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ความสัมพันธ์ระหว่างกรดแอบไซซิกและการแสดงออกของยีนแคลมอดูลินภายใต้ความเครียดจาก

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RELATIONSHIP BETWEEN ABSCISIC ACID AND CALMODULIN GENE EXPRESSION UNDER SALT STRESS IN RICE *Oryza sativa* L.

Ms. Sukhumaporn Saeng-ngam

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Program in Biological Sciences

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สุขุมาภรณ์ แสงงาม: ความสัมพันธ์ระหว่างกรดแอบไซซิกและการแสดงออกของยีนแคลมอดูลินภายใต้ความเครียดจากความเค็มในข้าว *Oryza sativa* L. (RELATIONSHIP BETWEEN ABSCISIC ACID AND CALMODULIN GENE EXPRESSION UNDER SALT STRESS IN RICE *Oryza sativa* L.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.ศุภจิตรา ชัชวาลย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร.ธีรพงษ์ บัวบูชา, ศ.ดร. ไพศาล สิทธิกรกุล, 170 หน้า.

งานวิจัยนี้มีจุดประสงค์ในการศึกษาความสัมพันธ์ระหว่างการตอบสนองต่อความเครียดจากความเค็มผ่านกรดแอบไซซิกและยีนแคลมอดูลินในข้าว (*Oryza sativa* L.) (*OsCam1-1*) ในการศึกษานี้ใช้ข้าวสองคู่สายพันธุ์ที่มีความสามารถในการทนเค็มต่างกันได้แก่ข้าวสายพันธุ์ไม่ทนเค็ม LPT123 และข้าวสายพันธุ์ทนเค็ม LPT123-TC171 และข้าวสายพันธุ์ไม่ทนเค็มขาวดอกมะลิ105 และข้าวสายพันธุ์ทนเค็ม FL530-IL การผลิตแอนติบอดีที่จำเพาะต่อกรดแอบไซซิกเพื่อตรวจวัดปริมาณกรดแอบไซซิกในข้าวในสภาวะเครียดจากความเค็มใช้แอนติเจนสองชนิดได้แก่กรดแอบไซซิกที่เชื่อมกับโปรตีน BSA ด้านคาร์บอนิลและกรดแอบไซซิกที่เชื่อมกับโปรตีน BSA ด้านคาร์บอกซิล พบว่าได้เซลล์ลูกผสมจำนวน 2 โคลนผลิตแอนติบอดีจำเพาะต่อกรดแอบไซซิกด้านคาร์บอกซิลและ 9 โคลนผลิตแอนติบอดีจำเพาะต่อกรดแอบไซซิกด้านคาร์บอนิล เซลล์ลูกผสมที่ผลิตแอนติบอดีที่มีความไวสูงสุดกับกรดแอบไซซิกนำมาใช้พัฒนาการตรวจวัดกรดแอบไซซิกด้วยวิธี competitive ELISA โมโนโคลนอลแอนติบอดีที่ผลิตได้สามารถตรวจวัดปริมาณกรดแอบไซซิกในพืชได้ในช่วง 0.024–0.756 พิโคโมลต่อมิลลิลิตร ข้าวสายพันธุ์ LPT123 และสายพันธุ์ LPT123-TC171 มีการสะสมปริมาณกรดแอบไซซิกในระดับที่คล้ายกันซึ่งมีการสะสมกรดแอบไซซิกที่เห็นได้ชัดเจนหลังจากได้รับความเครียดจากความเค็มเป็นเวลา 6 ถึง 9 วัน โดยพบการสะสมกรดแอบไซซิกในปริมาณสูงสุดในวันที่ 9 นอกจากนี้ในข้าวสายพันธุ์ FL530-IL มีปริมาณกรดแอบไซซิกสูงกว่าข้าวขาวดอกมะลิ105 อย่างมีนัยสำคัญซึ่งแสดงให้เห็นว่าระดับของกรดแอบไซซิกไม่ได้เป็นปัจจัยเดียวที่มีผลต่อการทนเค็มของข้าว การแสดงออกของยีนแคลมอดูลินในข้าวขาวดอกมะลิ105 เปรียบเทียบกับข้าวสายพันธุ์ FL530-IL พบว่าข้าวพันธุ์ FL530-IL มีการแสดงออกของยีน แคลมอดูลินในระดับที่เร็วกว่าและสูงกว่าข้าวขาวดอกมะลิ105 เมื่อได้รับภาวะเค็ม ซึ่งพบการแสดงออกของยีนแคลมอดูลินสูงกว่าภาวะปกติ 24 เท่าในขณะที่การแสดงออกของยีนแคลมอดูลินในข้าวขาวดอกมะลิ 105 สูงขึ้นประมาณ 7 เท่าหลังจากได้รับความเค็ม 30 นาที ข้าวขาวดอกมะลิ 105 ที่มีการแสดงออกของยีนแคลมอดูลินสูงตลอดเวลาภายใต้การควบคุมของโปรโมเตอร์ *35SCaMV* มีผลต่อการแสดงออกของยีน *9-*cis*-epoxycarotenoid dehydrogenase* (*NCED*) และยีน *ABA aldehyde oxidase* (*AAO*) ซึ่งเป็นยีนที่เกี่ยวข้องกับการสังเคราะห์กรดแอบไซซิกในพืช ข้าวขาวดอกมะลิ105 ที่มีการแสดงออกของยีนแคลมอดูลินสูงตลอดเวลามีผลต่อการเพิ่มขึ้นของปริมาณกรดแอบไซซิก เมื่อเปรียบเทียบกับข้าวในชุดควบคุมที่เป็นข้าวขาวดอกมะลิ105และข้าวทรานจีนิกส์ที่ไม่มีโครงสร้างของยีนที่มีการแสดงออกของยีน แคลมอดูลินเพิ่มขึ้น นอกจากนี้ข้าวขาวดอกมะลิ105 ที่มีการแสดงออกของยีนแคลมอดูลินสูงตลอดเวลาสามารถรักษาน้ำหนักแห้งของต้นได้ดีในภาวะเค็ม การศึกษาการแสดงออกของยีนแคลมอดูลินโดยเชื่อมต่อกับยีบีตา-กลูคูรอนิเดสซึ่งเป็นยีนรายงานผลภายใต้การควบคุมของโปรโมเตอร์ของยีนแคลมอดูลินในข้าวขาวดอกมะลิ105 พบว่าการให้กรดแอบไซซิกจากภายนอกมีผลทำให้การแสดงออกของยีนแคลมอดูลินมากขึ้นและการให้สารยับยั้งการสังเคราะห์กรดแอบไซซิก (อบามีนเอสจี) มีผลยับยั้งการแสดงออกของยีนแคลมอดูลิน ในภาวะเค็มการแสดงออกของยีนแคลมอดูลินถูกควบคุมโดยกรดแอบไซซิกในขณะเดียวกันการแสดงออกของยีนแคลมอดูลินยังมีบทบาทในการควบคุมปริมาณกรดแอบไซซิกในข้าว ซึ่งแสดงให้เห็นว่ากรดแอบไซซิกมีผลในการควบคุมการแสดงออกของยีนแคลมอดูลินและการส่งผ่านสัญญาณโดยแคลมอดูลินมีบทบาทต่อขบวนการสังเคราะห์กรดแอบไซซิกโดยแคลมอดูลินและกรดแอบไซซิกมีบทบาทควบคุมซึ่งกันและกัน ในการตอบสนองต่อภาวะเค็ม ระดับของแคลมอดูลินและกรดแอบไซซิกภายในเซลล์มีส่วนช่วยทำให้ข้าวมีความสามารถในการทนเค็มได้ดีขึ้น

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SUKHUMAPORN SAENG-NGAM: RELATIONSHIP BETWEEN ABSCISIC ACID AND CALMODULIN GENE EXPRESSION UNDER SALT STRESS IN RICE *Oryza sativa* L. THESIS ADVISOR: ASSOC. PROF. SUPACHITRA CHADCHAWAN, Ph.D., THESIS COADVISOR: ASSIST. PROF. TEERAPONG BUABOOCHA, Ph.D., PROF. PAISARN SITHIGORNGUL, Ph.D., 170 pp.

This research aims to characterize the relationships of salt responses to salt stress via abscisic acid (ABA) accumulation and the expression of salt-responsive *calmodulin* gene (*OsCam1-1*) in rice (*Oryza sativa* L.). Two pairs of rice cultivars/lines, which are different in salt-tolerant ability were used in these studies. Leung Pra Tew (LPT123) and Khao Dok Mali 105 (KDML105) rice cultivars are considered salt-sensitive ones while their isogenic lines, LPT123-TC171 and FL530-IL, are salt tolerant. In order to determine the level of ABA accumulation due to salt stress, monoclonal antibody (MAb) against (+)-ABA was produced. Two types of (±)-ABA-BSA conjugate, one with free carboxyl and the other with free carbonyl terminal, were used as the antigens in the antibody production. Hybridoma lines were then selected for specificity against (+)-ABA which is the natural form of ABA in plants. There were 2 clones producing MAb against the carboxyl terminal and 9 clones producing MAb recognizing the carbonyl end. The clone with the highest sensitivity of MAb for (+)-ABA detection was chosen to develop the competitive enzyme-linked immunosorbent assay (ELISA) method for ABA measurement. With this MAb, the endogenous ABA in the range of 0.024–0.756 pmol/ml could be detected. The ABA accumulation in rice leaves was clearly detected after 6–9 days after salt stress. The highest level of ABA content after salt stress in LPT123 and LPT123-TC171 was similar. The highest ABA was accumulated after 9 days of salt stress. On the other hand, FL530-IL rice showed the significant higher level of ABA accumulation than KDML105. These results suggest that the level of ABA accumulation may not the only factor that contributes to salt resistant ability in rice. *OsCam1-1* expression was studied in KDML105 and FL530-IL to investigate if it plays any roles in salt resistance. The faster and the higher response in *OsCam1-1* expression after salt stress were found in FL530-IL rice. A twenty-four-time higher level of *OsCam1-1* transcript was detected in the FL530-IL rice leaves after 30 minutes of salt stress, while only about seven times higher of the *OsCam1-1* transcript at the non-stress level was found in salt stress treated KDML105 cultivar at the same period of time. The transgenic KDML105 rice lines with over-expression of *OsCam1-1* was found to up-regulate the gene involving the ABA biosynthesis, *9-*cis*-epoxycarotenoid dehydrogenase* (*NCED*) and *ABA aldehyde oxidase* (*AAO*) genes, resulting in the higher level of ABA content in the transgenic lines, when compared to the wild type and the control transgenic lines without the over-expression construct. In addition, the over-expressing *OsCam1-1* transgenic plants were more tolerant to salt stress than the control plants as they exhibited better ability in maintaining shoot and root dry weights during salt stress. ABA actions on *OsCam1-1* promoter activity were investigated using the transgenic KDML105 rice containing β-*glucuronidase* gene regulated by *OsCam1-1* promoter. It was found that *OsCam1-1* promoter was activated by salt stress and exogenous ABA application. The ABA inhibitor, abamine SG, was found to inhibit *OsCam1-1* expression. These suggest that *OsCam1-1* expression was controlled by ABA during salt stress and at the same time ABA also played a role in *OsCam1-1* gene expression. These indicate that ABA is required for *OsCam1-1* expression and *OsCam1-1* signaling has an important role in the ABA biosynthesis. These two signal molecules regulate bidirectionally under salt stress response. The cellular level of both *OsCam1-1* gene expression and ABA accumulation contribute to salt resistance in rice.

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	Co-advisor's signature ……………………………….
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LIST OF ABBREVIATIONS

Ab	antibody
ABA	abscisic acid
APRT	adenine phosphoribosyl transferase
BSA	bovine serum albumin
°C	degree Celsius
Ca^{2+}	calcium ion
CADPR	cyclic ADP ribose
CaM	calmodulin
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CBL	calcinurin B-like protein
CPK	calcium dependent protein kinase
cyt	cytosol
Da	dalton
DAB	diaminobenzidine
DEPC	diethyl pyrocarbonate
DG	diacylglycerol
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	2′-deoxynucleoside 5′-triphosphate
DTT	dithiothreitol
EDC	1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide
EDTA	ethylene diamine tetraacetic acid

ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FBS	fetal bovine serum
FL530-IL	KDML105 introgression line
g	gram
GAM-HRP	goat anti-mouse immunoglobulin (H+L) horseradish peroxidase conjugated
GC	gas chromatography
h	hour
HA	*p*-aminohippuric acid
HAT	hypoxanthine, aminopterin and thymine
HCl	hydrochloric acid
HGPRT	hypohanthine-guanine phosphoribosyl transferase
HPLC	high performance liquid chromatography
Ig	immunoglobulin
IP_3	inositol-1,4,5-triphosphate
IMP	inosine monophosphate
kb	kilobase pairs in duplex nucleic acid, kilobases in single-standed nucleic acid
KCl	potassium chloride
kDa	kiloDalton
KDML105	Khao Dok Mali 105
K_ds	dissociating constants
KOH	potassium hydroxide
l	liter

LC-MS	liquid chromatography-mass spectrometry
LPT123	Leung Pra Tew123
LPT123-TC171	Leung Pra Tew123 isogenic line
Mg^{2+}	magnesium ion
μg	microgram
μl	microliter
μM	micromolar
M	mole per liter (molar)
mA	milliampere
MAb	monoclonal antibody
mg	milligram
min	minute
ml	milliliter
mM	millimolar
Mt	mitochondria
MU	7-hydroxy-4-methyl coumarin
4-MUG	4-methylumbelliferyl β-D-glucuronide
MW	molecular weight
N	normal
NCS	neuronal calcium ion sensor
ng	nanogram
NH_4Cl	ammonium chloride
NH_4OH	ammonium hydroxide
nm	nanometer
NMR	nuclear magnetic resonance

N-terminus	amino terminus
OA	ovalbumin
OD	optical density
OPD	*O*-phenelenediamino dihydrochoride
PAb	polyclonal antibody
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodieterase
PEG	polyethylene glycol
PIP_2	phosphotidyl inositol-4,5-bisphosphate
PKC	protein kinase C
Plast	plastid
PLC	phospholipase C
PM	plasma membrane
pmol	picomole
pNPG	para-nitrophenyl β-D-glucuronide
R	receptor
RT	room temperature
RT-PCR	real time polymerase chain reaction
	reverse transcription polymerase chain reaction
RBC	red blood cell
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
TK	thymidine kinase

TLC	thin layer chromatography
Tris	tris (hydroxyl methyl) aminomethane
UV	ultraviolet
V	voltage
v/v	volume by volume
w/v	weight by volume
X-Gluc	5-bromo-4-chloro-3-idolyl glucuronide

CHAPTER I

INTRODUCTION

Rice is the world's single most important food crop and primary food for more than a third of the world's population. Thailand is the world's biggest rice exporter. Rice is major agricultural exporting goods. Thailand has the fifth-largest area for rice cultivation in the world and has plans to further increase its land available for rice production, with a goal of adding 500,000 hectares to its already 9.2 million hectares of rice-growing areas in 2008. However, the salinity and drought stress are important problems of the rice cultivation in Thailand, which cause the decreasing of the rice production, especially in the northeast of Thailand. Salinity and drought are the most significant abiotic stresses affecting plant growth and limiting crop yield in the world (Jone and Corlett, 1992; Beltrano *et al*., 1999; Jiang and Zhang, 2004).

Water deficit stress causes cellular dehydration and consequently imposes an osmotic stress on plants, whereas soil salinity imposes both an ionic and osmotic stresses due to the high concentration of ions in root growing medium and the consequently low soil water potential. These stresses have an impact on growth of both the shoots and the roots. Regulatory processes are initiated within osmotically stressed plants to adjust their cellular metabolism in order to circumvent damage associated with the water loss. These processes are accompanied by changes in gene expression that are regulated by the phytohormone abscisic acid (ABA) (Creelman, 1989; Bray, 1993; Ingram and Bartels, 1996). ABA is an important signal in the salt and water stress. Both salt and water stresses can induce a rapid accumulation of ABA in plant tissues. This process itself is a cellular signaling cascade, in which the perception of salt signal or initial triggering for ABA accumulation is the most

important step. Therefore, ABA, as a plant growth regulator, is a ubiquitous in higher plants and has many physiological effects on the growth and differentiation of plants. It has been demonstrated that ABA plays important roles in stomatal regulation (Rock and Quatrano, 1995; Phillips *et al*., 1997), the stability of photosynthesis apparatus (Chandler and Robertson, 1994), and seed germination.

The content of ABA is increased when plants are under stress conditions including drought, salinity and cold. Plants challenged by drought and salt stress recruit ABA as an endogenous signaling to initiate adaptive processes (Zhu, 2002). Several genes and secondary messenger molecules that take part in ABA signal transduction have been identified over the past years for the plant survival in the stress conditions such as *LEA* gene that regulates the LEA protein synthesis in the seed (Galau *et al*., 1986; Vivekanada *et al*., 1992; Moons *et al*., 1997) and ABA also induces the expression of *pinII* gene that regulates the proteinase inhibitor II protein, which involves in the defense mechanism in plants (Pena-Cortes *et al*., 1997).

In previous study of gene expression profile in the early stage of two lines of rice, salt-tolerant line and salt-sensitive line were grown in the salt stress condition, and it was found that both rice lines have similar gene expression, but the salt-tolerant line responded more rapidly to salt stress than the salt-sensitive one (Kawasaki *et al*., 2001). These results indicated that the ability of salt tolerance in rice related to signal transduction. Although the components of the signal transduction pathway are difficult to identify, ABA is well known as one such component acting in one of the signal transduction pathways when the plants grow in the salt stress condition. Many studies have addressed that the ABA can enhance the plant tolerance to environmental stress by the application of the exogenous ABA to intact plants, or implication of the

higher level of endogenous ABA (Gibson *et al.*, 1991; Heschel and Hausmann, 2001; Xu *et al.*, 2002; Wang *el al.*, 2003; Li *et al.*, 2004). The level of ABA in plants increases upon their exposure to environmental stress (Zeevaart and Creelman, 1988; Xu *et al.*, 1995). The application of exogenous ABA to salt-tolerant rice line (LPT123–TC171) and salt-sensitive line (LPT123) that were genetically similar could induce salt tolerance in both lines (Klomsakul, 2004). This phenomenon occurs in parallel with the induction of proline accumulation in leaves, which suggests the role of proline accumulation in salt tolerance via ABA-dependent pathway.

The purpose of this study was to test the hypothesis that the endogenous ABA accumulating during salt stress reflects the salt resistance ability in rice. Therefore, in this study, the level of endogenous ABA in two rice lines/cultivars with different salt resistance will be determined. Two pairs of rice line/cultivar will be used. The salt-sensitive rice cultivars are Khao Dok Mali (KDML) 105 and Leung Pra Tew (LTP) 123, and the salt-tolerant rice lines with the same genetic background of the previous cultivars are FL530-IL, and LPT123-TC171, respectively. These two pairs of rice lines/cultivars are the Southeast Asian *Indica* rice. KDML105, commonly known as "Jasmine rice" or "Thai Hom Mali rice", is a photoperiod-sensitive cultivar and is well adapted to the rainfall environment in which the fields are normally low fertility, frequently experiencing drought and salt stress and also often attacked by diseases and insect pests. FL530-IL is the salt-tolerant rice line that was developed by the Rice Gene Discovery Unit, in collaboration with the National Center for Genetic Engineering and Biotechnology (BIOTEC) and the DNA Technology Laboratory (DANTEC) of Kasetsart University Kamphaeng Sean Campus. To obtain FL530 line for this research, the hybrid line between pokkali and IR29 rice 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, FL530-IL rice contains the genetic background similar to KDML105, but it has higher salt tolerance. The other line was LPT123-TC171 that is a somaclonal variation from LPT123 by plant tissue culture technique, therefore, LPT123-TC171 contains the genetic background similar to LPT123 and it has higher salt tolerance ability.

Since ABA has been proven to have vital physiological roles, analysis of trace amounts of ABA in plants is getting more and more important. To date, a number of analytical techniques have been developed for ABA determination such as gas chromatography (GC), GC-mass spectrometry (MS), high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), and radioimmunoassay (RIA) (Quarrie *et al.*, 1988; Philosoph *et al*., 1993; Andrew *et al*., 2004; Seo *et al*., 2005). In this study, one of the objectives is to develop the enzyme-linked immunosorbent assay (ELISA) technique to measure the level of endogenous ABA in plants by monoclonal antibody specific to (+)-ABA because this method is well suited for the assay of large numbers of samples and shows good specificity to ABA. Most of the above mentioned techniques need complicate and intensive purification protocols for ABA in plant samples and several problems exist with reported purification and quantification techniques. The requirement for several purification steps causes two problems. First, the procedure is time-consuming and tedious. Second, each step results in losses of ABA, so that total losses before quantification can be high up to 70% (Kerry and David, 1980).

Calmodulin (CaM) has been characterized to play an important role in salt stress signaling in several plant species including rice (Phean-o-pas *et al*., 2005, Boonburapong and Buaboocha, 2007). In order to clarify the signal transduction pathway involving ABA and CaM signaling during salt stress, the relationship between calcium sensor gene, *OsCam1-1*, the calmodulin induced by salt stress and ABA will be investigated.

This study consists of three parts. The first one is to develop the monoclonal antibody for endogenous ABA detection in plant extract. The second part is to determine the level of endogenous ABA in rice plants which have different salt-tolerant ability. The last one is to study the relationship between signal transduction pathway mediated by ABA and the expression of calcium sensor, such as calmodulin in rice under salt stress condition. The knowledge of the response of rice in the salt stress mediated by ABA is important for the understanding of the mechanism by which plants perceive environmental signals to cellular machinery to activate the adaptive responses in stress condition. It is also vital for continued development of rational breeding or transgenic strategies to improve stress tolerance in crops in the future. The objectives of this study are:

1. To develop the monoclonal antibody for endogenous ABA detection in plant extract.

2. To determine the level of endogenous ABA in rice plants which have different salt-tolerant ability.

3. To study the relationship between endogenous ABA level and the expression of calcium sensor, calmodulin, *OsCam1-1*, under salt stress condition.

CHAPTER II

LITERATURE REVIEW

1. Salinity stress and plant response in the salt stress

Abiotic stresses such as salinity and drought stresses are important factors that limit plant growth. High salinity causes hyperosmotic stress and ion disequilibrium that produce secondary effect or pathologies (Zhu, 2001). Typically, plants cope by either avoiding or tolerating salt stress, for example, plants are dormant during the salt episode or there must be cellular adjustment to tolerate the saline environment.

Tolerant mechanisms can be categorized as a function to minimize osmotic stress, an ion disequilibrium and allevation of the consequent secondary effects caused by the stress. The effects of salt stress have been caused by the chemicals of the saline solution. Initially, it establishes a water potential imbalance between the apoplast and symplast that leads to turgor pressure decrease and growth reduction. Growth cessation occurs when turgor pressure is reduced below the yield threshold of the cell wall and cellular dehydration begins when the water potential difference is greater than that can be compensated for by turgor loss. The cellular response to turgor reduction is osmotic adjustment.

Plants are classified as glycophytes or halophytes according to their capacity to grow on high salt medium. Most plants are glycophytes, they cannot tolerate salt stress because high salt concentration decreases the osmotic potential of soil solution causing a water stress in plants and causes severe ion toxicity, since Na^+ is not readily sequestered into vacuoles as in halophytes. The interaction of salt with mineral nutrition may result in nutrient imbalances and deficiencies. The consequence of these can lead to plant death as a result of growth arrest and molecular damage. In

halophytes, cytosolic and organellar mechanism is equivalently Na^+ and Cl^- sensitive, therefore, the osmotic adjustment is active in these compartments by accumulation of compatible osmolytes and osmoprotectants. The solutes that accumulate vary with the organism and even between plant species. A major organic osmotic solute consists of simple sugars (fructose and glucose), sugar alcohols (glycerol and methylate inositols) and complex sugars (trehalose, raffinose and fructans) and the other includes quaternary amino acid derivatives (proline, glycine betaine, β-alanine betaine, proline betaine) (Bohnert and Jensen, 1996). Na^+ and Cl^- movement into the vacuole might occur directly from the apoplast into the vacuole through membrane vesiculation or a cytological process that juxtaposes the plasma membrane to the tonoplast (Hasegawa *et al*., 2000).

When the plants face to the stress conditions such as salt and drought stress, they can close the stomata. This process occurs via the signal transduction of abscisic acid (ABA) (Taiz and Zeiger, 1998). ABA is considered to be a plant stress hormone because it is synthesized in response to many kinds of stress, including cold, salt, water, wounding and heat stress (Yabe *et al.*, 1994; Pastori and Foyer, 2002). There are some researches about plant response to salt and drought stress or osmotic stress in *Arabidopsis*. Plants can survive in the stress condition by changing the mechanism of some integral membrane proteins, activating the process of reactive oxygen species scavenging system, and/or accumulating the osmolytes (Shinozaki and Yamaguchi-Shinosaki, 1997). Salt stress and drought stress can induce the ABA synthesis in plant cell via the calcium binding protein and phosphoprotein cascade show in Figure 2.1 (Xiong and Zhu, 2003).

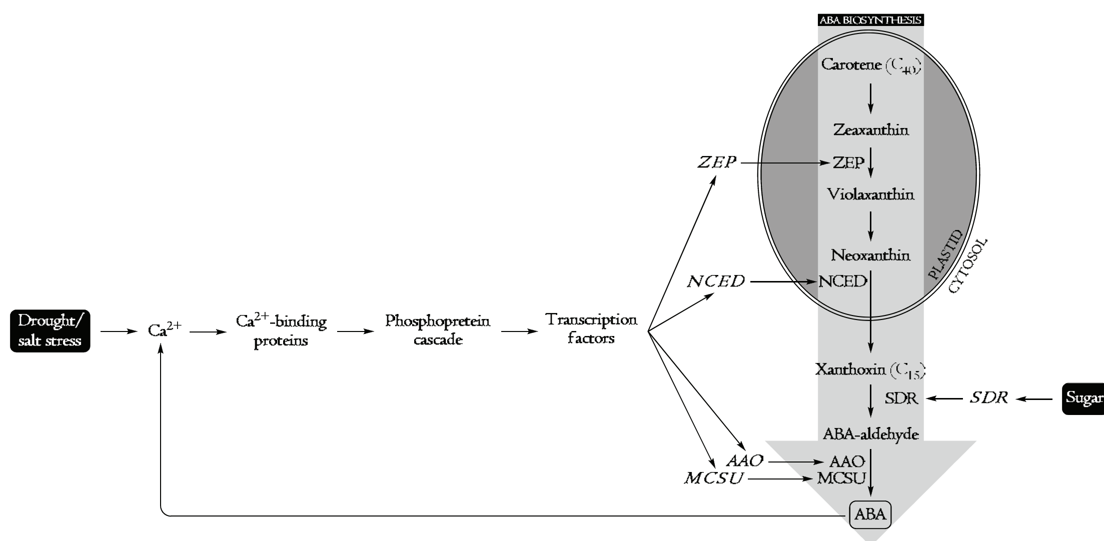


Figure 2.1 The effect of drought and salt stress on the ABA biosynthesis (Xiong and Zhu, 2003).

2. Abscisic acid

Abscisic acid (ABA) was discovered in a search for the factors that regulated the formation of abscission zone in fruits and leaves and it was a result of investigations on the regulatory signals controlling the onset of dormancy. ABA is sometimes considered to be a plant stress hormone because it is synthesized in response to many kinds of stress, including cold, salt, and water stresses (Yabe *et al.*, 1994; Pastori and Foyer, 2002). Only the *S*-(+)-enantiomorph of ABA is present in plant cells as shown in Figure 2.2. ABA in plant cell is also conjugated with glucose, called ABA-GE, which appears to be widespread in plants but has been identified conclusively only a few times. ABA limits Na^+ and Cl^- accumulation in leaves, which appears to be the result of a restriction of root-to-shoot ion transport via the transpiration stream because ABA is a stomatal regulator. Recently, the role of ABA in stress signal transduction has been investigated intensively and the results indicate that ABA can up-regulate many stress-responsive genes (Hasegawa *et al.*, 2000; Zhu,

2002). Several studies have shown that ABA accumulation is required for the development of stress tolerance in plants. However, the extent and the molecular basis of ABA involvement in stress-responsive gene expression and stress tolerance were not clear.

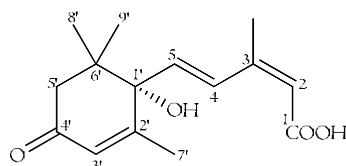


Figure 2.2 Structure of *S*-(+)-abscisic acid.

3. Abscisic acid synthesis may be regulated by stress.

ABA is synthesized in mature leaves and transported both to the root and throughout the shoot system, primarily via the phloem. Roots also can synthesize ABA and transport the hormone into the shoot in response to water stress. ABA is normally much more abundant in the phloem sap. Many studies suggested that ABA in higher plants was synthesized from an "indirect" pathway through the cleavage of a C_{40} carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via ABA-aldehyde (Taylor *et al*., 2000; Seo and Koshiba, 2002; Schwartz *et al*., 2003). The first step that is more specific to the ABA biosynthesis pathway is the epoxidation of zeaxanthin and antheraxanthin to violaxanthin, which occurs in plastids.

Increased ABA levels under drought and salt stress are mainly achieved by the induction of genes coding for enzymes that catalyze ABA biosynthetic reactions. The ABA biosynthetic pathway in higher plants is understood to a great extent. Zeaxanthin epoxidase (ZEP; encoded by *ABA1* in Arabidopsis and *ABA2* in tobacco

(Marin *et al.*, 1996) catalyzes the epoxidation of zeaxanthin and antheraxanthin to violaxanthin (Rock and Zeevaart, 1991). The 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes the oxidative cleavage of 9-*cis*-neoxanthin to generate xanthoxin (Schwartz *et al.*, 1997; Tan *et al.*, 1997). It is thought that xanthoxin is converted to ABA by a two-step reaction via ABA-aldehyde. The *Arabidopsis aba2* mutant is impaired in the first step of this reaction, and is thus unable to convert xanthoxin into ABA-aldehyde (Léon-Kloosterziel *et al.*, 1996). The *Arabidopsis aba3* mutant is defective in the last step of ABA biosynthesis, i.e. the conversion of ABA-aldehyde to ABA (Schwartz *et al.*, 1997), which is catalyzed by ABA-aldehyde oxidase (AAO) as shown in Figure 2.3. Mutations in either the aldehyde oxidase apoprotein (Seo *et al.*, 2000) or molybdenum cofactor biosynthetic enzymes (e.g. molybdenum cofactor sulfurase; MCSU) would impair ABA biosynthesis and lead to ABA deficiency in plants. In this ABA biosynthetic pathway, the rate-limiting step was thought to be the oxidative cleavage of neoxanthin catalyzed by NCED (Tan *et al.*, 1997; Liotenberg *et al.*, 1999; Qin and Zeevaart, 1999; Taylor *et al.*, 2000; Thompson *et al.*, 2000). The study of the expression of *ZEP*, *NCED*, *AAO3*, and *MCSU* gene indicated that these genes are all up-regulated by drought and salt stress (Audran *et al.*, 1998; Seo *et al.*, 2000; Iuchi *et al.*, 2000; Xiong *et al.*, 2001, 2002), although their protein levels were not examined in every case. Nevertheless, the mechanisms by which drought or salt stress that up-regulate ABA biosynthetic genes are not understood and how ABA regulates its own biosynthetic genes is not clear.

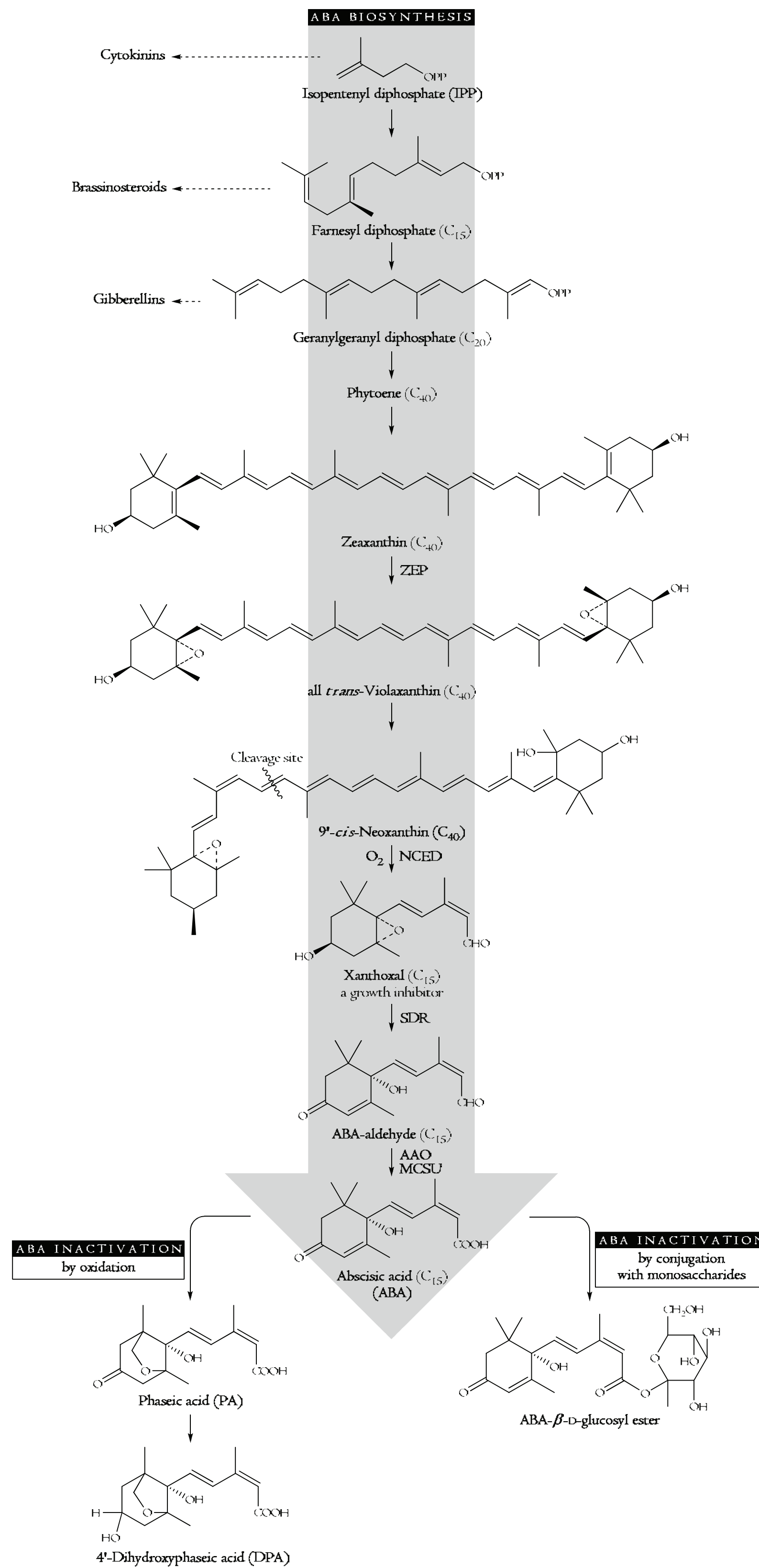


Figure 2.3 Pathway and regulation of ABA biosynthesis in higher plants (Modified from Taiz and Zeiger, 1998).

4. Signal transduction during drought/salinity

In cells of adult plants, there is a large overlap of ABA-, drought-, and salt-responsive gene expression (Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein *et al.*, 2002; Xiong *et al.*, 2002). Furthermore, characterization of stress gene promoters indicates that many promoters contain *cis*-acting elements that are separately responsible for abiotic stress and ABA. Because of their ABA-responsive promoter elements, these genes can be activated by stress-induced increase in ABA levels, as part of an ABA biosynthesis-dependent regulatory pathway. However, some apparent ABA-independent pathways may require ABA for full response as a result of cross talk between ABA and stress response pathways (Knight and Knight, 2001; Xiong *et al.*, 2002; Kim *et al.*, 2003).

ABA mediates some aspects of physiological responses to environmental stress such as osmotic- or drought-induced stomatal closure, the induction of the tolerance of salt, water, cold stress, and wound or pathogen response (Leung and Giraudat, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). In the salt stress condition, there are multiple pathways of signal transduction systems operating at the cellular level for gene regulation. Salts first decrease the osmotic potential of soil solution, resulting in the water stress in plants. Although the components of the signal transduction pathway are difficult to identify, ABA is well known as one such component acting in one of signal transduction pathways. The ABA-dependent and ABA-independent signal transduction pathways for the induction of stress related genes that occur in plants is shown in Figure 2.4.

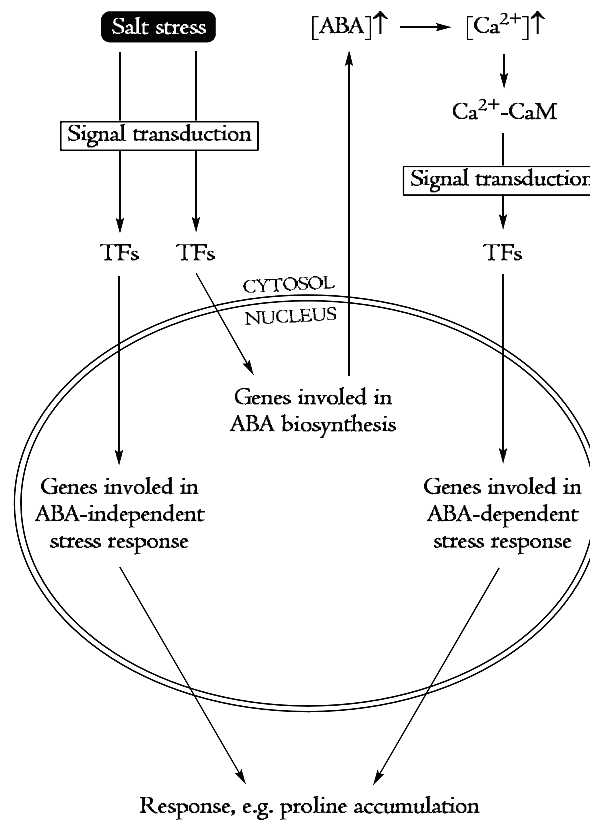


Figure 2.4 ABA-dependent and ABA-independent signal transduction in plants.

