000rpm, 4^{o}C for 5 minutes. The supernatant was removed as much as possible. The cell pellets was resuspended with 500µl cold 0.1M $CaCl_2$ by gently mixing and then centrifuged at 8,000rpm, 4^{o}C for 15 minutes. The supernatant was discarded. Then, the cells were resuspended with ½ of original volume cold 0.1M $CaCl_2$, 20 ml of cold glycerol, followed by incubation on ice for 30 minutes and harvested by centrifugation at 8,000rpm, 4^{o}C for 7 minutes. Let the cell pellet completely dry on room temperature. Finally, added 1/20 of original volume 0.1M $CaCl_2$ for resuspension cells and then derived it into 100µl aliquots and stored at -80^{o}C for later used.

5.4.2 Transformation (Heat-shock method)

The competent cells were gently thawed on ice. The 100µl of competent cells were mixed thoroughly with 10µl of each ligation reaction, and then the mixture was placed on ice for 30 minutes. The cells were heat-shocked at 42^{o}C for 90 seconds, then, the tubes were immediately chilled on ice for 3-5 minutes. After that, 400µl of LB broth were added into mixture and mix gently. The cell suspension was incubated at 37^{o}C with vigorous agitation for an hour. Finally, this cell suspension was spread on the LB agar plates, containing 100µg/ml amplicilin, 4µl of 1M Iso-1-thio-β-D-thiogalacto-pyranoside (IPTG), and 16µl of 20mg/ml 5-Bromo-4-chloro-3-indole-β-D-galactopyranoside (X-gal). The plates were incubated at 37^{o}C overnight.

5.5 Sequencing Analysis

Before DNA sequencing, the half of selected clones carrying the specific DNA fragment were proved by PCR amplification and endonuclease enzyme digestion. The correct clones were grown in LB broth containing 100µg/ml of amplicilin and incubated overnight at 37^{o}C with shaking 250rpm overnight. The cell suspension was kept in glycerol and stored at -80^{o}C. The 200µl of stock cells were used for DNA sequencing which was carried out at Invitrogen Corporation, China.

5.6 Plasmid DNA isolation

The selected clones of each construct were grown in 10 ml of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) containing 100µg/ml of amplicilin and incubated overnight at 37^{o}C with shaking 250rpm. The cells were spinned in a microcentrifuge at 8,000rpm for 7 minutes. The cells were resuspended in 100µl of Lysis buffer (Solution I: 50mM of Glucose, 25mM of Tris-HCl and 10mM of EDTA) and mixed by vortex. Then added 200µl of Alkaline-SDS solution (Solution II: 0.2N NaOH and 1%SDS) and allowed to stand on ice for 10 minutes. After that 300µl of High salt solution (Solution III: 3M of potassium acetate) was added to the mixture. The suspension was mixed gently and allowed to stand for 10 minutes on ice. The insoluble salt-genomic DNA precipitate was then removed by centrifugation at 10,000rpm for 10 minutes. The supernatant was transferred to a fresh microcentrifuge tube and the nucleic acid was precipitated by adding 2 volumes of isopropanol. The sample was mixed thoroughly and immediately centrifuged for 30 minutes to collect the precipitated DNA. The pellet was resuspended in 50µl of sterile water and the suspension was vortexed gently. 10µl of 3M sodium acetate, pH7 and 300µl of cold absolute ethanol was added to the mixture. The mixture was mixed and chilled on 4^{o}C overnight. The DNA was collected by centrifuging at 14,000rpm for

20 minutes at 4^{o}C. The pellet was rinsed with 300µl of 70% ethanol and allowed to dry for 10-15 minutes. The plasmid DNA was resuspended with 50µl of sterile water. The concentration was estimated by comparing with DNA marker.

5.7 Restriction enzyme digestion

The corrected DNA fragments were released from the pGEM®-T vector by restriction endonuclease that was used to cut DNA based on specific binding property and cleaving double-stranded DNA at a specific sequence. In this experiment, double digestion was performed. Typically, a reaction contains about 0.5-1µg of DNA. In a final volume of 20µl containing 1x enzyme reaction buffer and 2-5 units of restiction enzyme. This digested cDNA fragments were analysed by agarose gel electrophoresis. After that, the digested cDNA fragments and vector were purified from gel, according to manufacturer's protocol (StarPerp Gel Extraction Kit, GenStar Biosolutions Co., Ltd., China).

5.8 Ligase DNA fragment and expression vector

Following digestion, the cDNA fragment of each construct with the overhangs was fused in the same frame of vector, which can be controlled under promoter. The purified DNA fragments were ligated to the vector by T4 ligation. A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of PCR product (insert) used in ligation reaction, the following equation was described on above. The 10µl of ligation reaction was composed of 5µl of 2x ligase buffer, 4 units of T4 DNA ligase (Toyobo, Japan), and the appropriate amount of the DNA fragment and vector. The reaction was incubated at 16^{o}C overnight.

5.9 Bacterial Transformation (Heat-shock method)

E.coli transformation was continued as described on 5.4. The transformant cells were selected on the LB agar plates with contained antibiotic and incubated at 37^{o}C overnight.

