การแสดงออกของยีนและโปรตีนที่เกี่ยวข้องกับกลไกการทนความเค็มในข้าว *Oryza sativa* L. โดยการวิเคราะห์ด้วย cDNA-AFLP และโพรทีโอมิกส์

นางสาวรัตนาวดี วิชาจารณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EXPRESSION OF GENES AND PROTEINS INVOLVING SALT-TOLERANCE MECHANISMS IN RICE *Oryza sativa* L. ANALYZED BY cDNA-AFLP AND PROTEOMICS

Miss Rattanawadee Wichajarn

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Field of Study	Biotechnology
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นางสาวรัตนาวดี วิชาจารณ์ : การแสดงออกของยีนและโปรตีนที่เกี่ยวข้องกับกลไกการทนความเค็มในข้าว *Oryza sativa* L. โดยการวิเคราะห์ด้วย cDNA-AFLP และโพรทีโอมิกส์ (EXPRESSION OF GENES AND PROTEINS INVOLVING SALT TOLERANCE MECHANISMS IN RICE *Oryza sativa* L. ANALYZED BY cDNA- AFLP AND PROTEOMICS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร.วรวุฒิ จุฬาลักษณานุกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.สิทธิรักษ์ รอยตระกูล, 157 หน้า.

การตอบสนองต่อความเครียดจากสิ่งแวดล้อม ส่งผลให้เกิดการเปลี่ยนแปลงทางกายภาพและชีววิทยาของพืช โดยภาวะดินเค็มเป็นปัจจัยหนึ่งที่สำคัญต่อการเจริญเติบโตและพัฒนาการ ส่งผลให้ผลผลิตของข้าวลดน้อยลง โดยวิถีเมตาบอลิซึมต่อการตอบสนองความเครียดจากความเค็มมีความซับซ้อน ในการศึกษานี้ได้ค้นหาเครื่องหมายโมเลกุลที่เกี่ยวข้องกับความเครียดจากความเค็ม โดยทำการศึกษารูปแบบการแสดงออกของยีนที่แตกต่างกันในข้าวสายพันธุ์ทนเค็ม (Homjan) และข้าวสายพันธุ์ไม่ทนเค็ม (Pathumthani 1) โดยใช้เทคนิค RNA arbitrary-primed (RAP)-PCR หลังจากนั้นได้ใช้เทคนิค complementary DNA-Amplified fragment length polymorphism (cDNA-AFLP) ศึกษารูปแบบการแสดงออกของยีนที่แตกต่างกันใน Pokkali และ IR29 ซึ่งเป็นข้าวสายพันธุ์ทนเค็ม และไม่ทนเค็มตามลำดับ โดยพบยีนที่น่าสนใจจากวิธี RAP - PCR จำนวน 1 ยีนได้แก่ *protein kinase domain containing protein* และ จาก cDNA-AFLP จำนวน 7 ยีน ได้แก่ *lipin - N terminal conserve region (Lpin), ATP synthase subunit C (ATPsubc)* และ *cytochrome P450 monooxygenase (CYP450), hypothetical protein*$_{346}$, *hypothetical protein*$_{188}$ *hypothetical protein*$_{286}$ และ*hypotheticcl protein*$_{171}$ (*HPT*$_{171}$) โดยทำการตรวจสอบระดับการแสดงออกของยีน *Lpin, ATPsubc* และ *CYP450* ด้วยวิธี quantitative real-time RT–PCR และ *HPT*$_{171}$ ด้วยวิธี semiquantitative RT – PCR โดยมี *hypothetical protein*$_{111}$ (*HPT*$_{111}$) เป็นยีนควบคุม

จากการตรวจสอบรูปแบบของโปรตีนในใบของข้าวสายพันธุ์ Pokkali และ IR29 ด้วยวิธี one-dimensional gel electrophoresis และ LC-MS พบโปรตีนจำนวน 206 โปรตีนที่มีการแสดงออกที่เปลี่ยนแปลงและมีค่ับกรดอะมิโนเหมือนกับโปรตีนที่อยู่ในฐานข้อมูลจาก NCBI โดยจัดเป็นโปรตีนที่ไม่ทราบหน้าที่จำนวน 93 โปรตีน และเป็นโปรตีนที่ทราบหน้าที่จำนวน 113 โปรตีน ซึ่งพบโปรตีนดังกล่าวจำนวน 46 โปรตีน 24 โปรตีน และ 46 โปรตีนในข้าวสายพันธุ์ Pokkali สายพันธุ์ IR29 และทั้งสองสายพันธุ์ตามลำดับ เมื่อจัดกลุ่มโปรตีนที่พบตามลักษณะการทำงาน สามารถแบ่งได้เป็น 13 ประเภท เช่น กระบวนการเผาผลาญกายในเซลล์ ออกซิเดชันรีดักชัน กระบวนการสร้างสายอาร์เอ็นเอ โปรตีนโครงสร้าง กระบวนการสังเคราะห์แสง การป้องกันเซลล์ และโปรตีนขนส่ง เป็นต้น โดยได้เลือก cysteine protease (CTP) ซึ่งเป็นโปรตีนที่เกี่ยวข้องกับกระบวนการเผาผลาญกายในเซลล์ และมีการแสดงออกที่ลดลงอย่างชัดเจนในข้าว เมื่อได้รับความเค็ม มาตรวจสอบการแสดงออกด้วยวิธี semi-quantitative RT PCR

Semi-quantitative RT-PCR บ่งชี้ว่า *HTP*$_{171}$ มีการแสดงออกในใบของข้าวสายพันธุ์ IR29 ที่ไม่แตกต่างกันส่วนในสายพันธุ์ Pokkai พบว่ามีการแสดงออกที่สูงขึ้นอย่างมีนัยสำคัญทางสถิติ (P<0.05) ที่เวลา 3 ถึง 12 ชั่วโมงในภาวะที่ถูกความเค็ม สำหรับโปรตีน *CTP*$_{28}$ พบว่าในข้าวสายพันธุ์ IR29 มีการแสดงออกที่ลดลงที่เวลา 3 และ 24 ชั่วโมงหลังจากได้รับความเค็ม ส่วนในข้าวสายพันธุ์ Pokkali มีการแสดงออกที่เวลา 6 และ 12 ชั่วโมงหลังจากได้รับความเค็มสูงกว่าข้าวที่ไม่ได้รับความเค็มอย่างมีนัยสำคัญทางสถิติ (P<0.05)

Quantitative real-time PCR แสดงว่า การแสดงออกของยีน *Lpin* ในใบของข้าวสายพันธุ์ IR29 ที่เวลา 6 ชั่วโมงต่ำกว่าที่เวลา 24 ชั่วโมงหลังได้รับความเค็มอย่างมีนัยสำคัญทางสถิติ (P < 0.05) ส่วนในใบของข้าวสายพันธุ์ Pokkali ที่ไม่ได้รับความเค็มและหลังจากได้รับความเค็มเป็นเวลา 3 ชั่วโมง มีการแสดงออกของยีนดังกล่าว ต่ำกว่าข้าวที่ได้รับความเค็มเป็นเวลา 24 ชั่วโมงอย่างมีนัยสำคัญทางสถิติ (P<0.05) ส่วนการแสดงออกของยีน *ATPsubc* ในใบของข้าวสายพันธุ์ IR29 ที่เวลา 3 ชั่วโมงสูงกว่าในข้าวที่ไม่ได้รับความเค็มอย่างมีนัยสำคัญทางสถิติ (P < 0.05) โดยการแสดงออกของยีนดังกล่าวในใบของสายพันธุ์ Pokkali ไม่แตกต่างกัน ทั้งนี้ยีน *CYP450* ในใบของข้าว IR29 มีระดับการแสดงออกที่ 6 ชั่วโมง ต่ำกว่าที่ 24 ชั่วโมงหลังจากได้รับความเค็มอย่างมีนัยสำคัญทางสถิติ (P<0.05) ส่วนในสายพันธุ์ Pokkali มีการแสดงออกที่ 12 ชั่วโมงหลังจากได้รับความเค็มสูงกว่าในข้าวที่ไม่ได้รับความเค็มอย่างมีนัยสำคัญทางสถิติ (P<0.05)