Among these stress responses, the stomatal response are relatively fast, occur within minutes and the changing of activity of the various signaling molecules and ion channels has been involved in this process, whereas the rest are slower and require changes in gene expression. Furthermore, cell biological studies have implicated common classes of secondary messenger or components of phosphorylation cascade in both fast and slow response to ABA. There is substantial evidence for multiple redundant ABA perception and signal mechanism, such that no element would be absolutely required for all responses. A major avenue of biochemical studies of ABA signaling began with the identification of ABA-regulated genes (Rock, 1995; Busk and Pages, 1998). In maturing seed, ABA-regulated genes include genes required for the synthesis of storage reserves and the acquisition of desiccation tolerance, range

from relatively high abundance transcripts that are required for adaptation to stress or reverse synthesis, to low abundance transcripts that encode signaling components (Thomas, 1993; Rock, 1995).

Frandsen *et al*. (1996) reported an ABA-, salt- and desiccation-induced gene encoding a protein containing a conserved Ca^{2+} binding site, this result suggested that Ca^{2+} linked signaling occurs in osmotically stressed plants. Calcium represents a prime candidate for such cross talk nodes because it has been shown to serve as a second messenger for both ABA and stress responses.

5. Abscisic acid induces stomatal closure in the environmental stress

ABA can induce the closure of stomata within a few minutes. Water stress in the root can lead to as much as a 40-fold increase in the abscisic acid levels of the plant. Guard cells use turgor pressure to control the stomatal aperture. An increase in ion uptake triggered by light or changing in carbon dioxide concentration results in the osmotic uptake of water by the guard cells. The turgor deforms the guard cell and opens the stomata. The stomates close as a result of the loss of ions, leading to a loss of water and reduction in turgor pressure. Guard cell has an abscisic acid receptor on the plasma membranes, but no ABA receptors have been identified to date. Binding of abscisic acid to its receptor in the guard cell membrane opens ion channels and activates proton pump. Under such severe stress, there are two factors involve in this process, first, water can evaporate directly from the guard cells (hydropassive closure), causing them to loss turgor and close. Second, ABA is redistributed within the leaf from various pools to the guard cell (Taiz and Zeiger, 1998).

As water stress begins, some of ABA carried by the xylem stream is synthesized in roots that are in water potential of the soil causes any measurable change in the water status of the leaves. The pH of the xylem sap increases from about 6.3 to about 7.2. This condition is alkaline. Alkalanization of the apoplast favors formation of the dissociated form of ABA (ABA^-) which does not readily cross membranes. Therefore, less ABA enters the mesophyll cells and more reaches the guard cells via the transpiration stream. In the normal condition the xylem sap is slightly acidic, favoring the uptake of the undissociated form of ABA (ABAH) by the mesophyll cells (MacRobbie, 1981; Anderson *et al*., 1994).

Much evidence supports a role for calcium both in the promotion of stomatal closing and in the inhibition of stomatal opening. Ca^{2+}, inositol 1,4,5-triphosphate (IP_3), and H^+ are thought the act as secondary messengers that mediate the effects of ABA on the stomata. The stress condition, such as salt and drought, stimulates the release of ABA, which opens the calcium channels in the guard cell plasma membrane (Hamilton *et al*., 1999). The resulting influx of Ca^{2+} into the cytosol triggers of additional Ca^{2+} from intracellular stores. The elevated cytosolic Ca^{2+} concentration closes K^+ influx channel in the plasma membrane, while K^+ efflux channels are open. This change produces a net outflow of potassium ions and a decrease in turgor pressure in the guard cell.

6. The level of ABA in plants under stress condition

During seed maturation in many species, there are two peak of ABA accumulation. The studies of genetic in Arabidopsis demonstrate that the first ABA peak is maternally derived and immediately precedes the maturation phase (Karssen

et al., 1983). In some species such as *Brassica napus*, this peak is correlated with low germinability of isolated embryos, whereas in others such as, maize, there is no correlation (Rivin and Grudt, 1991). Studies with ABA-deficient mutants of tomato showed that mature seed had a significantly higher proportion of G2 cells (4CDNA) than other genotypes, providing further support for the idea that endogenous ABA is required for arrest in G1 (Liu *et al.*, 1994). ABA content is increased in seed maturation, because of ABA can induce the expression of cyclin-dependent kinase inhibitor (ICK1) (Wang *et al.*, 2001) that would lead to cell cycle arrest at G1/S transition. The second peak of ABA accumulation in wild type Arabidopsis seed depends on synthesis in the embryo itself.

The studies of ABA content in plants showed that stress induced efflux of ABA from mesophyll cells was related to the change in cell volume rather than to turgor change (Hartung *et al.*, 1983). The first experiment to demonstrate the concentration among salt tolerance, the researchers found that the level of endogenous ABA in salt treated roots and that of specific polypeptides whose synthesis in roots were induced by exogenous ABA (Moon *et al.*, 1997). The studies of ABA content of cassava under drought stress showed that leaves of all cassava genotype accumulated large amounts of ABA in response to 6 days of water deficit. In relation to control, in mature leaves accumulated 4–7 fold higher contents of ABA, expanding leaves accumulated 5–37 fold, and youngest leaves 2–4 fold. The expanding and folded (pre-expansion) leaves on stress plants had the highest ABA concentrations (2000–5000 pmol g^{-1} FW), while the mature leaves had lower concentrations (400–1200 pmolg^{-1} FW) (Alfredo and Tim, 2000). There are some researches reported that the endogenous ABA level in rice was higher in the anaerobic environment. Mapelli *et*

al. (1995) studied ABA in rice seed in the anaerobic environment. They found that ABA faster increased and it was released in the media not only in anoxia germinated seed but also in air germinated seedlings when those were subsequently subjected to anoxia, indicating that the free ABA release in the medium was induced in rice as a response to anoxia stress.

7. Calcium signaling

Calcium signaling plays an important role in plants for coordinating a wide range of developmental processes and responses to hormonal including ABA responses in which the Ca^{2+} signal is triggered by secondary messengers such as cyclic ADP ribose (cADPR), inositol 1,4,5 triphosphate (InsP3), myo-inositol hexakisphosphate (InsP6) or H_2O_2 (Schroeder *et al*., 2001) and environmental signals such as salinity, cold, light, drought, symbiotic and pathogenic elicitors. It appears that different stimuli elicit specific calcium signatures, generated by altering the kinetics, magnitude, and cellular source of the influx (Malhó *et al.*, 1998; Allen *et al.*, 2000, 2001; Evans *et al.*, 2001; Rudd and Franklin-Tong, 2001). Calcium (Ca^{2+}) is important in maintaining the stability of the cell wall, membrane and membrane bound protein, due to its ability to bridge chemical residues among these structure. (Nayyar, 2002). Research during the last two decades has clearly established that Ca^{2+} acts as an intracellular messenger in couping a wild range of extracellular signals to specific responses (Reddy, 2001). The concentration of Ca^{2+} in the cytoplasm of plants cells is maintained low as the nanomolar range (100–200 nM) by being actively pumped into intracellular compartments and extracellular spaces where [Ca^{2+}] is in the millimolar range (1–10 mM) as shown in Figure 2.5 (Reddy, 2001). The export of

Ca^{2+} ions from the cytosol to the extracellular space or into intracellular organelles is achieved by ATP-driven Ca^{2+}-pumps and antiporters (Vetter and Leclerc, 2003).

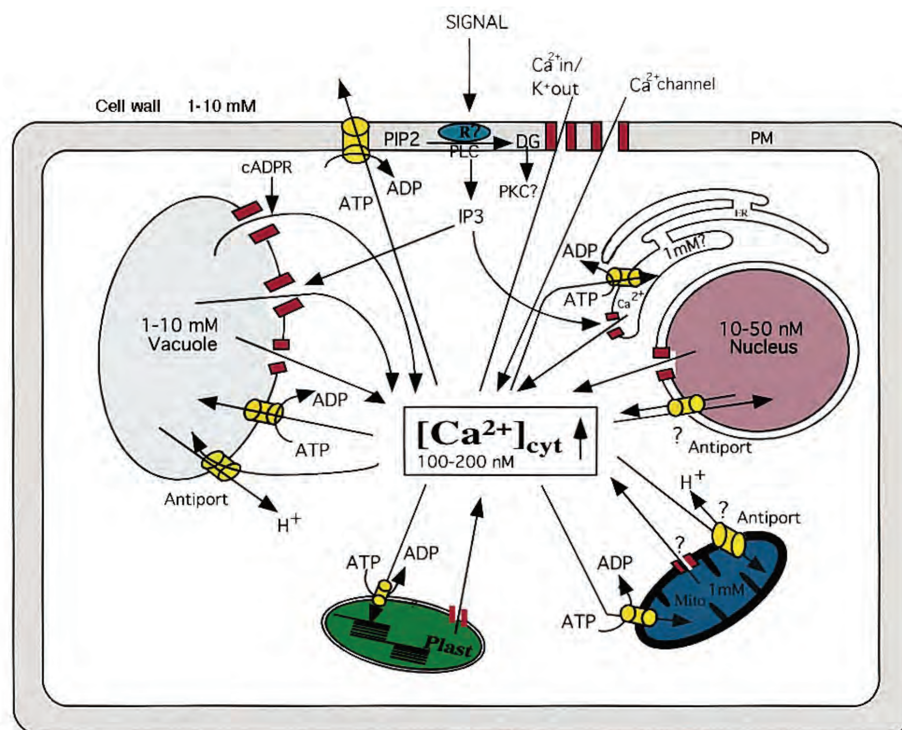


Figure 2.5 Schematic diagram illustrating the mechanisms by which plant cells elevate $[Ca^{2+}]_{cyt}$ in response to various signals and restore Ca^{2+} concentration to resting level. Ca^{2+} channels are shown in red, whereas Ca^{2+} ATPases and antiporters are indicated in yellow. Arrows indicate the direction of Ca^{2+} flow across the plasma membrane, and into and out of cellular organelles (vacuole, plastids, mitochondria, endoplasmic reticulum and nucleus). The estimated concentration of resting levels of Ca^{2+} in different organelles is indicated. Question marks indicate the lack of evidence. $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+}; PLC, phospholipase C; R, receptor, cADPR, cyclic ADP ribose, PIP_2, phosphotidyl inositol-4,5-bisphosphate, DG, diacylglycerol, PKC, protein kinase C, IP_3, inositol-1,4,5-trisphosphate; ER, endoplasmic reticulum; Mt, mitochondria; Plast, plastids; PM, plasma membrane (Reddy, 2001).

In ABA signaling, inositol triphosphate (IP_3), which is produced by phospholipase C activity acts as a secondary messenger regulating stomatal function and gene expression such as in Arabidopsis, the expression of only one of the six Arabidopsis *PLC* genes, *AtPLC1* is induced by ABA (Hirayama *et al.*, 1995). Different stimuli elicit Ca^{2+} transients which are distinct in their subcellular localization, amplitude, duration, frequency of oscillation and mode of spatial propagation (Snedden and Fromm, 2001). These properties are highly coordinated and regulated by the spatial distribution of Ca^{2+}-release channels and Ca^{2+} pump throughout the cell. The influx of Ca^{2+} ions are generated by voltage- and ligand-gated Ca^{2+}-permeable channels on the plasma membrane. In addition, several intracellular organelles function as Ca^{2+} stores, which can release Ca^{2+} upon stimulation by, for instance, inositol-1,4,5-trisphosphate (IP_3) or cyclic ADP-ribose (cADPR). The endoplasmic reticulum (ER) is a major Ca^{2+} stores, but mitochondria and the nucleus also participate actively in the release of Ca^{2+} through the IP_3-receptor. An important feature of the role of Ca^{2+} as a signal is the presence of repetitive Ca^{2+} transients. These transients may be generated both by first-round second messengers and by signaling molecules that may themselves be produced as a result of cascades of early Ca^{2+} signals as shown in Figure 2.6. These rounds of signals may have quite different signaling consequences and, therefore, physiological meaning (Xiong *et al.*, 2002).

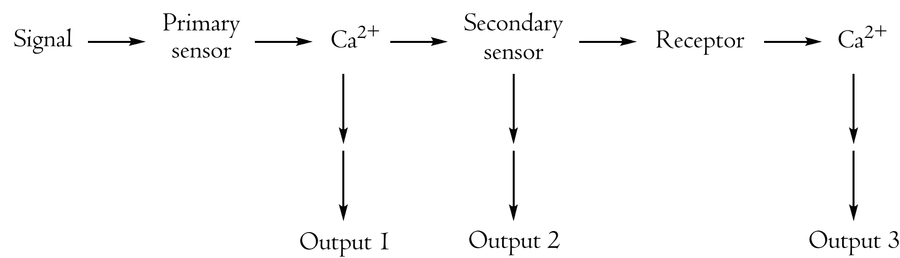


Figure 2.6 Repetitive Ca^{2+} transients upon the perception of a primary signal. The primary increase in cytosolic Ca^{2+} facilitates the generation of secondary signaling molecules, which stimulate a second round of transient Ca^{2+} increases, both locally and globally. These second Ca^{2+} transients may feedback regulate each of the previous steps (not shown). Ca^{2+} transients from different sources may have different biological significance and result in different outputs, as shown. Secondary signaling molecules such as ROS can also directly regulate signal transduction without Ca^{2+} (Output 2) (Xiong *et al.*, 2002).

8. Ca^{2+}-binding proteins

Transient Ca^{2+} increase in the cytoplasm in response to signals is sensed by several Ca^{2+}-binding proteins and decoded via Ca^{2+}-dependent conformational changes in these sensor polypeptides and interacting with target proteins. Once Ca^{2+} sensor decode the calcium elevation in the cytoplasm, Ca^{2+} efflux into the cell exterior and sequestration into cellular organelles such as vacuoles, ER and mitochondria restores its levels to resting state. A large number of Ca^{2+} sensors can be grouped into four major classes as shown in Figure 2.7. These include (A) Ca^{2+}-dependent protein kinase (CPK) that contains CaM-like Ca^{2+} binding domains and a kinase domain in a single protein. Each individual CPK protein is expected to detect changes in the Ca^{2+} parameters and translate these changes into the regulation of a protein kinase activity

(Roberts and Harmon, 1992). (B) Calmodulin (CaM) contains four EF-hand domains but have no enzymatic activity themselves and function by interacting with their target proteins (Zielinski, 1998). (C) Another EF-hand motif-containing Ca^{2+}-binding proteins are calcineurin B-like (CBL) proteins that are similar to both the regulatory B subunit of calcineurin and the neuronal Ca^{2+} sensor (NCS) in animals (Klee *et al.*, 1998) and (D) The last group is Ca^{2+}-binding proteins without EF-hand motifs. Members of the first three classes of Ca^{2+} sensors contain helix-loop-helix motifs that bind to Ca^{2+} with high affinity (Roberts and Harmon, 1992). However, different Ca^{2+}-binding proteins differ in the number of EF hand motifs and their affinity to Ca^{2+} with dissociating constants (K_ds) ranging from 10^{-5} to 10^{-9} M. Binding of Ca^{2+} to a Ca^{2+} sensor causes a conformational change in the sensor resulting in modulation of its activity or its ability to interact with and modulate function or activity of other proteins (Reddy, 2001).

9. Calmodulin

All eukaryotes, including higher plants, contain a small calcium-binding protein known as calmodulin (CaM). CaM is the most well characterized Ca^{2+} sensors among these groups of protein. It consists of only 148 amino acids, highly conserved, soluble, intracellular Ca^{2+}-binding protein ubiquitously found in animals, plants, fungi and protozoa, and it has four EF-hands that bind to four Ca^{2+} ion, two in the carboxyl-terminal domain and two in the amino-terminal domain. Many proteins involved in Ca^{2+} signal transduction alter their activity in response to changes in free Ca^{2+} levels, but are themselves not able to bind Ca^{2+} ion. Some of these proteins utilize CaM as a sensor and mediator of the initial Ca^{2+} signal. CaM is a multifunctional protein because of its ability to interact and regulate the activity of a

number of proteins. CaM relays the Ca^{2+} signal by binding free Ca^{2+} ions to its C- and N-terminal EF-hand pairs, which causes a conformational change and enables Ca^{2+}/CaM to bind to specific CaM-binding domains. The binding of Ca^{2+}/CaM to its target proteins alters their activity in a calcium dependent manner.

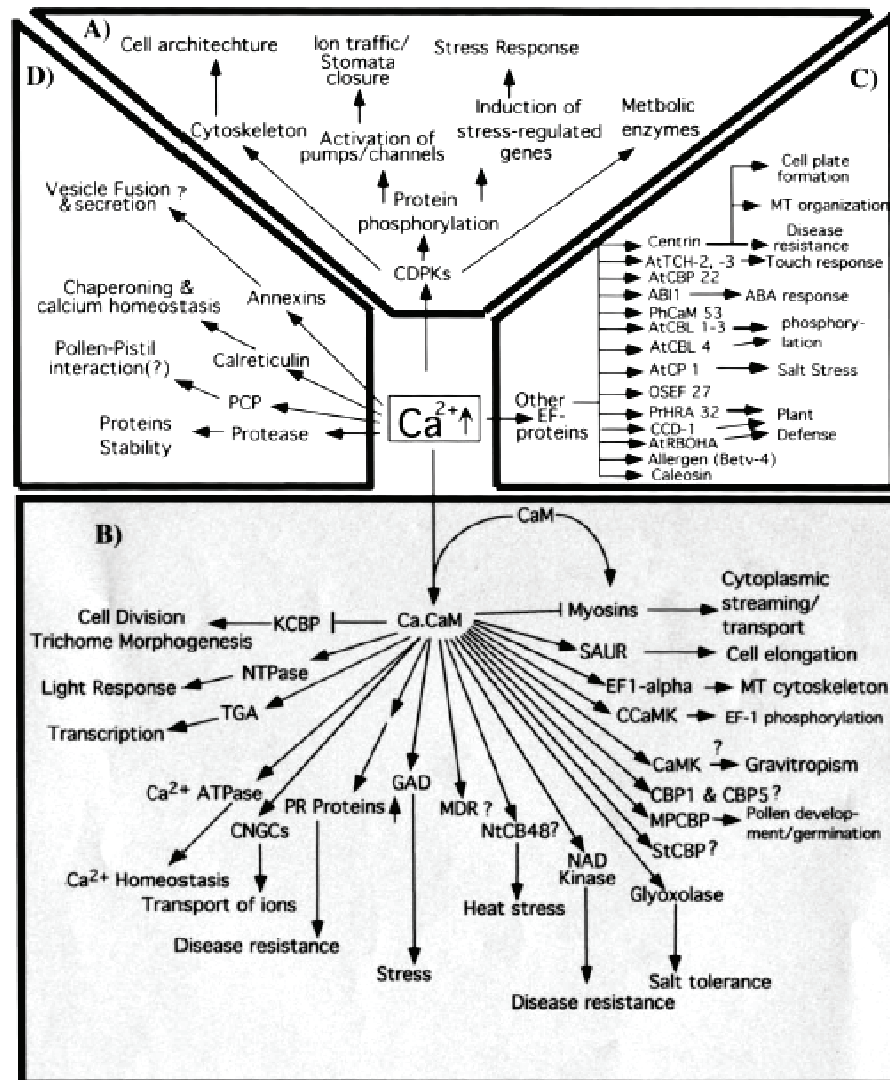


Figure 2.7 Ca^{2+} sensing proteins and their functions in plants. Four major groups of Ca^{2+} sensors (indicated in four boxes) have been described in plants: (A) Ca^{2+}-dependent protein kinase (CPK), (B) Calmodulin (CaM), (C) other EF-hand motif-containing Ca^{2+}-binding proteins and Calcineurin B-like (CBL) protein, (D) Ca^{2+}-binding proteins without EF-hand motifs (Reddy, 2001).

Biotic and abiotic signals are perceived by receptors, resulting in some cases, in transient changes in Ca^{2+} concentrations in the cytosol and/or organelles (e.g. nucleus). Increases in free Ca^{2+} concentrations originating from either extracellular pools or intracellular stores are capable of binding to Ca^{2+}-modulated proteins including calmodulin and calmodulin-related proteins. Structural modulations of these proteins enable them to interact with numerous cellular targets that control a multitude of cellular functions, such as metabolism, ion balance, cytoskeleton and protein modifications. In addition, Ca^{2+} and calmodulin might also regulate the expression of genes by complex signaling cascades or by direct binding to transcription factors. Rapid changes in cellular functions result from direct interactions of calmodulin and calmodulin-related proteins with their targets (within seconds to minutes) while slower responses require gene transcription, RNA processing and protein synthesis (variable times from minutes to days).

The EF hands in CaM are organized into two distinct globular domains, each of which contains one pair of EF hands. Each pair of EF hands is considered the basic functional unit. Pairing of EF hands is thought to stabilize the protein and increase its affinity toward Ca^{2+}. Although each globular domain binds Ca^{2+} and undergoes conformational changes independently, the two domains act in concert to bind target proteins. Upon increase of Ca^{2+} concentration to submicromolar or low micromolar levels, all CaM molecules are activated. Cooperative binding is required for this "on/off" mechanism to function efficiently. The cooperative Ca^{2+} binding ensures that full activation of the CaM occurs in a narrow region of calcium concentration during a signaling event. The selectivity of CaM toward Ca^{2+} also is an important factor in effective transduction of the Ca^{2+} signal. CaMs (and other EF hand-

containing proteins, including CBLs) have evolved as highly specific Ca^{2+} sensors (Luan *et al.*, 2002).

In plants, there are multiple *CaM* genes that code for either identical proteins or proteins containing a few conservative changes. These small changes in amino acid composition of CaM isoforms may contribute to differential interaction of each CaM isoform with target proteins. The striking example for differential regulation of CaMs comes from the studies with soybean CaM isoforms. In soybean there are five CaM isoforms (SCaM1 to -5). SCaM1, -2 and -3 are highly conserved compared to other plant CaM isoforms including *Arabidopsis* CaM isoforms whereas SCaM4 and -5 are divergent and showed differences in 32 amino acids with the conserved group (Lee *et al.*, 1995). Surprisingly, these divergent CaM isoforms are specifically induced by fungal elicitors or pathogen (Heo *et al.*, 1999). These results provided evidence for the differential regulation of CaM isoforms in plants. Soybean isoforms show differences in their relative abundance *in vivo*. The conserved isoforms are relatively abundant in their expression compared to the divergent forms. All CaM isoforms activate phosphodiesterase (PDE) but differ in their activation of NAD kinase, calcineurin and nitricoxide synthase indicating Ca^{2+}/CaM specificity between CaM isoforms and target proteins (Lee *et al.*, 1997). Different affinities for Ca^{2+}-CaM interactions with specific target proteins may be sufficient for the differential transduction of the Ca^{2+} signal (Luan *et al.*, 2002).

10. Calmodulin gene expression and environmental stress

Recent studies on *CaM* genes expression in response to different stimuli indicate that different CaM isoforms are involved in mediating a specific signal

(Zielinski, 1998). This indicates that *CaM* genes are differentially expressed in response to different stimuli such as drought, salinity, cold and parthogenic microorganism. Three of the six *Arabidopsis Cam* genes (*Cam1, -2* and *-3*) are inducible by touch stimulation (Zielinski, 1998) indicating the presence of different *cis*-regulatory elements in their promoters. In potato, only one of the eight CaM isoforms (PCaM1) is induced by touch (Takezawa *et al.*, 1995). In rice, the study of three CaM isoform: OsCam1, OsCam2 and OsCam3 showed that *OsCam1* and *OsCam3* gene are inducible by salinity and wounding. In contrast, *OsCam2* are not induced by these stimuli indicating that although the expression of genes encoding different calmodulin isoforms is ubiquitous, they are differentially regulated by various stress signals (Phean-o-pas *et al.*, 2005). The presence of multiple CaM isoforms adds further complexity to the Ca^{2+} mediated network in plants which suggests their important and diverse roles in calcium signaling.

11. Calmodulin in rice

Rice (*Oryza sativa*) was a cereal selected to be sequenced as a priority and has the status of a “model organism”. Rice with its relatively small genome size (~430 Mb), ease of transformation, well developed genetics, availability of a dense physical map and molecular markers (Wu *et al.*, 2002; Chen *et al.*, 2006), 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 is considered a model monocot system. Therefore, 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.

Boonburapong and Buaboocha (2007) studied the Ca^{2+}-binding proteins of rice by phylogenetic analysis based on amino acid sequences similarity and classified proteins with a high degree of identity as "true" CaMs that probably function as typical CaMs. They were named *OsCam1-1*, *OsCam1-2*, *OsCam1-3*, *OsCam*2 and *OsCam3*. *OsCam1-1*, *OsCam1-2* and *OsCam1-3* encode identical protein, whereas *OsCam2* and *OsCam3* encode a protein of only two amino acid differences and their sequences share 98.7% identity with those of *OsCam1* proteins. In addition, *OsCam1* amino acid sequences are identical to those of the typical CaMs from barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) reflecting the close relationships among monocot cereal plants. On average, OsCaM amino acid sequences share about 99%, 90% and 60% identity with those from plants, vertebrate and yeast, respectively.

12. Use of transgenic plants for characterization of gene functions

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most used method for introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. *Agrobacterium* introduces a plasmid, T-DNA, into the plant cell which integrates randomly in the plant genome. By replacing part of the T-DNA for the gene of a desired protein, the gene can be introduced stably in the plant cell as shown in Figure 2.8.

The first record on transgenic tobacco plants expressing foreign genes appeared at the beginning of the last decade (Herrera-Estrella *et al.*, 1983). Kawasaki *et al*. (2001) transformed the promoter of *RGS-38* gene, which encodes plastidic glutamine synthetase of *Oryza sativa* L., fused to a β-glucuronidase (GUS) reporter

gene, into tobacco plants. The reporter directed GUS expression, both in leaves and roots, and the expression of GUS was regulated by light. Until recently, *Agrobacterium*-mediated gene transfer into monocotyledonous plants was possible when reproducible and efficient methodologies were established on rice (Hiei *et al.*, 1994), corn (Ishida *et al.*, 1996) and wheat (Cheng *et al.*, 1997).

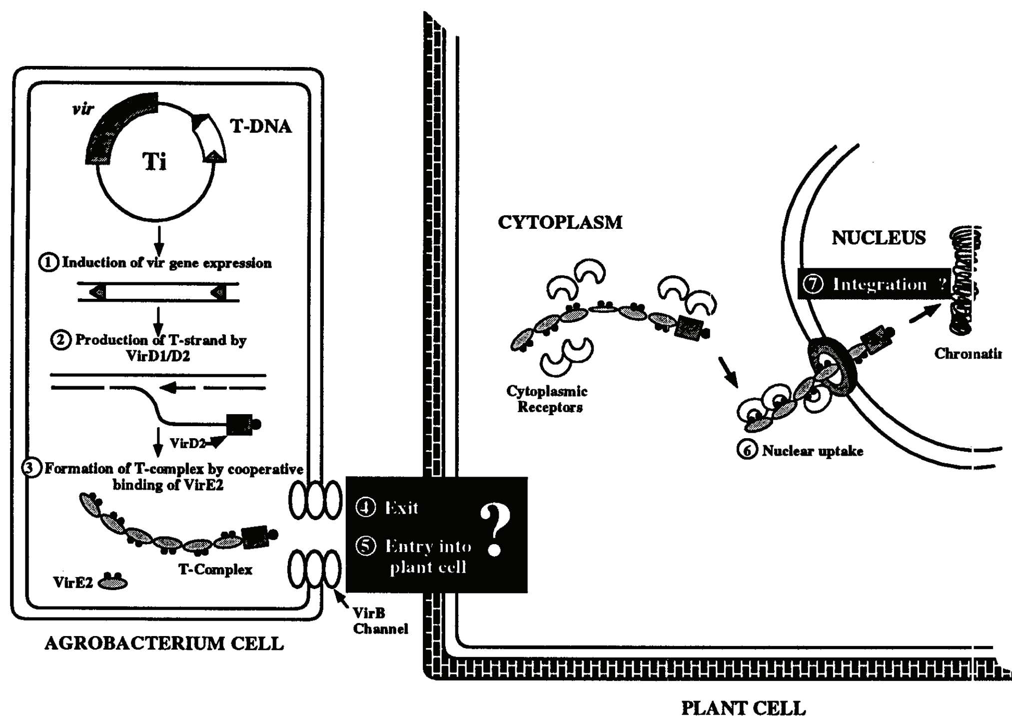


Figure 2.8 Basic steps in the transformation of plants cells by *A. tumefaciens* (Zupan and Zambryski, 1995).

To provide a clear indication that genetic transformation did take place, the vector pCAMBIA1301 uses β-glucuronidase (GUS) which is the most widely used system of reporter genes in plants. In general, reporter genes should have the following characteristics: 1) the genetic organization should be well described, 2) the gene products should not be present in the organism or tissue under study, 3) the gene products should be well characterized with regard to biochemical activity, 4) substrate

dependence and stability, and 5) the product of the reaction catalyzed by the reporter gene product should be stable, easily detectable, and quantifiable (Crazzolara *et al.*, 1995).

GUS is encoded by the *Escherichia coli uidA* gene (Jefferson *et al.*, 1987). The protein has a molecular weight of 68.2 kDa. The best substrate currently available for histochemical localization of β-glucuronidase activity in tissue and cells is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). The product of glucuronidase activity on X-Gluc is colorless. Instead, the indoxyl derivative product must undergo an oxidative dimerization to form the insoluble and highly colored indigo dye. This dimerization is stimulated by atmospheric oxygen, and can be enhanced by using an oxidation catalyst such as a potassium ferricyanide/ferrocyanide mixture. It is the efficient reporter that can be used to locate its expression in plant tissues and organs without the need to extract the respective tissue. However, the substrate for detection of GUS activity (X-Gluc) is expensive and the X-gluc-stained plant material is killed by the GUS assay the reaction is shown in Figure 2.9.

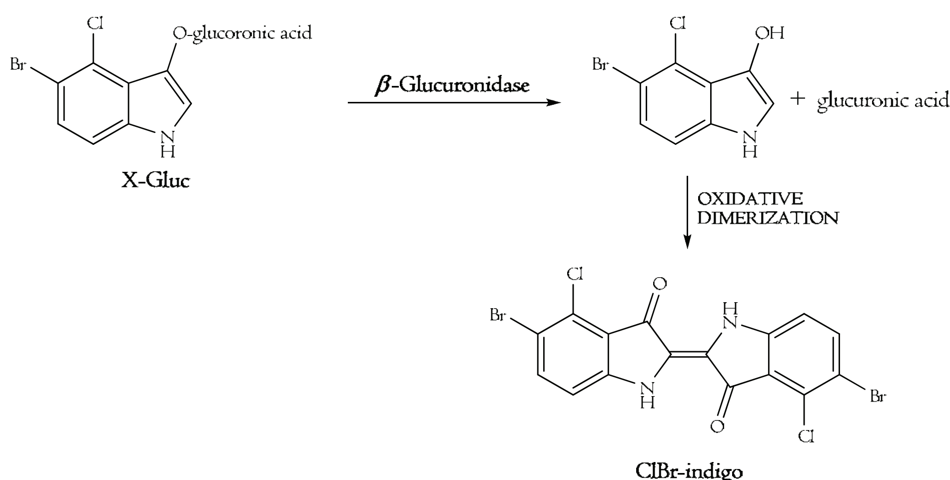
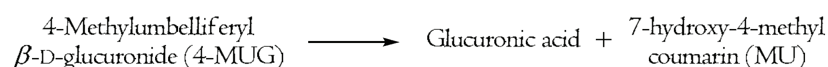


Figure 2.9 Chemical basis of X-Gluc GUS histochemical staining (Gallagher, 1992). Cleavage of X-Gluc produces the final insoluble blue precipitate ClBr-indigo.

13. Quantitation of GUS activity by fluorometry

The most widely used fluorogenic substrate for detection of *β*-glucuronidase activity *in vitro* is 4-methylumbelliferyl *β*-D-glucuronide (4-MUG) upon the hydrolysis by GUS. However, the fluorochrome 4-methylumbelliferone (7-hydroxy-4-methyl coumarin) is produce along with sugar glucuronic acid. Using excitation at 363 nm and measuring emission at 447 nm, background fluorescence from the substrate is negligible. In general, measurement of GUS activity using this fluorescent substrate are two to three orders of magnitude more sensitive than X-Gluc (5-bromo-4-chloro-3-indodyl *β*-D-glucuronide), that is a substrate for histochemical detection) or pNPG (para-nitrophenyl *β*-D-glucuronide) is a substrate for spectrophotometric detection.

The fluorometric assay on the hydrolysis of the MUG substrate by GUS is shown in this reaction.



Genetic screens used to date have been based on aberrant growth or gene expression response to ABA. Recent, reverse-genetics have been used to test a specific gene's functional role. Biochemical studies have identified a variety of gene promoter elements and transcription factor correlated with ABA response. The cell biological studies have tested the role of candidate secondary messengers and signaling intermediates in regulating cellular responses to ABA. Therefore, in this study, the mechanism of salt stress response mediated by ABA in rice (*Oryza sativa* L.) was studied for elucidation of the relationship between ABA and calmodulin gene expression under salt stress in rice. The transgenic rice line that harbours the over-

expression of *OsCam1-1* gene construct that fused with *gus* reporter gene were used.

14. Quantitation of ABA

There are various methods have been used for ABA measurement. For purification of ABA in crude extracts, HPLC has largely replaced TLC. Actual quantification is usually accomplished by GC with an electron capture detector, as well as by radio- or enzyme- immunoassays. Analytical technique for the quantification of ABA in plant extracts have been greatly improved in the past decade by immunoassay. Therefore, the amount of ABA as low as 10 pg can be detected by immunoenzymatic test (Weiler, 1980). This suggested that anti-ABA antibodies could be a very powerful tool for the detection of endogenous ABA in plants. Therefore, in this study preparation of MAb against ABA was performed for quantitation of endogenous ABA in rice under salt stress condition. The advantageous feature of using monoclonal antibodies against ABA for immunoassay is a large number of crude plant samples could be analyzed for ABA content in a short period of time with high specificity. Moreover this technique does not require complicate and intensive purification protocol (Weiler, 1980). And the last important thing is that the enzyme immunoassay of ABA using monoclonal antibodies is relatively cheaper than other available techniques for ABA quantitation.

15. Immunological techniques

Immunological techniques are widely used for diagnosis of many bacterial diseases in human medicine, food, agriculture and aquaculture. Nowadays, the MAbs have been used to detect the levels of plant hormones such as cytokinin and ABA by using enzyme-linked immunosorbent assay (ELISA) technique. In addition to high

sensitivity and specificity, immunological method can be simplified to obtain results quickly at relatively low cost (Sithigorngul *et al*., 2002).

16. Monoclonal antibodies

Antibodies (also known as immunoglobulin) are a class of protein molecules produced by B lymphocytes of the adaptive immune system which act as flexible adapters between the infectious agents and phagocytes. They are produced and present in the serum when an animal encounters an antigen either by infection or by deliberate injection. Antibodies as used in immunological test systems are powerful tools for the specific detection and identification of virus. Monoclonal antibodies are antibodies produced by on expanded clone of B-lymphoid cell or plasma cell. All any molecules have the same amino acid sequence and the same class of immunoglobulins with an identical antigen binding site (Dreesman, 1983). They are directed to a single epitope on the antigen and they are available in essentially unlimited quantities. Monoclonal antibodies (MAbs) are important reagents used in biomedical research, in diagnosis of diseases and in treatment of diseases such as infections and cancer.

In 1975, Kohler and Milstein developed the technique for producing hybridoma cells that could secrete antibodies with defined specificity. In this technique, an antibody-secreting cell isolated from an immunized animal is fused with a myeloma cell, to produce a type of malignant immunoglobulin secreting cell. B lymphocyte hybridomas are made by fusing B lymphocytes with myeloma cells. The lymphocytes contribute specific antibody producing genes to the hybridoma while the myelomas provide genes that allow the hybridoma to divide indefinitely and continue to express the immunoglobulin gene.

17. Principle of monoclonal antibody production

The fusion between an antibody secreting cell and myeloma cell can be facilitated by fusogens. Fusogens can be lysolecithin, or Sendai virus or polyethyleneglycol (PEG). PEG is commonly used for hybridoma fusion. When the cells are treated with PEG, their membranes fuse and then multinucleated cells called heterokaryons are formed. At the next cell division, the nuclei of the heterokaryons fuse, and the daughter cells possess a more or equal share of the genetic material. The resulting hybrid cells are not genetically stable and there is a strong tendency for loss of chromosomes. If one of the chromosomes that carry the rearranged immune-globulin heavy or light chain genes in lost, production of the antibodies will end.

In the most efficient fusion, 1% of starting cells are fused and 1 of 10^5 forms viable hybrids. The cells from the immunized animals do not continue to grow in tissue culture and the unfused myeloma cells must be subsequently killed by the selection procedure. What is needed in this step is a way to suppress the myeloma growth but ensure that only the hybrids will grow. By far, the popular selective systems are the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and the adenine phosphoribosyl transferase (APRT) selective system (Littlefield, 1964).