5.10 Fusion vector preparation

The half of selected clones carrying the specific DNA fragment were proved by PCR amplification and endonuclease enzyme digestion. The transformant cells were grown in selective medium for a night. The number of cells were increased in LB broth containing antibiotic and incubated overnight at 37^{o}C with shaking 250rpm for 14-16 hours. The cell suspension was kept in glycerol and stored at -80^{o}C.

6. Particle bombardment and Laser-scanning microscope

6.1 Onion tissue preparation

The outer papery layers and dry tip of the onion were removed. Split the onion lengthwise into quarters. Then, discard one or two innermost layers of leaves to expose the fleshy medium-sized leaves. Begin to separate the adaxial epidermis from the underlying tissue by using the point forceps pull it off completely. And then, place the strip onion epidermis on MS agar plate added 3% sucrose with the adaxial surface of the tissue face down. Make incisions as needed so that the epidermis makes good contact with the medium. The plates were kept in dark for 2-3 hours.

6.2 Preparation of gold particles

Each bombardment, consist of 34µl of gold particles, 14µl of 5µg plasmid DNA, 34µl of 2.5M calcium chloride and 14µl of 0.2M spermidine (free base), was mixed by vortex and incubated for 20 minutes on wet ice with occasional vortex. The mixture was added 200 µl of 80% ethanol and centrifuged at 13,000rpm for 20 seconds.

The supernatant was discarded, and then wash the pellet by 20-30µl of absolute ethanol and vortex vigorously. Then, 200µl of absolute ethanol was added into mixture. The mixture was centrifuged at 13,000rpm for 20 seconds and discarded the supernatant. The pellet was washed repeat 2-3 times. The washed particles were resuspended in 60µl of ethanol. The 12µl of suspension was spread onto macrocarrier and used for bombardment.

6.3 Bombardment

Transformation of each fragment in onion epidermal cells by particle bombardment (Bio-Rad) was performed according to manufacturer's protocol. The localization of fusion proteins was observed using a Leica laser-scanning confocal microscope (Leica Microsystem) after the incubation of the transformed cells on the MS medium added 3% sucrose at 25^{o}C in the dark for 18-30 hours. The GFP fluorescence was induced by excitation at 480nm with an argon laser.

7. RNA extraction

Total RNA was extracted by the standard hot-phenol method (Thikart *et al*., 2006). *Oryza sativa* L. tissues were ground in liquid nitrogen using chilled mortars and pestles. The plant material was kept frozen and ground to a fine powder. Then, the ground tissues were added into 500µl of hot extraction buffer (80^{o}C) (phenol, 0.1M LiCl, 100mM Tris-HCl (pH 8.0), 10mM EDTA and 1% SDS). After that, the mixtures were homogenized by vortex for 30 seconds. Consequentially, 250µl of phenol: chloroform: isoamyalcohol (25:24:1) was added and mixed gently. The mixture was centrifuged at 14,000rpm for 5 minutes at 4^{o}C. The upper aqueous phase was transferred to a fresh microcentrifuge tube. RNA was precipitated by addition of two volumes of absolute ethanol and stored at -20^{o}C for 30 minutes. The mixture was centrifuged at 10,000rpm for 10 minutes at 4^{o}C. After centrifugation, the pellet was washed with 80% ethanol and briefly air-dried at

room temperature. The pellet was dissolved in 160µl DEPC-treated TE buffer. RNA solution was added 40µl of 10M LiCl and stored overnight at -20°C. The mixture was left at room temperature for 5-10 minutes and centrifuged at 14,000rpm for 20 minutes at 4°C. After centrifugation, the pellet was dissolved in 20µl DEPC-treated TE buffer. The quality of RNA was performed by agarose electrophoresis. Whereas the concentration was estimated by measuring the optical density at 260nm, and calculating in µg/ml unit, using the following equation:

$$[\text{RNA}] = A_{260} \times \text{dilution factor} \times 40^*$$

*The absorbance at 260 nm (A_{260}) of 1.0 corresponds to the RNA of approximately 40µg/ml (Sambrook *et al.*, 1989)

8. *DNase* treatment and reverse transcription

8.1 *DNase*I Treatment

Ten micrograms of the total RNA sample were added to the *Dnase*I treatment mixture (1xDNase buffer and 1 unit of RQ1 *DNase*). The reaction was incubated at 37°C for an hour. After incubation, the mixture was added 100µl of 40 mM Tris-HCl (pH 8.0) and one volume of phenol: chloroform: isoamylalcohol (25:24:1), then centrifuged at 12,000rpm for 5 minutes at 4°C. The upper aqueous phase was transferred to a fresh microcentrifuge tube. RNA was precipitated by the addition of 0.1 volumes of 3M NaOAc (pH 5.2) and 0.6 volumes of cooled isopropanol and stored at -20°C for 30 minutes. The mixture was centrifuged at 12,000rpm for 10 minutes at 4°C. After centrifugation, the pellet was washed with 80% ethanol and briefly air-dried at room temperature. The DNA-free RNA pellet was resuspended in 10µl DEPC-treated water.

8.2 Reverse Transcription

One to three microgram of the *DNase*-treated RNA was added to the reverse transcription mixture (1x M-MLV Reverse Transcriptase buffer, 100ng of oligo$(dT)_{15}$ primer, 100ng of the dNTP mix, and 200 units of M-MLVRT). The reaction was incubated at room temperature for 10 minutes, then at 42^{o}C for an hour and immediately placed in boiling water for 3 minutes and quick-chilled on wet ice to terminate the reaction. The first strand cDNA was stored at -20^{o}C or used to assemble PCR reactions immediately on ice.