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต รัตนาวดี วิชาจารณ์

ปีการศึกษา.....2552..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม

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KEYWORDS : SALT TOLERANCE / RICE / cDNA-AFLP / RT-PCR / PROTEOMICS

RATTANAWADEE WICHAJARN: EXPRESSION OF GENES AND PROTEINS INVOLVING SALT-TOLERANCE MECHANISMS IN RICE *Oryza sativa* L. ANALYZED BY cDNA-AFLP AND PROTEOMICS. THESIS ADVISOR: ASSOC. PROF. WARAWUT CHULALAKSANANUKUL, Ph.D, THESIS CO-ADVISOR: SITTIRUK ROYTRAKUL, Ph.D, 157 pp.

Plants respond to environmental stress with a number of physiological and biochemical changes. High salinity is one of the most important stresses severely limiting growth and development of rice (*Oryza sativa* L.), and thus reducing its crop productivity. The metabolic pathways for salt stress responses are likely to be very complicated. In this study, molecular markers associated with salt stress of *O. sativa* were identified. Differential expression profiles of transcripts in salt-tolerant (Homjan) and salt-susceptible (Pathumthani 1) varieties under a time course of salt stress treatment were examined by RNA arbitrary-primed (RAP)-PCR. Subsequently, salt-tolerant (Pokkali) and salt-susceptible (IR29) varietjes were used for complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) analysis. Several specific/differential expression transcripts were observed. One transcripts from RAP-PCR and seven transcripts from cDNA-AFLP were cloned and sequenced. A RAP-PCR fragment significantly match *protein kinase domain containing protein* while three cDNA-AFLP fragments significantly similar to *lipin-N terminal conserve region (Lpin), ATP synthase subunit C (ATPsubc)* and *cytochrome P450 monooxygenase (CYP450), hypothetical protein*$_{240}$, *hypothetical protein*$_{186}$, *hypothetical protein*$_{206}$ *and hypothetical protein*$_{171}$, respectively. The expression levels of three known transcripts and that of *hypothetical protein*$_{171}$ *(HTP*$_{171}$*)* (from cDNA-AFLP) upon salt stress treatment in both Pokkali and IR29 varieties were examined by quantitative real-time PCR and semi-quantitative RT-PCR, respectively. *Hypothetical protein*$_{217}$ *(HTP*$_{217}$*)* were used for internal standard.

Proteomic studies based on one-dimensional gel electrophoresis and LC-MS/MS of proteins in leaves of Pokkali and IR29 were carried out. A total of 206 differential expressed proteins significantly matched previously deposited sequences in the NCBI database. Of these, 113 proteins matched those having known functions and 93 proteins significantly similar to hypothetical proteins. In total, 43, 24 and 46 proteins were found in Pokkali, IR29 or both varieties, respectively. These proteins could be categorized to 13 functional categories including those, for example, in cellular metabolism, oxidation reduction, RNA processing, structural protein, photosynthesis, cell rescue and defence, transport and binding proteins etc. Cysteine protease (CTP) was down–regulated during salt stress. This expression of a transcript encoding this protein was further examined by semi-quantitative RT PCR.

Semi-quantitative RT-PCR revealed that the expression level of *hypothetical protein*$_{171}$ was not significantly different in leaves of IR29 ($P > 0.05$). In contrast, that of this gene in Pokkali was significantly up-regulated at 3 to 12 hpt ($P < 0.05$). *CTP*$_{201}$ was significantly down-regulated at 3 to 24 hpt in IR29 but that in Pokkali was significantly higher than control at 6 and 12 hpt ($P < 0.05$).

Quantitative real-time PCR indicated that the expression of *lipin N-terminal conserved region family protein* in IR29 at 6 hpt was significantly lower than that at 24 hpt ($P < 0.05$). The expression levels of this gene in Pokkali at 0 and 3 hpt were significantly lower than that at 24 hpt ($P < 0.05$). *ATP synthase subunit C family protein* in leaves of IR29 at 3 hpt was significantly greater than that of the control ($P < 0.05$) but its expression in Pokkali was comparable during the stress treatment ($P > 0.05$). *Cytochrome p450 monooxygenase* was less abundantly expressed at 6 hpt than that at 24 hpt in IR29 ($P < 0.05$). In Pokkali, its expression level at 12 hpt was significantly higher than that of the control ($P < 0.05$).

Field of Study :.....Biotechnology..... Student's Signature Rattanawadee Wichajarn.

Academic Year :.....2009..... Advisor's Signature Warawut Chulalaksananukul

Co-Advisor's Signature Sittiruk Roytrakul

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CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Page

Page

Page

Page

Page

Page

Page

Page

LIST OF ABBREVIATIONS

bp	base pair
°C	degree celcius
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
Kb	kilobase
kDa	kilodaltan
M	molar
$MgCl_2$	magnesium chloride
mg	milligram
ml	millilitre
mM	millimolar
ng	nanogram
OD	optical density

PCR	polymerase chain reaction
pI	isoelectric point
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolution per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
Tris	tris (hydroxyl methyl) aminomethane
µg	microgram
µl	microlitre
µM	micromolar
UV	ultraviolet

CHAPTER I

INTRODUCTION

1.1 Background information

Rice (*Oryza sativa* L.) is the world most important food crop and a primary source of carbohydrate for more than half of the world population. More than 90% of the world rice is grown and consumed in Asia. Thailand is the one of most country which leader rice export as commercial crop (Table 1.1). However, most rice cultivars have been reported as salt-sensitive character. Rice can grow in wide range of environments such as irrigated, rained, upland and flood prone. Nevertheless, the plant growth is obstructed under abiotic stresses; such as, salinity of soil, drought, excess of water, mineral deficiencies, soil toxicity, and unfavorable temperatures. It has been reported that around 5% of the cultivated land is affected by salinity (Akashi *et al.,* 2006). Moreover, it has been predicted that increasing salinization in agricultural fields will reduce the land available for cultivation by 30% within the next 25 years, and up to 50% by the year 2050 (Wang *et al.*, 2003).