The main biosynthetic pathways of purine and pyrimidine can be blocked by the folic acid antagonist, aminopterine, and that of purine can be blocked by glutamine antagonist, azaserine. As a self guard for survival, the normal cells possess alternative salvage pathways as shown in Figure 2.10 to overcome this biosynthesis. In these pathways, the recycle synthesis is carried out from the single building stones which include diphosphoribose phosphate, glutamine, glycine and other free bases

from nucleic acid degradation via the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) which catalyzes the formation of inosine monophosphate (IMP) and guanylate (GMP) from hypoxanthine and guanine, respectively. The enzyme adenine phosphoribosyl transferase (APRT) catalyzes the formation of adenylate (AMP) from free adenine bases. Salvage pathway also depends on the enzyme thymidine kinase (TK); but selection by TK is more difficult, because the occurrence of TK negative variant is rare events (Goding, 1983). In selective system, the myeloma cells which are defective in the enzyme HGPRT ($HGPRT^-$), die in cell culture medium containing hypoxanthine (H), aminopterine (A) and thymidine (T) or HAT (Figure 2.11). They cannot multiply in the presence of aminopterine, even when supplied with hypoxanthine and thymidine. The cell which produces enzyme TK and HGPRT can survive. Therefore, only the hybridoma cells survive in the selective medium, since the myelomas provided the ability to grow in medium culture and spleen cells contributed the functional HGPRT enzyme necessary to overcome the aminopterin block. In fact, this system is a half-selective medium, since the spleen cells which are normal cells die off naturally.

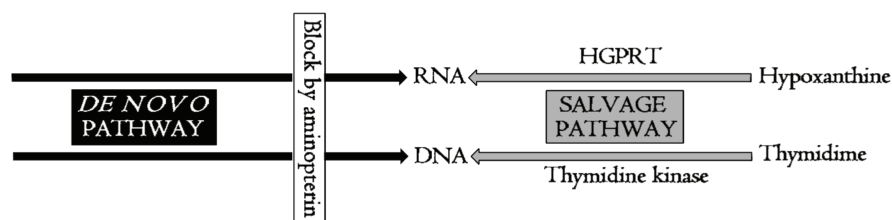


Figure 2.10 Principle of the HAT selective system: Main biosynthetic pathway are blocked by aminopterine. Cells can synthesize nucleic acid using the salvage pathways if hypoxanthine and thymidine are provided. Cells which lack the salvage pathway enzymes namely, the HGPRT or thymidine kinase cannot synthesize nucleic acid, and die off.

Another selective system was based on the property of the enzyme APRT. The medium contains azaserine to block the *de novo* purine synthesis. Myeloma cell line lacking this enzyme ($APRT^-$) cannot complete their nucleic acid synthesis and die off. Mutant myeloma cells lacking HGPRT are selected by the use of toxic drug to incorporate into DNA of the myeloma cells via HGPRT. The HGPRT producing cells die and only the mutant which is defective in HGPRT will survive for selection. Production of HGPRT deficient cells is relatively easy, since the enzyme is coded for by a gene on X-chromosome, and thus only a single mutation is needed to result in total loss of the enzyme.

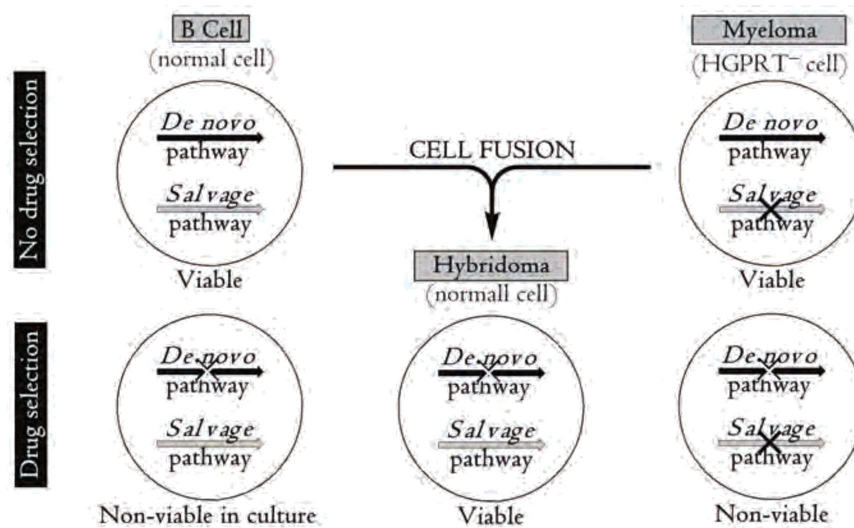


Figure 2.11 Drug selection for viable hybridomas synthesis (Harlow and Lane, 1998).

18. Monoclonal antibody structure and diversity

The mouse B cell repertoire of producing over 10 million different antibody molecules with dissociation constants for specific antigen ranging from 10^{-5}–10^{-10} mol/l. Most monoclonal antibodies currently employed in biotechnology are of murine origin usually mouse IgG antibodies. The diverse binding specificity of an IgG molecule is a consequence of the unique structure of the IgG molecule and the

amino acid contain within two identical binding pockets at the N-terminal end. An IgG molecule is symmetrical and consists of one pair of heavy (H) 55 kDa polypeptides chains and one pair of light (L) 25 kDa polypeptides chains linked by interchain disulfide bonds. The H and L chains are woven into a tight barrel- like structure formed by repeating B-pleated sheets that juxtapose variable and hypervarriable stretches of amino acids and fold to form two identical antigen combining sites (Vetterlein, 1989).

Vetterlein (1989) found that the advantages of monoclonal antibodies were (a) each clone B cell produces only one type of antibody of predefined specificity, (b) impure antigens can be used to generate monoclonal antibodies, (c) MAbs can be generated against rare and weakly immunogenic antigen, (d) MAbs have a predefined isotype and thus variable effecter functions, and (e) reproducible antibody can be made in unlimited amounts.

19. Fusion of myeloma cells and immune cells

Spontaneous fusion of cell is usually rare except that of sperms, eggs, osteoblasts, myotubes, foreign body giant cells or certain virus infection cells (syncytium formation) (Springer, 1985). However normal cell fusion can possibly be made by addition of cell fusogen(s) such as Sandai virus and polyethyleneglycol (PEG) or lysolecithin. PEG is now the reagent of choice. Therefore, when cells are treated with fusogen, their membranes fuse and multinucleate cells, called heterokaryons, are formed and then the nuclei of heterokaryons fused. At the next cell division, the hybrid cells possess a more or less equal share of the genetic material.

The most monoclonal antibodies used in research, medicine and biotechnology are of mouse origin (Vetterlein, 1989). An outline of the experimental protocol for the production of monoclonal antibodies is shown in Figure 2.12.

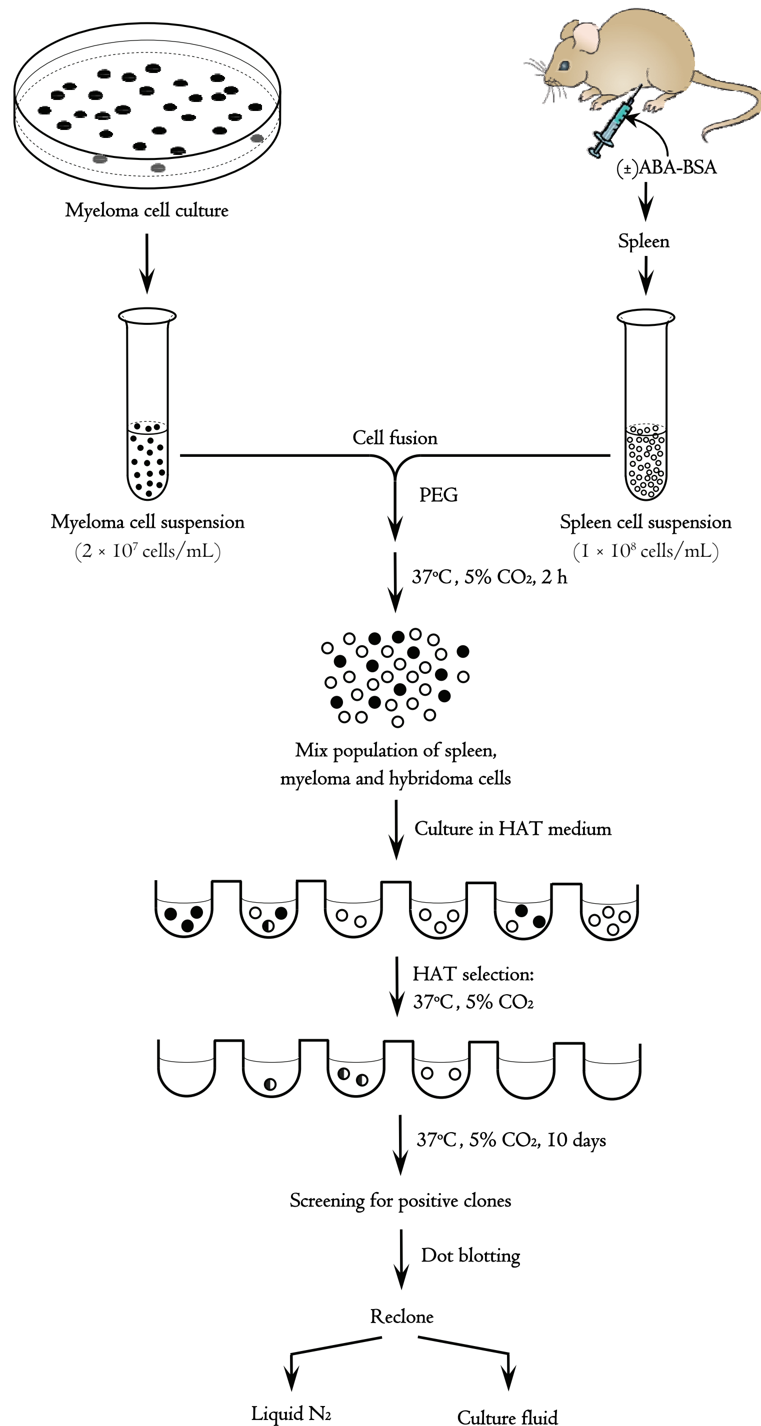


Figure 2.12 Monoclonal antibodies production. (Modified from Sithigorngul *et al*., 2002.)

20. Immunization

Primary injection of antigen emulsified in complete Freund's adjuvant is injected intraperitoneallly (other injection site such as intramuscular hind legs and hind foot-pads are also commonly employed). Boost injections are given using incomplete Freund s adjuvant at 2–3 week intervals. A final injection is often given 2–3 days before spleen or lymphoid cells are removed (Steward, 1984).

21. Screening of hybridoma

The growing hybridoma are screened for their productions of antibody by appropriate sensitive assay e.g. Enzyme-linked immunosorbent assay (ELISA), Radioimmunoassay (RIA), Dot-blot (DO), Western blot (WB) and Immunohistochemistry (IHC) and the antibody producing hybridoma are cloned in soft agar or by limiting dilution method for increasing the quantity of the antibody (Steward, 1984).

22. Reclone positive clones

Two widely methods are used to achieve this; cloning in soft agar and cloning by limiting dilution (Steward, 1984).

a. Cloning in soft agar

High dilutions of the cells are made in agar and appropriate colonies are picked out from the soft agar and grown in culture.

b. Cloning by limiting dilution

High dilutions of the cells are made so that individual cells can be transfer to tissue culture medium in microtiter plates and grown. Wells containing one colony are then grown in culture. Hybridoma can be grown in tissue culture and up to 10 µg/ml of specific antibody may be obtained.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Plant materials

Rice (*Oryza sativa* L.) cultivar Khao Dok Mali 105 (KDML105)

Rice (*Oryza sativa* L.) cultivar FL530-IL (FL530; an introgression line of KDML105)

Rice (*Oryza sativa* L.) cultivar Leung Pra Tew 123 (LPT123)

Rice (*Oryza sativa* L.) cultivar Leung Pra Tew 123-TC171 (LPT123-TC171; an isogenic line of LPT123)

OsCam1-1::uidA overexpressed KDML105 line

35S::OsCam1-1 overexpressed KDML105 line

1.2 Instruments

1.2.1 Equipment for ABA-BSA conjugated antigen synthesis

Aluminium foil

Autopipette tip

Balance: Sartorius CP423s (Scientific Promotion Co. USA)

Dialysis bag

Eppendorf tubes 1.5 ml

Grass bottle 50 ml

Grass bottle 100 ml

Liquid nitrogen tank

Micropipette

Rotary evaporator

Round flask

1.2.2 Equipment for monoclonal antibody production

Blood collecting tube

Centrifuge

Centrifuge tubes 50 ml

CO_2 incubator oven

Cotton

Cryotube

Eight channel pipette

Forceps

Grass bottle

Inverted microscope

Liquid nitrogen tank

Microculture plate 96, 24 and 6 well

Microplate reader

Rubber tube and mouth piece

Sterile pasture pipette

Sterile Petri dish

Syringe 22 G

1.2.3 Equipment for dot bolt ELISA

Forceps

Micropipette

Nitrocellulose membrane

Plastic box

Ruler

Scissors

1.2.4 Equipment for plant growing

Grass bottle 50 ml

Grass bottle 100 ml

Plastic tray 8 × 12 $inch^2$

Plastic tray 500 × 100 cm^2

Sand

1.2.5 Equipment for collecting plant growth

Aluminium foil

Balance: Sartorius CP423s (Scientific Promotion Co. USA)

Forceps

Hot-air oven

Ruler

Scissors

1.2.6 Equipment for ABA extraction

Autopipette tip

Centrifuge (Universal16, Hettich, Germany)

Centrifuge tubes 50 ml

Deep freezer –80ºC

Eppendorf tubes 1.5 ml

Icebox

Mortars and pestles

Refrigerator

Spatula

Vortex mixer

1.2.7 Equipment for ABA determination by competitive ELISA

Eight channel pipette

Micropipette

Microplate reader

Plastic box

96-well microculture plate

1.2.8 Equipment for ABA determination by HPLC

Beaker

250 mm × 4 mm column packed with 5 µm ODS Hypersil (Shandon Runcorn, UK)

Eppendorf tubes 1.5 ml

HPLC (Agilent Technologies Series 1100)

Nipro disposable syringe 5 ml

Rotary evaporator

Round flask

Vacuum pump

Vial 5 ml

1.2.9 Equipment for RNA extraction

Cuvette

Deep freezer –80ºC

DNA-RNA Horizontal gel eletrophoresis apparatus

Eppendorf tubes 1.5 ml

Gel document (Gel Doc^{TM} 2000, BIO-RAD)

Microwave oven

Mortar and pestle

pH meter

Refrigerated centrifuge

Spatula

Spectrophotometer

Vortex mixer

Water bath

1.2.10 Equipment for determination of gene expression by RT-PCR

Gel document (Gel Doc^{TM} 2000, BIO-RAD)

PCR equipment

PCR thin wall microcentrifuge tube 0.2 ml

Pipette tips 10, 100, 1000 µl

1.2.11 Equipment for determination of gene expression by real time-PCR

PCR thin wall microcentrifuge tube 0.2 ml

Pipette tips 10, 100, 1000 µl

Real-time PCR equipment

1.2.12 Equipment for GUS assay

Eight channel pipette

Eppendorf tubes 1.5 ml

Microplate reader

Mortar and pestle

Plastic box

2. Methods

2.1 Development of the monoclonal antibody for endogenous ABA detection in plant extract

As ABA molecule is too small to induce the immune system in animal, it need to be conjugated with other protein molecules, such as bovine serum albumin (BSA). Two types of ABA conjugated BSA were synthesized according to Weiler (1980) with some modification.

2.1.1 Antigen preparation

2.1.1.1 Synthesis of the (±)-ABA conjugated with BSA at the carboxy terminal

The (±)-ABA was conjugated with BSA in the carboxyl terminal by using the NHS esterification in PBS solution. (±)-ABA (Sigma,cis-trans isomers, 99%) was coupled through C_1 to BSA using 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide-HCl (EDC). (±)-ABA (66 mg), dissolved in 1.5 ml solution of water-dimethyl-formamide (1:2), and then it was added dropwise to an ice cold solution of 125 mg BSA in 5 ml H_2O, while keeping the pH > 8.1 with 1N NaOH. EDC (105

mg) was divided into four parts and then added over a period of 2 h. The solution was stirred under N_2 at 4°C in the dark overnight. The conjugate (ABA-BSA) was dialysed for 4 days against distilled water and stored at –20°C. The reaction of synthesis the (±)-ABA conjugate with BSA in the carboxyl terminal was done by using the NHS esterification which is shown in Figure 3.1.

(±)-ABA-ovalbumin conjugated and aminohippuric acid BSA conjugates were prepared in the same manner and used for determination of MAb specificity.

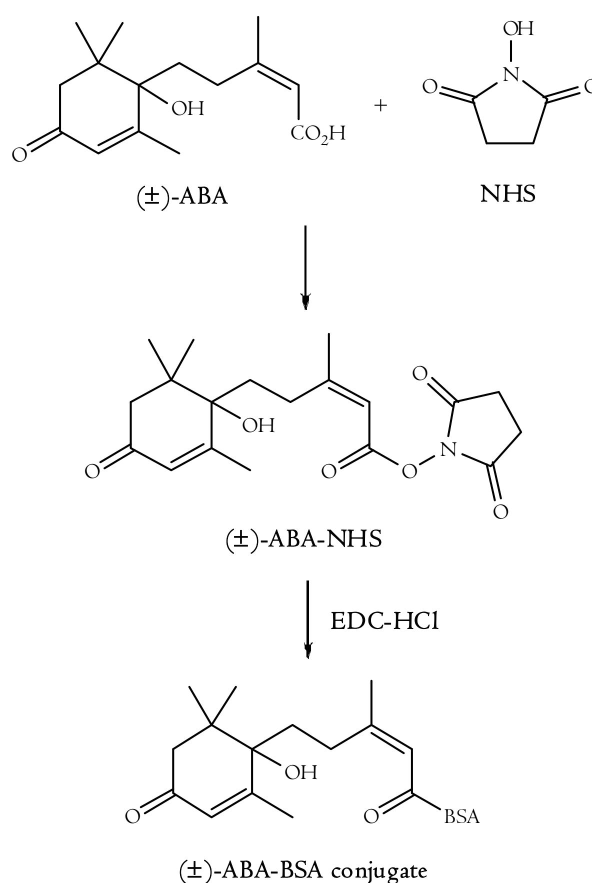


Figure 3.1 The reaction of (±)-ABA conjugated with BSA at the carboxyl terminal.

2.1.1.2 Synthesis of the (±)-ABA conjugated with BSA at the carbonyl terminal

The (±)-ABA conjugate with BSA in the carbonyl terminal by using the tyrosinehydrasine with *p*-aminohippuric acid BSA in PBS solution. (±)-ABA was coupled through C_4 to BSA by the tyrosinehydrasine with amino-hippuric acid in PBS solution as shown in Figure 3.2. The conjugate (ABA-C_4-BSA) was dialysed for 4 days against water and stored at –20°C. This method was slightly modified from Weiler (1980).

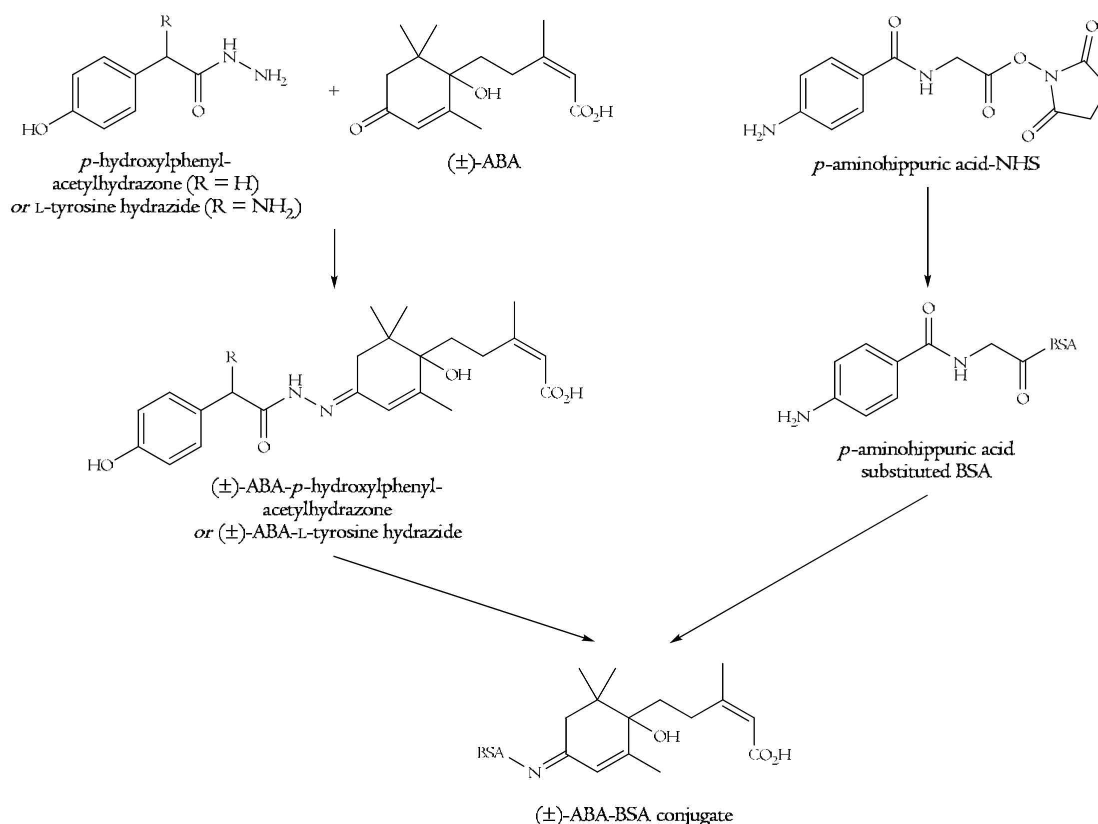


Figure 3.2 The reaction of (±)-ABA conjugated with BSA at the carbonyl terminal.

2.1.2 Monoclonal (+)-ABA antibody production

2.1.2.1 Immunization of mice

The antigen (±)-ABA conjugated with BSA at the carboxyl terminal was used for injection the mice for production of antibody specific to carbonyl terminal of ABA. Four 6-week-old Swiss albino mice were first immunized intraperitoneally with antigen performed in the 2.1.1. Antigen (±)-ABA-BSA conjugate emulsified in complete Freund's adjuvant (Sigma) for a total volume of 100 µl per mouse (antigen:adjuvant 1:1 v/v; 50 µg/mouse). The mice were boosted twice with the same antigen mixed with incomplete Freund's adjuvant (Sigma). One week after the third injection, antisera were collected, absorbed with hippuric acid-BSA conjugated in PBS overnight at 4°C and tested for immunoreactivity by dot blot. The best performing mouse was boosted 3 days before hybridoma production.

The (±)-ABA conjugated with BSA at the carbonyl terminal was also used to production of antibody specific to carboxyl terminal of ABA as the same method described above.

2.1.2.2 Serum specificity test and screening of antiserum

The antiserum from each mouse was tested against (±)-ABA-BSA, HA-BSA, (±)-ABA-OA, HA-OA and (+)-ABA-BSA. The best response was used for spleen donor in hybridoma production (Appendix C2).

2.1.2.3 Hybridoma production

Hybridomas were produced according to Kohler and Milstein (1976) with slight modification. P3X myeloma was cultured in RPMI 1640 media supplemented with 10% calf bovine serum (CBS) in a humidified incubator in the presence of 5% CO_2 at 37ºC. The myelomas were maintained in long-phased growth by means of subculturing everyday for one week prior to fusion. The media volume in the petri dishes was maintained at approximately 15 ml.

Spleen was clipped off and minced using a 5 ml syringe plunger through a sterile grid. The suspension was aspirated through the grid several times by pipetting. Ten ml of spleen suspension was added to a 50 ml polypropylene tube containing approximately 10^8 cells of P3X myeloma. The cell mixture was centrifuged for 5 min at 200x g and the supernatant was discarded. To facilitate fusion, one ml of pre-warmed (37ºC) 40% (w/v) polyethene glycol (PEG 1500, Sigma) was added to the cell suspension using a pipettor (1000 µl), and simultaneously the tube was slightly agitated. This step was performed for exactly one min. Thirty-nine ml of HAT media (Appendix A1.5) was added into the suspension, and it was then incubated in a humidified incubator for 2 hrs. The cell suspension was suspended in HAT medium. One hundred µl of this mixed cell suspension was dispensed into each well of the thirty 96-well culture plates, and they were kept in the humidified incubator.

2.1.2.4 First screen of hybridomas for anti-ABA antibodies by dot ELISA

Six days after fusion, macroscopic growths were examined. The cell supernatants were screened for anti (±)-ABA monoclonal antibody by dot ELISA.

The nitrocellulose membrane of 0.45 µm average pore size (Schleicher and Schull®, Dassel, Germany) was used as a solid phase in the dot ELISA. The antigen (±)-ABA-BSA was applied into the nitrocellulose membrane as 1 µg/spot and the antibody from each hybridoma was added to the well, resulting the ¼ dilution of the antibody (Appendix C2).

Subsequently, the nitrocellulose membrane disks were incubated for 3 h at room temperature with horseradish peroxidase conjugated goat anti-mouse IgG heavy and light chain specific antibody (GAM-HRP : Bio-Rad®) at 1:1500 dilution in 0.5% BLOTTO. Then the nitrocellulose membrane was washed 4 times with PBS and incubated substrate containing 0.03% DAB (3,3-diaminobenzidine tetrahydrochloride, Sigma), 0.01 % H_2O_2 and 0.05% $CoCl_2$ in PBS at RT for 5 min, and washed with distilled water. The results were scored as positive or negative after visual observations of dark gray spot on the nitrocellulose membrane.

2.1.2.5 Cloning of hybridomas by the limiting dilution method

The positive hybridoma cells were recloned by the limiting dilution method to obtain single clone per well. From each positive well, 10 µl of the culture suspension containing approximately 100 variable cells were drawn off and thoroughly mixed with 7.5 ml of HAT media. One hundred µl of the mixture was

dispensed into 48 wells of 96 well microtiter plate and more 2.5 ml of the HAT media was added into the remaining mixture to lower the concentration of hybridoma cells. The plates were incubated in the humidified incubator for 7 days. Cell growth in the bottom of the wells was monitored using and inverted microscope. Wells that contained a single colony were further propagated in 20% FCS RPMI 1640 medium and cell supernatants were secondary screening tested by dot ELISA with the (±)-ABA-BSA antigen as described in 2.1.2.4.

2.1.2.6 Cryopreservation

The mid-log phase proliferating hybridomas were harvested and centrifuged at 200X g for 5 min at RT. The hybridoma pellets were mixed with cold freezing media (Appendix A1.6.) (4ºC) at approximately 5×10^6 cells/ml. About 0.5 ml of the cell suspension was transferred into cryopreservation tubes (NUNC®), slowly froze in the deep freeze at –80 ºC overnight and then transferred into liquid N_2 (–196 ºC).

2.1.2.7 Characterization of monoclonal antibodies

Isotypes and subisotypes of mouse immunoglobulins produced by the hybridomas were determined by sandwich ELISA using Zymed's Mouse Mono Ab ID kit (HRP). The procedure was conducted following the manufacturer's recommendation with slight modification. Briefly, 50 µl of goat anti-mouse IgG dilution were added to the microtiter wells. After the plates were incubated in a humidified box at 4ºC overnight, they were washed with 150 µl of 0.5% BLOTTO for 4 times. Each MAb (50 µl at 1:50 dilution) was added to 8 wells of each column, incubated for 4 h and washed as before. Fifty µl of each class and subclass specific

rabbit anti-mouse immunoglobulins (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, IgM, κ, and λ) were added into 12 wells of each row, respectively, incubated for 4 h and washed as before. The goat anti rabbit-horseradish peroxidase conjugated (GAR-HRP) was added into each well (50 µl), incubated for 4 h and washed as before. To develop the reaction, 80 µl of substrate mixture (1 mg/ml *O*-phenylenediamine dihydrochloride (OPD), 0.006% H_2O_2 in citrate buffer pH 4.5) was added to each well for 5 min and the reaction was stopped by 80 µl of 1 N H_2SO_4/well. The OD was measured at 450 nm using an ELISA reader (Titertek Multiskan® PLUS, Finland). The flow chart of the step in isotypes and subisotypes determination is shown in Figure 3.3.

2.2 Sensitivity test of monoclonal antibody

2.2.1 Dot blotting

The sensitivity of the monoclonal antibody was determined by dot blotting. The (±)-ABA-BSA or (+)-ABA-BSA were serially dilution (10 fold) and spotted onto each square nitrocellulose membrane (1 µl/spot) and incubated with antibody from each clone at 1:20 dilution in 5% BLOTTO for 8 h. After extensive washing with 0.5% BLOTTO, the membrane was incubated in GAM-HRP at 1:1500 dilution for 8 h then washed extensively in 0.5% BLOTTO and incubated in a substrate mixture as described in 2.1.2.4. The last visualized spot of each MAb was determined.

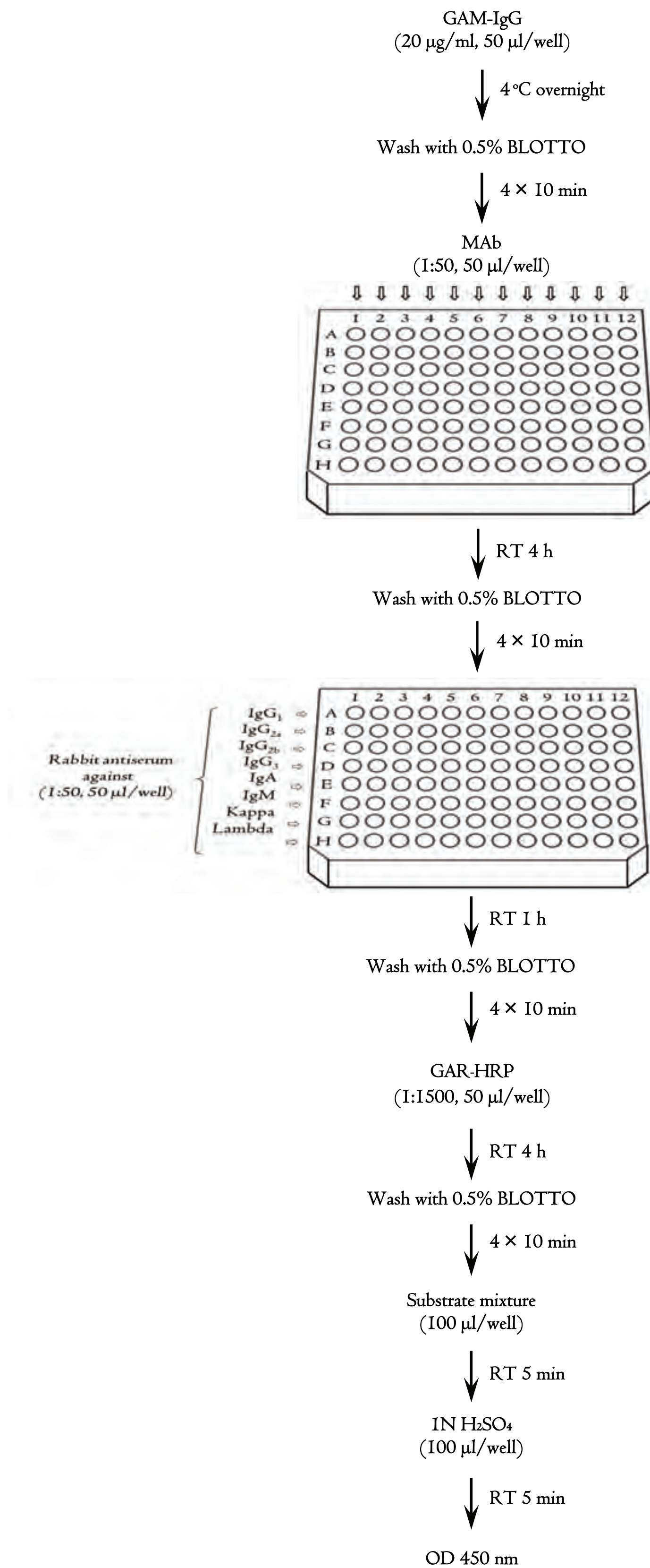


Figure 3.3 A flow chart for determination of antibody isotypes and subisotypes preparation (Sithigorngul *et al*., 2000).

2.2.2 Indirect ELISA

2.2.2.1 Sensitivity test of monoclonal antibody in different concentration of antigen

The sensitivity of monoclonal antibody was confirmed by indirect ELISA. Each row of a microplate was coated with (±)-ABA-BSA (50 µl) at various concentrations: 0.08, 0.016, 0.032, 0.16, 0.8, 4 and 8 µg/ml for overnight at 4ºC. After washing and blocking with BLOTTO as before, each MAb at 1:100 dilution (50 µl) was added to each column and processed for indirect ELISA as described in 2.1.2.7. Absorbance at 450 nm was determined with automated microplate reader. The MAb that gave high absorbance at the lowest concentration of (±)-ABA-BSA was determined.

2.2.2.2 Titer determination

The selected concentration of the antigen (150 ng/ml) in 2.2.2.1 was used for determination suitable titer of MAb in indirect ELISA. The (±)-ABA-BSA (50 µl) was coated on the bottom of microtiterplate and processed for indirect ELISA as described above using MAb at 1:10, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:4000, 1:5000 and 1:10,000 dilutions.

2.2.3 Determination of competitive ELISA sensitivity for ABA quantitation

The antigen (±)-ABA-BSA (0.15 µg/ml: 50 µl) and MAb at the selected dilution determined in 2.2.2 were used to develop competitive ELISA.

(+)-ABA or (±)-ABA at concentration of 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.10, 0.05, 0.025 and 0.125 µg/ml were added to each well, followed by 50 µl of anti-(+)-ABA MAb at the selected dilution then processed as in described in indirect ELISA described above. Two replicates were determined for each dilution.

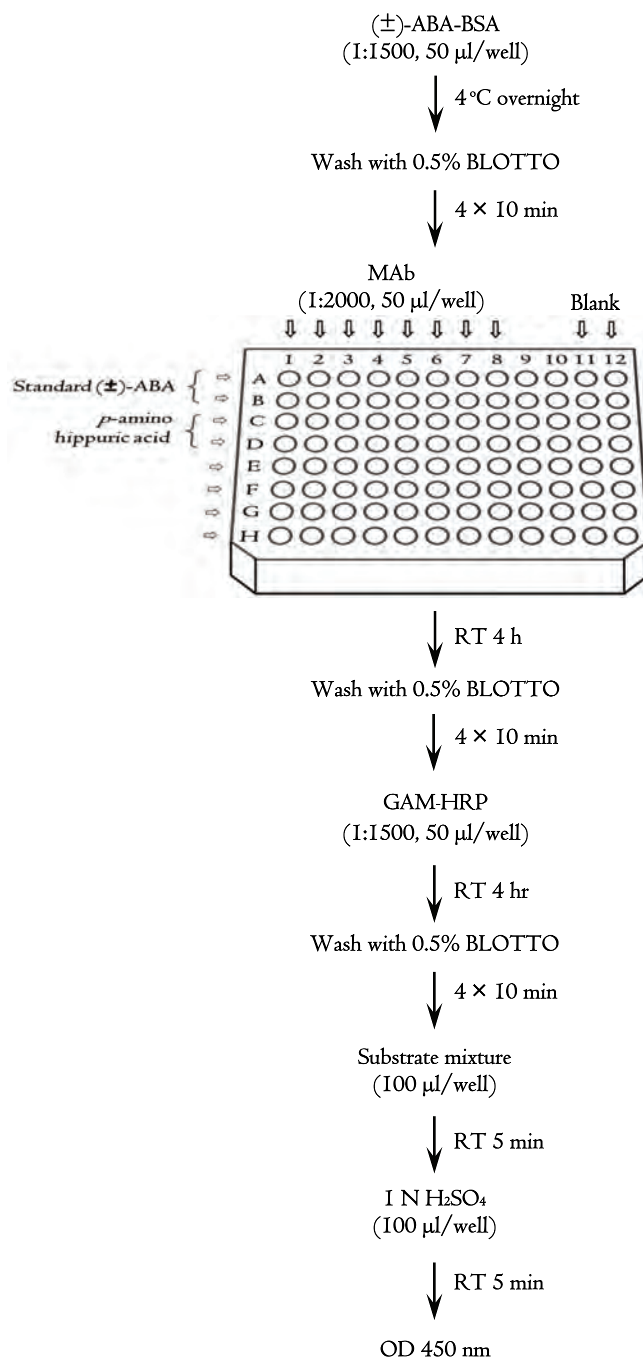


Figure 3.4 A flow chart for determination of ABA in plant extract by indirect competitive ELISA.

2.2.4 The effect of methanol extraction buffer on the sensitivity of monoclonal antibody by indirect ELISA

To study of the effect of methanol extraction buffer on the sensitivity of antibody by indirect ELISA, the 10%, 20% and 30% methanol extraction buffer were used. The standard (±)-ABA concentration of 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.10, 0.05, 0.025 and 0.125 µg/ml were dissolved in 10%, 20% and 30% methanol extraction buffer. The method was done as described in 2.2.3.1. (±)-ABA and (+)-ABA were also tested in the same manner.

2.3 Detection of the endogenous ABA in the rice tissues, when grown under normal/salt stress condition by the ELISA and HPLC method

2.3.1 Plant samples and growing condition

Rice seeds (*Oryza sativa* L.) of salt sensitive lines (KDML105 and LPT123) and salt tolerant line (FL530-IL and LPT123-TC171) were germinated on sand for 1 week. The one week old seedlings, then were transferred to WP No.2 nutrient solution (Vajrabhaya and Vajrabhaya, 1991). Plants were grown in the green house with normal light and the temperature was between 32 ºC during the day and 29ºC during the night.