9. Real-time polymerase chain reaction

The specific primer sequences for each gene were designed. The parallel amplification of *OsACTIN* and *AtEF-1α*, the housekeeping control gene in rice and Arabidopsis, was used to normalize gene expression. Real-time PCR technique was used for quantitatively determine the expression profile of the *OsNUC1*. The quantitative real-time PCR was conducted on the Opticon2 Continuous Fluorescence Detector System (MJ research, USA). At least 3 independent real-time PCR reactions were performed on the same cDNA preparation. Each reaction contained 10µl of 2x SYBR Green Master Mix Reagent (Toyobo), 1µl of cDNA samples, and 200nM gene-specific primers in a final volume of 20µl. The thermal cycle used was as follows: 94^{o}C for 20 min, than 40 cycles of 94^{o}C for 20 sec, x^{o}C for 20 sec and 72^{o}C for 20 sec, then a final extension at 72^{o}C was done for 10 min. x is the annealing temperature which is depend on melting temperature of oligonucleotide primer.

9.1 Calculation of relative gene expression level

The level of gene expression was determined in comparison with the housekeeping gene (reference gene) expression in reference to the expression on Day0 of the treatment (control). For the detection in various rice organs, the gene expression was calculated in reference to the expression level of young leaf blade tissues. The relative expression ratio of target gene was calculated based on PCR efficiency (E) and the CP deviations. CP was defined as the point at which the fluorescence rises appreciably above the background fluorescence (Pfaffl, 2001).

$$\text{Relative gene expression level} = \frac{(E_{target})^{\Delta CPtarget\ (control-sample)}}{(E_{ref})^{\Delta CPref\ (control-sample)}}$$

10. ABA quantitative analysis

10.1 ABA extraction

The methods for extraction of abscisic acid ((±)-ABA) were modified from those described by Walker-Simmons (1987). The frozen tissues were extracted with methanol extraction solution (methanol containing 0.1mg ml^{-1} butylated hydroxyl-toluene and 0.5mg ml^{-1} citric acid monohydrate) at the ratio of 1g fresh weight tissue: 50ml methanol extracting solution. The extracts were shaken in dark for 16 hours and then centrifuged at 4800rpm at 4^{o}C for 15 minutes. The supernatant was evaporated to dryness using rotary evaporator and resuspended with methanol for ABA quantification by HPLC (Agilent Technologies Series 1100).

10.2 Quantitation of ABA by HPLC

ABA was quantified using HPLC (Agilent Technology Series 1100). HPLC instrument equipped with a UV absorbance detector operating at 254nm was used. Column 250nm x 4mm i.d, was packed with 5µm ODS Hypersil, Shandon Runcorn, UK), 50µl of sample was loaded and it was eluted at a flow rate of 1ml/min using different

proportion of methanol and 0.05M acetic acid: 30% methanol for 6 minutes, 30 to 50% linear gradient of methanol over 15 minutes and ABA was measured by liquid chromatography-mass spectrometry (LCA-MS) (Agilent Technologies Series 1200 (LC), microTOF BRUKER (MS)) for detection the fraction of plant extract. ABA content is expressed on the basic of dry weight. Quantitative analyses of ABA were performed with (±)-*cis-trans* ABA (Sigma) as a standard. The ABA level was the mean of triplicate measurement.

11. Plant transformation via *Agrobacterium tumeficiens*

11.1 Agrobacterium Transformation

11.1.1 Agrobacterium competent cell preparation

Agobacterium tumefaciens GV3101 cells were cultured overnight in 10ml LB broth containing 25µg/ml of rifampicin and 50µg/ml of gentamycin. *Agrobacterium* GV3101 has rifampicin resistance (chromosomal marker) and gentamycin resistance (Ti plasmid marker). Use overnight culture 1ml to inoculate LB 200ml and grown at 28^{o}C for 10 hours. Grow bacteria to a density of 0.5 to 0.6 OD_{600}. Collected pellet cells by 4^{o}C centrifugation at 5000rpm for 10 minutes, discard supernatant. Resuspend each pellet in 5ml of cold TE. Pellet cells were spined to 5000 RCF for 5 minutes at 4^{o}C, discard supernatant. Resuspend in a total volume of 20ml cold LB. Competent cells were quick freezed in N_2 liquid with aliquots of 250µl and stored at -80^{o}C.

11.1.2 Transformation (Heat-shock method)

Thaw competent *Agrobacterium* GV3101 on ice (use 250µl per transformation reaction) and then, added 10µl of ligation reaction (100ng/µl), mix it thoroughly. The mixture was kept on ice 5 minutes, then transfer to liquid nitrogen for 5 minutes. After that, the mixture was incubated for a further 5

minutes in a 37°C water bath. Added 1ml LB to each reaction, sealed well, and placed the tubes at 28°C with vigorous agitation for 4 hours. Collected the cells by centrifuging 5000rpm 5 minutes and then discarded the supernatant. Resuspend pellet in 200µl LB medium and spread 100µl of cells on LB agar plates containing antibiotic (transformant cell selection), 25µg/ml of rifampicin and 50µg/ml of gentamycin (*Agrobacterium* GV3101 strain selection). The cells were incubated for 3 days at 28°C. Before the next step, the half of selected clones carrying the specific DNA fragment were proved by PCR amplification. And then, checked DNA fragment by agarose gel electrophoresis.