Table 1.1 Rice : Harvested area, production and yield of major countries, 2005-2007

Country	Harvested area (1000 rai)			Production (1000 tons)			Yield per rai (Kgs)		
	2005	2006	2007	2005	2006	2007	2005	2006	2007
World Total	**966,883**	**977,464**	**981,230**	**631,869**	**644,490**	**654,413**	**654**	**659**	**667**
China	181,978	184,154	182,688	182,059	184,128	185,490	1,000	1,000	1,015
India	272,874	272,606	275,000	137,690	139,137	141,134	505	510	513
Indonesia	73,994	73,665	76,035	54,151	54,455	57,049	732	739	750
Bangladesh	65,775	70,000	70,000	39,796	43,504	43,504	605	621	621
Viet Nam	45,808	45,778	45,656	35,791	35,827	35,567	781	783	779
Myanmar	43,800	50,875	51,250	25,364	30,600	32,610	579	601	636
Thailand	**63,906**	**63,532**	**66,681**	**30,292**	**29,642**	**32,099**	**474**	**467**	**481**
Philippines	25,440	26,000	26,563	14,603	15,327	16,000	574	590	602
Brazil	24,474	18,568	18,133	13,193	11,527	11,080	539	621	611
Japan	10,663	10,550	10,488	11,342	10,695	10,970	1,064	1,014	1,046
Others	15,8171	161,1745	158,736	87,588	89,648	88,910	554	554	560

Source : Food and Agriculture Organization of the United Nation

Soil salinity is a soil containing some water soluble salt. Excess salt in soil interferes with several physiological and biochemical processes, resulting in problems such as osmotic stress, ion imbalance, ion toxicity, and oxidative stress; these conditions ultimately interact with several cellular components, including DNA, protein, lipids and pigment in plants (Zhu., 2002). The protection of crops against salinity-induced damage is, therefore, a global challenge for the near future. Plants can employ numerous physiological and biochemical strategies to cope with adverse conditions by altering the functioning of a number of genes. Therefore, the identification of genes involved in biotic or abiotic stress responses is a fundamental step in understanding the molecular mechanisms of stress responses and developing transgenic plants with enhanced tolerance to stress.

Gene expression profiling has become an important tool to investigate how an organism responds to environmental changes. Sometimes, these transcriptional changes are successful adaptations leading to tolerance while in other instances the plant ultimately fails to adapt to the new environment and is labeled as sensitive to that condition. Expression profiling can define both tolerant and sensitive responses.

Rice has 12 chromosomes with a much smaller genome, estimated at 420 Mbp. The assembled sequence covers 93% of whole genome. Gene predictions on the assembled sequence suggested that the genome contains 32,000 to 50,000 genes. Of these, 98% are homologous to the known maize, wheat, and barley proteins are found (Goff *et al.,* 2002).

Large-scale genomic and cDNA sequencing projects have identified numerous genes for which no biological function is known. Genome-wide expression analysis is a valuable tool for determining the functions of genes and their spatial and temporal expression patterns, as well as elucidating the genetic networks in which they participate.

The widely used cDNA-AFLP method and related techniques have been employed for gene discovery (Durrant *et al.,* 2000; Qin *et al.,* 2001) but not, so far, for global, quantitative gene expression analysis. The original cDNA-AFLP method (Bachem *et al.,* 1998) can be improved and adapted to permit its use as a robust and

reproducible tool for quantitative transcript profiling. Therefore, the method can be used to perform genome-wide expression studies.

Proteomic technique is a powerful and widely used method for analysis of protein mapping and expression of interesting expressed proteins in various cells and tissues of organisms. Proteomic techniques provide the basic information on protein expression profiles and post-translational modification of interesting proteins

The first major difference between genome and proteome analysis is that the genome is static, while the proteome of each living cell is dynamic, altering in response to the individual cell's metabolic state and reception of intracellular and extracellular signal molecules. Thus while the genome enables a prediction of the proteome simply as the gene products, this cannot be described as the proteome, since it is not known which genes are expressed at any specific moment in time, and many of the proteins which are expressed will be post-translationally altered, by one or more of approximately 200 modifications (Mann and Jensen *et al.*, 2003).

1.2 General introduction

1.2.1 Taxonomy of rice

Rice is a member of the family *Poaceae*, tribe *Oryzeae*. The genus *Oryza* contains approximately 22 species. Of which, 20 are wild species and two, *O. sativa* and *O. glaberrima*, are cultivated (Vaughan., 1994). The two cultivated rice varieties, *O.sativa* L. and Steud. are diploid (2n = 24), whereas wild species are often tetraploid. *O.sativa* L. is more economically important than *O. glaberrima* Steud, which is locally grow in West Africa (Grist., 1975).

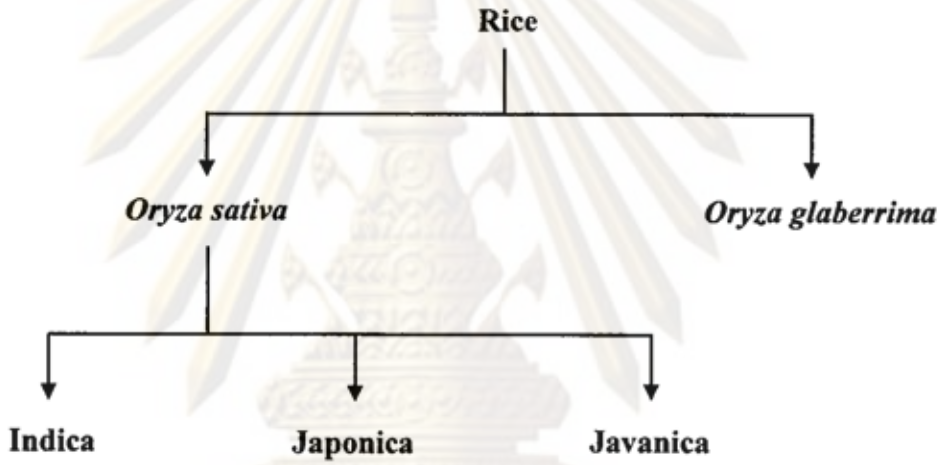

Figure 1.1 Varieties of wold cultivated rice

The *Oryza sativa* L. is divided by distinct ecotypes into 3 varieties: indica, japonica, and javanica. Among ecotype varieties, the indica rice is cultivated in wide regions and now rapid substituted other varieties because of the good growing and cooking properties. The indica rice has a high tillering capability. A clump is tall, so it exhibits considerably drought tolerance and resistance to insect pests and diseases. The grain is medium to long, with high amylose content, resulting in dry and fluffy cooked rice that shows little disintegration, moreover, they are composed of aromatic flavor and highly valued. Most of the indica subspecies are grown in Indian, Southern China, South America and Thailand. Javanica subspecies are primarily grown in Indonesia, Malaysia and Philippines, located in the rice terraces of the Philippines and

the mountainous regions of Madagascar. However, these subspecies are gradually disappeared due to the rapid spread of the modern indica subspecies which are high yield cooking quality and high adaptability to field condition. (Christou., 1994).