2.3.2 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 0.1 mg ml^{-1} butylated hydroxyl-toluene and 0.5 mg ml^{-1} citric acid monohydrate) at the ratio of 1 g fresh

weight tissue in 50 ml methanol extracting solution. The extracts were shaken in dark for 16 hrs and then centrifuged at 4800 g at 4ºC for 15 min. The supernatant was evaporated to dryness using rotary evaporator and re-suspended with methanol for ABA quantification by HPLC.

For the ABA determination experiment, twenty-four-day old seedlings were transferred to the WP. No.2 nutrient solution, containing 0.5% (w/v) NaCl. For non-stressed control plants, they were grown in the same nutrient solution without NaCl. The 500–1000 mg of leaf tissues were harvested and frozen immediately in the liquid N_2 for ABA quantification at 0, 0.5, 1, 2 and 4 hours after treatment. For the longer response, the leaf tissues were collected on day 0, 1, 3, 5, 7, 9 and 11 after treatment.

2.3.3 Experimental design and statistical analysis

To determine the difference level of ABA accumulation during salt stress condition in various rice lines which have different salt tolerant ability, the completely randomized design (CRD) with 3 replicates was performed the analysis of variance (ANOVA) was used to distinguish the mean difference and mean comparison was done with Duncan's Multiple Range Test (DMRT).

2.4 Detection of the endogenous ABA in rice tissues, when grown under normal/salt stress condition by ELISA method

2.4.1 Plant sample and growing condition

Plant samples were prepared as indicated in 2.3.1

2.4.2 Experimental design and statistical analysis

Experimental design and statistical analysis were performed as indicated in 2.3.3.

2.4.3 ABA extraction

The frozen tissues were extracted with methanol extraction buffer as indicated in 2.3.2. The 100% methanol extraction buffer was diluted at 10% methanol in PBS buffer for ABA quantitation by indirect competitive ELISA method.

2.4.4 Quantitation of ABA by ELISA method

The samples were loaded onto (±)-ABA-C_1-BSA conjugated precoated 96 well microtiter plate as indicated in 2.2.3. Tissue sample for each dilution was done in triplicate. The indirect competitive ELISA was performed in the similar protocol as indicated in 2.2.3. The duplicate of standard (±)-ABA at various concentrations was incubated in the same plate.

2.5 Quantitation of ABA by HPLC

ABA was quantified using HPLC (Agilent Technologies Series 1100). HPLC instrument equipped with a UV absorbance detector operating at 254 nm was used. Column 250 mm x 4 mm i.d, was packed with 5 µm ODS Hypersil, Shandon Runcorn, UK), 50 µl of sample was loaded and it was eluted at a flow rate of 1 ml/min using different proportion of methanol and 0.05 M acetic acid : 30% methanol for 6 min, 30 to 50% linear gradient of methanol over 20 min, 50% methanol for 6 min and 50 to 100% methanol over 15 min and ABA was identified by liquid

chromatography-mass spectrometry (LC-MS) (Agilent Technologies Series 1200 (LC), microTOF BRUKER (MS)) for detection the fraction of plant extract.

ABA content is expressed on the basis 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.

2.6 The relationship between the *OsCam1-1* gene expression and ABA accumulation during salt stress condition

2.6.1 The effect of salt stress on *OsCam1-1* gene expression: Determination of the *OsCam1-1* gene expression in the KDML105 and FL530-IL in the salt stress condition by using real-time-polymerase chain reaction (real-time PCR)

Rice plant materials were prepared as indicated in 2.3.1. After the salt treated on twenty-four-day old seedling, leaf tissues was harvested at 0, 0.5, 1.0, 1.5, 2.0, and 4.0 hours after treatment for *OsCam1-1* gene expression detection. The non-stressed plants were also collected as controls.

2.6.1.1 Total RNA preparation

Total RNA was isolated by hot phenol procedure as described by Thikard *et al*. (2005). *Oryza sativa* L. tissues were ground in liquid nitrogen using chilled mortar and pestle to fine powder. Then, 500 µl of hot extraction buffer (80ºC) (phenol, 0.1 M LiCl, 100 mM Tris-HCl (pH 8.0), 10 mM EDTA and 1% SDS) was added to the frozen powder. The mixtures were homogenized by vortexing for 30 seconds. Then, it was centrifuged at 14,000 rpm for 5 minutes at 4 ºC. The upper aqueous phase was transferred at a fresh microcentrifuge tube. RNA was precipitated

by the addition of 0.2 volume of 10 M LiCl and stored overnight at –20ºC. The mixture was left at room temperature for 5–10 minutes and centrifuge at 14,000 rpm for 20 minutes at 4ºC. After centrifugation, the pellet was dissolved in 20 µl DEPC treated water. The concentration was estimated by measuring the optical density at 260 nm and calculation of the concentration in µg/ml unit was performed using the following equation:

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

* The absorbance at 260 nm (A_{260}) of 1.0 corresponds to the RNA of approximately 40 µg/ml (Sambrook *et al.*, 2001).

The quality and quantity of RNA sample were checked on the 1%(w/v) agarose gel in TBE buffer.

2.6.1.2 Real-Time-PCR Amplification of mRNA sequences

a) DNase Treatment

Ten micrograms of the total RNA sample were added to the DNase (Takara, Japan) treatment mixture according to manufacturer protocol. The reaction was incubated at 37ºC for 1 hour. Phenol-chloroform extraction was performed by addition of 50 µl sterile water and 100 µl of phenol-chloroform solution. After vortexing, it was centrifuged at 14,000 rpm for 10 minutes at 4ºC. The supernatant was added with 10 µl (0.1 volumes) of 3 M NaOAc and 200 µl (2 volume) of isopropanol and stored overnight at –20ºC. The mixture was left at room temperature for 5-10 minutes, before centrifugation at 14,000 rpm for 20 minutes at 4ºC, and then the supernatant was discarded and the pellet was washed with 300 µl of 80% ethanol and air dried. The total RNA was resuspended with 20 µl of diethyl pyrocarbonate (DEPC)-treated water.

b) Reverse Transcription

Two micrograms of the DNase-treated RNA was added to the reverse transcription mixture (1X M-MLVRT buffer, 100 ng of oligo$(dT)_{15}$ primer, 100 ng of the dNTP mix, and 200 units of M-MLVRT). The reaction was incubated at room temperature for 10 minutes and 42ºC for 1 hour.

c) Real-Time-PCR Amplification

Real-time-PCR amplification was carried out by using forward and reverse oligonucleotide primers that provided by Assist. Prof. Dr. Teerapong Buaboocha. The *OsActin* primers were used as the internal standard. The sequences were as follows:

*OsCam1-1*F	5´-ACCGTGCATTGCCGTATTAG-3´
*OsCam1-1*R	5´-GCAAGCCTTAACAGATTCAC-3´
OsActin-F	5´-CTGCCGAGCGGGAAATTGTC-3´
OsActin-R	5´-CTGGCGGAGCGACAACCTGT-3´

Real-Time-PCR amplification by iQSYBR Green Super mix for *OsCam1-1* and *actin* gene was performed as follows: predenaturation at 95ºC for 30 second, denaturation at 95ºC for 30 second, annealing at 55ºC for 30 second, and extension at 72ºC for 30 second 35 cycles.

2.6.2 The effect of exogenous ABA and abamine SG application on *OsCam1-1* (*Calmodulin 1-1* in *Oryza sativa* L.) promoter activity

2.6.2.1 Transgenic rice lines harboring a *gus* reporter gene under the *OsCam1-1* promoter

Seeds of the transgenic rice, the *indica* rice cultivar (*Oryza sativa* L.) KDML105 that harbors a *gus* reporter gene under the control of the *OsCam1-1* (*Calmodulin 1-1* in *Oryza sativa* L.) promoter was provided by Assist. Prof. Dr. Teerapong Buaboocha.

The construct of the *gus* reporter gene under the control of the *OsCam1-1* promoter as shown in Figure 3.5 was produced using the pCAMBIA1391Z vector which contains a gus (*uidA* with an intron) as a reporter gene and a hygromycin-resistant gene (*hpt*) under the control of the *35S* promoter from cauliflower mosaic virus (CaMV) as a plant selectable marker within the T-DNA. The pCAMBIA1391Z vector also contains a kanamycin-resistant gene (*npt II*) gene that could be used as a bacterial selectable marker. The construct of the *gus* reporter gene under the control of the *OsCam1-1* promoter was transformed into the rice calli by co-cultivation with *Agrobacterium*.

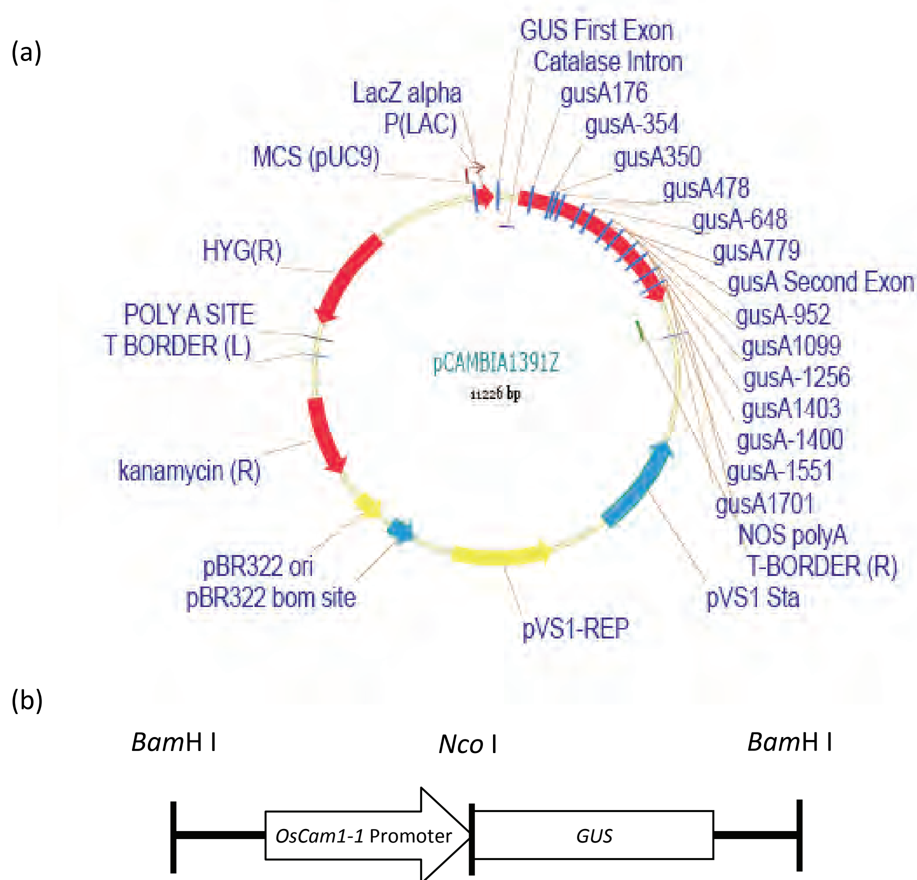


Figure 3.5 Schematic diagrams of (a) pCAMBIA 1391Z carrying the promoter *gus* reporter gene and (b) the construct of the *gus* reporter gene under the control of the *OsCam1-1* promoter.

2.6.2.2 Charaterization of the transgenic rice lines by histochemical analysis for *β*-glucuronidase (GUS) activity for homozygosity determination

To selected the transgenic rice plants for the experiment, the homozygous transgenic lines were used. Transgenic rice plants harboring the *gus* reporter gene under the control of the *OsCam1-1* promoter were grown in normal conditions for determination of the homozygosity of the *gus* reporter gene. Homozygosity of positively stained T_1 generation plants was verified by segregation analysis of their T_2 generation plants via histochemical GUS assays (Appendix A5.1).

The leaf tissues were placed into microcentrifuge tubes and 500 μl of histochemical (X-Gluc) straining solution composed of 1 mM X-Gluc, 100 mM NaH_2PO_4/Na_2HPO_4, 0.5 mM K Ferrocyanide, 0.5 mM K Ferricyanide, 0.1% (v/v) Triton X-100 and 10 mM EDTA was added, and then incubated at 37ºC overnight befored formation of blue color could be detected.

2.6.2.3 Treatment of the transgenic rice lines with ABA

Two lines of the homozygous transgenic rice were used. In the treatment of exogenous ABA application, 100 μM (±)-ABA was soakly sprayed onto the rice leaves early in the morning. The rice sprayed with H_2O and buffer of the ABA solution were used as controls. Rice seedlings were germinated and grown in hydroponic culture solution WP No.2 (Vajrabhaya and Vajrabhaya, 1991) in the greenhouse. Four replicates was used for all experiments. Twenty-four-day old rice seedlings grown in WP No.2 solution and WP containing 0.5% (w/v) NaCl were used for determination of the *OsCam1-1* promoter activity in the leaf tissue at –2, 0, 1.0, 2.0, and 4.0 hours after stress treatment.

2.6.2.4 Treatment of the transgenic rice lines with abamine SG

Two lines of the transgenic rice were used. In the treatment of abamine SG, which is an ABA inhititor application, 100 μM abamine SG was soakly sprayed onto the rice leaves early in the morning. The rice sprayed with H_2O and buffer of the abamine SG solution were used as controls. Rice seedlings were germinated and grown in hydroponic culture solution WP No.2 (Vajrabhaya and Vajrabhaya, 1991) in the greenhouse. Four replicates were used for all experiments.

Twenty-four-day old rice seedlings grown in WP No.2 solution and WP containing 0.5% (w/v) NaCl were used for determination of the *OsCam1-1* promoter activity in the leaf tissue at –2, 0, 1.0, 2.0, and 4.0 hours after stress treatment.

2.6.2.5 Determination of GUS activity in the transgenic rice lines

a) Determination of protein concentrations

Concentration of the proteins extracted from the transgenic rice lines were determined by the BCA protein assay kit (Pierce®) (Appendix A5.2). The 25 µl of each protein standard or plant extract was pipetted into a microplate well (working range 20–2,000 µg/ml). Two hundred µl of sample with protein concentration in the working range was added to each well, mixed for 30 seconds and incubated at 37ºC for 30 min. After plates were cooled to RT, the absorbance at 540 nm was measured using the microplate reader. The averaged 540 nm absorbance values of the blank replicates were subtracted from the 540 nm absorbance values of all individual BSA standards and unknown sample replicates. Standard curve was prepared by plotting the subtracted absorbance at 540 nm of each BSA standard against its concentration in µg/ml. The standard curve was used to determine the protein concentration of each unknown sample.

b) GUS activity of the transgenic rice lines

The *OsCam1-1* promoter activity was detected via the GUS activity assay. The leaf tissue was ground in 0.2 ml of GUS extraction buffer in a microcentrifuge tube. The homogenate was centrifuged in a microcentrifuge at 10,000 g for 5 min and the supernatant was taken and placed on ice. In a

microcentrifuge tube, 0.1 ml of sample extract was mixed with 0.4 ml of 1 mM 4-MUG and incubated at 37ºC. The fluorescence with excitation at 355 nm and emission at 460 nm was measured against time, and the rate of accumulation of the fluorescent product was calculated.

2.6.3 The effect of *OsCam1-1* over-expression in the transgenic rice lines on ABA accumulation

2.6.3.1 Plant material

Transgenic rice lines harboring the *OsCam1-1* gene under the control of a *35SCaMV* promoter were used for this experiment in overexpression with KDML105 wild type and the transgenic line containing no *OsCam1-1* overexpression construct.

Seeds of the *indica* rice cultivar (*Oryza sativa* L.) KDML105 that harbors the construct of *OsCam1-1* gene under the control of the *35SCaMV* promoter and the transgenic rice lines that were transformed with only the vector (pCAMBIA1301) construct were provided by Assist. Prof. Dr. Teerapong Buaboocha.

A schematic diagram of pCAMBIA1301 is shown in Figure 3.6(a). pCAMBIA1301 contains a *35SCaMV-OsCam1-1-nos* cassette, that was constructed in pGEM vector. The ligation product was transformed into *E. coli* XL1-Blue for selection of the recombinant plasmid which contains the expression cassette consisting of the *35SCaMV* promoter, the *OsCam1-1* coding sequence and the nos terminator. The *Agrobacterium tumefaciens* was transformed with the pCAMBIA1301-*35SCaMV-OsCam1-1-nos* as shown in Figure 3.6(b) and

pCAMBIA1301 without the gene construct was used to generate control plants. The wild type plants of KDML105 and the transgenic rice lines that were transformed with only the vector were used as controls.

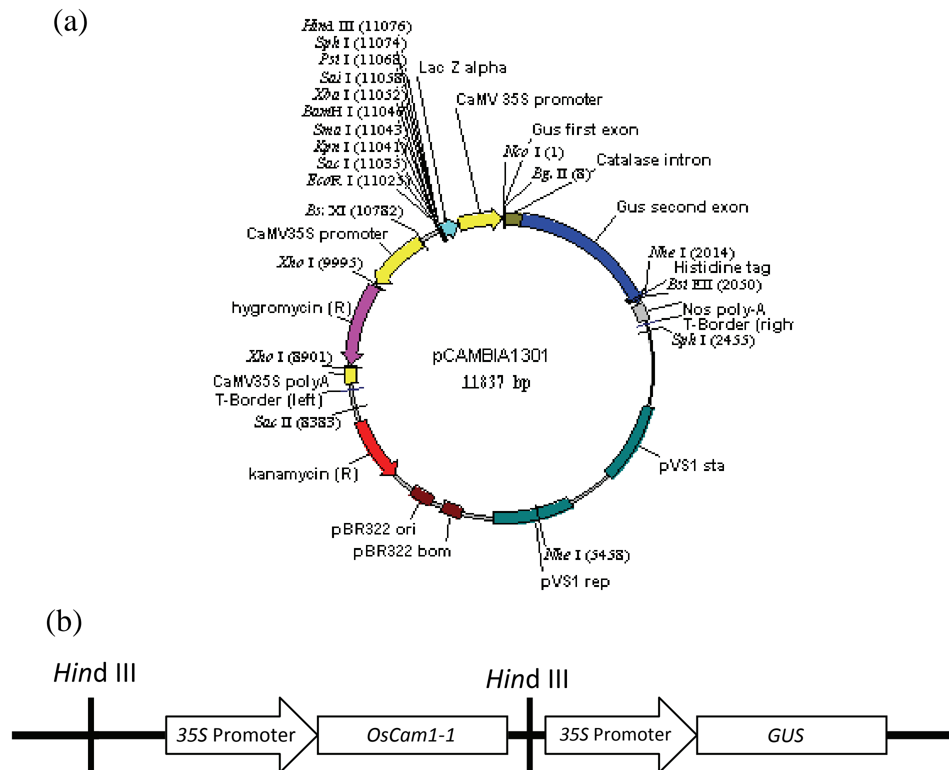


Figure 3.6 Schematic diagrams of (a) construct of pCAMBIA1301 and (b) the construct of the *gus* reporter gene under the control of the *35SCaMV* promoter.

2.6.3.2 Charaterization of the transgenic rice lines by histochemical analysis for *β*-Glucuronidase (GUS) activity for homozygosity determination

In order to avoid the effect of transgene copy number, the homozygous transgenic lines were used in all experiments. Transgenic rice lines harboring the construct of the *OsCam1-1* gene under the control of the *35SCaMV* promoter and the transgenic rice lines that were transformed with only the vector

(pCAMBIA1301) were grown in normal conditions for selection of the homozygosity of the *gus* reporter gene. Homozygosity of the positively plants stained T_1 generation plants was verified by segregation analysis of their T_2 generation plants via histochemical GUS assays (Appendix A5.1) as indicated in 2.6.2.2.

2.6.3.3 Determination of the endogenous ABA level in the transgenics rice lines

The endogenous ABA level in the transgenic rice lines homozygous for the *gus* reporter gene and the wild type rice (KDML105) was determined by HPLC method as indicated in 2.5. Rice seedlings were germinated and grown in WP No.2 nutrient solution (Vajrabhaya and Vajrabhaya, 1991) in the greenhouse. Twenty-four-day old rice seedlings grown in WP No.2 nutrient solution and WP No.2 nutrient solution containing 0.5% (w/v) NaCl were used for endogenous ABA quantification in the leaf tissues. The samples were collected for ABA detection on day 0, 1, 3, 5, 7, 9 and 11 after stress treatment. Plants were prepared with 3 replicates and the ABA measurement of each sample was performed in triplicates.

2.7 The relationship between the *OsCam1-1* gene expression and genes involving in ABA biosynthesis in transgenic rice lines containing the over-expression of *OsCam1-1* gene under salt stress condition

To study the effect of the overexpression of *OsCam1-1* gene expression on the expression of gene involving in ABA biosynthesis, *NCED* and *AAO* genes were chosen as they were previously reported that Ca^{2+} concentration may affect their gene expression (Xiong *et al*., 2002).

2.7.1 The effect of the overexpression of *OsCam1-1* gene in the transgenic rice lines in the normal condition

2.7.1.1 Transgenic rice line and growing condition

Rice seedlings of three lines of transgenic rice, that harboring the construct of *OsCam 1-1* gene under the control of *35SCaMV* promoter and the transgenic rice lines that were transformed with only the vector (pCAMBIA1301) construct and KDML105, were germinated and grown in WP No.2 nutrient solution (Vajrabhaya and Vajrabhaya, 1991) in the greenhouse. Twenty-four-day old rice seedlings were used for detection of *NCED* and *AAO* gene expression in leaf tissues at 0, 3, 6 and 9 days after salt stress treatment.

2.7.1.2 Detection of *NCED* and *AAO* gene expression

2.7.1.2.1 Total RNA preparation

Total RNA was isolated by hot phenol procedure (Thikard *et al*., 2005) as indicated in 2.6.1.1.

2.7.1.2.2 Amplification of targeted genes to determine the level of expression

Total RNA sample, was treated with DNase I as indicated in 2.6.1.2 and the reverse transcription was performed in the similar way as indicated previously. RT-PCR amplification was carried out by using forward and reverse oligonucleotide primers. The sequences were as follows:

OsNCED-F	5´-GGGTTCCAATCCACTGTTTG-3´
OsNCED-R	5´-GCTATCACCCGTGTCGAGAT-3´
OsAAO-F	5´-AGGAATGGAAGAAGCTGACC-3´
OsAAO-R	5´-ACAGGACGCTGCAACTTA-3´
OsActin-F	5´-CTGCCGAGCGGGAAATTGTC-3´
OsActin-R	5´-CTGGCGGAGCGACAACCTTG-3´

RT-PCR amplification of *NCED* by Taq polymerase was performed as follows: predenaturation at 95ºC for 2 minutes, denaturation at 95ºC for 1 minute, annealing at 54ºC for 1 minute, and extension at 72ºC for 2 minutes 35 cycles. *AAO*: predenaturation at 95ºC for 2 minutes, denaturation at 95ºC for 1 minutes, annealing at 55ºC for 1 minute, and extension at 72ºC for 2 minutes 30 cycles. *Actin*: predenaturation at 95ºC for 2 minutes, denaturation at 95ºC for 1 minutes, annealing at 55ºC for 1 minute, and extension at 72ºC for 2 minutes 28 cycles.

The RT-PCR products were separated on 1% agarose gel (Appendix B2) and the stained with ethydium bromide. The visualization of DNA fragment was done using UV transilluminator and photographed.

2.7.2 The effect of the overexpression of *OsCam1-1* gene in the transgenic rice lines in the salt stress condition

2.7.2.1 Transgenic rice and growing condition

The transgenic rice lines used in 2.7.1.1 were also used for this study, but when they were twenty-four-day old rice seedlings, they were subjected to salt stress by addition of 0.5%NaCl to the nutrient solution.

2.7.2.2 Detection of *NCED* and *AAO* gene expression

The expression was perform in the similar ways as listed in 2.7.1.2.

CHAPTER IV

RESULTS AND DISCUSSION

1. Development of monoclonal antibody for ABA detection in plant extract

1.1 Production of polyclonal antibody against to carbonyl terminal of ABA

One week after completion of the fourth booster using (±)-ABA-BSA carboxy terminal conjugated, the antisera from four mice were collected and preabsorbed with BSA-hippuric acid (HA) conjugated (HA-BSA) at the ratio of 1:10. Ten fold serial dilutions (1 mg/ml to 10^{-5} mg/ml) of seven antigens; (±)-ABA-BSA, HA-BSA, (±)-ABA-OA, HA-OA, (+)-ABA-BSA, BSA and OA were tested with antiserum at 1:500 dilution. All antisera from four mice demonstrated strong immunoreactivity to (±)-ABA-BSA, (±)-ABA-OA and (+)-ABA-BSA, weak immunoreactivity to HA-BSA and BSA. No immunoreactivity was observed with HA-OA and OA (Figure 4.1). This result demonstrated that all antisera contained major antibodies specific to (±)-ABA and (+)-ABA , and minor antibodies against BSA. The second and third mice demonstrated strongest immunoreactivity, therefore, the third mouse was used as spleen donor for hybridoma production.

1.2 Production of polyclonal antibody specific to carboxyl terminal of ABA molecule

One week after completion of the fourth booster using (±)-ABA-BSA for antibody production specific to carboxyl terminal of ABA molecule, antisera from 7 female ICR mice (mouse number 1-7) could recognize (±)-ABA-BSA in the carboxyl terminus. The similar specificity test for the antisera was performed, using eight antigens, (±)-ABA-BSA, (±)-ABA-OA, (+)-ABA-OA, hippuric acid-BSA, hippuric

acid-OA, (+)-ABA-BSA, BSA and OA. Antisera from every mouse bound antigens, (±)-ABA-BSA, (+)-ABA-OA, hippuric acid-BSA, (+)-ABA-BSA and BSA but could not bind to hippuric acid-OA and OA as shown in Figure 4.2, which suggested that the antisera obtained were specific to (+)-ABA, (±)-ABA and BSA. The antisera from mouse number 5 showed the highest sensitivity against (+)-ABA-BSA, when compared to others therefore, mouse number 5 was chosen as the spleen donor for hybridoma production.

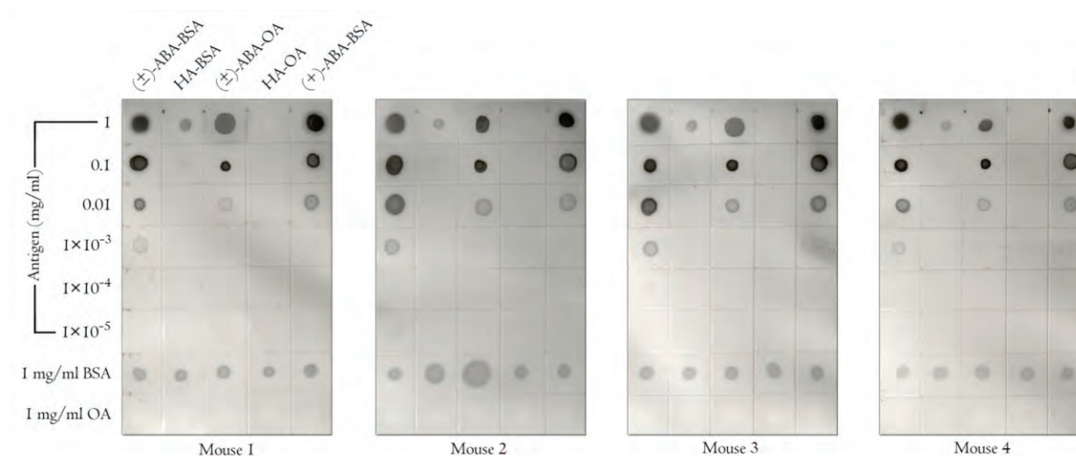


Figure 4.1 Specificity of antisera obtained from 4 mice immunized with (±)-ABA-BSA. (±)-ABA-BSA: (±)-ABA conjugated with bovine serum albumin, HA-BSA: hippuric acid conjugated with bovine serum albumin, (±)-ABA-OA: (±)-ABA conjugated with ovalbumin, HA-OA: hippuric acid conjugated with ovalbumin, (+)-ABA-BSA: (+)-ABA conjugated with bovine serum albumin, BSA: bovine serum albumin, OA: ovalbumin.

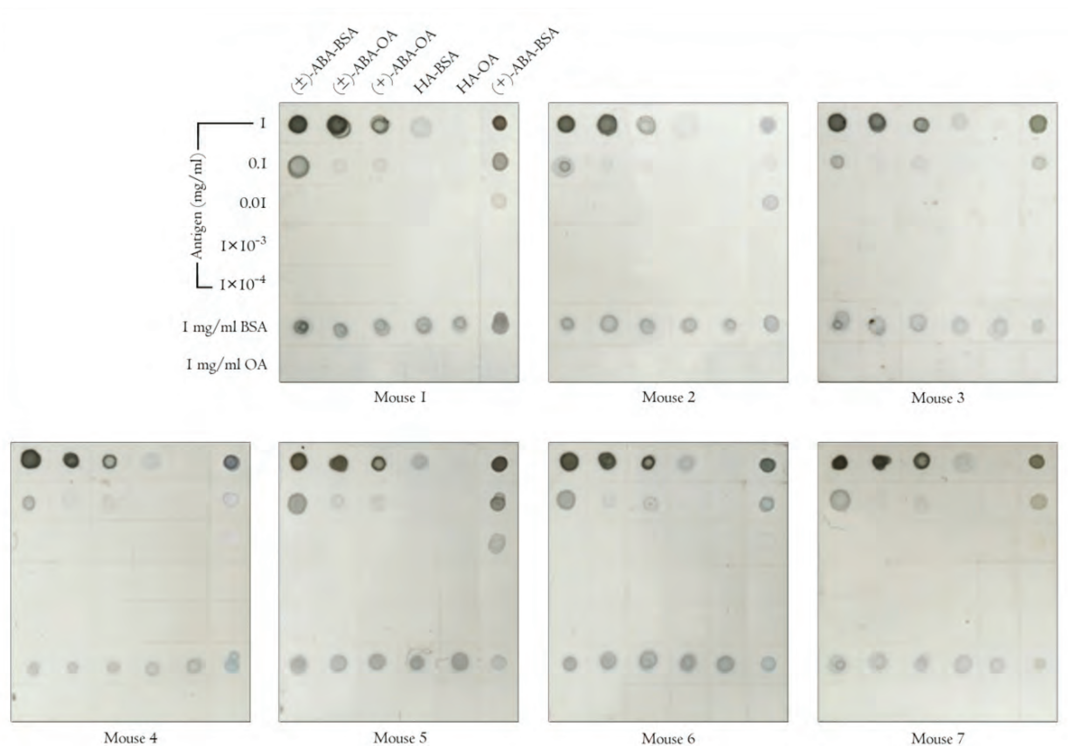


Figure 4.2 Specificity of antisera obtained from 7 mice immunized with (±)-ABA-BSA. (±)-ABA-BSA: (±)-ABA conjugated with bovine serum albumin, (±)-ABA-OA: (±)-ABA conjugated with ovalbumin, (+)-ABA-OA: (+)-ABA conjugated with ovalbumin, HA-BSA: hippuric acid conjugated with bovine serum albumin, HA-OA: hippuric acid conjugated with ovalbumin, (+)-ABA-BSA: (+)-ABA conjugated with bovine serum albumin, BSA: bovine serum albumin, OA: ovalbumin.

1.3 Production of monoclonal antibodies carbonyl terminal of ABA molecule

From one fusion, the cell mixture was laid in 30 microculture plates and about half (1,500 culture wells) contained hybridoma colonies (1 to 3 colonies of the hybridoma per well). The first screening by dot blotting against (±)-ABA, 20 hybridoma clones demonstrated strong immunoreactivity. The second screening was

performed by dot blotting against (±)-ABA-BSA, (±)-ABA-OA, (+)-ABA-BSA, OA, BSA and HA-BSA. Ten hybridoma clones producing antibodies specific to ABA were selected. The hybridoma clones could be divided into 3 groups according to their specificities. Group 1 consisted of one clone (E8-6) producing MAb specific to (–)-ABA since no immunoreactivity was observed with (+)-ABA. The second group of hybridomas consisted of 2 clones (A4-7-1 and A5-20-2) produced MAbs that bound strongly to (±)-ABA and bound slightly to (+)-ABA. The third group consisted of 7 clones producing MAbs that bound well to both (±)-ABA and (+)-ABA. This evidence demonstrated that the MAbs in the third group bound well to both form of ABA (Figure 4.3).

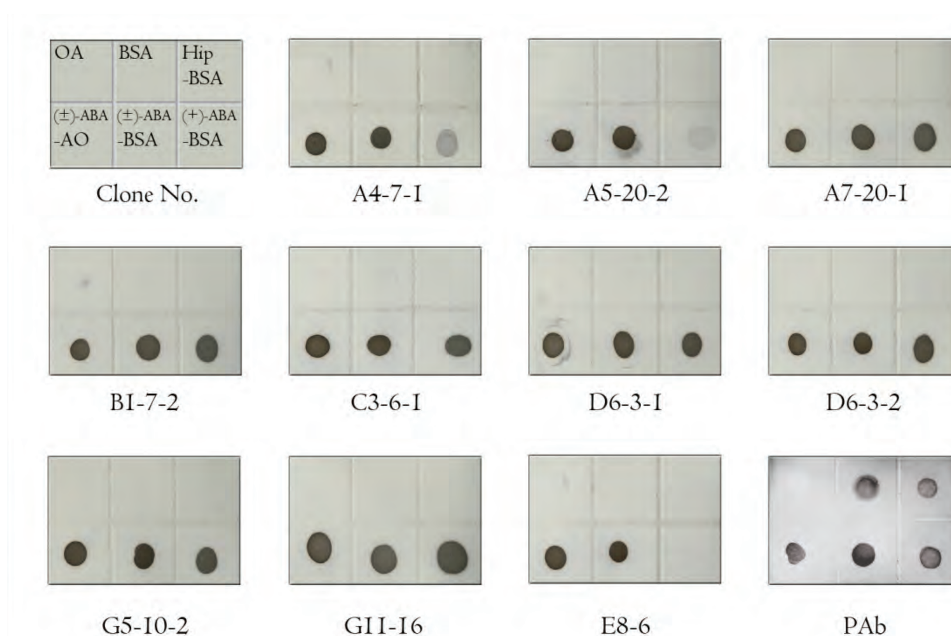


Figure 4.3 Specificity of MAbs tested by dot blotting. The polyclonal antibody (PAb) was used as the positive control. OA: ovalbumin, BSA: bovine serum albumin, HA-BSA: hippuric acid conjugated with bovine serum albumin, (±)-ABA-OA: (±)-ABA conjugated with ovalbumin, (±)-ABA-BSA: (±)-ABA conjugated with bovine serum albumin, (+)-ABA-BSA: (+)-ABA conjugated with bovine serum albumin.

Culture fluids from the wells that showed strong cross reaction of these clones were selected for subsequent cloning by limiting dilution method for monoclonal antibody production.

1.4 Production of monoclonal antibodies carboxyl terminal of ABA molecule

One fusion trial with 30 microculture plates yielded approximately 327 culture wells. About 10% of them gave 1 to 3 colonies of the hybridoma per well. The culture fluids containing antibodies that reacted with (±)-ABA-BSA in carboxyl terminal side were determined by dot blotting. The result showed that two monoclones, clone number 44 and 369 could produced antibodies against (±)-ABA-OA for first screening and after secondary screening by dot blotting, for selection the monoclonal antibodies specific to carboxyl terminal of (+)-ABA. These two clones produced MAbs that bound to (±)-ABA-OA, (+)-ABA-BSA but light cross reaction against HA-BSA. This result suggested that these two clones produced the antibodies that could recognize the carboxyl terminal of (+)-ABA as shown in Figure 4.4.

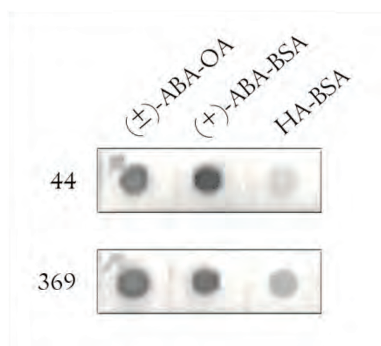


Figure 4.4 Specificity of MAbs tested by dot blotting. (±)-ABA-OA: (±)-ABA conjugated with ovalbumin, (+)-ABA-BSA: (+)-ABA conjugated with bovine serum albumin, HA-BSA: hippuric acid conjugated with bovine serum albumin.

1.5 Sensitivity testing

1.5.1 Sensitivity testing of MAbs specific to carbonyl terminal of (+)-ABA

Sensitivity of 9 MAbs were tested against (±)-ABA-BSA and (+)-ABA-BSA. MAbG11-16 and A5-20-2 demonstrated the highest sensitivity when tested with (±)-ABA-BSA (1.56 µg/ml) (Figure 4.5). However, MAb G11-16 demonstrated the highest sensitivity with (+)-ABA-BSA (0.39 µg/ml) (Figure 4.6). The sensitivity of MAb in the first group (E8-6) was not tested since (-)-ABA was not available.