11.2 Plant transformation

11.2.1 Plant preparation

Arabidopsis thaliana L., ecotype Columbia, was used as the wild type. Seeds were surface-sterilized in 1.125% NaClO solution for 15 minutes and washed 4-6 times with sterilized water. Sterilized seeds were plated on Murashige & Skoog (1962) (MS) medium containing 1% sucrose and incubated in darkness at 4°C for 2 days. The plates were then transferred to an incubator with the temperature of 22°C under a 16:8 hour light: dark photoperiod for a week. After that, Arabidopsis seedlings were grown on soil when plants contained numerous unopened floral.

11.2.2 Floral dipping

Transgenic plants were generated by *Agrobacterium*-mediated transformation by the floral dip method (Bechtold and Pelletier, 1998). Stopped watering the plants and allowed the soil to dry out a little. Three days prior to plant transformation, inoculated a 40ml liquid LB culture of *Agrobacterium* carrying

a recombinant vector and incubate at 28^{o}C with vigorous agitation, 2 days. The LB medium was contained antibiotics that selected for both the Ti and the T-DNA plasmid. Then, transferred 40ml preculture LB into 200 ml LB and incubated again with vigorous agitation for a further 24 hours at 28^{o}C. After that, the *Agrobacterium* cell pellet was collected by centrifugation at 3000 rpm on room temperature for 20 minutes and discarded supernatant. The cells were resuspended again in 50ml of ½ MS (5% sucrose) and then, added 10µl of selvet into cell suspension. Before inoculated *Agrobacterium* into plant cells, the inflorescence shoots that be opened or will be opened flower were removed. Inverted a pot of plants and dipped the inflorescence shoots into the cell suspension and allowed the plants to soak with cell suspension for 15 minutes. After dipping, placed a transparent cover over them for the next 24 hours and grown in dark. Finally, they were returned to the normal growing conditions.

11.3 Transgenic Arabidopsis selection

T1 generation and progenies were plated on half-strength MS medium containing an antibiotic for putative transgenic plant selection. This experiment, kanamycin plate, 50µg/ml kanamycin (Sigma-Aldrich, St. Louis, MO) was supplemented.

12. Proline content determination

Proline determination was performed according to Bates *et al*. (1972). L-Proline (Sigma) was used to standardize the procedure for quantifying sample values. Acid-ninhydrin was prepared by warming 0.625 grams of ninhydrin in 30ml glacial acetic acid and 20ml 6M phosphoric acid, with agitation, until dissolved. Approximately 0.25g of plant material was homogenized in 2.5ml of 3% aqueous sulfosalicylic acid and the homogenate filtered through filter paper# 1. One milliliter of filtrate was reacted with

0.5ml acld-ninhdrin and 0.5ml of glacial acetic acid in a test tube for 1 hour at 100°C, and the reaction terminated in an ice bath. The reaction mixture was extracted with 2.5ml toluene, mixed vigorously with a test tube stirrer for 15-20 seconds. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520nm using toluene for a blank. The proline concentration was determined from a standard curve and calculated on a fresh weight basis.

APPENDIX C

Cloning vector

1. pGEM®-T vector

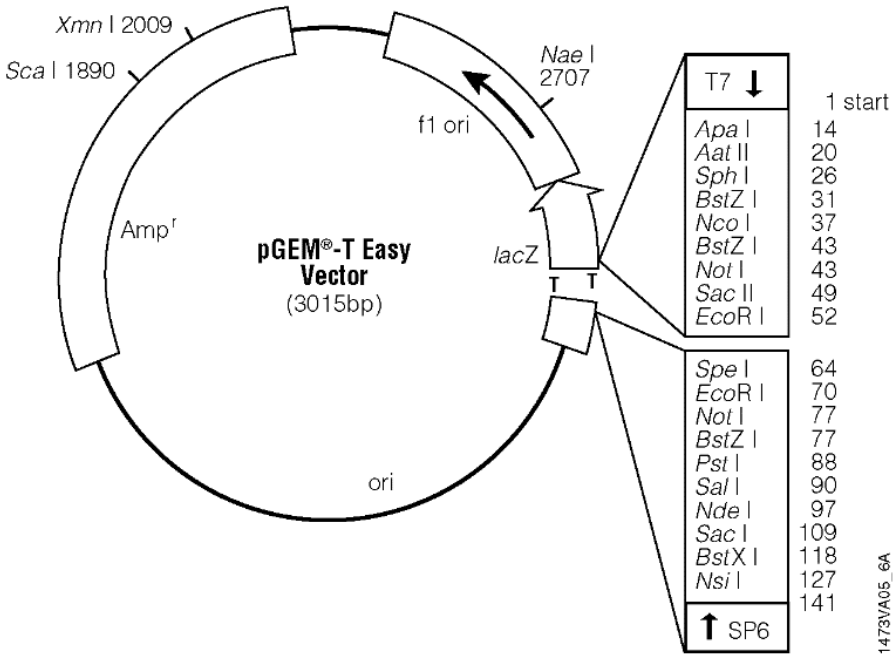


Figure C.1 The schematic diagram of cloning sites in pGEM-T Easy vector.