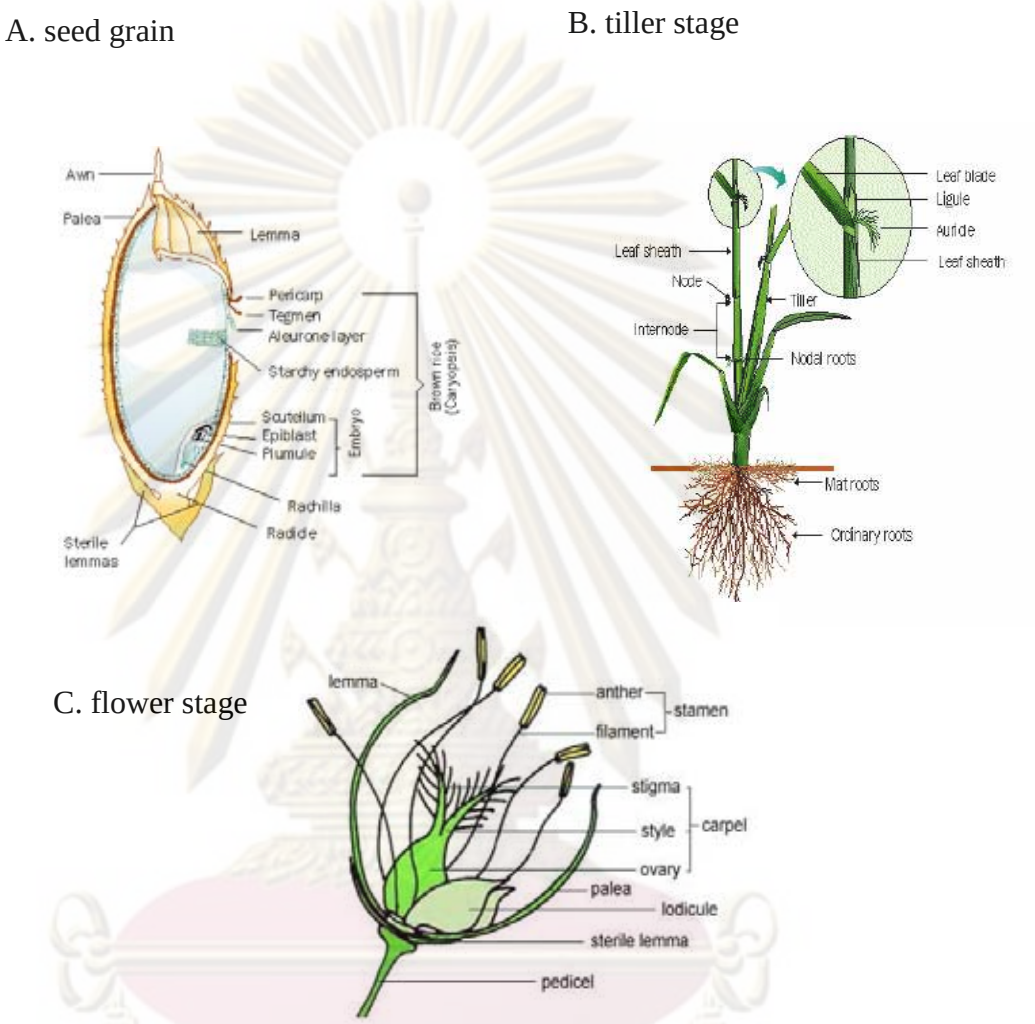

Figure 1.2. Morphology of the rice grain (a) vegetative parts (b) and reproductive parts of rice (c) (Maclean *et al.*, 2002)

1.1.2 Saline environment

A saline soil is defined as having a high concentration of soluble salts, high enough to affect plant growth. Salt concentration in a soil is measured in terms of its electrical conductivity, as described in the section below on measurements. The USDA Salinity Laboratory defines a saline soil as having an EC_e of 4 dS/m or more. EC_e is the electrical conductivity of the 'saturated paste extract', that is, of the solution extracted from a soil sample after being mixed with sufficient water to

produce a saturated paste. However, many crops are affected by soil with an EC_e less than 4dS/m. The moisture content of a drained soil at field capacity may be much lower than the water content of its saturated paste. Further, under dryland agriculture, the soil water content might drop to half of field capacity during the life of the crop. The actual salinity of a rain-fed field whose soil had an EC_e of 4 dS/m could be 8-12 dS/m, this would severely limit yield of most crops.

1.2.3 Diversity of salt-tolerant species and the criteria for salt stress tolerance

In general, plants have different degrees of salt tolerant traits which are divided into 4 types. Firstly, glycophytes are the species that are sensitive to elevated root zone salinity, and do not accumulate high concentration of ions in their growing tissues. Growth and development rates of plants decrease when expose to salinity level higher than 10 mM. Secondly, halophytes or salt tolerant species means the species that tolerate relatively high soil salinity up to 50 mM and are capable of accumulating relatively high quantities of Na^+ and Cl^- in their tissues before the reduction in growth occurs. Thirdly, obligate halophytes are the species that tolerate moderate salinity level of 50 mM. And lastly, facultative halophytes are the species that show glycophyte traits, but the halophytic metabolism occurs when exposured to moderate salinity. For example, ice plant (*Mesembryanthemum crystallinum*) automatically switched from C3 to crassulacean acid metabolism (CAM) photosynthesis when exposed to salt stress (Orcutt and Nilsen., 2000; Sairam and Tyagi., 2004).

Mostly, halophytes can tolerate to salinity in seawater level or 500 mM NaCl while, ghycophytes commonly are sensitive to salinity greater than 50 mM NaCl. Rice was reported as a glycophyte that yield reduction of 50% when exposured at 40 Mm NaCl (Orcutt and Nilsen., 2000). However, rice shows wide range of salt tolerance ability. Among rice cultivars, the level of salt tolerance was divided into salt resistant rice (e.g. Pokkali), moderately salt tolerant rice (e.g. IR4595-4-1-13 and IR98884-54-3-1E-P1), and salt sensitive rice (e.g. IR29) (Gregorio *et al,* 2002).

“Pokkali” is a well known salt tolerant donor in classical breeding and is commonly grown in coastal area of Kerala, India. It is a traditional, tall, photoperiod

sensitive rice cultivar that is susceptible to lodging and has low tillering capacity with long, broad, dark, and droopy leaves. Moreover, the leaves senescence occurs quickly after flowering. The grain has pericarp and poor cooking quality (Kawasaki *et al.*, 2001; Gregorio *et al.*, 2002) showed that Pokkali continued growing at a low photosynthetic rate after 7 days of salt stress, plant biomass approximately doubled. It was known that Pokkali maintained water content in the shoot during a 6-week stress under the conditions of 150 mM NaCl. In contrast, salt sensitive IR29 showed a slightly slower response to the shock treatment, and the plants wilted irreversibly after 24 h. These results indicated that Pokkali achieved tolerance by rapidly expressing mechanisms for efficiently withstanding salt stress more than the salt sensitive IR29.

Mostly, rice cultivated varieties are very sensitive to salt stress. Once, salt tolerant plants differ from salt sensitive by the low rate of Na+ and Cl- absorption through root, these ions move to the leaves, and are detoxified by compartmenting in vacuoles. Thus, salt tolerant ability is the capability to avoid the ion toxicity and prevent the increase of salt related-ion in cytoplasm or cell wall.

1.2.4 Salinity stress and plant response

Salt stress imposes both osmotic and ionic stresses on plants. Both glycophytic and halophytic plants cannot tolerate large amount of salts in the cytoplasm. They either restrict the excess salts in the vacuole or compartmentalize the ions in different tissues to facilitate their metabolic functions (Iyengar and Reddy., 1996; Zhu., 2002). Leave organs are more vulnerable than roots to Na^+, because Na^+ (and Cl^-) mostly accumulates to higher concentration in shoots than in roots. Na^+ is transported to shoots by the rapidly moving along with transpiration stream of the xylem (Tester and Davenport., 2003).

Osmotic damages such as osmotically driven removal of water from cells could occur resulting of the build up of high Na^+ concentrations in the leaf apoplast, since Na^+ entered into the xylem stream. The cellular toxicity of Na^+ caused of osmotic problem. Plants needed to maintain internal water potential below for keeping optimum turgor and water uptake. So it required an increase in osmotic, either by uptake of soil solutes or by synthesis of compatible solutes. Various compatible osmolytes such as proline, glycinebetaine, and polyols greatly reduced stress damage

to plant cells. An increased production of osmolytes was a general phenomenon in all plants in response to salt stress in order to reduce the osmotic potential of the cytosol for facilitating water uptake. Many compatible osmolytes had additional functions such as protecting proteins from misfolding and alleviating the toxic effect of reactive oxygen species generated by salt stress (Smirnoff and Cumber., 1989).