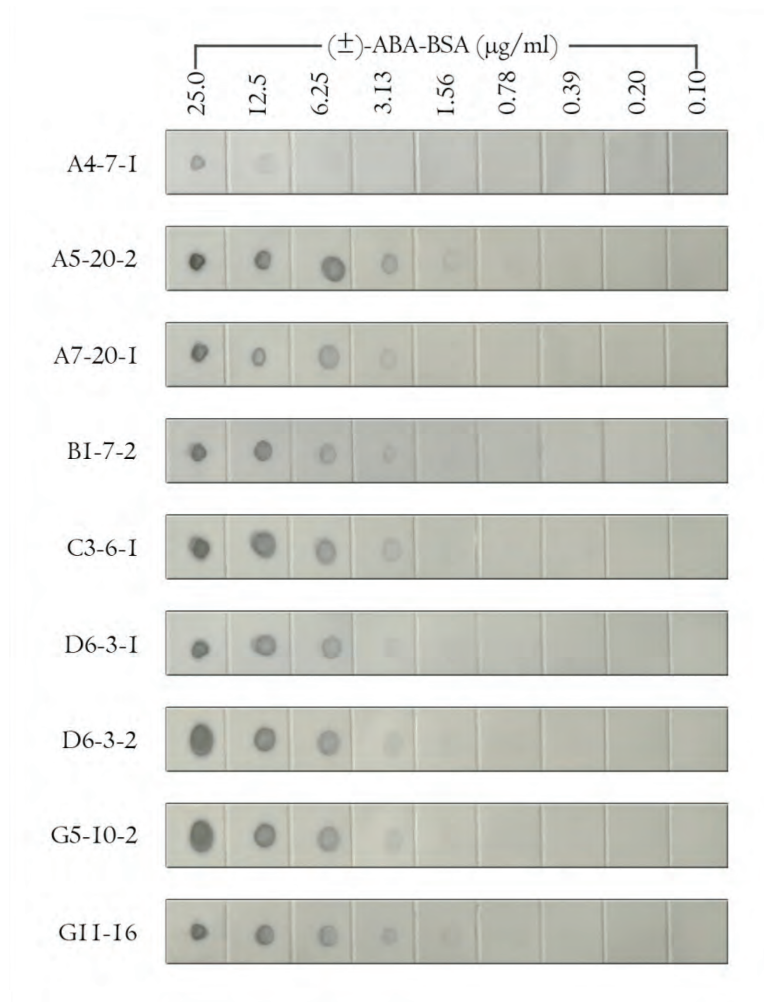


Figure 4.5 Sensitivity test of monoclonal antibodies against carbonyl terminal of (±)-ABA-BSA by dot blotting.

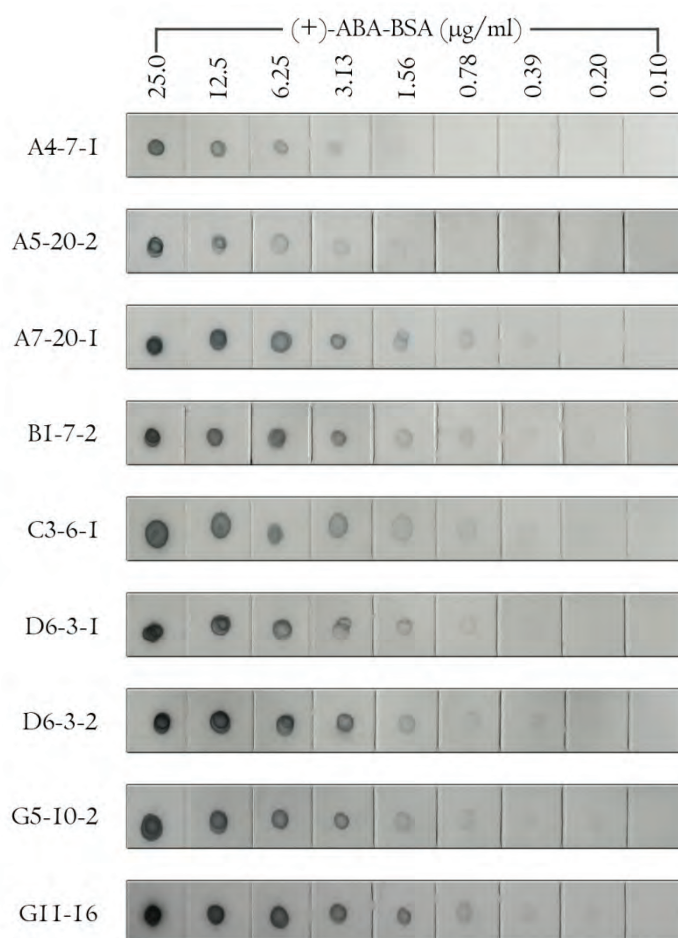


Figure 4.6 Sensitivity test of monoclonal antibodies against carbonyl terminal of (+)-ABA-BSA by dot blot ELISA.

1.5.2 Sensitivity testing of MAbs specific to carboxyl terminal of (+)-ABA

Sensitivity of 2 MAbs were tested against (+)-ABA-BSA. MAb44 and 369 demonstrated the highest sensitivity when tested with (+)-ABA-BSA (1.56μg/ml) (Figure 4.7). This result indicated that the sensitivity of these MAbs (MAb44 and 369) was lower than the sensitivity of MAbs specific to carbonyl terminal of ABA therefore, MAbs specific to carbonyl terminal of ABA were used for development of competitive ELISA for quantification of ABA.

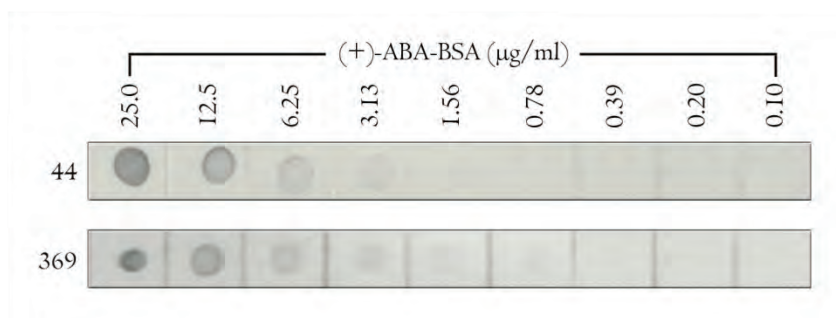


Figure 4.7 Sensitivity test of monoclonal antibodies against carboxyl terminal of (+)-ABA-BSA by dot blot ELISA.

1.6 Isotype and subisotype determination

MAbs specific to carbonyl terminal of ABA, all 10 MAbs were belong to IgG class in which 9 of them combined with kappa light chain only one MAb conmbined with lamda light chain. The Ig subclass of each MAbs were shown in Table 4.1. MAbs specific to carboxyl terminal of ABA, 2 MAbs were belong to IgG class and kappa light chain as shown in Table 4.2.

Table 4.1 Isotype and subisotype determination of the MAbs against carbonyl terminal of (±)-ABA.

MAbs	**Isotype and Subisotype**	**Light chain**
A4-7-1	IgG_{2b}	kappa
A5-20-2	IgG_{2a}	lambda
A7-20-1	IgG_1	kappa
B1-7-2	IgG_1	kappa

Table 4.1 (continued) Isotype and subisotype determination of the MAbs against carbonyl terminal of (±)-ABA.

MAbs	Isotype and Subisotype	Light chain
C-3-6-1	IgG_1	kappa
D6-3-1	IgG_{2b}	kappa
D6-3-2	IgG_{2b}	kappa
G11-16	IgG_{2b}	lambda
E8-6	IgG_{2b}	lambda

Table 4.2 Isotype and subisotype determination of the MAbs against carboxyl terminal of (±)-ABA.

MAbs	Isotype and Subisotype	Light chain
44	IgG_1	kappa
369	IgG_1	kappa

2. Development of competitive ELISA for quantification of ABA

2.1 Selection of MAb by indirected immunoperoxidase ELISA

Three MAbs (G11-16, A7-20-1, A5-20-2) with demonstrated strong immunoreactivity in dot blotting were tested by indirect ELISA using various concentrations of (±)-ABA-BSA. MAb G11-16 showed lower concentration of (±)-

ABA-BSA than that of MAbs A7-20-1 and A5-20-2 (Table 4.3). Therefore, MAb G11-16 was selected for quantitative determination of ABA. The optimal dilution of the MAb was 1:2000 and the minimal concentration of (±)-ABA-BSA was 0.15 µg/ml (Table 4.4).

Table 4.3 Binding of MAbs to (±)-ABA-BSA at various concentration using indirect ELISA.

(±)-ABA-BSA (µg/ml)	**Antigen**		
	G11-16	**A7-20-1**	**A5-20-2**
8	1.275 ± 0.009	1.221 ± 0.004	1.339 ± 0.038
4	1.262 ± 0.035	1.245 ± 0.016	1.323 ± 0.011
0.8	1.258 ± 0.004	1.207 ± 0.075	1.213 ± 0.101
0.16	1.148 ± 0.095	1.094 ± 0.082	0.860 ± 0.050
0.032	0.731 ± 0.015	0.791 ± 0.198	0.409 ± 0.018
0.016	0.394 ± 0.279	0.340 ± 0.253	0.118 ± 0.004
0.008	0.206 ± 0.006	0.168 ± 0.003	0.110 ± 0.004
1 mg/ml BSA	0.106 ± 0.033	0.082 ± 0.004	0.088 ± 0.006

Table 4.4 Determination of optimal dilution of MAb using indirect ELISA.

Antibody (G11-16) dilution	(±)-ABA-BSA (μg/ml)		
	0.15	**0.2**	**0.3**
1:10	2.542	2.596	2.611
1:50	2.524	2.588	2.544
1:100	2.458	2.498	2.504
1:200	2.422	2.487	2.464
1:500	2.169	2.188	2.114
1:1000	1.836	1.867	1.818
1:2000	1.764	1.738	1.726
1:4000	1.248	1.267	1.247
1:5000	1.221	1.281	1.268
1:10,000	0.823	0.834	0.811

2.2 Determination of suitable methanol concentration for sample extraction using Competitive ELISA.

MAb at dilution of 1:2000 and (±)-ABA-BSA at 0.15 μg/ml was used for determination the methanol concentration in extraction buffer on the sensitivity

competitive ELISA. Three concentration of methanol (10%, 20% and 30%) were used to dissolved (±)-ABA at 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.10, 0.05, 0.025 and 0.125 μg/ml in competitive ELISA. The ranges of (±)-ABA dissolved in 10% methanol demonstrated wider and higher sensitivity than that of (±)-ABA dissolved in 20% and 30% methanol; 0.05-6.4 μg/ml (10% methanol), 0.2-6.4 μg/ml (20% methanol), 0.4-6.4 μg/ml (30% methanol) (Figure 4.8).

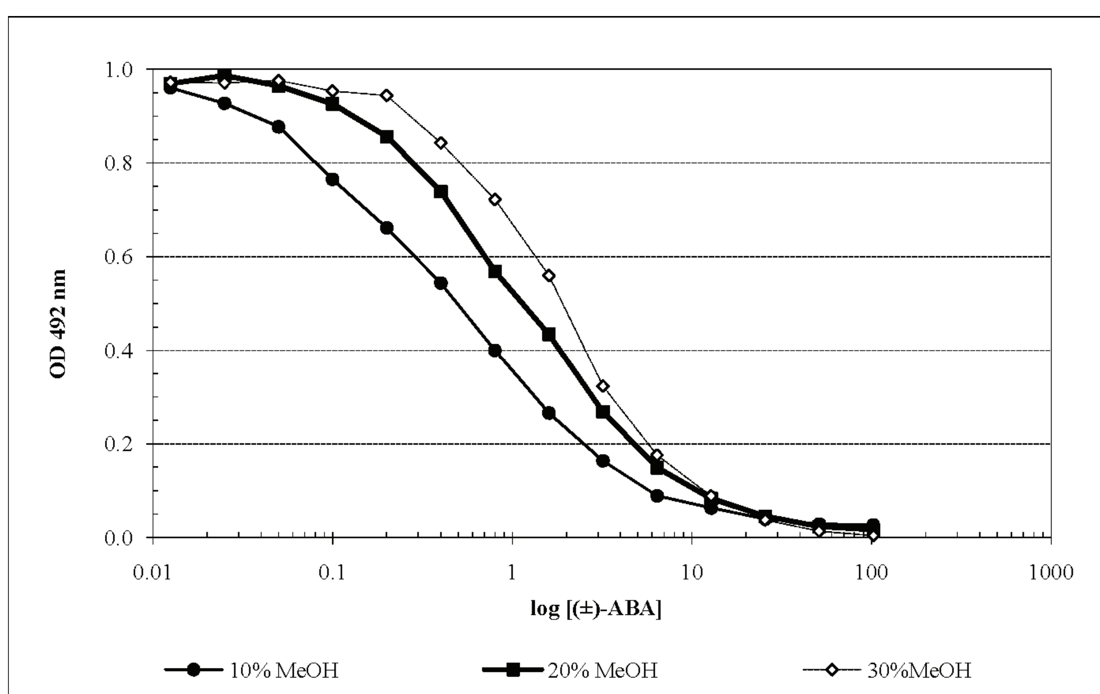


Figure 4.8 Competitive ELISA of (±)-ABA using 3 concentrations of methanol as extraction buffer.

2.3 Comparative competitive ELISA between (+)-ABA and (±)-ABA

The (+)-ABA and (±)-ABA at concentration of 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.10, 0.05, 0.025 and 0.125 μg/ml dissolve in 10% methanol buffer were used in competitive ELISA. The binding of MAb to both ABA forms were almost the same. This evidence indicated that the MAb could bind to both forms of ABA equally

and could not differentiate them (Figure 4.9).

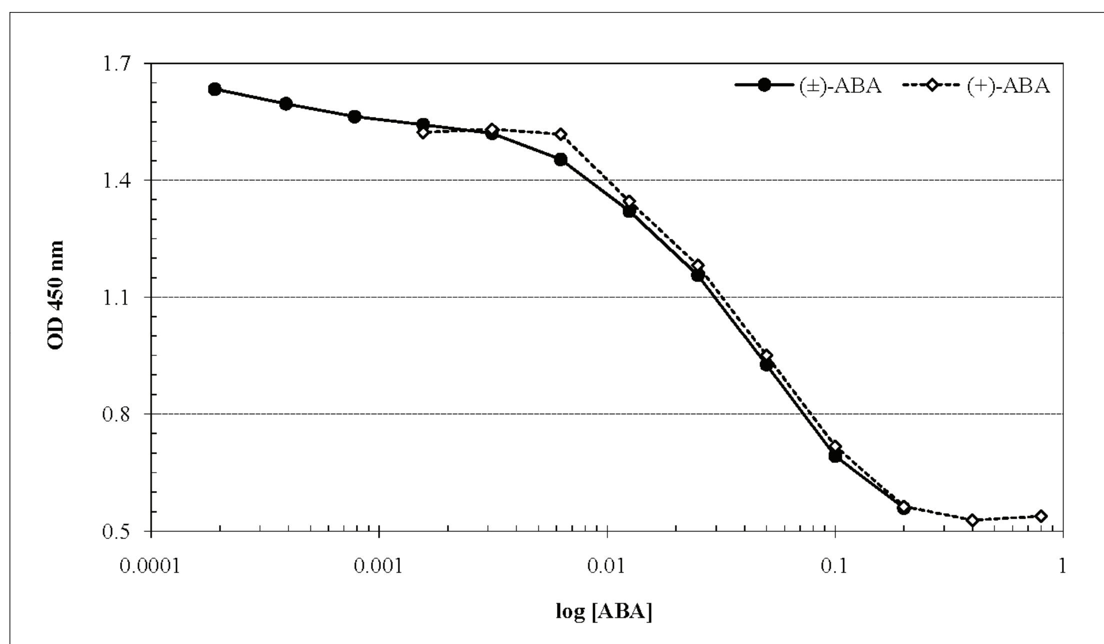


Figure 4.9 Competition of (±)-ABA and (+)-ABA in competitive ELISA.

3. Sensitivity of HPLC on determination of ABA in comparison with competitive ELISA

Determination of the area under the peak of (±)-ABA at various concentrations showed that the area was directly related to concentration of (±)-ABA (Figure 4.10) and the minimal concentration of (±)-ABA that could be determined was 0.156 µg/ml (Table 4.5) which was over ten times higher concentration than the minimal concentration of (±)-ABA determined by competitive ELISA 0.0125 µg/ml (Table 4.6, Figure 4.11).

Table 4.5 Peak area of standard (±)-ABA by HPLC method.

Standard (±)-ABA (µg/ml)	Peak Area (mAU*s)			
	R1	R2	R3	Average
0.156	10.09	10.59	10.34	10.34
0.312	29.66	41.08	31.75	34.16
0.625	85.98	89.72	86.15	87.28
1.25	170.64	239.68	185.51	198.61
5	1019.24	998.78	1101.54	1039.86
10	2215.43	2254.33	2273.52	2247.76
50	11201.00	11129.47	11338.11	11222.86
100	22477.61	20198.55	23100.23	21925.46

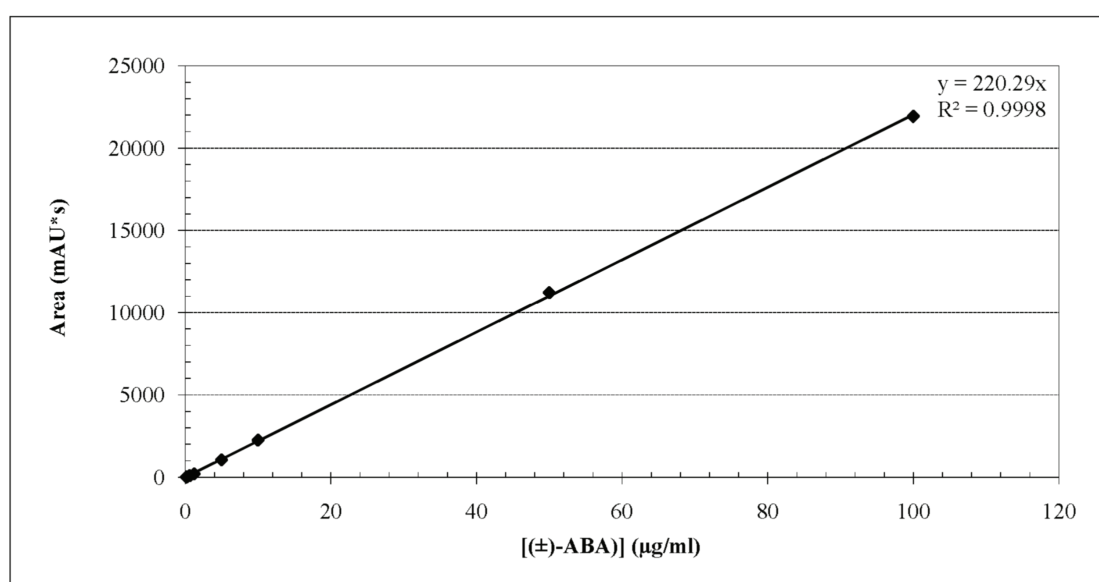


Figure 4.10 Standard curve of (±)-ABA by HPLC method.

Table 4.6 Optical density of standard (±)-ABA by indirect competitive ELISA method.

(±)-ABA (μg/ml)	OD 450 nm			
	R1	**R2**	**Average**	**OD-Blank**
0.2	0.794	0.694	0.744	0.559
0.1	0.844	0.91	0.877	0.692
0.05	1.082	1.14	1.111	0.926
0.025	1.331	1.352	1.342	1.157
0.0125	1.502	1.51	1.506	1.321
0.00625	1.653	1.624	1.639	1.454
0.003125	1.724	1.686	1.705	1.520
0.00156	1.712	1.743	1.728	1.543
0.00078	1.769	1.727	1.748	1.563
0.00039	1.8	1.762	1.781	1.596
0.00019	1.832	1.805	1.819	1.634

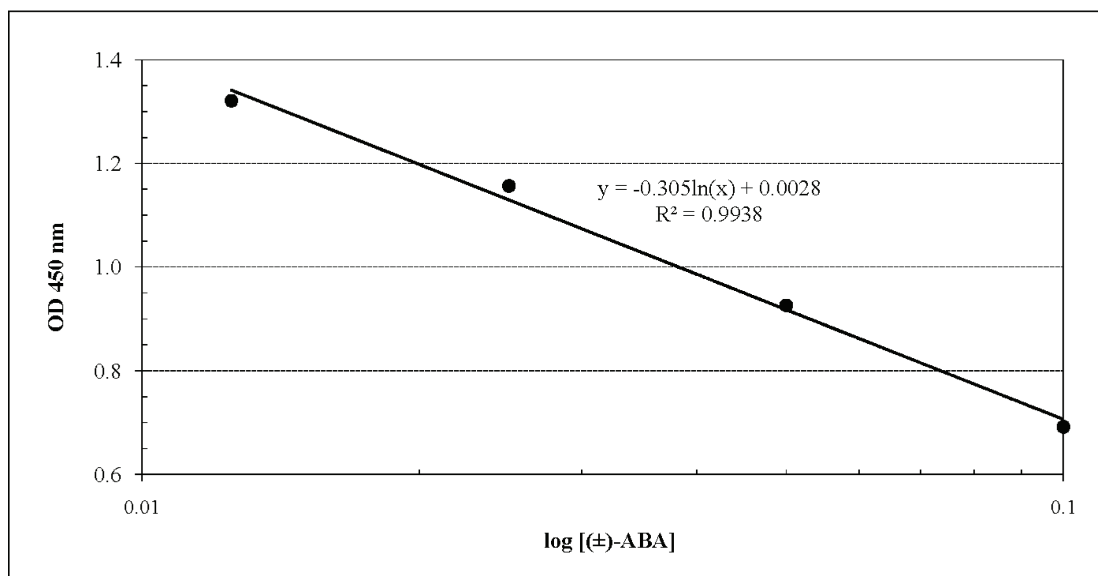


Figure 4.11 Standard curve of (±)-ABA by indirect competitive ELISA method.

4. Detection of the endogenous ABA in the rice tissues, when grown under normal/salt stress condition by HPLC method

During the first four hours after salt stress, no significant increase in ABA content was found in both rice cultivar/line tested. However, ABA content in the salt sensitive cultivar, LPT123 tend to increase after 1 hour of salt stress treatment, while in the salt resistant line, LPT123-TC171, ABA content tended to be increase in 2 hours after salt stress treatment. After 4 hours for salt stress, the ABA content in LPT123-TC171 was significant higher than the ABA content in LPT123 (Figure 4.12).

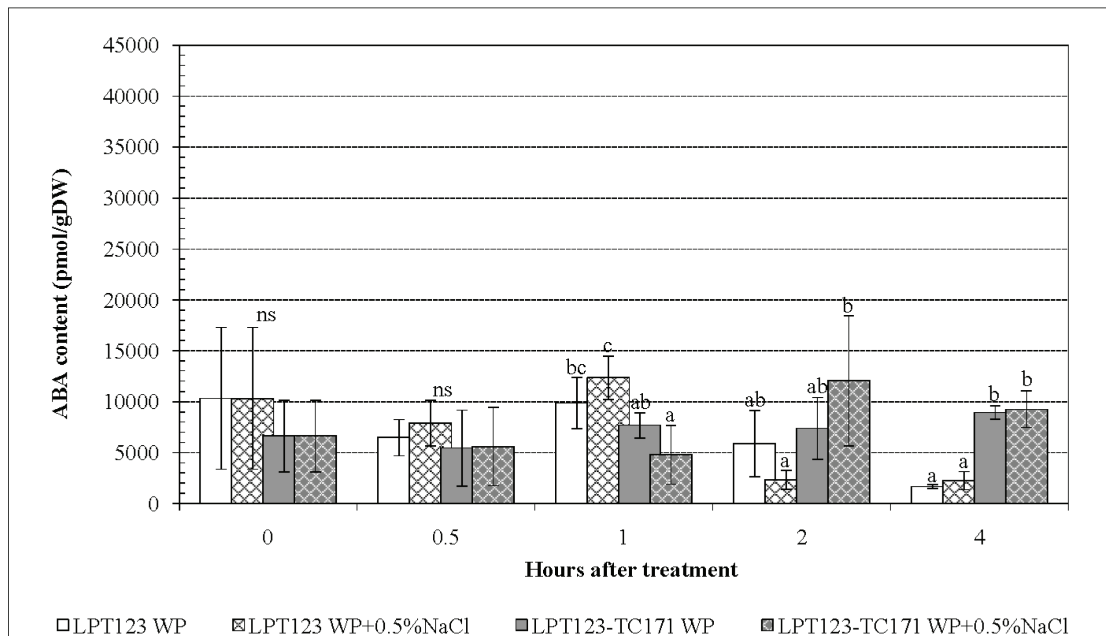


Figure 4.12 ABA content in leaves of salt sensitive rice line (LPT123) and salt tolerant rice line (LPT123-TC171) under normal and salt stress condition detect by HPLC method.

The similar result of ABA accumulation during the first 4 hours of salt stress was found in KDML105 and FL530-IL rice. At the beginning of the experiment ABA content in FL530-IL was significant higher than ABA content in KDML105. The increase in ABA content in the salt resistant rice FL530-IL was not found during the first from hours of salt stress condition. In contrast, salt stress induced significant ABA accumulation in KDML105 after 2 hours of stress (Figure 4.13).

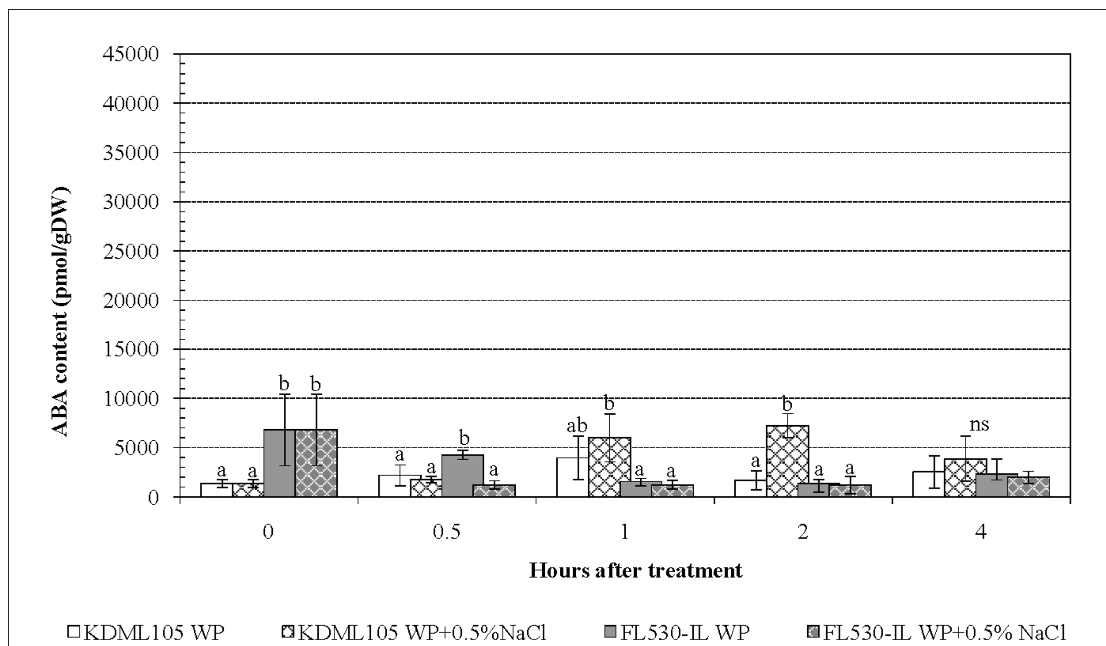


Figure 4.13 ABA content in leaves of salt sensitive rice line (KDML105) and salt tolerant rice line (FL530-IL) under normal and salt stress condition detect by HPLC method.

Salt stress induced ABA accumulation in leaf tissue in both salt sensitive (LPT123) and salt resistant (LPT123-TC171) lines. ABA content was highest after 9 days under salt stress. ABA content accumulated in LPT123 rice was three times higher than the ABA accumulation in the p1ants grown in normal condition, while LPT123-TC171 showed twenty times higher in ABA content, when compared to the normal grown plants. The highest level of ABA content in both LPT123 and LPT123-TC171 rice were not significantly different. The high accumulation of ABA was decreased to the normal level after 11 days of the treatment in salt sensitive cultivar, LPT123, but even the reduction in ABA level in the salt resistant line, LPT123-TC171 on day 11 after salt stress, its ABA content was significantly higher than the ABA content in the normal grown LPT123-TC171. After 13 days, the ABA

content level was at the normal level (Figure 4.14).

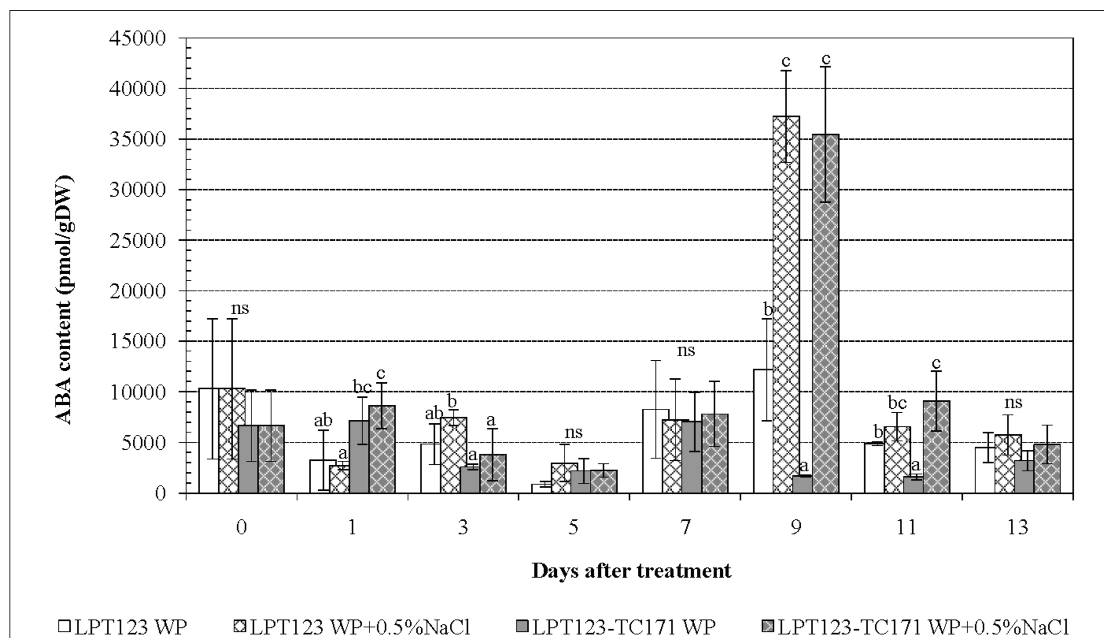


Figure 4.14 ABA content in leaves of salt sensitive rice line (LPT123) and salt tolerant rice line (LPT123-TC171) under normal and salt stress condition detect by HPLC method.

Similarly, salt stress induced ABA accumulation in KDML105 and FL530-IL rice. After 7 days of salt stress, ABA content in salt stress treated plants of both KDML105 and FL530-IL was higher than the ABA content in the normal growth plants and ABA content of KDML105 was highest. At this time point, salt stressed FL530-IL rice accumulated the highest level of ABA accumulation after 9 days of salt stress. The significant higher level of ABA accumulation in salt-stressed FL530-IL was found until the end of the experimental period (13 days), when compared to the normal grown plants (Figure 4.15).

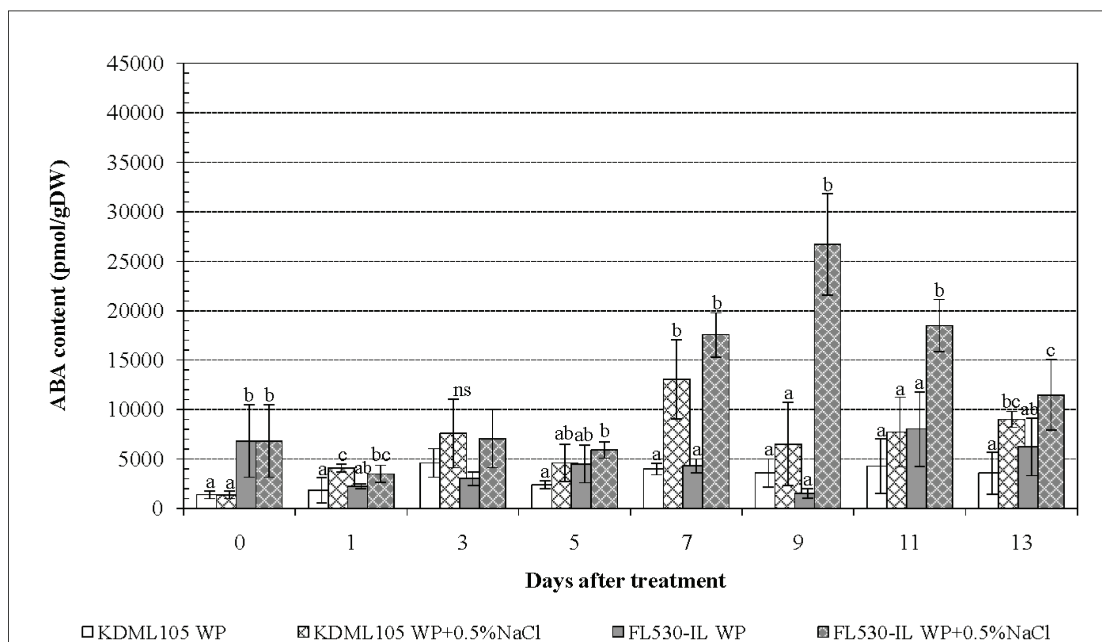


Figure 4.15 ABA content in leaves of salt sensitive rice line (KDML105) and salt tolerant rice line (FL530-IL) under normal and salt stress condition detect by HPLC method.

5. Detection of the endogenous ABA in rice tissues, when grown under normal/salt stress condition by ELISA method

The ABA content that found in salt sensitive rice line (LPT123) and salt tolerant rice line (LPT123-TC171) under the salt stress condition showed that ABA content was not significantly increased in both lines of rice, LPT123 and LPT123-TC171 at 0.5, 1, 2 and 4 hours after salt stress treatment (Figure 4.16).

The level of ABA content was in the range of 154.597 – 275.323 pmol/gDW. The similar result was also found when the measurement of ABA was performed with KDML105 and FL530-IL rice (Figure 4.17). However, the level of ABA content was higher. It was range from 298.468 – 456.126 pmol/gDW.

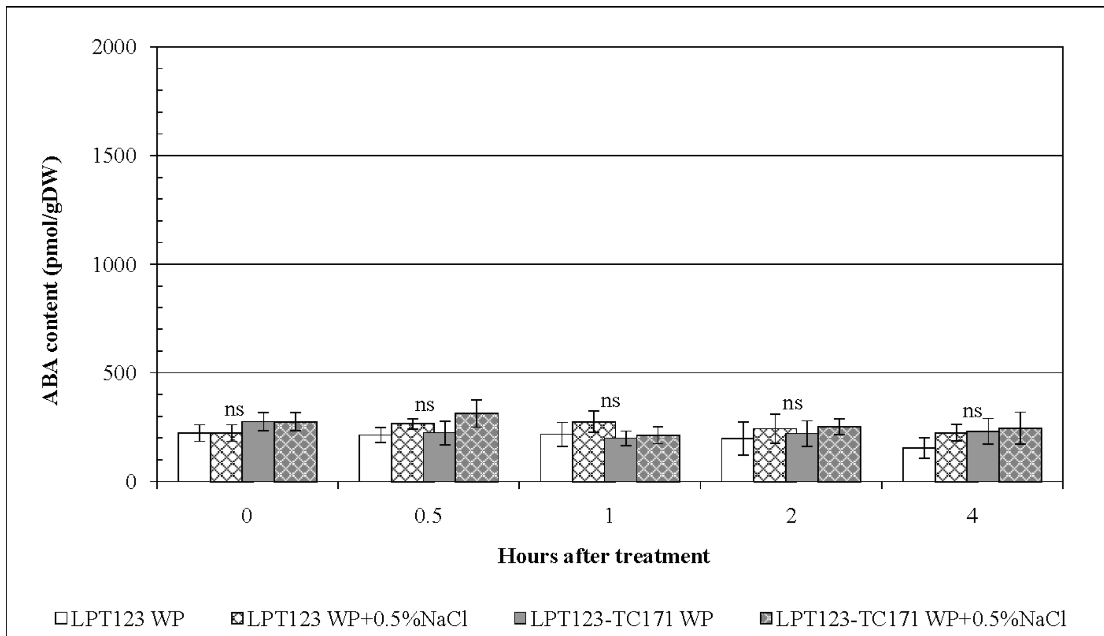


Figure 4.16 ABA content in leaves of salt sensitive rice line (LPT123) and salt tolerant rice line (LPT123-TC171) under normal and salt stress condition by ELISA method.