2. pJim19 vector

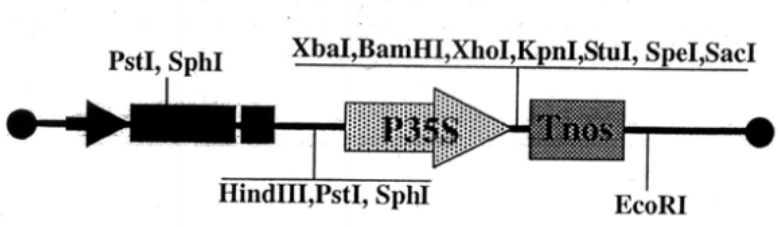


Figure C.2 The schematic diagram of cloning *pJim19* vector for Arabidopsis.

APPENDIX D

Standard curve

1. Size of DNA fragment

DNA marker size (bp)	Distance migration (cm)
8611.02	1.4
7332.18	2.6
5733.63	4.1
5200.78	4.6
4667.93	5.1
3921.94	5.8

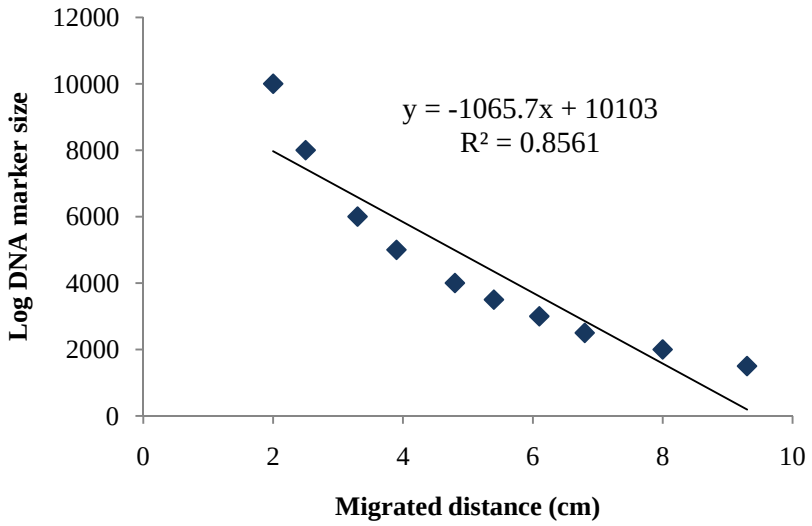


Figure D.1 Size of DNA

2. ABA standard

Area of peak	ABA concentration (nmole)
1675.95	1.95
3504.63	3.9
7426.45	7.8
9909.83	15.6

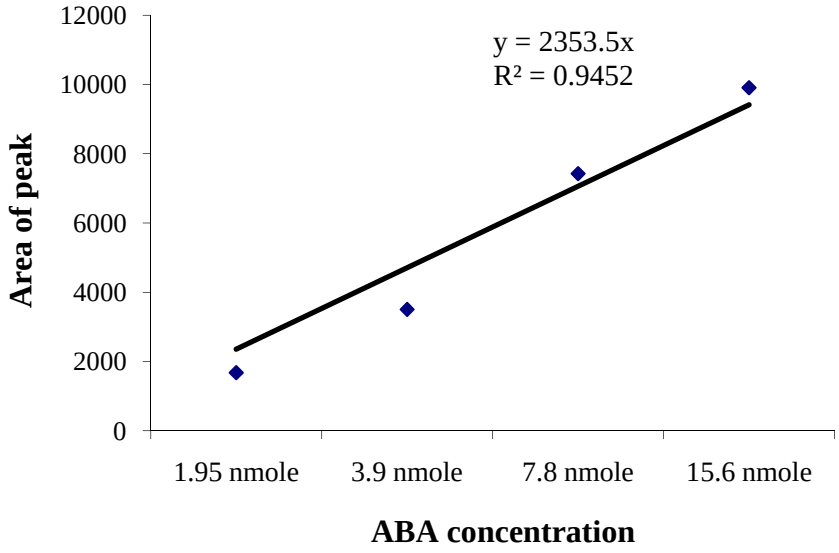


Figure D.2 Standard curve of ABA accumulation.