Under salt stress, water and osmotic potential of plants became more negative value, whereas turgor pressure increased with increasing salinity (Hernandez *et al.*, 1995; Meloni *et al.*, 2001; Romeroaranda *et al.*, 2001). Leaf water potential and osmotic potential decline depended on the osmotic potential of the root zone and the mode of stress imposition. A greater decline of osmotic potential compared with the total water potential leaded to turgor maintenance in plants under progressive or prolonged NaCl stress (Rajasekaran *et al.*, 2001). Ionic damage from excessive sodium ions, once entered into the cytoplasm, had a strong inhibitory effect on the activity of many enzymes. Because of the similar physico-chemical properties of sodium and potassium ions, sodium at a high concentration had a strong inhibitory effect on root potassium uptake. For example, K^+ uptake *via* Arabidopsis KUP1 (Kim *et al*, 1998; Fu and Luan, 1998), which mediated both high and low affinity K^+ transport, was inhibited by 5 mM or higher concentrations of NaCl (Fu and Luan., 1998). It was a general phenomenon that salt treatment caused a decrease in cellular K^+ content, which might be partly responsible for reducing growth and vigour under salt stress. Salinity enhanced Na^+, Ca^{2+}, and Cl^- contents and the ratio of K^+/Na^+ decreased in a number of plants. Moreover, an increase of Na^+ and Cl^- content induced proline accumulation and decreased both the activity of proline dehydrogenase (PDH; a catabolic enzyme of proline) and the total Ca^{2+} and water-soluble Ca^{2+} content. These results suggested that in the marine algae *Ulva fasciata*, a loss of cellular Ca^{2+} associated with NaCl induced-proline accumulation *via* an inhibition of PDH activity (Lee and Liu., 1999).

Plants employed several ways to combat ionic stress that imposed by high salinity. These included restricting salt uptake, increase extrusion and compartmentalization, and controlling long distance transport to aerial parts. Moreover, salt stress affected all the plant major processes such as morphology, physiology and biochemistry. Firstly, morphological symptoms were indications of

the injurious effects of salt stress. Salinity might directly or indirectly inhibit cell division and enlargement of growing point. Chloride induced elongation of the palisade cells, which leaded leaves becoming succulent. Salt stress also resulted in a considerable decrease on the fresh and dry weights of leaves, stems, and roots (Chartzoulakis and Klapaki., 2000).

In case of rice, salinity also reduced seedling growth, grain yield, spikelets number per panicle, panicle length, panicle weight, number of primary branches, seed set, and yield components such as spikelet number and tiller number (Lutts *et al.*, 1995; Abdullah *et al.*, 2001). Secondly, plants under salt conditions showed alterations of physiological processes. This involved diversion in photosynthesis, respiration, and hormonal regulation, accumulation of solutes as well as various defense and adaptive mechanisms were regulated at the molecular level including gene expression (Greenway and Munns., 1980; Weimberg and Shannon., 1988). However, the dominant physiological process was photosynthesis. Plant growth as biomass production was a measure of net photosynthesis and, therefore, environmental stresses affecting growth also influenced photosynthesis process. Salt stress caused either short or long-term effect. The short-term outcome occurred after a few hours or within 1 or 2 d of the onset of exposure and this response was important as a complete cessation of carbon assimilation. The long-term effect happened after several days of exposure to salt causing the reduction of carbon assimilation due to the salt accumulation in developing leaves. The reduction of photosynthetic activity depended on two aspects of salinization; i.e. the total concentration of salt and their ionic composition.

High salt concentration in soil and water created high osmotic potential, which reduced the availability of water to plants. Decrease of water potential caused osmotic stress, which reversibly inactivated photosynthetic electron transport *via* shrinkage of intercellular space due to efflux of water through water channels in the plasma membrane (Allakhverdiev *et al.*, 1999). Increase of osmotic potential under high salt conditions caused Na^+ ions leakage into the cytosol (Papageorgiou *et al.*, 1998) and inactivated both photosynthetic and respiratory electron transport (Allakhverdiev *et al.*, 1999). High salt (NaCl) uptake competed with the uptake of other nutrient ions, especially K^+ leading to potassium deficiency (Ball., 198). Under such conditions of

high salinity and K^+ deficiency, a reduction in quantum yield of oxygen evolution due to malfunctioning of photosystem II occurred (Ball., 1988). Finally, under saline conditions, alteration in biochemical processes happened by the change of the pattern of gene expression, and both qualitative and quantitative protein synthesis adjustment.

1.3 Molecular genetic approaches used in this thesis

1.3.1 Polymerase Chain Reaction (PCR)

The introduction of the polymerase chain reaction (PCR) has opened a new approach for molecular genetic studies. This is a technique for enzymatically replicating DNA without using a living organism, such as *E. coli* or yeast. PCR is a method for an *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA by two oligonucleotide primers, usually 18-27 nucleotides in length. The target DNA sequence can be synthesized from a low amount of starting DNA template within a few hours.

The PCR reaction components constitute DNA template, a pair of primers for the target sequence, dNTPs (dATP, dCTP, dGTP and dTTP), appropriate buffer and heat-stable DNA polymerase (usually *Taq* polymerase). The amplification reaction usually consists of three steps; denaturation of double stranded DNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal temperature, and extension of the annealed primers by the heat-stable DNA polymerase. The cycle is repeated for 30-40 times (Figure 1.3). The amplification product is determined by gel electrophoresis.