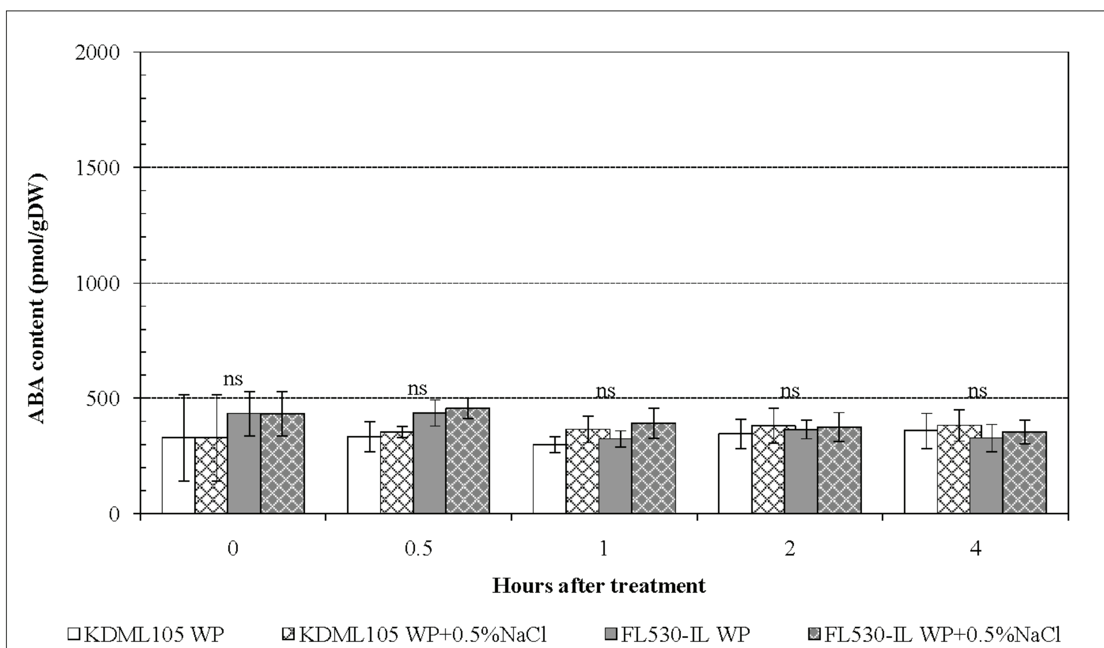


Figure 4.17 ABA content in leaves of salt sensitive rice line (KDML105) and salt tolerant rice line (FL530-IL) under normal and salt stress condition detect by ELISA.

Salt stress induced ABA accumulation in leaf tissue in both salt sensitive (LPT123) and salt resistant (LPT123-TC171) line. ABA content was highest after 9 days under salt stress. ABA content accumulated in LPT123 rice was 3 times higher than the ABA accumulation in the plants growth in normal condition, similar to LPT123-TC171, when compared to the normal growth plants. The highest of ABA accumulation in both LPT123 and LPT123-TC171 was not significantly different. The high accumulation of ABA was decreased to the normal level after 11 days of the treatment in salt sensitive cultivar, LPT123 and salt tolerant, LPT123-TC171 and ABA content in the both of rice lines was not significantly different after 11 and 13 days of the salt stress treatment (Figure 4.18).

Similarly, salt stress induced ABA accumulation in KDML105 and Fl530-IL rice after 3 days of salt stress, ABA content in salt stress treated plants of both KDML105 and FL530-IL was higher than the ABA content in the normal growth plants and ABA content of FL530-IL was significant higher than ABA content of KDML105 after 1 day of salt stress treatment.

After 7 days of salt stress, ABA content in salt stress treated plants of both KDML105 and FL530-IL was higher than the ABA content in normal growth plants. At this times point, salt stressed FL530-IL rice accumulated the highest level of ABA accumulation after 9 days of salt stress. The significant higher level of ABA accumulation in salt-stressed FL530-IL was found until the end of the experimental period (13 days), when compared to the normal growth plants (Figure 4.19).

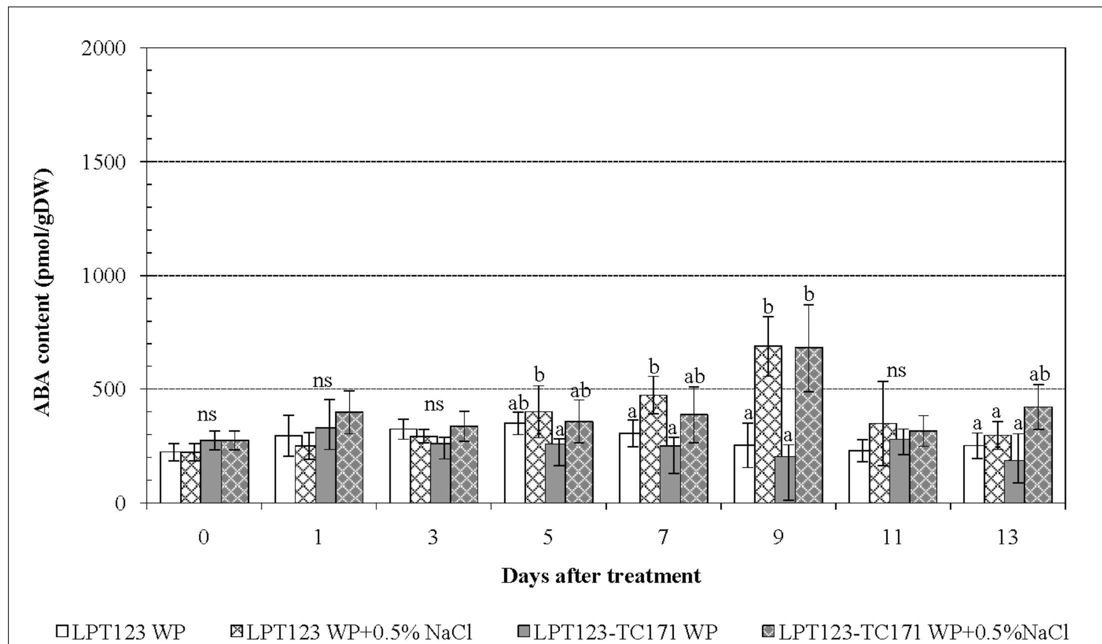


Figure 4.18 ABA content in leaves of salt sensitive rice line (LPT123) and salt tolerant rice line (LPT123-TC171) under normal and salt stress condition detect by ELISA method.

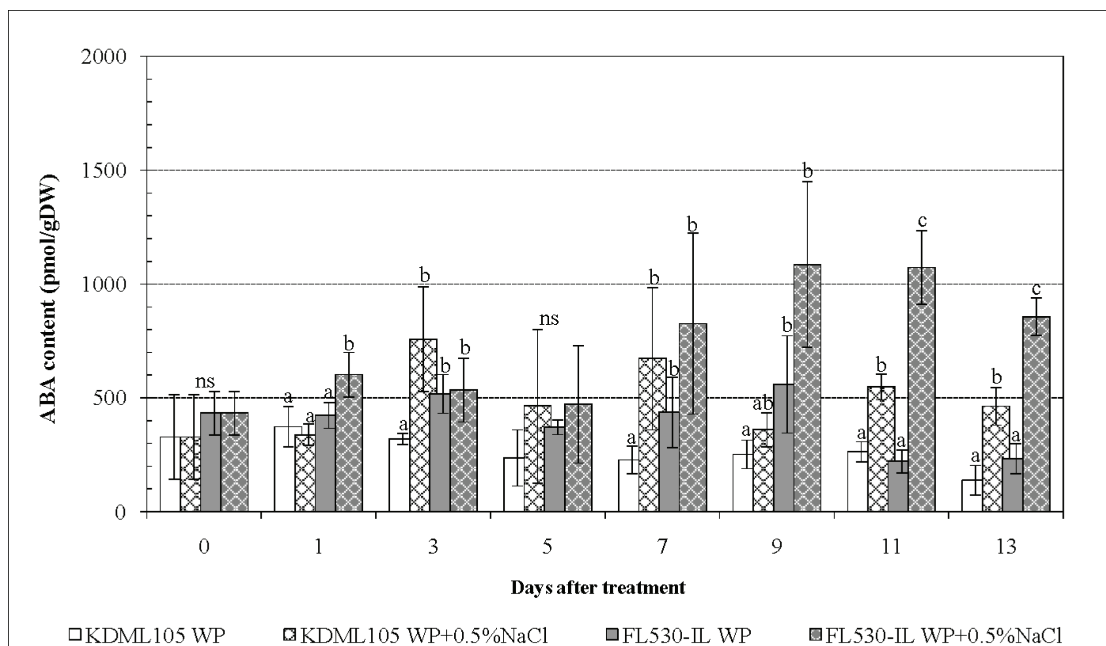


Figure 4.19 ABA content in leaves of salt sensitive rice line (KDML105) and salt tolerant rice line (FL530-IL) under normal and salt stress condition detect by ELISA method.

Growth of salt tolerant rice line (LPT123-TC171) and salt sensitive rice line (LPT123) was decreased when the both plants grew under salt stress condition when compared to the both rice line grew in normal condition. The dry weight of salt tolerant rice line (LPT123-TC171) grew under salt stress was significantly lower than the dry weight of plants grew under normal condition on day 7 to day 13. However, the dry weight of salt stressed LPT123-TC171 was significantly highest than the dry weight of salt stressed LPT123 after 13 days of salt stress reduce growth by 38 % in LPT123 and 27 % in LPT123-TC171 (Figure 4.20).

Growth of salt tolerant rice line (FL530-IL) and salt sensitive rice line (KDML105) was decreased when the both plants grew under salt stress condition when compared to the both rice lines grew in normal condition. This was similar to growth of salt sensitive line (LPT123) and salt tolerant rice line (LPT123-TC171) in the same condition. After 7 days of salt stress, dry weight of salt sensitive KDML105 rice was significant lower than the dry weight of plants grown in normal condition, while FL530-IL was able to maintain the dry weight after 7 days of salt stress. However, the significant decrease in dry weight due to the salt stress was found in FL530-IL after 13 days of the treatment. At the end of the experiment (13 days), salt stress caused 46 % of dry weight reduction in KDML105 rice, while in FL530-IL dry weight was reduced by 32 % (Figure 4.20).

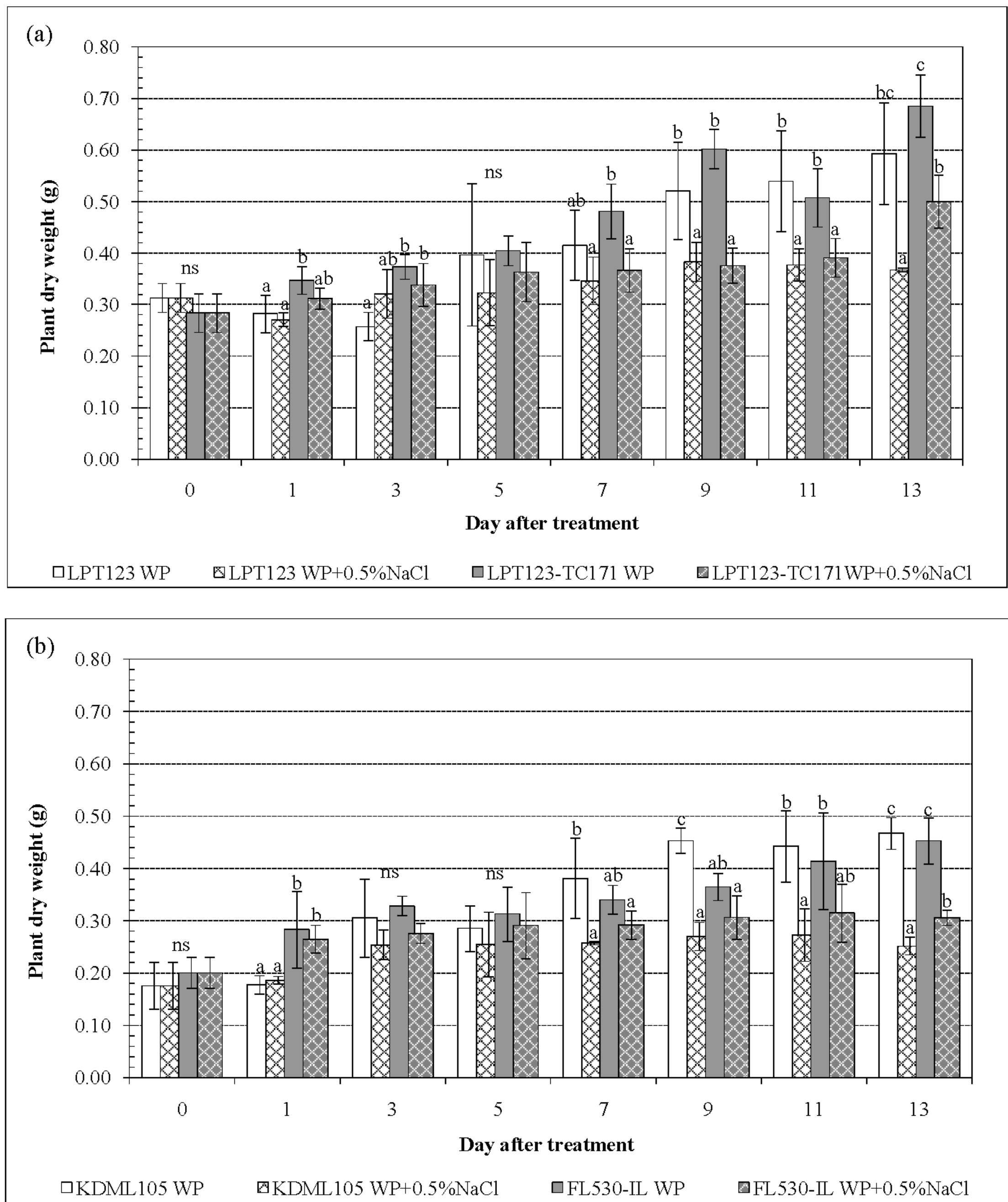


Figure 4.20 Growth of two pair of rice lines salt sensitive rice line and salt tolerant rice line (a) LPT123 and LPT123-TC171 and (b) KDML105 and FL530-IL under normal and salt stress condition.

ABA is readily soluble in variety of solvents. The most common used for extraction from plant tissues have been acidified (0.5-2% acetic acid) acetone (70-100%) or aqueous methanol (80-90%). Moreover, in this study methanol: acetic acid was used for extraction of ABA from rice plant tissues. To prevent ABA oxidation during the extraction, BHT was added into the extraction solution, which is 0.05 M acetic acid in methanol. This extraction method for ABA was shown to have high efficient recoveries of ABA (Weiler, 1980). In this study the two pairs of rice line/cultivar, the salt sensitive line (LPT123 and KDML105) and the salt tolerant line (LPT123-TC171 and FL530-IL) which have a different ability of salt tolerant were used for study the ability of ABA accumulation in salt stress condition. Therefore, in this study the monoclonal antibody specific to ABA was performed for endogenous ABA determination in rice leaf tissue in both of rice lines, salt sensitive rice line (LPT123, KDML105) and salt tolerant rice line (LPT123-TC171 and FL530-IL) when grew under salt stress condition. The two types of monoclonal antibody specific to ABA was performed, that were monoclonal antibody specific to carbonyl terminal of ABA and monoclonal antibody specific to carboxyl terminal of ABA. The monoclonal antibody specific to the carbonyl terminal of ABA was used for ABA determination because the monoclonal antibody specific to carboxyl terminal of ABA showed the lower sensitivity than this monoclonal antibody. This antibody could detect (+)-ABA in range of 0.2 to 0.00625 µg/ml (0.756 – 0.0236 pmol/ml) while the ABA detection kit (Phytodetex ABA Test Kit) by monoclonal antibody against ABA in plant extract could detect (+)-ABA in range of 0.16 – 0.0064 pmol/ml. However, ABA Kit antibody showed four times higher sensitivity than MAb was produced in this study. Therefore, MAb specific to carbonyl terminal of ABA was used for endogenous ABA determination in the two pairs of rice line/cultivar.

The first pair of rice line, salt sensitive rice line, LPT123 and salt tolerant rice line, LPT123-TC171 showed that the ability of ABA accumulation of salt tolerant rice line, LPT123-TC171 was higher than the ability of ABA accumulation of salt sensitive rice line, LPT123 when endogenous ABA was determined by HPLC and ELISA method. Similar to the ABA accumulation of salt sensitive rice line, KDML105 and salt tolerant rice line, FL530-IL. ABA accumulation of salt tolerant rice line, FL530-IL, was higher than salt sensitive rice line, KDML105 on day 7 to 13 after salt stress treatment.

Indirect competitive ELISA was selected as the method of endogenous ABA detection in rice tissues because of its high specificity and sensitivity toward ABA (Weiler, 1980), and due to the small amounts of plant tissue. The method for detection of endogenous ABA in rice leaf tissue by HPLC and indirect competitive ELISA method in this study indicated that the sensitivity of competitive ELISA method showed about 10 times higher than the sensitivity of HPLC method.

The trend of ABA content in rice leaf tissue of salt sensitive rice line (LPT123) and salt tolerant rice line (LPT123-TC171) by indirect competitive ELISA was similar to the ABA content by the HPLC method. The level of ABA was 50 times less than the level detected by HPLC. The phenomenon occurred because the ELISA method has more specificity than HPLC method. The peak area at the retention time of ABA may resulted from the related substance of ABA, which leads to the higher level of ABA detected, while the monoclonal antibody was specific to ABA only. Although in a crude extract ABA is a minor component, and considerable purification is required before identification and quantitation is possible but crude extract was used in this study was not purified before ABA determination because

preliminary test for suitable protocol of ABA extraction by purification and endogenous ABA level was determined by HPLC, result showed that there was no ABA peak area signal although the more plant sample was used (1.5 g FW) (data not shown). In contrast crude plant sample (1.5 g FW) without purification was used for ABA determination as the same method showed the ABA peak area signal this indicates that the purification of plant extract affects to the quantity of ABA.

In this study the pH of methanol extraction buffer was in range of 2.5-5.5 therefore, in this range could extract ABA and related compounds with carboxyl groups such as phaseic acid (PA), 4-dihydrophaseic acid (DPA) and the ABA-1-4-dioles from rice plant tissues (Ciha *et al*., 1977). These compounds can lead to gross overestimates of ABA concentration. Moreover, determination of endogenous ABA using HPLC method detected by UV absorbance has some disadvantage such as the absorbance spectra of ABA and related compounds are non-specific. Many primary and secondary metabolites can absorb in the range 200-300 nm. Even a combination of retention time and UV spectra cannot be taken as a good evidence for the presence of ABA. Therefore, in this study the fraction of rice crude extract was also collected and determined endogenous ABA by LC-MS.

Moreover, ABA is unstable at the high temperature more than 70ºC and plant extract should receive the minimum exposure to light to prevent isomerization of ABA to its isomer this can lead to errors in quantifying ABA (Leroux et al., 1985; Morris at al., 1988).

While method for ABA determination by indirect competitive ELISA was highly specific to ABA than other molecule which is similar ABA structure because

monoclonal antibody specific to carbonyl terminal of ABA would not bind to hippuric acid, which is similar ABA structure. This indicates that the antibody is specific to ABA.

However the trend of ABA level in salt tolerant rice line (LPT123-TC171) and salt sensitive rice line (LPT123) was similar when it was determined by HPLC method.

The level of ABA content in salt tolerant rice line (LPT123-TC171) is increased higher than salt sensitive rice line (LPT123) in salt stress condition similar to the ABA content in the other pair of rice line, FL530-IL and KDML105. The highest ABA accumulation in LPT123 and LPT123-TC171 was found on day 9 after salt stress, and similar level of ABA was found, while in rice with KDML105 genetic background showed the difference in the peak day of ABA accumulation and also the level of accumulation.

The ABA accumulation in both rice lines/cultivars salt sensitive LPT123 rice and salt tolerant rice, LPT123-TC171 was highest on day 9 after salt stress treatment it may resulted from the rate of ABA synthesis and ABA degradation, ABA-GE or PA may be change to ABA for plant response to salt stress condition and after that on day 11 ABA may be change to inactive form such as ABA-GE and PA and storage in the plant cells (Hartung *et al*., 2002; Sauter *et al*., 2002).

The salt sensitive KDML105 showed the highest ABA level faster than the salt resistant line, but the lower of ABA accumulation was found. This result indicated that in two pairs of rice line /cultivar which have the same genetic background salt sensitive rice line KDML105 and salt tolerant line FL530-IL, and

other pair has the LPT123 genetic background the original LPT123 is concerned as the salt sensitive one, and LPT123-TC171 which is the salt resistant line have the different of ABA accumulation ability when plants response to salt stress condition.

This result indicates that salt stress can induce the ABA biosynthesis in plants and ABA help the plants to adjust to water deficit by closing the stomata and ABA is also known to increase in plants under salt stress and to promote plants growth (Thomas and Bohnert, 1993) as observed in this study and under salt stress, the whole-plant response to salt is also regulated by an ABA-dependent pathway. ABA also regulates gene expression in wide range of cells to induce changes that help the plant to adjust to water deficit and ion toxicity (Zeevarrt and Creelman, 1988).

From this result it has been suggested that certain environmental signals such as salt and drought stress conditions that most dramatically activated ABA biosynthesis. However, salt and drought stress caused the increasing of endogenous ABA in rice plants resulted mainly from increased de novo biosynthesis (Hasegawa *et al*., 2000). In this study rice grew under the salt stress condition the water potential in plants cell is decreased so the water stress occurred in this condition. Water stress negatively affects plants, and plants reacts in different ways. One of this is a lessening of the rate of growth of stressed plants as compared with controls, and an increased in endogenous ABA. Cramer (2002) suggested that this increased in ABA may be responsible for growth inhibition to stressed plants.

ABA content of LPT123 and LPT123-TC171 rice was dramatically reduced after 11-13 days of salt stress, which was different from ABA content in KDML105 and FL530-IL rice that was gradually declined after the highest ABA content had

reached. This occurred because the LPT123 and LPT123-TC171 had more dry leaves after 11-13 days of salt stress when compared to KDML105 and FL530-IL rice. These dry matters could not accumulate ABA, resulting the drastic reduction of ABA content in this rice line/cultivar.

In contrast, under non stressful condition, ABA in plant cells remains at low level. Some low level of ABA may be required for normal plants growth, as evidenced by reduced vigor observed in ABA-deficient mutant plants that can be restored to the wild type level of growth by exogenous ABA because all ABA deficient mutants still have certain basal levels of ABA that are not dramatically lower than those in wild type under normal growth condition, and it is difficult to determine the cellular processes that required a very small amount of ABA (Xiong and Zhu, 2003). It was reported that exogenously applied ABA ameliorate the NaCl-induced reduction of rice growth (Klomsakul, 2004). It is inferred that the content of the endogenous ABA in rice plants might be changed under salt stress and it was similar to ABA content found in salt sensitive line (KDML105 and LPT123) and salt tolerant rice line (FL530-IL and LPT123-TC171) grown under salt stress condition in this study.

Under salt stress condition, the reduction in plants dry matter might have been due to a slower growth rate and slower development as a result of osmotic stress imposed by salinity, or due to inhibition of photosynthesis as a result of direct effects of salinity on the photosynthesis apparatus (Xu *et al.*, 1995). The salt sensitive rice line, LPT123 showed lower leaves and root dry weight than salt tolerant rice line, LPT123-TC171 and the greater reduction in leaves and root dry weight, in response to salt stress suggest that the growth of rice is sensitive to salinity and salt tolerant rice

line (LPT123-TC171 and FL530-IL) is better maintained the matter for ability to survival in the stress condition, in comparison with salt sensitive rice line (LPT123 and KDML105).

The salt resistance found in our study here is consistent with the previous study in which a significant difference between these two rice lines was indicated by their salt-tolerant scoring, relative water content (RWC) and Na^+/K^+ ratio, with the lower salt-tolerant scoring and Na^+/K^+ ratio and the higher RWC found in the FL530-IL rice (Suriya-arunroj *et al.*, 2004).

The salt resistant line showed the higher level of ABA accumulation after salt stress. It was previously shown that the increase in ABA level was at least partially the result of transcriptional regulation of the enzymes in the ABA biosynthesis pathway. The main step of the biosynthetic pathway for ABA is the conversion of zeaxanthin to violaxanthin catalyzed by the enzyme zeaxanthin epoxidase (ZEP) (Audran *et al.*, 1998). The enzymes in the biosynthesis pathway including 9-cis epoxycarotenoid deoxygenase (NCED), ABA aldehyde oxidase (AAO) and molybdenum cofactor sulfurylase (MCSU) were shown to be transcriptionally induced by drought or salt-stress (Audran *et al.*, 1998; Xiong *et al.*, 2001). Therefore, it was assumed that these up-regulation of these genes occurred in these rice lines. It has been known that ABA controls the plant adaptive response to NaCl stress by altering expression of some salt-tolerant genes (Narusaka *et al.*, 2003; Rakwal and Komatsu, 2004; Zhu *et al.*, 2005).

The higher level of the ABA content and the prolonged period of ABA accumulation may be one of the phenotypes contributing to the salt resistance

character in rice. The salt tolerant rice cell line under salt stress conditions accumulated a higher level of endogenous ABA than the salt-susceptible one (Perales *et al*., 2005). A similar phenomenon was also found in the naturally selected populations of Blue Panicgrass (*Panicum antidotale* Retz.). Higher accumulation of the free ABA content was found to be highly correlated with the degree of adaptability to the saline environment (Ahmad *et al*., 2009). Therefore, it is consistent with our results, which have shown that accumulation of ABA was higher in the salt-resistant FL530-IL line, than the salt-sensitive KDML105. In previous studies, exogenous ABA application was shown to induce salt-stress resistance by induction of some salt responsive genes (Bueno *et al*., 1998; Hasegawa *et al*., 2000; Zhu, 2002; Verslues and Zhu, 2007). In agreement with our results, these findings suggest the role of ABA in salt resistance in plants. Therefore, the salt sensitive rice line KDML105 and salt tolerant rice line FL530-IL which have the different pattern of ABA accumulation were used for elucidating the relationship between ABA accumulation and *OsCam1-1* gene expression when plant grown under salt stress condition.

6. The relationship between the *OsCam1-1* gene expression and ABA accumulation during salt stress condition

6.1 The effect of salt stress on *OsCam1-1* gene expression

6.1.1 Determination of the *OsCam1-1* gene expression in the KDML105 and FL530-IL in the salt stress condition using real-time-polymerase chain reaction (real-time PCR)

Comparison of the *OsCam1-1* gene expression in the KDML105 and FL530-IL rice leaves during salt-stress condition showed that the significant increase in *OsCam1-1* gene expression was found in FL530-IL after 30 min of salt stress, while in KDML105, the induction of *OsCam1-1* was detected after 2 hours of salt stress.

The highest level of *OsCam1-1* gene in the salt resistant line, FL530-IL was higher than the highest level of gene expression found in the salt sensitive cultivar, KDML105 rice (Figure 4.21).

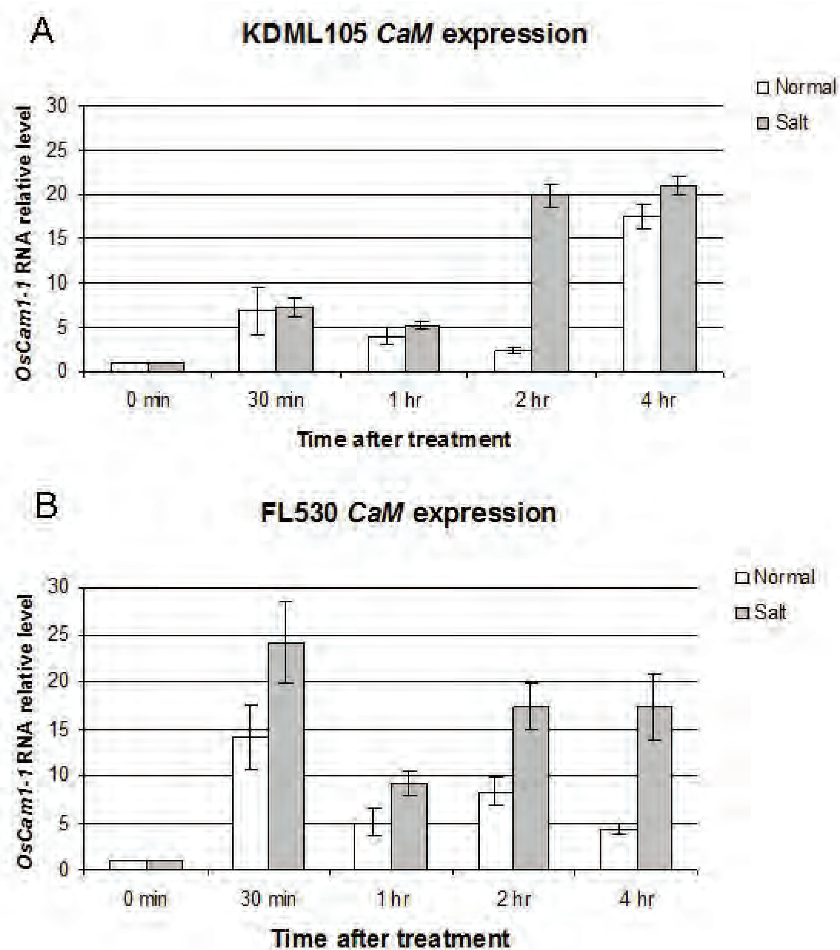


Figure 4.21 Calmodulin, *OsCam1-1* gene expression pattern in salt sensitive rice line (KDML105) (A) and salt tolerant rice line (FL530-IL) (B).

The increase in *OsCam1-1* gene expression was also found in the normal grown plants of both line /cultivar when compared the expression level at the beginning of the experiment (0 min). This suggested that *OsCam1-1* may induce to other factors, such as mechanical stimulation. It was reported that calmodulin gene in rice could be induced by touching (Phean-o-pas *et al*., 2005). However, the salt effect seems to be stronger for *OsCam1-1* gene induction in both KDML105 and FL530-IL rice.

Calcium-dependent signaling plays an important role in signal transduction of a wide range of stimuli, such as light, gravity, pathogen, osmotic stress and salinity stress (Zielinski, 1998; Reddy, 2001; Rudd and Franklin-Tong, 2001; Snedden and Fromme, 2001; Fasano *et al*., 2002). The *OsCam1-1* was previously shown to be up-regulated by salt stress during the first four-hours of salt treatment (Phean-o-pas *et al*., 2005). This is consistent with our results found here. Interestingly, *OsCam1-1* gene expression was also induced in the control seedlings, which received no salt stress. This induction was likely caused by the transfer of seedlings to the fresh nutrient solution without NaCl as a control. The transfer process may present a mechanical stimulus that induces *OsCam1-1* gene expression in rice. However, salt stress showed an ectopic effect over the mechanically-induced *OsCam1-1* gene expression (Figure 3 A and B). The higher *OsCam1-1* expression level and the faster induction in the FL530-IL rice seedlings suggests that the salt-tolerant FL530-IL rice line may have a higher sensitivity to salt stress than the salt sensitive KDML105 rice line.

In rice, Boonburapong and Buaboocha (2007) reported that *OsCam1-1* is identical to the OsCaM proteins encoded by two other *Cam* genes, *OsCam1-2*, and

OsCam1-3. As it was clearly induced by salt stress, *OsCaM1-1* is suggested to play an important role in salt stress signaling in rice. It was reported that *OsCam1-1* was also induced by mannitol and wounding (Phean-o-pas *et al*., 2005). Moreover, CaM was also shown to involve in the ABA-induced antioxidant defense in tomato during water stress (Hu *et al*., 2008). With the consistency in the level and the sensitivity of the *OsCam1-1* response and the salt resistance of the rice lines in this experiment, it suggests that *OsCaM1-1* may play a role in the salt-stress signaling cascade and its level and sensitivity of expression may contribute to salt resistance in rice.

6.2 The effect of exogenous ABA and abamine SG application on *OsCam1-1* (*Calmodulin 1-1* in *Oryza sativa* L.) promoter activity

6.2.1 The effect of exogenous ABA application on *OsCam1-1* gene expression under salt stress condition

The effect of exogenous ABA application on *OsCam1-1* gene expression by using *gus* reporter gene under the control of *OsCam1-1* promoter was studied. Two transgenic KDML105 rice lines, carrying a *gus* (*uidA* with an intron) as a reporter gene, regulated with *OsCam1-1* promotor and a hygromycin-resistant gene (*hpt*) as a plant selectable marker, were obtained via *Agrobacterium* transformation (Takpirom, 2007).

In this study, the exogenous 100 µM ABA was sprayed on the rice leave 2 hours before salt-stress treatment to avoid the interaction of mechanical stress and salt stress on the *OsCam1-1* promoter activity. The result showed that in normal condition, in transgenic rice line 95, *OsCam1-1* gene expression level could be induced by 100 µM ABA application, when grown in normal condition. The up-

regulation of *OsCam1-1* was found in 1 hour after the transfer to the new nutrient solution or 3 hours after ABA application. The increased of *OsCam1-1* expression was found up to 4 hours after seedling transfer. The induction in *OsCam1-1* promotor of the seedling treated with no ABA was detected after 4 hours of transferring to the fresh nutrient solution. This suggested the induction of *OsCam1-1* gene expression due to mechanical stimuli this result similar to the previous study reported that the touching stress could induce the higher expression of *OsCam1-1* gene (Phean-o-pas *et al.*, 2005). And the pattern of *OsCam1-1* gene expression of another transgenic rice line, line 96, was similar to what found in line 95, except the earlier of *OsCam1-1* induction when sprayed with 100 µM ABA (Figure 4.22).

Under salt stress condition, the results showed that after the transgenic rice exposed to salt stress, the expression of *OsCam1-1* gene was increased at 0, 1 and 2 hours after salt stress treatment and then after 4 hours, it was decreased, with the application of the exogenous ABA in salt stress treatment, the rice seedling showed the lower level of *OsCam1-1* gene expression, when compared to the plants sprayed with 0 µM ABA (Figure 4.23). And the comparison of the *OsCam1-1* gene expression between plant sprayed with buffer in normal condition and in salt stress condition, the *OsCam1-1* expression of plants in salt stress condition was higher than the expression of plants in normal condition. The pattern of *OsCam1-1* expression of transgenic rice line 95 was similar to transgenic rice line 96 (Figure 4.24).

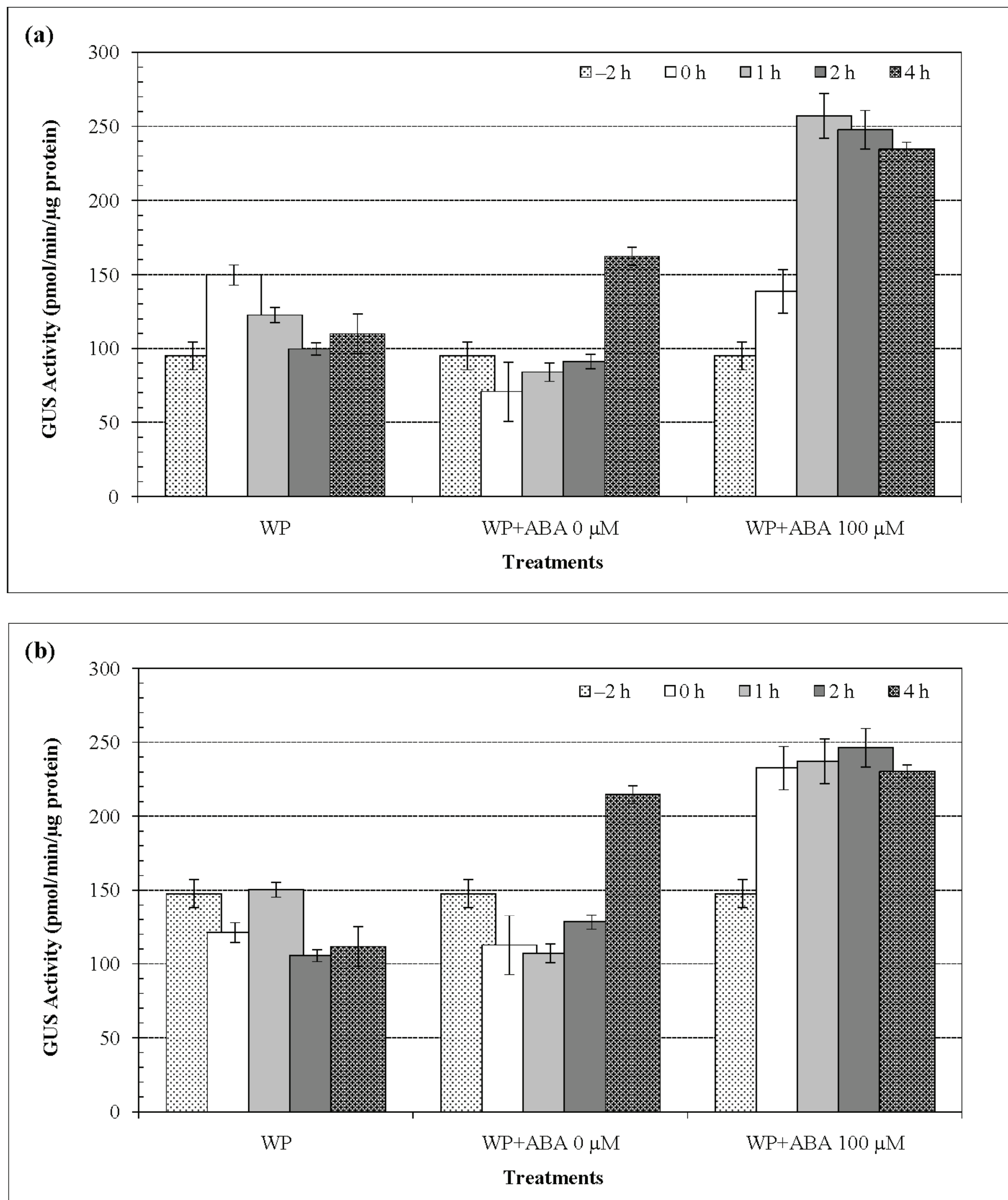


Figure 4.22 The effect of exogenous ABA on GUS activity under control of *OsCam1-1* gene promoter in transgenic rice line 95 (a) and 96 (b).