3. Proline standard

OD520	Proline content (mg)
0.00268	0
0.25119	0.0781
0.47749	0.1562
1.0495	0.3125

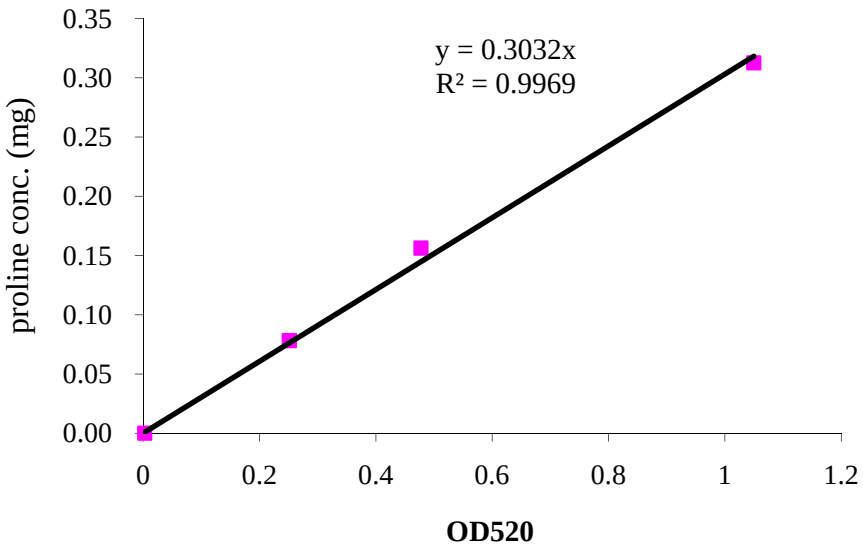
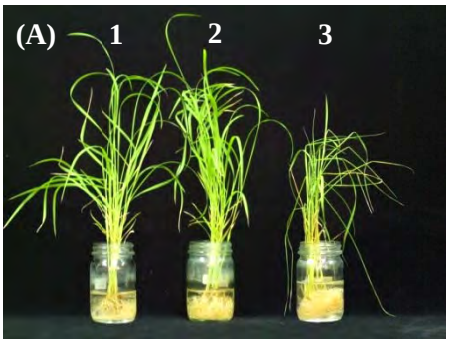


Figure D.3 Standard curve of proline accumulation.

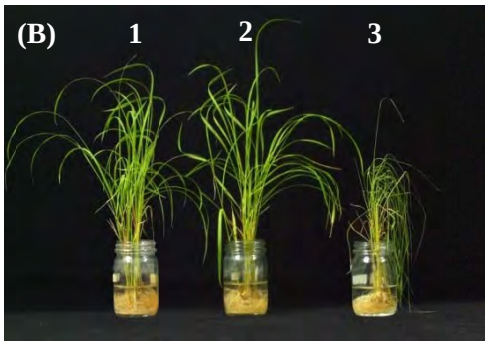
APPENDIX E

Phenotype of rice seedlings

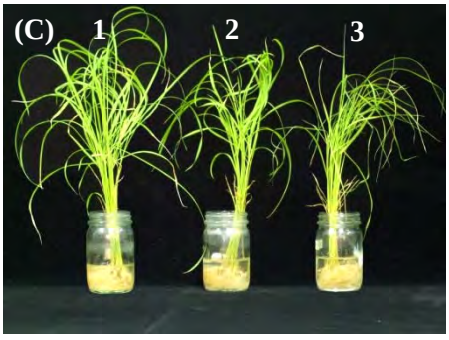
LPT123-Day1



LPT123-Day2



LPT123-TC171-Day1



LPT123-TC171-Day2

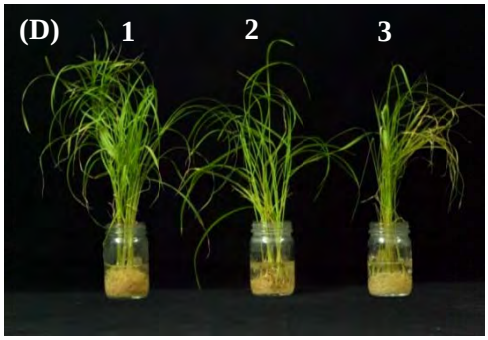
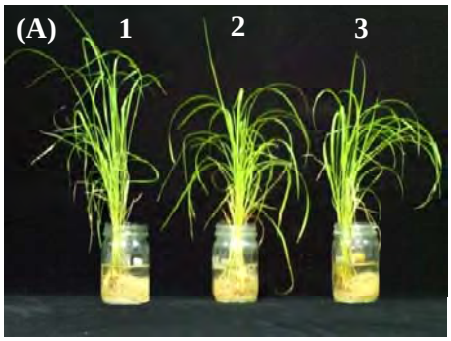
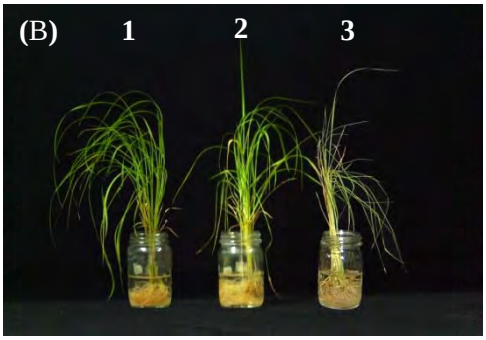


Figure E.1 3-week-old salt-sensitive LPT123 (A and B) and salt-resistant LPT123-TC171 (C and D) rice seedlings after grown in WP No.2 solution (1) and treated with 0µM abamine SG as control (2) or 50µM abamine SG (3), a day (A and C) and two days (B and D) under normal condition.

LPT123-Day1



LPT123-Day2



LPT123-TC171-Day1

LPT123-TC171-Day2

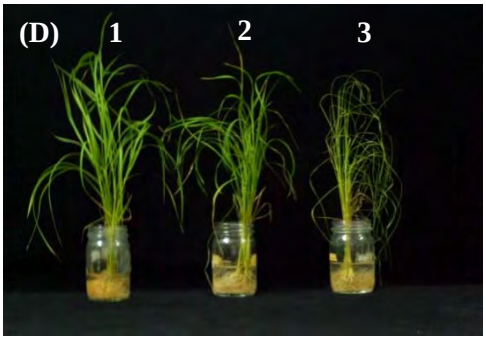


Figure E.2 3-week-old salt-sensitive LPT123 (A and B) and salt-resistant LPT123-TC171 (C and D) rice seedlings after grown in WP No.2 solution (1) and treated with 0µM abamine SG as control (2) or 50µM abamine SG (3), a day (A and C) and two days (B and D) under salt-stress condition.