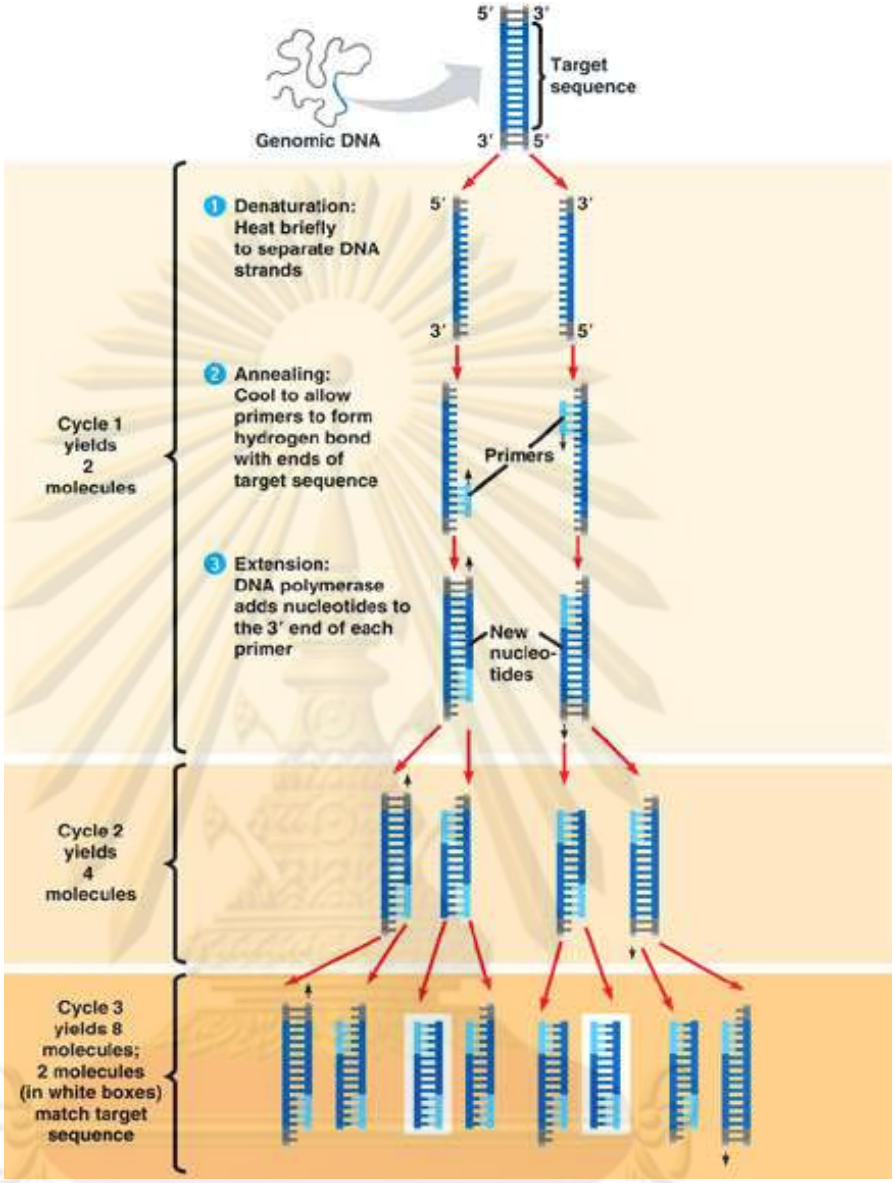

Figure 1.3 General illustration of the polymerase chain reaction (PCR) for amplifying DNA segments.

1.3.2 DNA sequencing

Polymorphism at the DNA level can be studied by several methods but the direct strategy is determination of nucleotide sequences of a defined region. DNA sequencing is the process of determining the exact order of the bases A, T, C and G in a piece of DNA. There are two general methods for sequencing of DNA segments: the "chemical cleavage" procedure (Maxam and Gilbert., 1977) and the "chain termination" procedure (Sanger *et al.,* 1977). Nevertheless, the latter method is more

popular because chemical cleavage procedure requires the use of several hazardous substances.

DNA sequencing provides high resolution and facilitating interpretation. DNA fragments generated from PCR can be directly sequenced or alternatively, those fragments can be cloned and sequenced. This eliminates the need to establish a genome library and searching of a particular gene in the library. However, sequencing of a large number of individuals using conventional method is extremely tedious and prohibitively possible. The enzymatic sequencing approach has presently been developed to the automated method (Figure 1.4).

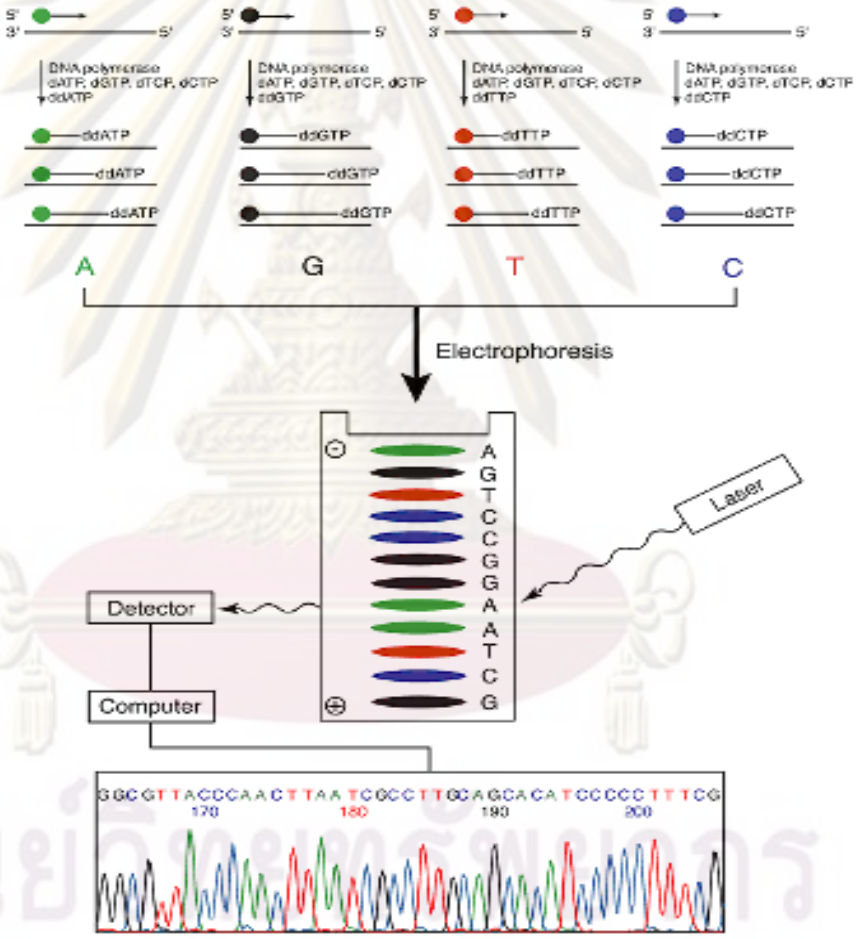

Figure 1.4 Automated DNA sequencing.

DNA sequences can be detected using a fluorescence-based system following labeling of a sequencing primer or incorporated nucleotides with a fluorescence dye. At present, automated DNA sequencing is commonly used. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

1.3.3 RNA arbitrarily primed-polymerase chain reaction (RAP-PCR)

RNA arbitrarily primed PCR (RAP-PCR; Welsh and McClelland., 1990) is a simple version of the Differential Display PCR DD-PCR. This technique is generally comparable to RAPD-PCR with the exception that the first-strand cDNA synthesized from total RNA (or mRNA) was used as the template (Figure 1.5). It is believed that the first strand cDNA synthesized from a random primer allow better possibility to detect the inner part of the transcripts.