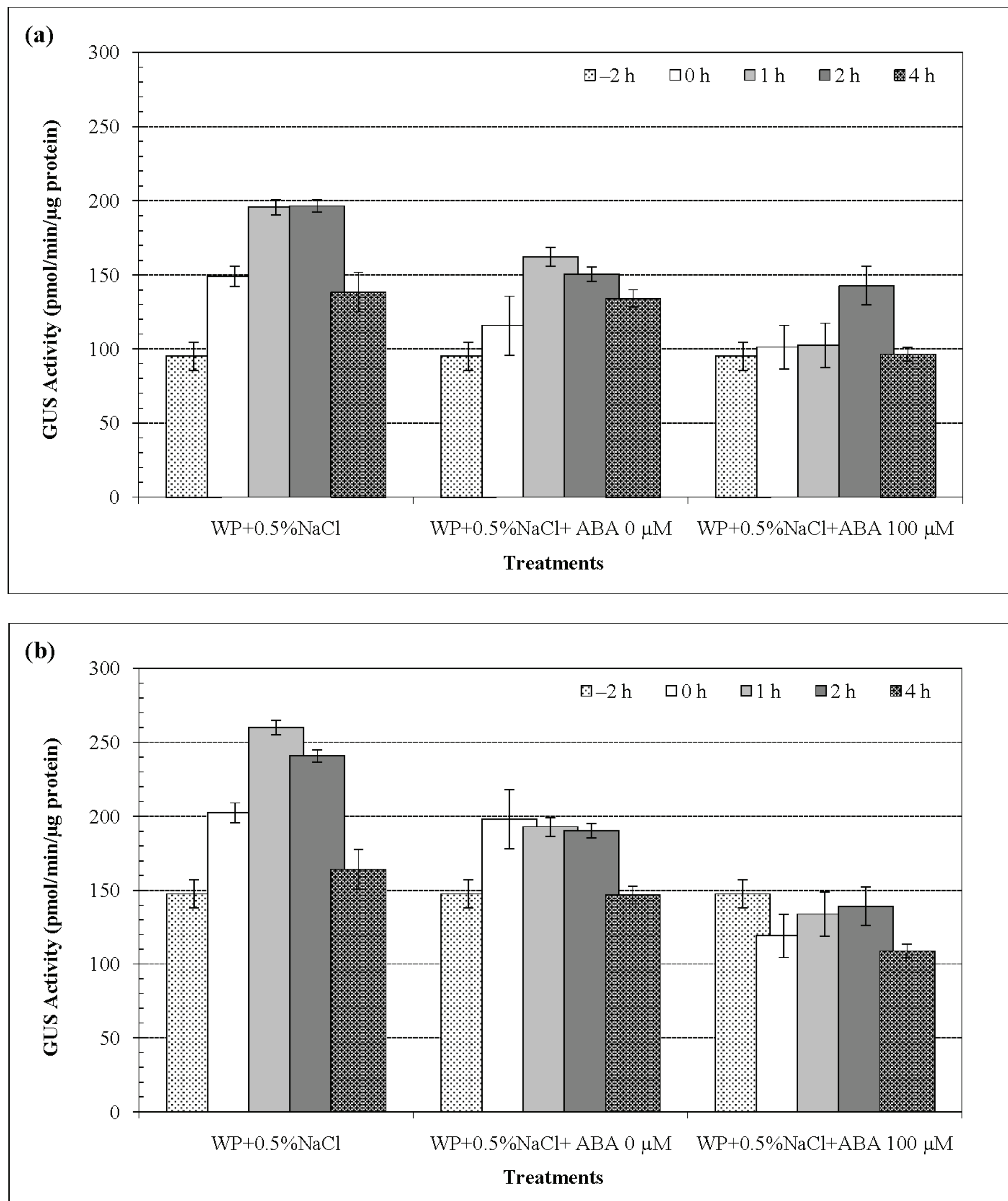


Figure 4.23 The effect of exogenous ABA on GUS activity under control of *OsCam1-1* gene promoter in transgenic rice line 95 (a) and 96 (b).

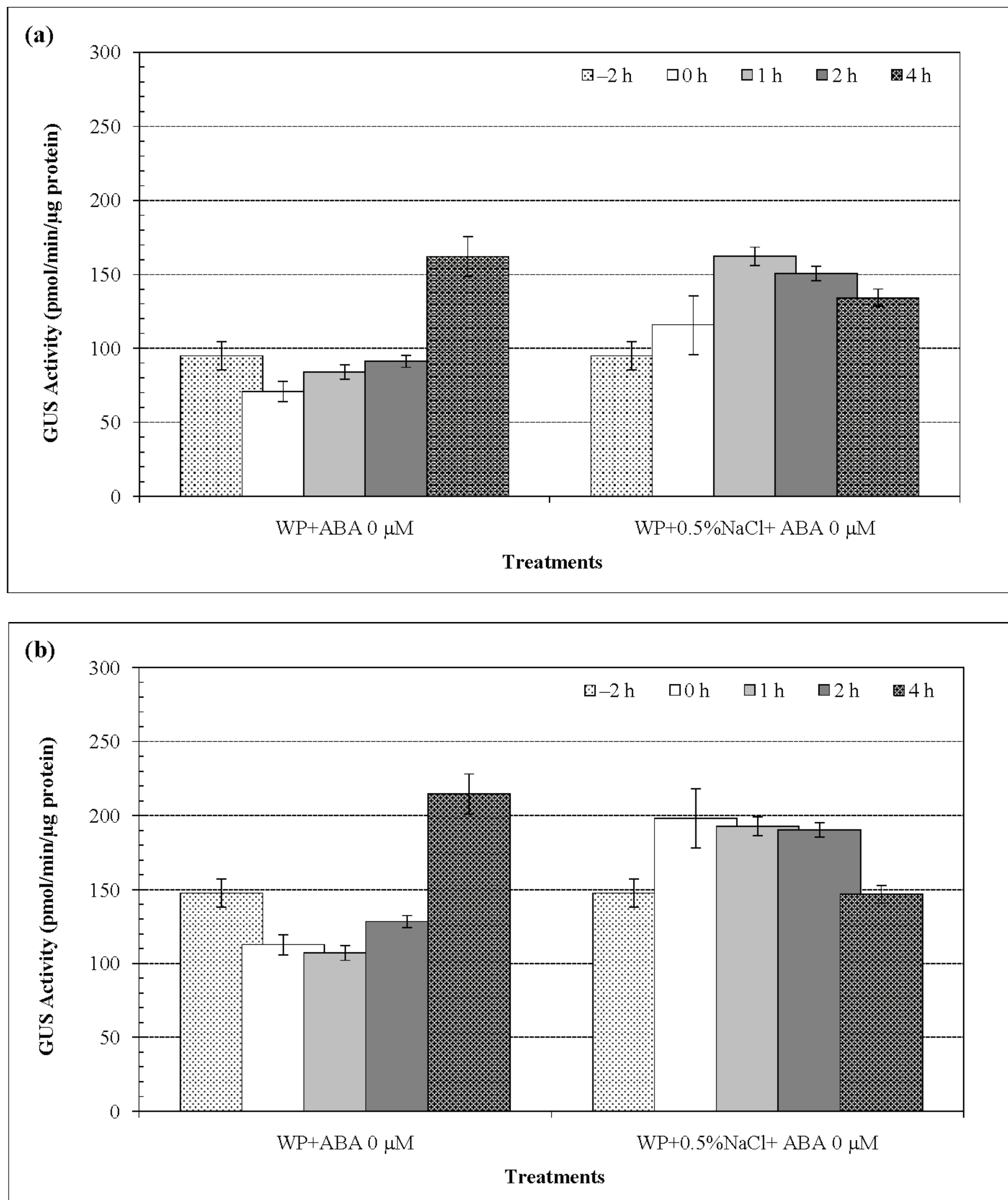


Figure 4.24 The effect of exogenous ABA on GUS activity under control of *OsCam1-1* gene promoter in transgenic rice line 95 (a) and 96 (b) grown under normal and salt stress.

Under salt stress condition, when exogenous ABA spraying and salt stress treatment were applied, the expression of *OsCam1-1* gene was lower than sprayed with only buffer (0 μM ABA) in salt stress condition. This result may be due to the effect of ABA on the stability of *gus* reporter gene during salt stress condition. Although, *OsCam1-1* promotor could be induced by the exogenous ABA, salt may increase the turn-over rate of GUS protein when plant grow under salt stress, leading to the lower level of *gus* expression in the salt stress grown seedling treated with ABA.

The expression of *OsCam1-1* gene that found in this study is similar to the expression of *OsCam1-1* gene expression in the salt sensitive rice line (KDML105) in the previous study. Boonburapong (2006) found that under salt stress, the expression levels of *OsCam1-1* gene found in salt sensitive rice line (KDML105), increased in 1 to 2 hours after salt stress treatment.

6.2.2 The effect of ABA inhibitor (abamine SG) application on *OsCam1-1* gene expression under salt stress condition

In order to study the role of ABA requirement for *OsCam1-1* gene expression, the inhibitor of ABA biosynthesis pathway, abamine SG (Sun-Young *et al*., 2004), was used to block ABA accumulation. The abamine SG was sprayed on the rice 2 hours before the treatment by transferring to fresh medium with /without NaCl.

The result showed that in normal condition, in transgenic rice line 95, *OsCam1-1* gene expression level could be induced by 100 μM abamine SG application. The up-regulate of *OsCam1-1* was found in 0 hour and then *OsCam1-1*

gene expression level was decreased in 1, 2 and 4 hours after treatment and the pattern of *OsCam1-1* gene expression was similar to transgenic line 96 (Figure 4.25).

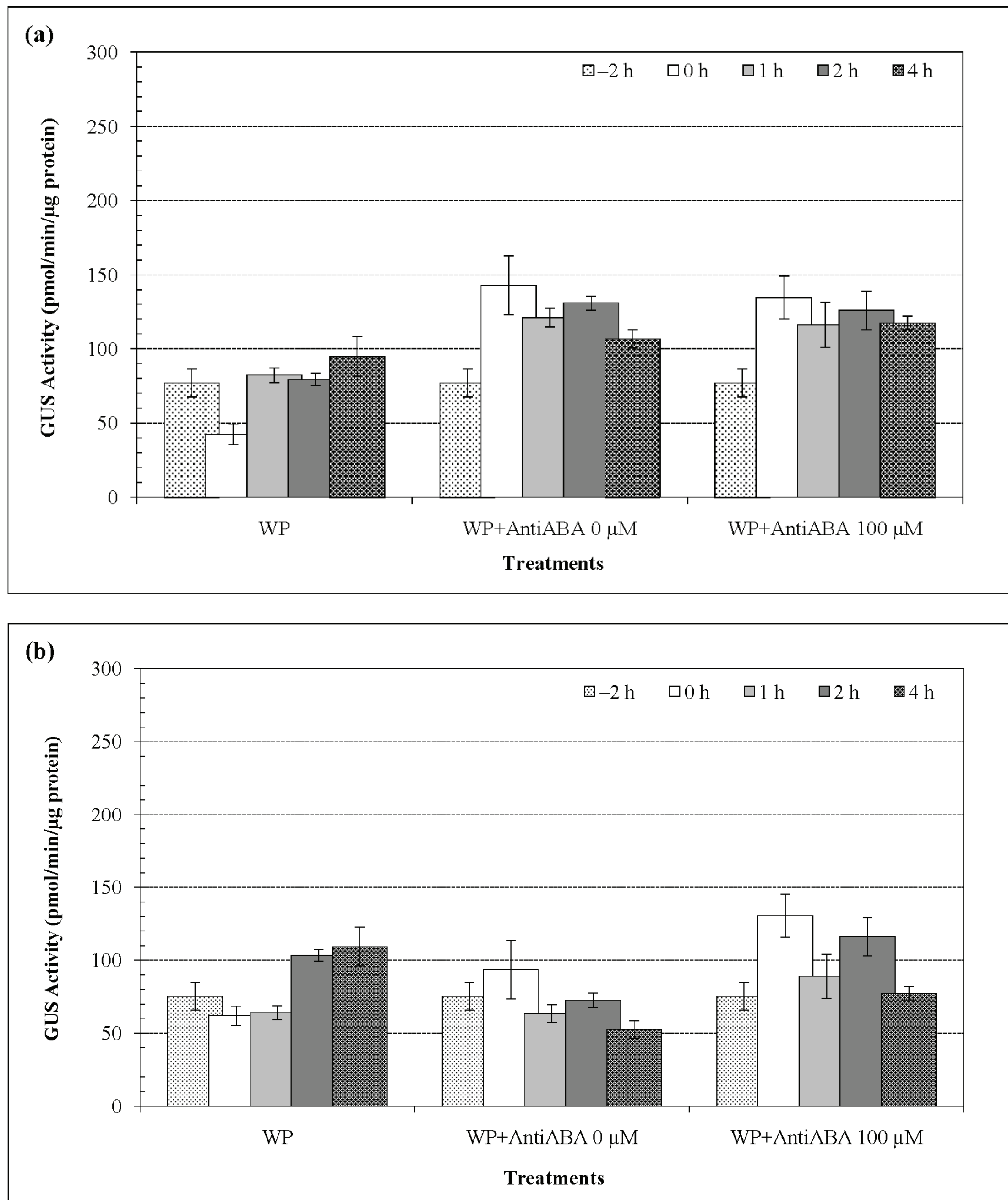


Figure 4.25 The effect of ABA inhibitor (abamine SG) on GUS activity under control of *OsCam1-1* gene promoter in transgenic rice line 95 (a) and 96 (b).

Under salt stress condition, when the transgenic rice, line 95, exposed to salt stress, the expression of *OsCam1-1* gene was increased at 0, 1, 2 and 4 hours after salt treatment when the buffer was sprayed on to the plant in salt stress condition. The up-regulation of *OsCam1-1* gene expression was found since hour 0. This suggested that spraying may cause the touching response of *OsCam1-1* gene expression.

The similar pattern was found in both lines of transgenic rice, line 95 and line 96 (Figure 4.26). The application of abamine SG was found to inhibit *OsCam1-1* gene expression during salt stress after 4 hours of salt stress treatment. The consistence of this response was also detected in line 96.

Abamine SG is a novel *NCED* inhibitor of the oxidative cleavage of 9-cis-epoxycarotenoids in ABA biosynthesis with an amine moiety (Sun-Young *et al.*, 2004). It was the most potent and specific ABA biosynthesis inhibitor targeting to *NCED*, which catalyzes the oxidative cleavage of 9-*cis*-neoxanthin to generate xanthoxin (Schwartz *et al.*, 1997; Tan *et al.*, 1997). It is thought that xanthoxin is converted to ABA by a two-step reaction via ABA-aldehyde which is catalyzed by ABA-aldehyde oxidase (*AAO*) (Schwartz *et al.*, 1997). The rate-limiting step was thought to be the oxidative cleavage of neoxanthin catalyzed by *NCED* (Tan *et al.*, 1997; Liotenberg *et al.*, 1999; Qin and Zeevaart, 1999; Taylor *et al.*, 2000; Thompson *et al.*, 2000) and the resulting of its role, ABA-aldehyde oxidase (*AAO*), is an enzyme covert the ABA-aldehyde to ABA was also inhibited by abamine SG (Sun-Young *et al.*, 2004).

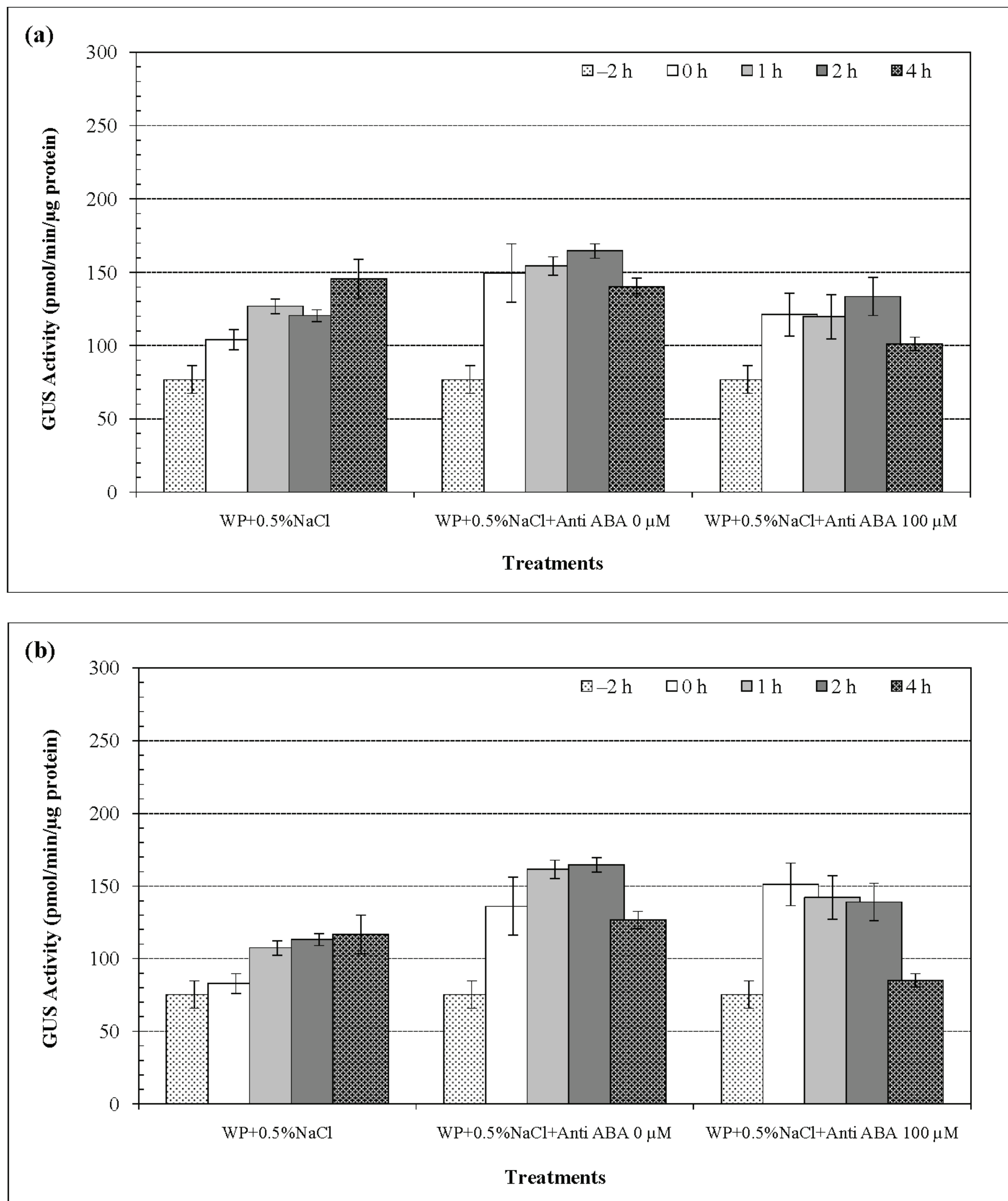


Figure 4.26 The effect of ABA inhibitor (abamine SG) on GUS activity under control of *OsCam1-1* gene promoter in transgenic rice line 95 (a) and 96 (b).

The application of either abamine SG buffer or abamine SG solution caused the increase in *OsCam1-1* gene expression after the transfer to the fresh medium. This up-regulation of *OsCam1-1* gene in this case was earlier than the up-

regulation found when treated with ABA buffer or ABA solution.

Between these two experiments, the difference was the solvent for ABA and abamine SG, DMSO was used as the solvent for abamine SG while 100% methanol was used as the solvent in preparation of ABA stock solution at the concentration of 10 mM. Then the stock solution was dilute to the working concentration as indicated. Therefore, the increase in *OsCam1-1* gene expression may be due to the induction by the low level of DMSO, the solvent of abamine SG.

This result indicated that the *OsCam1-1* gene expression is related to the ABA signal in the plants cell. When ABA biosynthesis pathway is blocked by abamine SG, the ABA accumulation in plant cells was decreased resulting in the lower level of *OsCam1-1* gene induction. Therefore, this suggested that ABA plays a role in *Cam1-1* gene expression.

6.3 The effect of Os*Cam1-1* over-expression in the transgenic rice lines on ABA accumulation

The relationship between *OsCam1-1* gene expression and the level of endogenous ABA accumulation in rice was studies. The transgenic rice lines harboring the construct of the *OsCam1-1* gene regulated by the *35SCaMV* promoter was used for ABA quantification during normal and salt stress condition. The content of ABA in transgenic rice line was measured in leaf tissues by using high performance liquid chromatography technique (HPLC) as described in the material and method. The transgenic line without the *OsCam1-1* over expression cassette, and the wild type KDML105 rice were used as controls.

Endogenous ABA level that found in the *OsCam1-1* over expression line grown in normal condition was significantly higher than the ABA level found in the controls, and both control showed the similar level of ABA content (Figure 4.27).

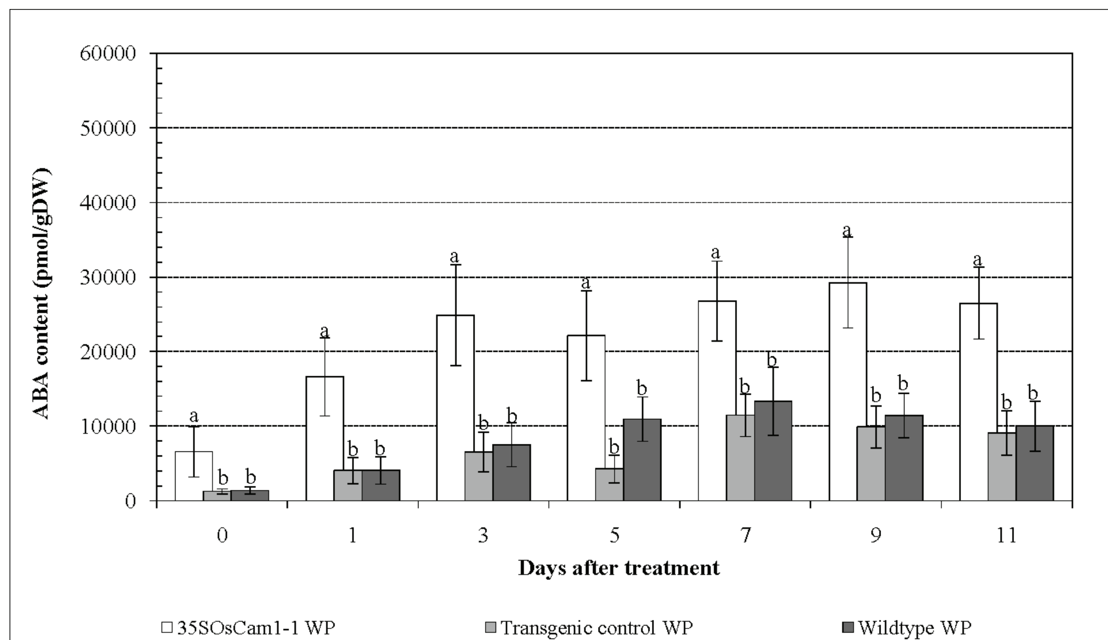


Figure 4.27 ABA content in transgenic rice over-expression of *OsCam1-1* gene in non stress condition.

Under salt stress condition, the level of endogenous ABA in the over-expression *OsCam1-1* gene transgenic rice line was significantly higher than the other two rice lines that were used as controls and the ABA content was highest after of salt stress treatment 7 days after salt stress treatment and then slightly decreased after 9-11 days (Figure 4.28). In particular, when the salt stress was applied to rice plants, transgenic rice line which harboring the *OsCam1-1* gene under the control of the *35SCaMV* promoter showed the higher level of endogenous ABA than the non-stress condition, but the level of endogenous ABA of transgenic rice that over-expression of *OsCam1-1* gene under salt stress condition was not significantly different when compared with the ABA level in the transgenic line grown in non-stress condition as

shown in Figure 4.29.

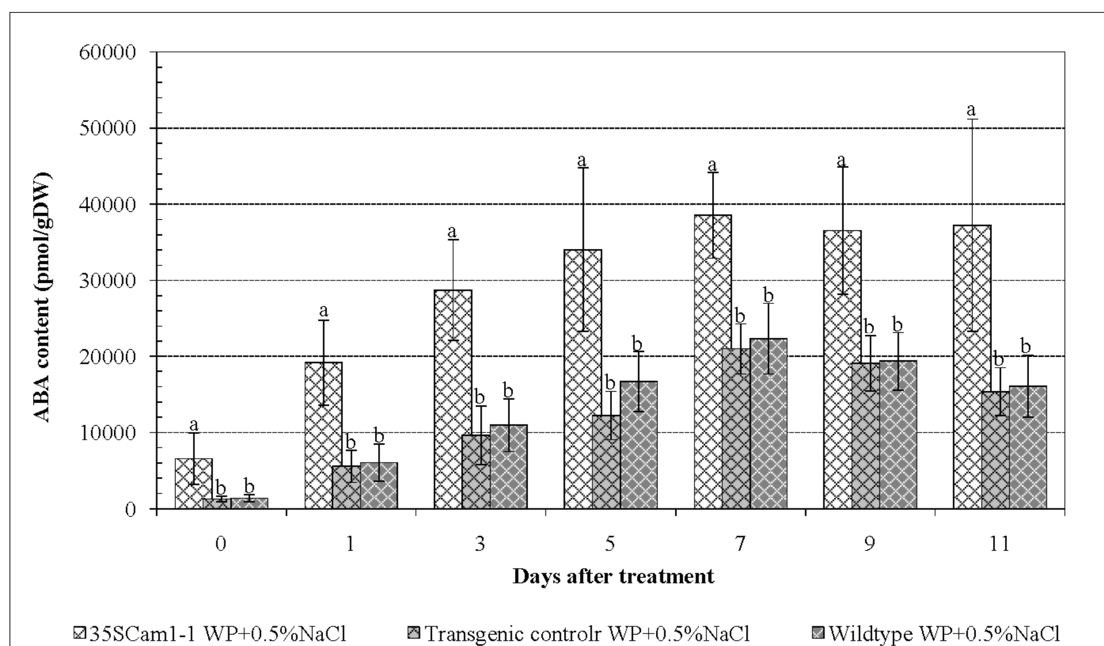


Figure 4.28 ABA content in transgenic rice over-expression of *OsCam1-1* gene in salt stress condition.

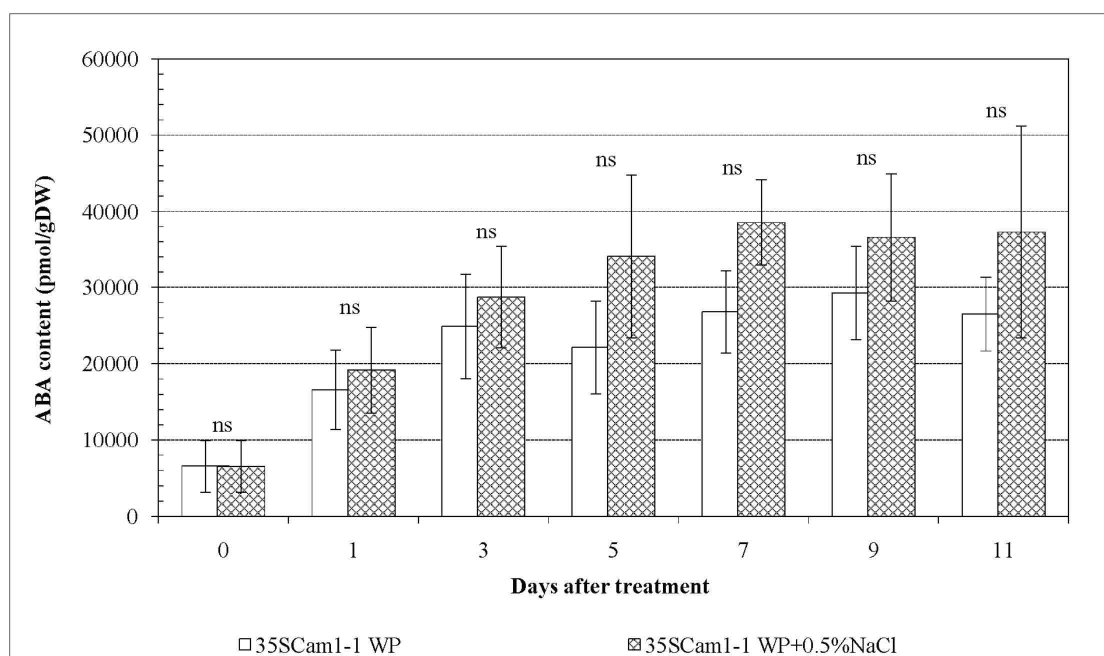


Figure 4.29 ABA content in transgenic rice over-expression of *OsCam1-1* gene in non stress and salt stress condition.

Growth of *OsCam1-1* over-expression transgenic rice line was determined by measuring the whole plant dry weight, and the transgenic line without the over-expression construct was used as a control. Under salt stress condition, the significant reduction in dry weight after salt stress condition was found in control transgenic line after 7 days of salt stress, while the *OsCam1-1* over-expression line could maintain dry weight. After 11 days of salt stress, both transgenic line had the significant lower dry weight when compared to the normal grown plants. However, the *OsCam1-1* over-expression line had the higher level of dry weight (Figure 4.31).

It is well known that endogenous ABA level commonly increases in response to salt stress (Wang *et al*., 2001). Similar result was observed in this study, the changes of endogenous ABA levels in transgenic rice plant that was *OsCam1-1* gene over-expression under salt stress were affected by the influence of calcium binding protein content in rice plant. The calmodulin (CaM) has been reported as a small calcium binding protein that transduces secondary messenger signals into a wide array of cellular responses. Expression of some, but not all, of the multiple CaM isoforms in plant species have been reported to be induced by particular stress signal including signal produced by stimuli such as salinity, cold, wind, wounding and pathogenic attack (Yamakawa *et al*., 2001). The CaM isoform, *OsCam1-1* gene has been reported that this CaM isoform expressed in rice (*Oryza sativa* L.) under salt stress condition (Phean-o-pas, Punteeranurak and Buaboocha, 2005). This result indicated that the over-expression of *OsCam1-1* gene in transgenic rice plants could increase ABA accumulation level in plant cells in both non-stress and salt-stress conditions, because CaM is involved in signal transduction of ABA-dependent pathway.

The up regulation of *OsCam1-1* by salt stress and ABA application was supported by the analysis of *OsCam1-1* promoter sequence (Figure 4.30). The promoter contains 2 ABRE elements (ACGTG and AACGCGT) at -993 to -989 and -378 to -372 upstream of the start codon.

Moreover, dehydration sequence element / c-repeat (core motif of DRE/CRT) was also found in the promoter as shown in Figure 4.30. These supported the response of *OsCam1-1* gene expression during salt stress and ABA application.

AATCCTCCCTGCTGATGTTGCCGGAGCAAGCGATCCAAATCTCCTCTCCCAGGCTTTTCCCAAAGAAAGGTGAAAGG

ATGCAAAGCG**ACCGAC**CGCCATGAGCGACCTCGCGGCTCCTTGCTCTCTGATCAAGAAGTTCGAAGCCTCAAACAGA

GAAGAGGAAGGGCTCGTGGGCTTCTGGCGATGTCAACTTGGGGTACGTTACGTTCTATTCCTGACAAGCGACAACGG

CATGATGGGCCGGCTGAACACACTGGCCCAGGGCCCATCAGAAGTATTCACGATCTGCGCTGGTG***ACGTG***GCACCGG

CCGGATTTGCAGCTTTGAATAATTTTTTTGGAAAGTGGAAAAATAAAAGAGGGAAATCATCACTCCGCGTCCAGACC

GCGTCCGGGGTAGTACGGTAATCCCCGGCCTCCTGGAAAACTGACACGGAGCCGGTCCCTCTTTTGGAAGTCATGGG

CAGGCTGACTGTACCACATGGCAGCACGTTCCCGCAATTCACAGGCCTTGCCCCTCGACGTTGCACCGTGTGACCAA

AATGTCCTCGCATGTTGCCCTGTGTCCTACTTGCTCGGTGCTCGGTATAATGATGCCATCCATGCACGTCATACATA

TATAGAACTTATACAAGTCCAGATAAAACATAACACCGCGGAGAAAAAAAACGCTAGTAAGAGCCAAATCATGATGG

ATTGATGGGGTGCACATGTATATACATACCTTCAGACGGCAAGAAGATATTCATGCACTACAAATGTTACAAAATCA

CATCCTAACATAGTACCAACGACGCTGACAACACCACCGCGCGAGCCAGCGATGGACTCGCGCACGTCGTCGTGGAC

TCGTGGCGCCCATGTCGCGTCGCGGTCGGTGACCGCTTTGCCCGTCGCACGTTGCGAGCATCGC**AACGCGT**CCTCCA

CTCCAGCCTGTGCCAACGAGACGAGGGACATTAGGAAAGTACAGCCGCGCATACCTTGGTTGATTGATCAGGAGGAG

ACGGGGGAGTGAGAGGGAGAGAGAGTAATTGAAGGGGGGGTAGTTTGGGGAATTCGCGGGGGCGCGACGTTAACGGG

AGGCGTGGACGGCGGAACGGGCCGGTCGGGGAACGGGCGGGCGGGTATAAAGGCCGGCGAAATTCCCCGGACGCGAG

GCCACAGATCGCTCCCAGCCATTCTCTCCGCGACGGTCTCGTCTTCCCCACCCCTCGCCTCCTCGCGCGCTCGGTGA

GAGAAGCGAAGAAGAAGAAGAGGAGGAGGAAGAAGCCAGGCTAAGCCCCGCGGCATGG

Figure 4.30 Cis-acting elements of *OsCam1-1* promoter analyzed by PLACE program. ACGTG, ABRE-like sequence and AACGCGT, ABRERATCAL, ABRE-related sequence required for etiolation-induced expression of erd1 (early responsive to dehydration), ACCGAC, DRECRTCOREAT (core motif of DRE/CRT) sequence required for dehydration (Appendix E).

The increase in endogenous ABA content that was found in transgenic rice line which overexpressed *OsCam1-1* gene that resulted in the increase in salt resistant as shown in the better ability to maintain dry weight during salt stress (Figure 4.31) implied that the ABA could help plants to adapt to survive under salt stress condition. These mechanism may include the induction of the stomata closure to reduce water loss during salt stress, the induction of osmolyte accumulation (Wang *et al*., 2001), and induction of other genes that are involved in cell protection during salt stress. The study of CaM and the effect of exogenous ABA in plant under salt stress condition have been reported. The effects of exogenous ABA applied to the plant in stress condition showed that when the *calmodulin* gene in plant was knocked out, the level of endogenous ABA in mutant line was lower than wild type and after sprayed exogenous ABA on the mutant line, the results found that the ability of plant response decreased under the stress condition. The signal transduction pathway of ABA has been elucidated that ABA is a signal in calcium signaling pathway in order to induce the transcription factor that involved genes regulated ABA biosynthesis and this process helps plant response under environmental stress (Xiong and Zhu, 2003).

7. The relationship between the *OsCam1-1* gene expression and genes involving in ABA biosynthesis in transgenic rice lines containing the over-expression of *OsCam1-1* gene under salt stress condition

To study the effect of the over-expression of *OsCam1-1* gene expression on the expression of genes involving in ABA biosynthesis, *NCED* and *AAO* genes were chosen as they were previously reported that Ca^{2+} may effect their gene expression (Xiong *et al.*, 2002).

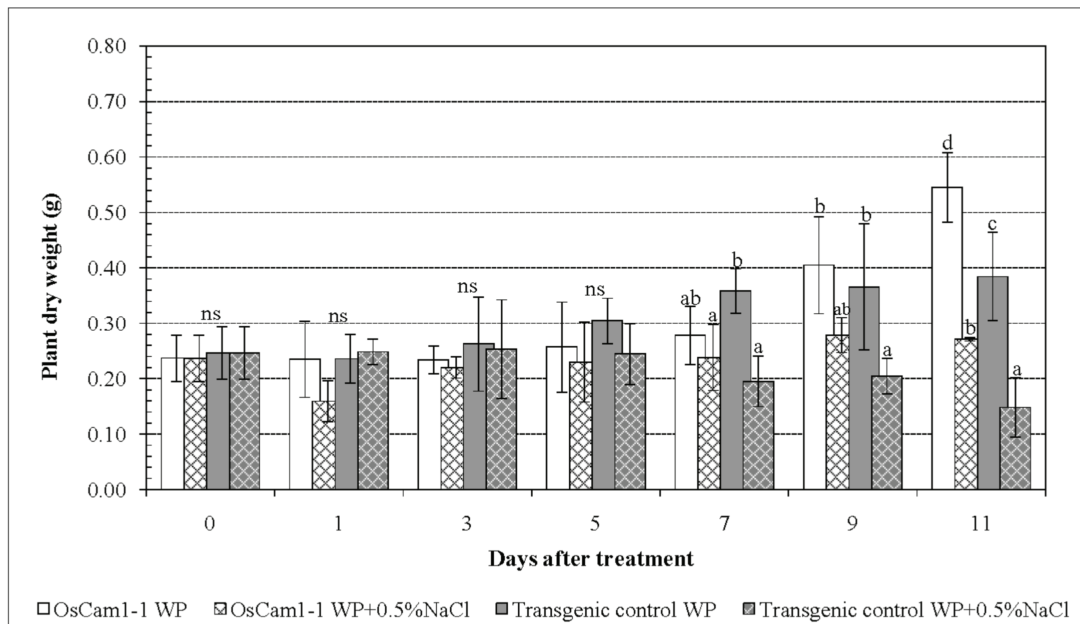


Figure 4.31 Plant dry weight of transgenic rice lines and control of transgenic rice line under non stress and salt stress condition.

The strategy to determine the relationship of *OsCam1-1* gene expression and gene involving ABA biosynthesis was to detect *NCED* and *AAO* gene expression in the *OsCam1-1* over-expression line and the transgenic line without the over-expression cassette and the wild type KDML105 which were used as controls.

7.1 The effect of the over-expression of *OsCam1-1* gene in the transgenic rice lines in the normal condition

The effect of over-expression of *OsCam1-1* gene on the expression of genes involving in ABA biosynthesis, *NCED* gene (9-cis-epoxycarotenoid dioxygenase) and *AAO* gene (ABA-aldehyde oxidase) in transgenic rice lines on the normal stress showed that the expression of *NCED* mRNA and *AAO* mRNA levels were expressed in the higher level in the transgenic rice which *OsCam1-1* over-expression cassette (lane 1-3 Figure 4.32) than the level in transgenic rice without the

expression cassette (lane 4-6 Figure 4.32).