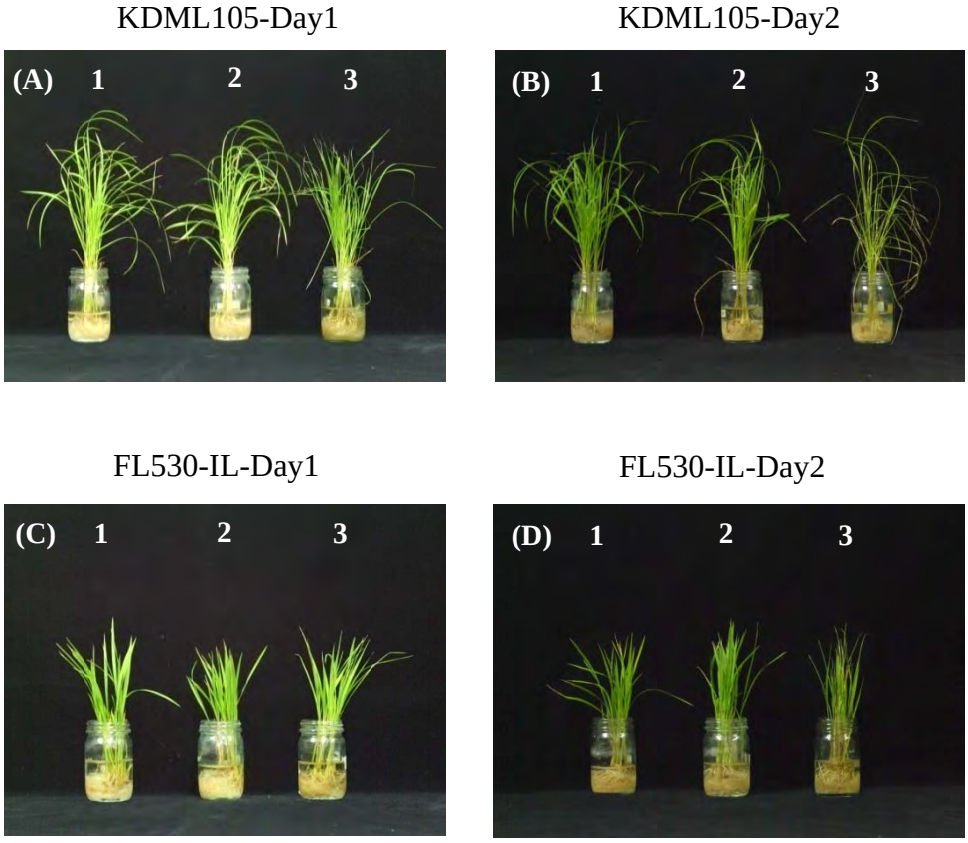
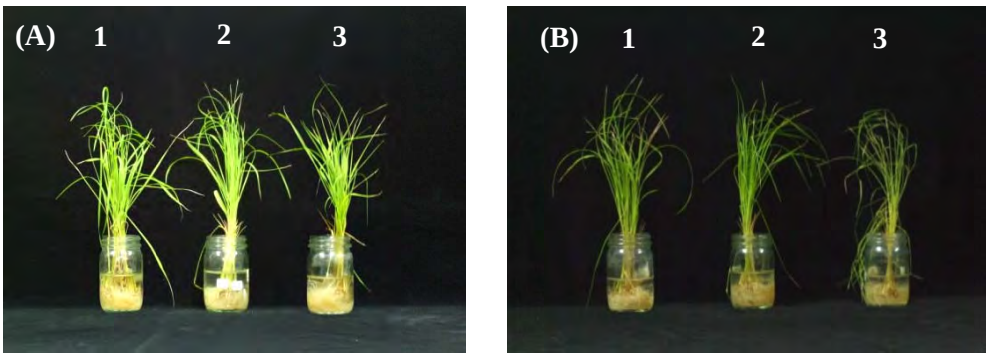


Figure E.3 3-week-old salt-sensitive KDML105 (A and B) and salt-resistant FL530-IL (C and D) rice seedlings after grown in WP No.2 solution (1) and treated with 0µM abamine SG as control (2) or 50µM abamine SG (3), a day (A and C) and two days (B and D) under normal condition.

KDML105-Day1 KDML105-Day2



FL530-IL-Day1 FL530-IL-Day2

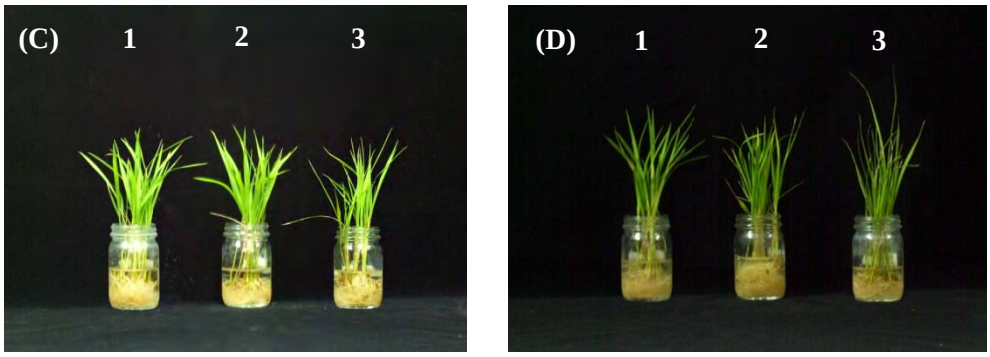


Figure E.4 3-week-old salt-sensitive KDML105 (A and B) and salt-resistant FL530-IL (C and D) rice seedlings after grown in WP No.2 solution (1) and treated with 0µM abamine SG as control (2) or 50µM abamine SG (3), a day (A and C) and two days (B and D) under salt-stress condition.