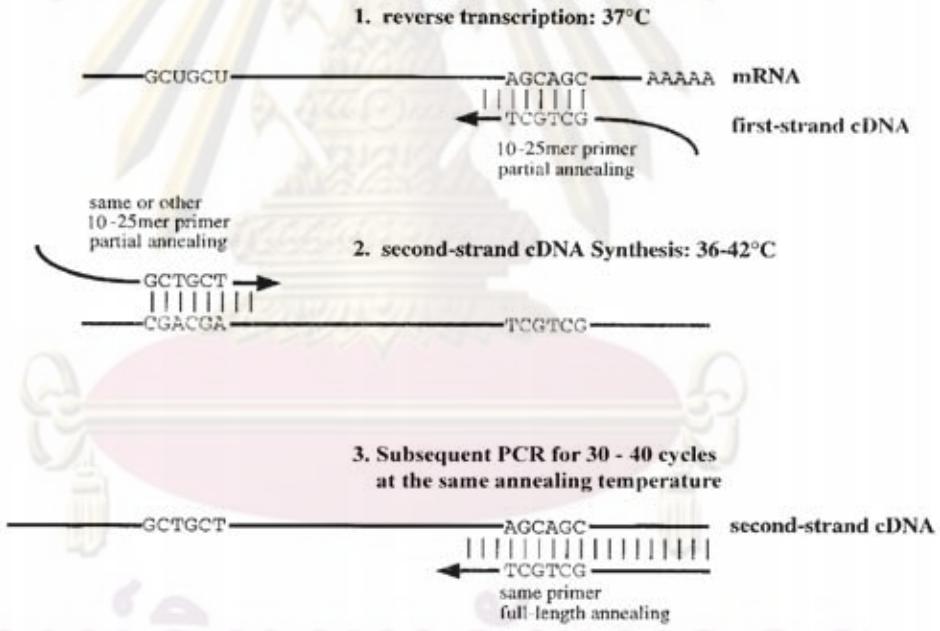

Figure 1.5 Overall concept of RAP-PCR. (1) During the first-strand cDNA synthesis, a single 10 – 25 base arbitrary primer anneals and extends from sites present within the mRNA. (2) Synthesis of the second strand cDNA primed by the same, or another, 10 – 25 base arbitrary primer proceeds during a single round of low-stringency DNA synthesis catalyzed by *Taq* polymerase. The result of the two enzymatic steps is the synthesis of a collection of molecules that are flanked at their 3′ and 5′ ends by sequences completely matching the sequences of the arbitrary primers. (3) These DNA fragments serve as templates for PCR amplification. (http://pubs.nrc-cnrc.gc.ca/ispmb/ispmb19/R00-060.pdf)

PCR is carried out using combination of two different arbitrary primers. Differentially expressed transcripts are detected after size-fractionated through denaturing polyacrylamide gels by silver staining. Fragments from DD-PCR and/or RAP-PCR should be characterized by cloning and sequencing. Primers can be designed and tested for the expression levels of interesting fragments.

1.3.4 Complementary DNA-Amplified fragment length polymorphism (cDNA-AFLP)

The cDNA-AFLP is an improvement of traditional differential display techniques. It is a PCR-based method which starts with cDNA synthesis from total or mRNA followed by the conventional AFLP approach. Accordingly, the cDNA-AFLP technique is essentially identical to AFLP with the exception that cDNA rather than genomic is used as the starting material. In this study, single strand cDNA was synthesized from total RNA of each sample using a SMART^{TM} PCR cDNA synthesis kit. The resulting single-stranded (ss) cDNA contains the 5' end of the mRNA, as well as sequences that are complementary to the SMART Oligonucleotide. The second strand cDNA was synthesized by PCR (Figure 1.6). The resulting cDNA was then manipulated using a typical AFLP approach described previously.

By increasing the stringency of the PCR amplification (adding more additional nucleotides to the primers), the sensitivity of the analysis can be increased. In this way, genes with a low expression level can also be detected. The fragments that are amplified are roughly 100-400 bp. These fragments are separated on high-resolution gels. The differences in the intensity of the bands that can be observed and provide a preliminary measure of the relative differences in the levels of gene expression. Further characterization of interesting transcripts is often required. The sensitivity and specificity of the method allows the detection of poorly expressed genes and the determination of subtle differences in transcriptional activity. Therefore, cDNA-AFLP can generate a global overview of gene expression under investigated conditions.

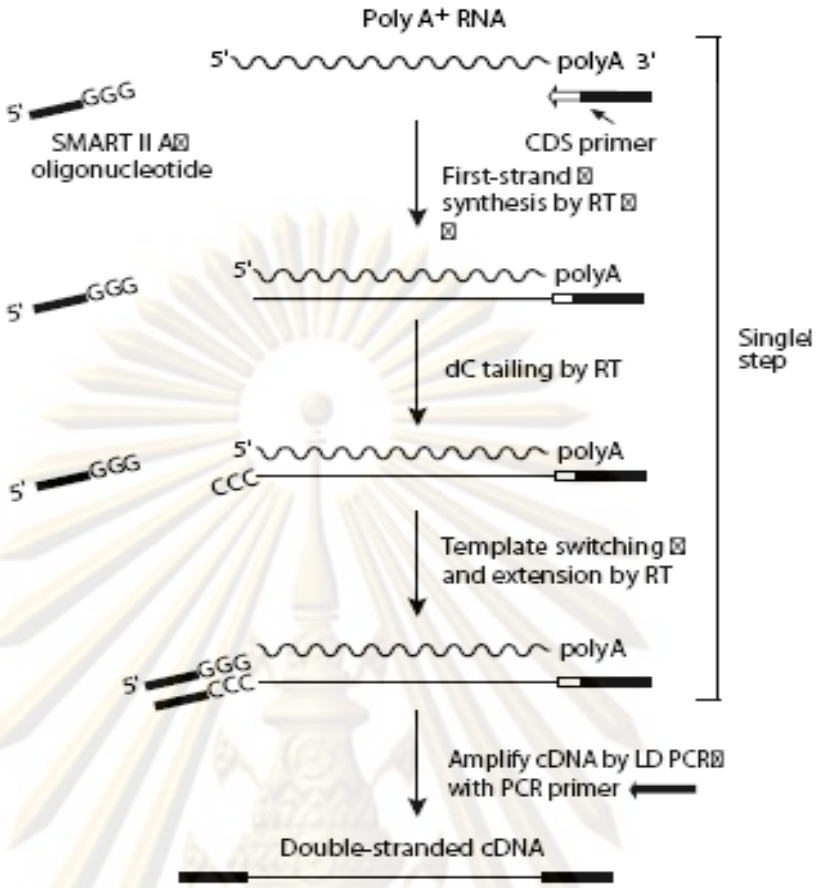

Figure 1.6 A flow chart illustrating cDNA synthesis using a SMART™ PCR cDNA Synthesis kit**.** The SMART II A Oligonucleotide, 3' SMART CDS Primer II A, and 5' PCR Primer II A all contain a stretch of identical sequence. (http://www.clontech.com/images/pt/PT3041-1.pdf)

1.3.6 Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR)

RACE-PCR is the common approach used for isolation of the full length of characterized cDNA. Using SMART (Switching Mechanism At 5′ end of RNA Transcript) technology, terminal transferase activity of Powerscript Reverse Transcriptase (RT) adds 3 - 5 nucleotides (predominantly dC) to the 3′ end of the first-strand cDNA. This activity is harnessed by the SMART oligonucleotides whose terminal stretch of dG can anneal to the dC-rich cDNA tail and serve as an extended template for reverse transcriptase. A complete cDNA copy of original mRNA is synthesized with the additional SMART sequence at the end (Fig. 1.7).