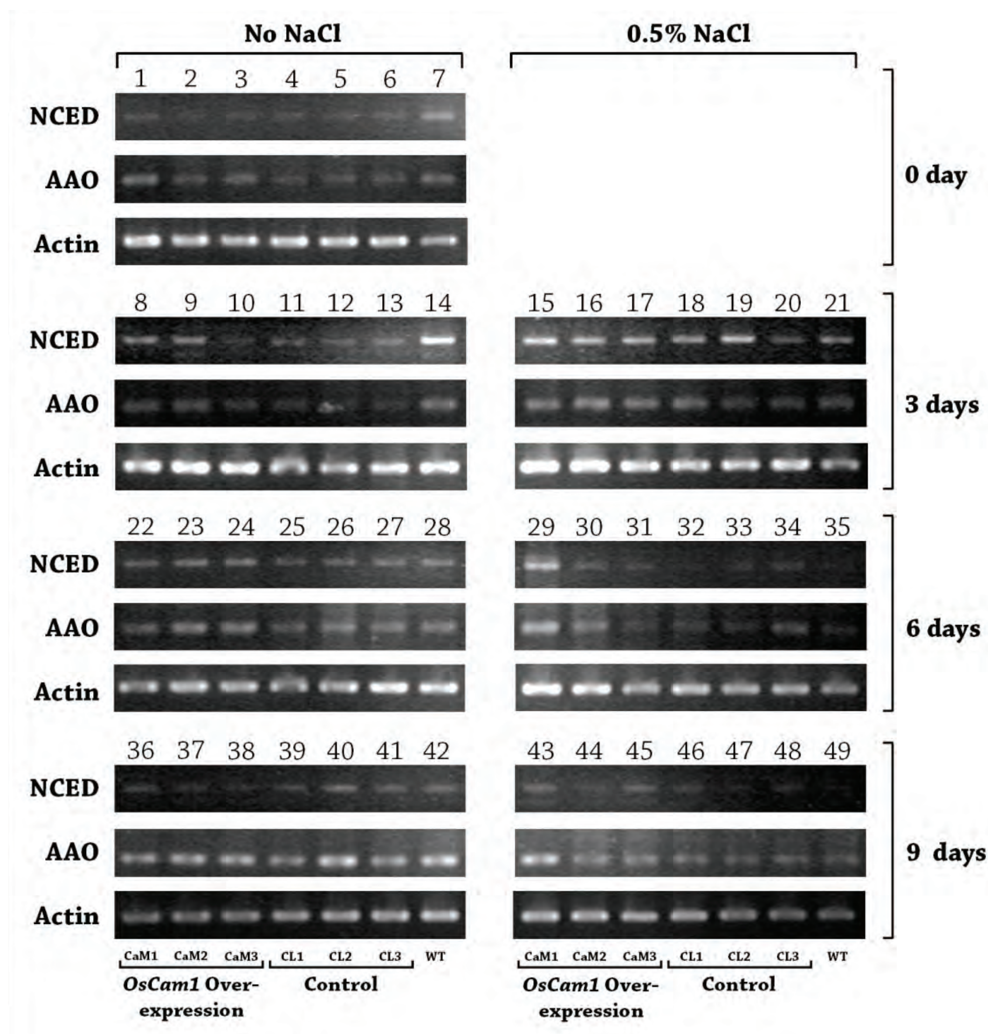


Figure 4.32 Expression of *NCED* and *AAO* gene involved ABA biosynthesis pathway in transgenic rice line under normal and salt stress condition.

However in some sample, KDML105 rice showed higher level of *NCED* gene expression, when compared to the *OsCam1-1* over-expression lines. This may be due to the variation occurs in KDML105 rice.

ABA is synthesized in mature leaves and transported both to the root and throughout the shoot system. ABA is normally much more abundant in the phloem sap. In many studies suggested that ABA in higher plants is synthesized from an

"indirect" pathway through the cleavage of a C_{40} carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via ABA-aldehyde (Taylor *et al.*, 2000, Seo and Koshiba, 2002, Schwartz *et al.*, 2003). The increasing of ABA levels under drought and salt stress are mainly achieved by the induction of genes coding for enzymes that catalyze ABA biosynthetic reactions. The 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) catalyzes the oxidative cleavage of 9-*cis*-neoxanthin to generate xanthoxin (Schwartz *et al.*, 1997; Tan *et al.*, 1997). It is thought that xanthoxin is converted to ABA by a two-step reaction via ABA-aldehyde which is catalyzed by ABA-aldehyde oxidase (*AAO*) (Schwartz *et al.*, 1997). In this ABA biosynthetic pathway, the rate-limiting step was thought to be the oxidative cleavage of neoxanthin catalyzed by *NCED* (Tan *et al.*, 1997; Liotenberg *et al.*, 1999; Qin and Zeevaart, 1999; Taylor *et al.*, 2000; Thompson *et al.*, 2000). The study of the expression of *NCED* and *AAO* gene indicated that these genes are all up-regulated by drought and salt stress (Audran *et al.*, 1998; Seo *et al.*, 2000; Iuchi *et al.*, 2001; Xiong *et al.*, 2001, 2002), although their protein levels were not examined in every case. Therefore, in this study *NCED* and *AAO* gene were chosen because these genes were previously reported that Ca^{2+} may effect their gene expression (Xiong *et al.*, 2002).

The effect of over-expression of *OsCam1-1* gene on the expression of genes involving in ABA biosynthesis in transgenic rice lines on the normal condition showed that the expression of *NCED* mRNA and *AAO* mRNA levels were higher in the transgenic rice with *OsCam1-1* over-expression cassette than transgenic control line and wild type (KDML105). This result indicated that the high abundance of calmodulin in the transgenic rice lines regulated the ABA biosynthesis which resulted from the up-regulation of *NCED* gene that translate to the 9-*cis*-epoxycarotenoid

dioxygenase which is the late limiting step enzyme of ABA biosynthesis pathway. In addition, the over-expression of *OsCam1-1* gene effect on the higher expression of *AAO* gene in transgenic rice lines, the *AAO* gene translates to ABA-aldehyde oxidase. This enzyme catalyzed ABA-aldehyde to convert to ABA in plant cells. This result indicated that *NCED* and *AAO* mRNA level are affected by *OsCam1-1* mRNA level.

7.2 The effect of the over-expression of *OsCam1-1* gene in the transgenic rice lines in the salt stress condition

The effect of over-expression of *OsCam1-1* gene on the expression of genes involving in ABA biosynthesis, *NCED* gene and *AAO* gene in transgenic rice lines during salt stress showed that the expression of *NCED* mRNA and *AAO* mRNA levels were higher in the transgenic rice with the over-expression cassette when compared to the transgenic controls.

Salt stress induced both *NCED* and *AAO* in all plant lines tested after 3 days of salt stress treatment. These led to the comparable level of gene expression among the *OsCam1-1* over-expression line (lane 15-17) the control transgenic lines (lane 18-20) and the wild type (lane 21). However, after 6 days of salt stress, the *NCED* and *AAO* expression in the control plants (lane 32-35) was more decreased than the OsCam1-1 over-expression lines (lane 29-31). After 9 days of salt stress, *NCED* gene expression in all plants tested was decreased, while the *AAO* expression stayed unchanged.

The over-expression of one of the CaM isoforms in Arabidopsis that binds to a transcription factor MYB2 resulted in enhancing its DNA binding activity and salt tolerance in Arabidopsis (Jae *et al*., 2005). CaM was also shown to involve in the

ABA-induced antioxidant defense in tomato during water stress (Hu *et al*., 2008). With the consistency in the level and the sensitivity of the *OsCam1-1* response and the salt resistance of the rice lines in our experiment, it suggests that *OsCaM1-1* may play a role in the salt-stress signaling cascade and its level and sensitivity of expression may contribute to salt resistance in rice.

The up-regulation of *NCED* and *AAO* genes after salt stress was consistent with the previous study showing that both genes were transcriptionally up-regulated by salt stress (Audran *et al*., 1998; Iuchi *et al*., 2000; Seo *et al*., 2000; Xiong *et al*., 2001, 2002). However, the over-expression line, which had the higher level of NCED and AAO gene expression than others showed the higher level of ABA accumulation after salt stress. These results suggest that the higher ABA content in the control transgenic rice line is at least partially if not absolutely caused by up-regulation of *NCED* and *AAO* gene expression under both normal and salt-stress conditions.

In poplar species, *Populus euphratica*, the salt-resistant poplar species have relatively higher ABA and CaM concentrations under a high salinity condition, when compared to the susceptible cultivar/species, *P. nigra* cv. Italica and *P. popularis* (Chang *et al*., 2006). In *Arabidopsis*, ABA accumulation leads to changes in expression or activity of HDACs, which in turn regulate growth under stress (Chinnusamy and Zhu, 2009). Similarly in these experiments, the higher level of ABA content during salt stress in the control transgenic rice line was consistent with the ability in maintenance of the shoot dry weight during salt stress, when compared to the transgenic control line. Taken together, these results suggest that the *OsCam1-1* over-expression confers better salt-tolerant ability on the transgenic plants possibly via ABA accumulation.

These data indicate that the higher capacity to synthesize stress signals, such as ABA and CaM contributes to better salt resistance. Our experiments have revealed the *OsCam1-1*over-expression effect on the up-regulation of *NCED* and *AAO* gene expression and the ABA content level, which suggests the function of the calcium-dependent OsCaM1-1 protein in the signaling cascade for ABA biosynthesis during salt-stress conditions. However, this process should not be in the same pathway of the signal transduction cascade for stomatal signaling previously described as the function of ABA action via CaM signaling, because the ABA accumulation occurred much slower than the stomatal closure signal process. The enhancement of *OsCam1-1* gene expression on ABA accumulation is suggested to be a slow response that leads to expression of other salt resistant genes. The sensitivity to salt stress via calmodulin, *OsCam1-1* and ABA accumulation ultimately contributes to salt resistance in rice.

CHAPTER V

CONCLUSION

1. Monoclonal antibodies (MAbs) specific to the carboxyl terminal or carbonyl terminal of abscisic acid (ABA) were produced. The MAbs against carbonyl terminal has the higher sensitivity for ABA detection than the MAbs specific to the carboxyl terminal of ABA. Therefore, the MAbs against carbonyl terminal of ABA with the highest sensitivity was used for determination of endogenous ABA in rice tissues. This MAb could detect ABA in the range of 0.024-0.756 pmol/ml (0.0063 – 0.2 μg/ml). The required rice leaf tissues were about 0.5-1.0 g FW for the detection. The sensitivity of this MAb for ABA is comparable to the commercial ABA detection kit (Phytodetex ABA Test Kit), which can detect ABA in the range of 0.0064-0.16 pmol/ml. When compared to the sensitivity of ABA detection by HPLC, the MAb specific to carbonyl terminal of ABA has ten times higher sensitivity.

2. Salt stress induced ABA accumulation of all four rice lines/cultivars tested, LPT123, LPT123-TC171, KDML105, and FL530-IL. The highest level of ABA accumulation was detected after 9 days under salt-stress condition. The highest level of ABA was equivalent in the salt sensitive, LLPT123, and the salt resistant line, LPT123-TC171. On the other hand, the salt resistant line, FL530-IL, showed the significant higher level of ABA content than the salt sensitive KDML105 after 9 days in salt-stress condition. This suggested that ABA accumulation ability may contribute to salt resistant ability. And of course, in the case of LPT123 and LPT123-TC171, there are

other factors leading to salt resistance that are more important than ABA content.

3. The expression of the rice calmodulin gene responsive to salt stress, *OsCam1-1* was studied in KDML105 and FL530 rice. Salt stress induced *OsCam1-1*gene expression in both rice line/cultivar, but the *OsCam1-1*gene expression in FL530-IL responded faster and higher than the expression in KDML105. The faster and the higher expression of *OsCam1-1*gene expression may contribute to higher salt resistant ability in rice.

4. To test if the level of *OsCam1-1*gene expression contributes to salt resistance and ABA accumulation level, *OsCam1-1*gene was over-expressed in KDML105 rice by *35SCaMV* promoter regulation. The over-expressed *OsCam1-1*transgenic lines showed the higher level of ABA in both normal and salt-stress condition, when compared to the transgenic lines without the *OsCam1-1*over-expression cassette, and wild type. It was showed that the over-expressed *OsCam1-1*transgenic lines had higher level of *9-cis-epoxycarotenoid dioxygenase* (*NCED*) and *abscisic aldehyde oxidase* (*AAO*) gene expression in normal and salt-stress condition. Moreover, the over-expressing transgenic line was more resistant to salt stress than the transgenic control. This supports that *OsCam1-1*gene expression regulates ABA biosynthesis and contributes to salt resistant ability in rice.

5. The effects of ABA on *OsCam1-1*gene expression was studied using the transgenic KDML105 rice, containing *β-glucuronidase* (*gus*) gene regulated by *OsCam1-1*promoter. Salt stress and exogenous ABA induced *OsCam1-1*

promoter activity, while the application of ABA inhibitor, abamine SG, blocked the up-regulation of *OsCam1-1*during salt stress. This suggests that ABA is required for *OsCam1-1*gene expression during salt stress.

6. Taken together, ABA induced *OsCam1-1*gene expression and over-expression of *OsCam1-1*gene up-regulating genes involving in ABA biosynthesis, resulting in the higher accumulation of ABA lead to the conclusion that ABA and *OsCam1-1*gene expression function in the bidirectional interaction to each other, and contribute to salt resistant ability in rice.

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APPENDICES

APPENDIX A

REAGENT RECIPES

1. Reagents for hybridoma production

1.1 RPMI medium

RPMI 1640 (Gibco BRL, USA)	10.4 g
D-Glutamine (Sigma)	3.6 g
L-Glutamine (Sigma)	0.2923 g
Sodium pyruvate ($C_3H_3O_3Na$) (Sigma)	1.1005 g
$NaHCO_3$	2.0160 g
HEPES (N-2-Hydroxyethylpiperazine- N-2-ethanesulfonic acid, Sigma)	5.9525 g

Adjust volume to 1000.0 ml with Meri Q water

The solutions of penicillin G, streptomycin and kankmycin were added to the final concentrations of 20,000 units, 200 mg and 200 mg per liter, respectively. The medium was sterilized by Millipore (pore size 0.22 µm) filtration and stored at 4 ºC.

1.2 100X HT supplement (Gibco BRL, USA)

Sodium hypoxanthine	10.0 mM
Thymines	1.6 mM

1.3 RPMI medium with serum

RPMI medium (1.1)	80.0 ml
Fetal calf serum (FCS, Starrate, Australia)	20.0 ml
or bovine calf serum (BCS, Starrate, Australia)	
100X HT supplement (Gibco BRL, USA)	1.0 ml

1.4 Hybridoma selective medium (HAT medium)

RPMI medium (1)	80.0 ml
FCS	20.0 ml
100X HT supplement	1.0 ml
50X Aminopterin (Sigma)	2.0 ml
Mouse red blood cell	1% v/v

1.5 Fusion solution (40% polyethylene glycol)

Polyethylene glycol (PEG)	4.0 g

Adjust volume to 10 ml with RPMI medium (1.1)

The solution was incubated at 37 ºC in CO_2 incubator before use.

1.6 Freezing medium (12% DMSO)

Dimethylsulfoxide (DMSO, Sigma)	12.0 ml
RPMI medium (1.1)	88.0 ml

The medium was stored in cold (4 ºC) before use.

2. Reagent for determination of isotype and subisotype of monoclonal antibodies using antibody captured on anti-Ig antibodies

Hybridoma sub-isotyping kit, mouse (Zymed) contents:

a) Rabbit anti – Mouse IgG_1 (γ1 chain specific)

b) Rabbit anti – Mouse IgG_{2a} (γ2a chain specific)

c) Rabbit anti – Mouse IgG_{2b} (γ2b chain specific)

d) Rabbit anti – Mouse IgG_3 (γ3 chain specific)

e) Rabbit anti – Mouse IgA (α chain specific)

f) Rabbit anti – Mouse IgM (μ chain specific)

g) Rabbit anti – Mouse kappa light chain

h) Rabbit anti – Mouse lambda light chain

i) Negative Control: Normal Rabbit Serum

j) Positive Control: Monoclonal Mouse IgG_1 in RPMI-1460 with 10% FBS

k) 10X Subtrate buffer (1 M citric acid, pH 4.2; 0.03% H_2O_2)

l) 50X ABTS substrate (2,2-azino-di [3-ethylbenzthiazoline sulfonic acid])

m) 50X Blocking solution (25% BSA in PBS and 0.5% NaN_3)

n) 50X HPR-Goat anti-Rabbit IgG (H+L)

o) 50X HPR-Goat anti-Mouse IgGAM (0.5% mg/mL in PBS containing 10% glycerol and 0.05% NaN_3)

p) 50% Tween 20

3. Buffers and reagents for ELISA assay

3.1 0.15 M Phosphate buffered saline (PBS) pH 7.2

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.2 g
Na_2HPO_4 (or $Na_2HPO_4 \cdot 7H_2O$)	1.15 g (2.15 g)

Adjust volume to 1000.0 ml with H_2O (distilled water)

3.2 1% Merthiolate

Thimerosal (Sigma)	1.0 g

Adjust volume to 100.0 ml with H_2O (distilled water)

3.3 5% BLOTTO solution (Johnson *et al.*, 1984)

Skimmed milk	5.0 g
1% Merthiolate (Sigma)	1.0 ml
Triton X-100 (Sigma)	0.1 ml

Adjust volume to 100.0 ml with 0.15 M PBS pH 7.2

3.4 Washing solution (0.5% Blotto)

5% BLOTTO solution (3.3)	50.0 ml
0.15 M PBS pH 7.2	950.0 ml

3.5 0.1 M Citrate buffer pH 4.5

Sodium citrate	29.4 g
1% Merthiolate	10.0 ml

Adjust volume to 1000.0 ml with H_2O (distilled water)

3.6. 1 N H_2SO_4

Conc. H_2SO_4	27.0 ml
H_2O (distilled water)	973.0 ml

3.7 Bradford reagent (Bradford, 1976)

Coomassie brilliant blue G250	10.0 mg
Methanol	5.0 ml
Phosphoric acid 85% (w/v)	10.0 ml
Distilled water	100.0 ml

4. WP Solution (WP No.2 1991) (Vajrabhaya and Vajrabhaya, 1991)

Chemicals	Content (mg/l)
Macroelements:	
Potassium nitrate (KNO_3)	580
Calcium sulfate ($CaSO_4$)	500
Magnesium sulfate ($MgSO_4 .7H_2O$)	450
Triple super phosphate	250
Ammonium sulfate ($(NH_4)_2SO_4$)	100

Chemicals	Content (mg/l)
Microelements:	
di-sodium ethylene diamine tetraacetate (Na_2EDTA)[a]	160
Ferrous sulfate ($FeSO_4 . 7H_2O$)[a]	120
Manganese sulfate ($MnSO_4 . H_2O$)	15
Boric acid (H_3BO_3)	5
Zinc sulfate ($ZnSO_4 .7H_2O$)	1.5
Potassium iodine (KI)	1.0
Sodium molybdate ($Na_2MoO_4 .2H_2O$)	0.1
Copper sulfate ($CuSO_4.5H_2O$)	0.05
Cobalt chloride ($CoCl_2.6H_2O$)	0.05

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

1. Weight Na_2EDTA 40 g and $FeSO_4.7H_2O$ 30 g.

2. Dissolve Na_2EDTA 40 g in distilled water 500 ml and $FeSO_4.7H_2O$ 30 g in distilled water 500 ml at 70-90 ºC.

3. Add the Na_2EDTA into $FeSO_4.7H_2O$ solution mix and then prepare this solution under the O_2 at the room temperature for 3-4 hours until the clear solution.

5. Reagents of GUS assay

5.1 β-Glucoronidase (GUS) assays—Staining Solution

100 mM NaH_2PO_4/Na_2HPO_4 buffer pH 7.5	10 ml of 0.2 M
0.1% Triton X-100	200 µl of 10% (v/v)
10 mM EDTA	400 µl of 0.5 M
1 mM X-Gluc	1 ml of 20 mM (0.104 g/10 ml DMF)
0.5 mM K Ferricyanide	1 ml of 10 mM (33 mg/10 ml H_2O)
0.5 mM K Ferrocyanide	1 ml of 10 mM (44 mg/10 ml H_2O)
H_2O	6.4 ml

A time-saving alternative

Mix phosphate buffer, EDTA, Triton X-100, and H_2O in the propotions given above and store at room temperature. Just before using, prepare the K-Ferricyanide and K-Ferrocyanide stocks. Mix the components in the following proportions.

Component	ml/ 5 ml solution
Buffer Stock	4.25
K-Ferro Stock	0.25
K-Ferri Stock	0.25
X-Gluc Stock	0.25

5.2 BCA Protein Assay Kit

5.2.1 Preparation of diluted albumin (BSA) standards

Dilution scheme for standard test tube protocol and microplate procedure (Working Range 20-2,000 µg/ml).

Albumin standard ampules, 2 mg/ml, containing bovine serum albumin (BSA) at 2.0 mg/ml in 0.9 % saline and 0.05 % sodium azide

Vial	Volume of dilution	Volume and source of BSA	BSA concentration
A	0	300 µl of stock	20,000 µg/ml
B	125 µl	375 µl of stock	1,500 µg/ml
C	325 µl	325 µl of stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml

5.2.2 Preparation of the BCA working reagent (WR)

BCA reagent A, 1,000 ml (in Product No. 23225), containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide

BCA reagent B, 25 ml, containing 4 % cupric sulfate

Prepare WR by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1, reagent A:B). Reagent B is first added to reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Microplate procedure (Sample to WR ratio 1:8).

6. Reagents for RNA extraction

6.1 0.2 M Phosphate buffer stock:

Stock A: Na_2 $HPO_4.7H_2O$ 26.8 g

Dissolve in a final volume of 500 ml ddH_2O

Stock B: $NaHPO_4.H_2O$ 13.8 g

Dissolve in a final volume of 500 ml ddH_2O

Place solution A in a 1000 ml beaker with a stir bar on a magnetic stirrer. Add solution B with stirring until the pH reaches 7.0.

6.2 DEPC-treated H_2O:

H_2O	100.0 ml
DEPC	0.5 ml

Shake vigorously and leave open under a hood overnight and autoclave.

6.3 RAN extraction buffer

100 mM Tris pH 9

100 mM NaCl

20 mM EDTA

1% lauryl sacrosinate

0.1% (v/v) β-mercaptoethanol

0.1% DEPC (diethyl pyrrocarbonate)

6.4 TE

10 mM Tris pH 8

1 mM EDTA

APPENDIX B

AGAROSE GEL ELECTROPHORESIS

1. Reagents

1.1. 5X TBE

Tris-base	54 g
Boric acid	27.5 g
0.5 M EDTA pH 8	20 ml

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

30% glycerol in water

0.25% bromophenol blue

0.25% cylene cyanol

1.3 Ethidium bromide stock:

Ethidium Bromide	1 g
H_2O	100 ml

Stir several hours with a magnetic stirrer and store in a dark bottle at 4 °C.

2. Method

Agarose gel electrophoresis is the standard method used for separation of DNA fragments on their molecular weight and used for rough estimation of DAN on its direct relationship between amount of DNA and the level of the fluorescence after ethidium bromide staining. The ladder obtained from restriction enzyme digestion

was analyzed according to their size. Agarose was solubilized by heating in a microwave oven and allowed to cool to 50-60 ºC before pouring into a plastic gel former with a present well-forming comb. The concentration of agarose gel was used varies with the size of the DNA fragment to be prepared. Generally 1.0-1.8% gel in Tris-borate-EDTA (TBE) buffer was used.

The DNA were mixed with 10% (v/v) of DNA gel loading buffer (0.1 M EDTA/NaOH pH 7.5, 50% (v/v) of glycerol, 1% (w/v) of SDS, 0.5 (w/v) of xylene cyanol FF, and 0.5 (w/v) of Bromophenol blue). The mixture was load onto slots of the gel that was submerged in electrophoretic chamber fill with TBE. Electrophoresis was carried out at constant 100 volts, the duration of the running time was depended on the size of DNA. Gel was run until the bromophenol blue reached approximately ¾ of the gel length. After that the gel was stained with ethidium bromide solution (5-10 µg/ml in distilled water) for 5-10 minutes and the destained with an appropriate amount of water with gently shaking for 10 minutes to remove unbound ethidium bromide from agrose gel. The DNA fragment were visualized as fluorescent bands under an UV transilluminator and photographed.

APPENDIX C

1. Selection of antibody

The selected mouse was then euthanized by cervical dislocation. It was sprayed liberally with 70% alcohol, and the spleen was removed aseptically. Three separated sets of sterile surgical utensils were prepared. The first set was for opening the skin ventrally and making the abdominal wall exposed, the second set making a small incision in the abdominal wall, the third set pulling out the spleen. Carefully the skin and loose hair was kept well away from the work surface.

The spleen was placed and soaked in two sterile petri dishes containing PBS. To minimize the chance of contamination, the spleen was taken and extensive soaked in a sterile Petri dishes containing RPMI 1640 media. Each soak was for a minimum of 5 min. During the extensive soak as much the connective tissues was carefully removed as possible. Prior to performing the fusion, the Petri dishes containing PBS, and containing RPMI 1640 media were placed overnight in a humidified incubator.

2. First screen of antibody

The nitrocellulose membrane and then placed in shallow plastic box containing 30 ml of 5 % blotto in PBS for 30 min. After transfered to a PBS buffer without 5% blotto each antigen spot on the membrane was cut and place in each well of microtiter plate, contain 5% blotto. The antibody from each hybridoma was added to the well, resulting the ¼ dilution of the antibody.

APPENDIX D

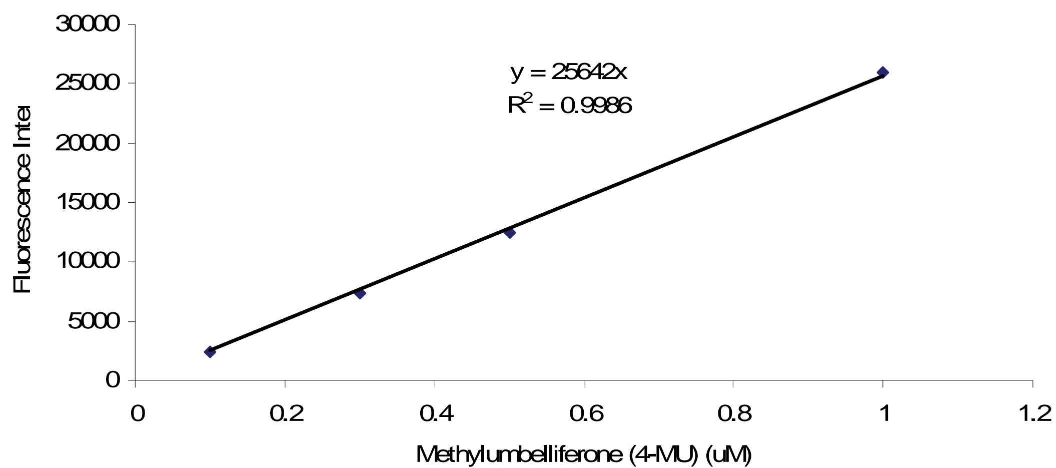

Figure D.1 Standard curve showing the linear rise in fluorescence with increasing concentrations of MU in phosphate buffer.

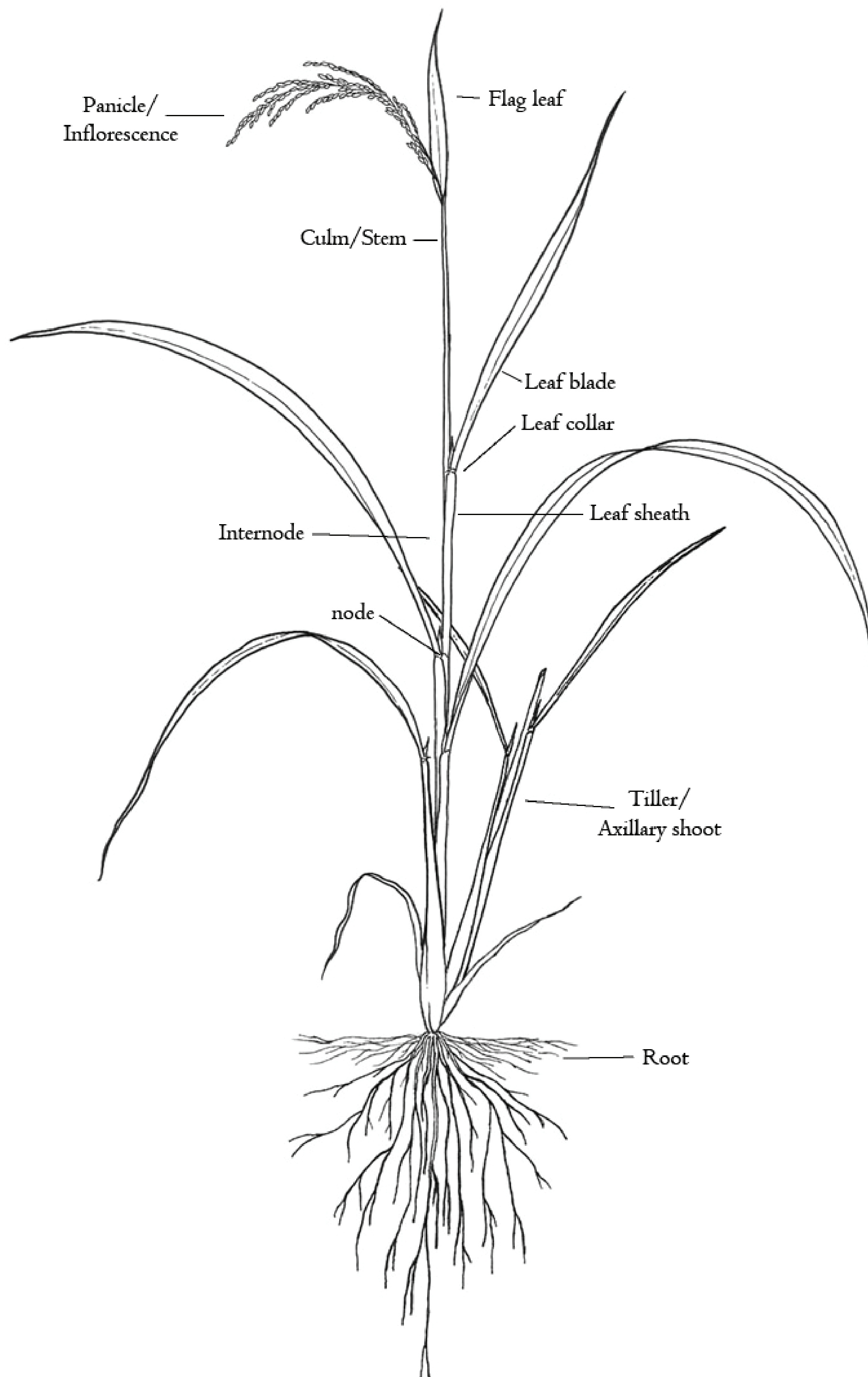

Figure D.2 Morphology of rice (*Oryza sativa* L.).

APPENDIX E

OsCam1-1 promoter analysis by PLACE program

AATCCTCCCTGCTGATGTTGCCGGAGCAAGCGATCCAAATCTCCTCTCCCAGGCTTTTCCCA
AAGAAAGGTGAAAGGATGCAAAGCGACCGACCGCCATGAGCGACCTCGCGGCTCCTTGCTCT
CTGATCAAGAAGTTCGAAGCCTCAAACAGAGAAGAGGAAGGGCTCGTGGGCTTCTGGCGATG
TCAACTTGGGGTACGTTACGTTCTATTCCTGACAAGCGACAACGGCATGATGGGCCGGCTGA
ACACACTGGCCCAGGGCCCATCAGAAGTATTCACGATCTGCGCTGGTGACGTGGCACCGGCC
GGATTTGCAGCTTTGAATAATTTTTTTGGAAAGTGGAAAAATAAAAGAGGGAAATCATCACT
CCGCGTCCAGACCGCGTCCGGGGTAGTACGGTAATCCCCGGCCTCCTGGAAAACTGACACGG
AGCCGGTCCCTCTTTTGGAAGTCATGGGCAGGCTGACTGTACCACATGGCAGCACGTTCCCG
CAATTCACAGGCCTTGCCCCTCGACGTTGCACCGTGTGACCAAAATGTCCTCGCATGTTGCC
CTGTGTCCTACTTGCTCGGTGCTCGGTATAATGATGCCATCCATGCACGTCATACATATATA
GAACTTATACAAGTCCAGATAAAACATAACACCGCGGAGAAAAAAAACGCTAGTAAGAGCCA
AATCATGATGGATTGATGGGGTGCACATGTATATACATACCTTCAGACGGCAAGAAGATATT
CATGCACTACAAATGTTACAAAATCACATCCTAACATAGTACCAACGACGCTGACAACACCA
CCGCGCGAGCCAGCGATGGACTCGCGCACGTCGTCGTGGACTCGTGGCGCCCATGTCGCGTC
GCGGTCGGTGACCGCTTTGCCCGTCGCACGTTGCGAGCATCGCAACGCGTCCTCCACTCCAG
CCTGTGCCAACGAGACGAGGGACATTAGGAAAGTACAGCCGCGCATACCTTGGTTGATTGAT
CAGGAGGAGACGGGGGAGTGAGAGGGAGAGAGAGTAATTGAAGGGGGGGTAGTTTGGGGAAT
TCGCGGGGGCGCGACGTTAACGGGAGGCGTGGACGGCGGAACGGGCCGGTCGGGGAACGGGC
GGGCGGGTATATAAAGGCCGGCGAAATTCCCCGGACGCGAGGCCACAGATCGCTCCCAGCCATT
CTCTCCGCGACGGTCTCGTCTTCCCCACCCCTCGCCTCCTCGCGCGCTCGGTGAGAGAAGCG
AAGAAGAAGAAGAAGAGGAGGAGGAAGAAGCCAGGCTAAGCCCCGCGGCATGG

ACGTG, ABRE-like sequence (from -199 to -195) required for etiolation-induced expression of erd1 (early responsive to dehydration) in Arabidopsis.

MACGYGB, ABRERATCAL, "ABRE-related sequence" or "Repeated sequence motifs" identified in the upstream regions of 162 Ca^{2+}-responsive upregulated genes, M=C/A; Y=T/C; B=T/C/G.

ACGTGKC, ACGTABREMOTIFA2OSEM, experimentally determined sequence requirement of ACGT-core of motif A in ABRE of the rice gene, DRE and ABRE are interdependent in the ABA-responsive expression of the rd29A in Arabidopsis; K=G/T.

ACGT, ACGTATERD1, sequence (from -155 to -152) required for etiolation-induced expression of erd1 (early responsive to dehydration) in Arabidopsis.

ACACNNG, DPBFCOREDCDC3, a novel class of bZIP transcription factors, DPBF-1 and 2 (Dc3 promoter-binding factor-1 and 2) binding core sequence; found in the carrot (D.c.) Dc3 gene promoter; Dc3 expression is normally embryo-specific, and also can be induced by ABA; the Arabidopsis abscisic acid response gene ABI5 encodes a bZIP transcription factor; abi5 mutant have a pleiotropic defects in ABA response; ABI5 regulates a subset of late embryogenesis-abundant genes; GIA1 (growth-insensitivity to ABA) is identical to ABI5.

RCCGAC, DRECRTCOREAT, core motif of DRE/CRT (dehydration-responsive element/C-repeat) *cis*-acting element found in many genes in Arabidopsis and in rice; R=G/A; Os DREB1A bound to GCCGAC more preferentially than to ACCGAC whereas At DREB1A bound to both GCCGAC and ACCGAC efficiently; Maize ZmDREB1A bound to DRE, HaDREB2 in *Helianthus annuus* (sunflower), HaDREB2 physically interact with HaHSFA9 in vitro.

GAAAAA,GT1GMSCAM4, DE "GT-1 motif" found in the promoter of soybean (*Glycine max*) CaM isoform, *SCaM-4*; plays a role in pathogen- and salt-induced *SCaM-4* gene expression.

CATGCA, RYREPEATBNNAPA, DE "RY repeat" found in RY/G box (the complex containing the two RY repeats and the G-box) of *napA* gene in *Brassica napus* (B.n.); found between -78 and -50; required for seed specific expression; dist B ABRE mediated transactivation by ABI3 and ABI3-dependent response to ABA; a tetramer of the composite RY/G complex mediated only ABA-independent

transactivation by ABI3; B2 domain of ABI3 is necessary for ABA-independent and ABA-dependent activation through the dist B ABRE.

TAAAG,TAAAGSTKST1,TAAAG motif found in promoter of *Solanum tuberosum* (S.t.) KST1 gene; Target site for trans-acting StDof1 protein controlling guard cell-specific gene expression; KST1 gene encodes a K^+ influx channel of guard cells.

BIOGRAPHY

Ms. Sukhumaporn Saeng-ngam was born on December 18, 1978 in Nakhonsawan. After she finished school in 1995 from Satree Angthong School in Angthong, she was enrolled in the Department of Biology at Srinakharinwirot University and graduated with the degree of Bachelor of Science in 2000 and graduated with the degree of Master of Science in 2004 from Department of Botany at Kasetsart University. She has gotten the scholarship from Collaborative Research Network, Office of the Higher Education Commission to study for the degree of Doctor of Philosophy in Biological Science program, Faculty of Science, Chulalongkorn University since 2004.