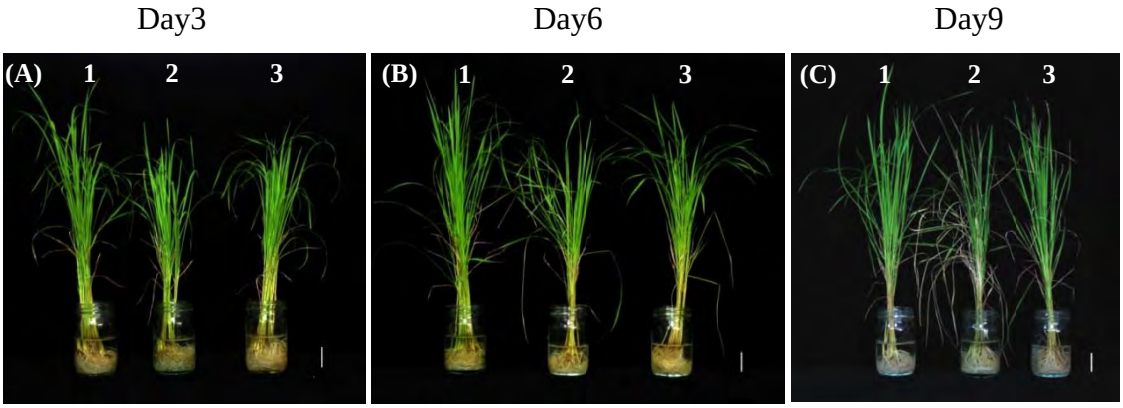


Figure E.5 3-week-old salt-sensitive LPT123 rice seedlings after grown in WP No.2 solution (1) or salt-stress (0.5%NaCl) (2) or treated by 100µM calmodulin inhibitor, W-7 (3) on day3 (A), day6 (B) and day9 (C) of treatments under salt-stress condition.

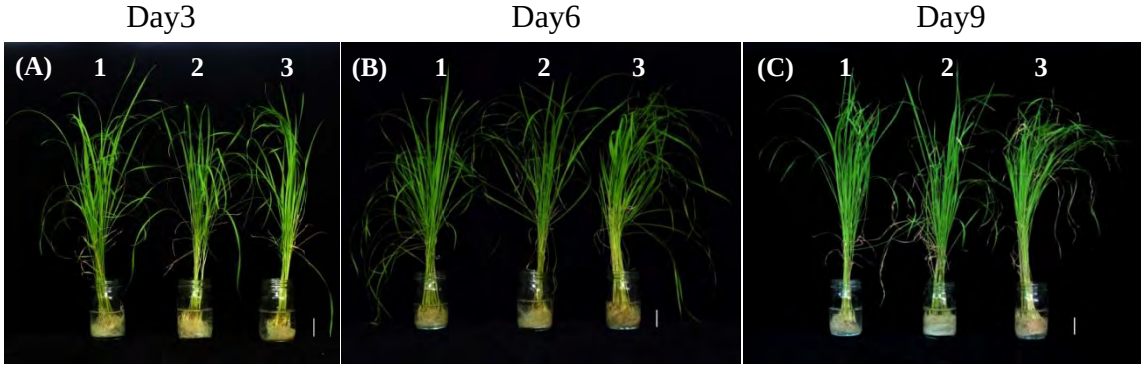


Figure E.6 3-week-old salt-resistant LPT123-TC171 rice seedlings after grown in WP No.2 solution (1) or salt-stress (0.5%NaCl) (2) or treated by 100µM calmodulin inhibitor, W-7 (3) on day3 (A), day6 (B) and day9 (C) of treatments under salt-stress condition.

BIOGRAPHY

Ms. Siriporn Sripinyowanich was born on July 16, 1983 in Bangkok. After she finished high school in 2000 from Satree Samutprakarn School in Samutprakarn, she was enrolled in Major of Agricaultural Biotechnology, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus and graduated with the 1st class honor in the degree of Bachelor of Agricultural Biotechnology in 2004. She has gotten the scholarship from Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadaj to study for the degree of Doctor of Philosophy in Biological Sciences Program, Faculty of Science, Chulalongkorn University since 2005. She was supported by Commission on Higher Education via the exchange student program in 2008. Then in 2010, she got the Food Security Center's scholarship program "Sandwich Scholarships for visiting PhD students from developing countries" from the University of Hohenheim, which is part of the DAAD (German Academic Exchange Service) program "exceed" and is supported by DAAD and the German Federal Ministry for Economic Cooperation and Development (BMZ) and in cooperation with the College of Life Sciences at Peking University.