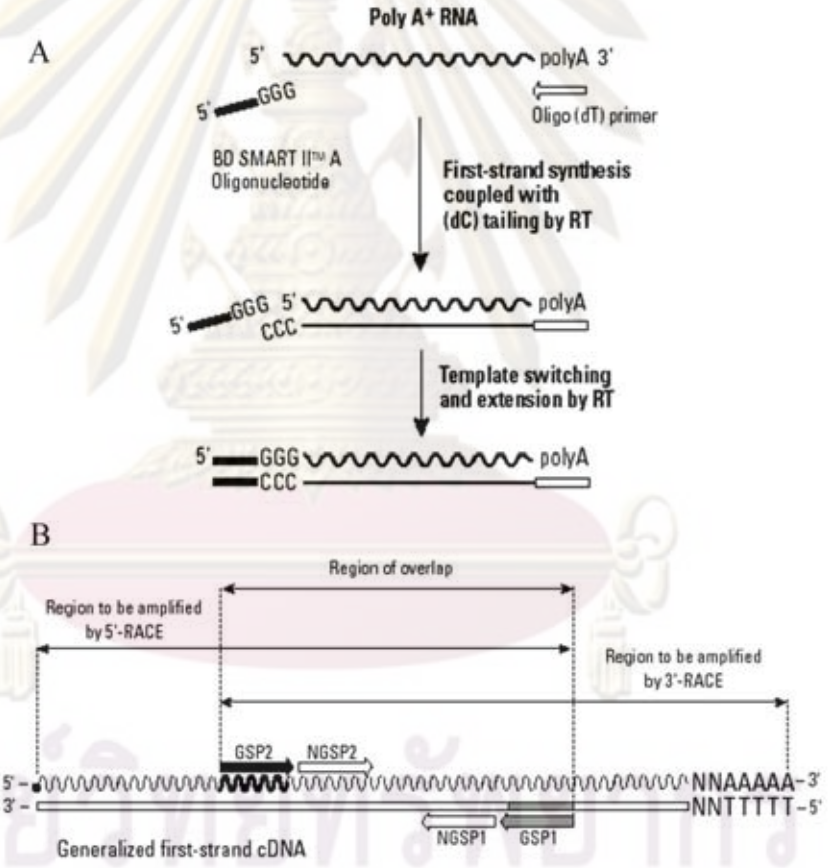

Figure 1.7 Overview of the SMART™ RACE cDNA Amplification Kit. A. Mechanism of SMART cDNA synthesis. First strand synthesis is primed using a modified oligo (dT) primer. After reverse transcriptase reaches the end of the mRNA template, it added several dC residues. The SMART II A Oligonucleotide annels to the tail of the cDNA and serves as an extended template for PowerScriptRT. B. Relationships of gene-specific primers to the cDNA template. This diagram shows a generalized first strand cDNA template.

The first strand cDNA of 5′ and 3′ RACE is synthesized using a modified oligo (dT) primers and serve as the template for RACE PCR reactions. Gene specific primers (GSPs) are designed from interested gene for 5′- RACE PCR (antisense primer) and 3′-RACE PCR (sense primer) and used with the universal primer (UPM) that recognize the SMART sequence. RACE products are characterized. Finally, the full length cDNA is constructed.

1.3.5 Reverse transcription-polymerase chain reaction (RT-PCR) and semi-quantitative RT-PCR

RT-PCR is a variant of polymerase chain reaction (PCR)**.** RT-PCR but the first strand cDNA instead of genomic DNA is used as the template in the amplification reaction (Figure 1.8). It is a direct method for examination of gene expression of known transcripts in the target species. The template for RT-PCR can be the first stranded cDNA synthesized from total RNA or poly A^+ RNA using oligo (dT) or random primers and reverse transcriptase. The product is then subjected to the second strand synthesis using gene-specific forward and reverse primers.

RT-PCR can also be used to identify homologues of interesting genes by using degenerate primers and/or conserved gene-specific primers from the original species and the first strand cDNA of the interesting species is used as the template. The amplified product is further characterized by cloning and sequencing**.**

Semi-quantitative RT-PCR is a quantitative approach where the target genes and the internal control (e.g. a housekeeping gene) were separately or simultaneously amplified using the same template. The internal control (such as *β-actin*; *elongation factor*, *EF-1α* or *Glyceraldehyde-3-Phosphate Dehydrogenase,G3PDH*) is used under the assumption that those coding genes are transcribed constantly and independently from the extracellular environment stimuli and that their transcripts are reverse transcribed with the same efficiency as the product of interesting transcript.

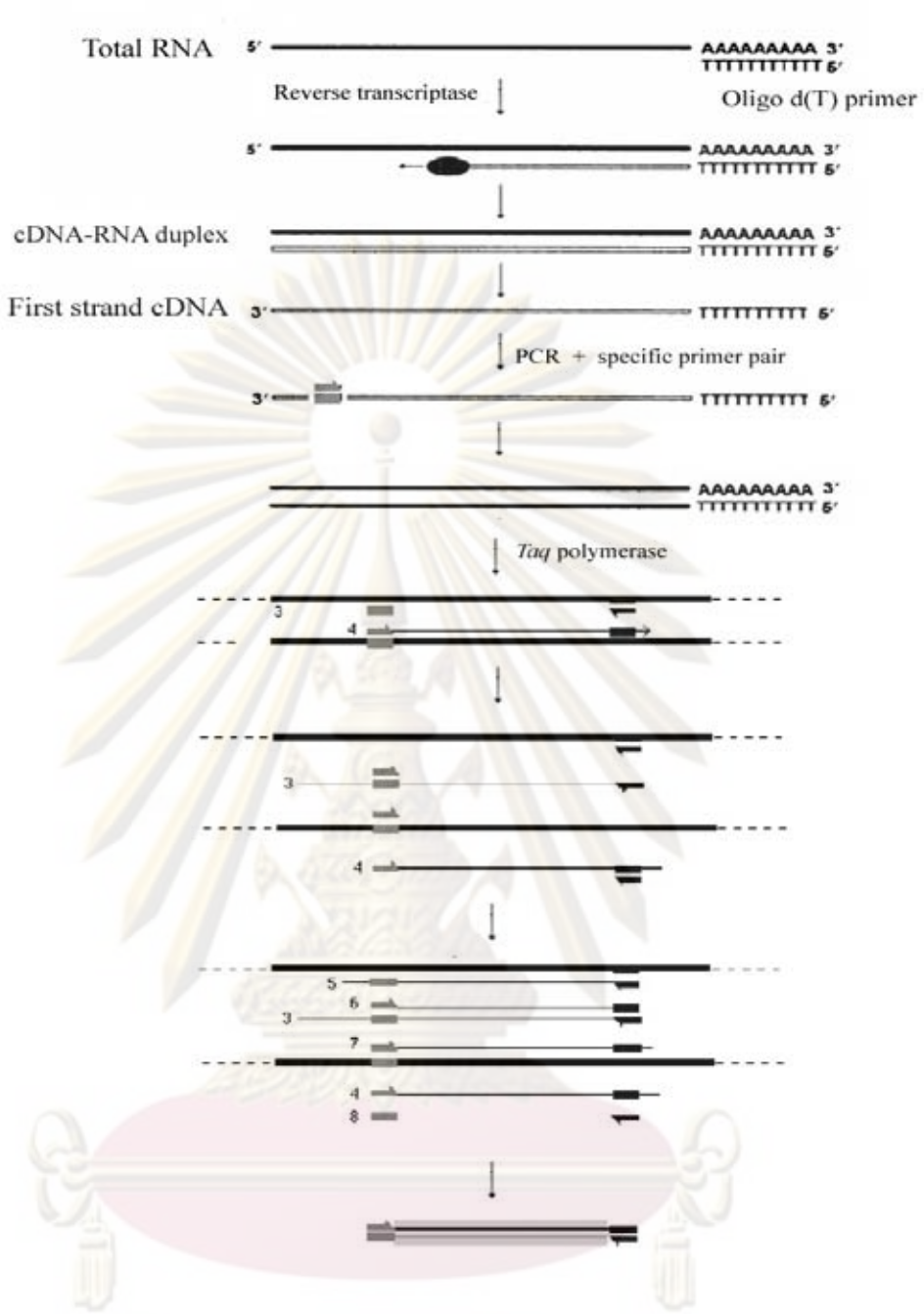

Figure 1.8 Overall concept of the RT-PCR procedure. During the first strand cDNA synthesis, an oligo d(T) primer anneals and extends from sites present within the total RNA. Second strand cDNA synthesis primed by the 18–25 base specific primer proceeds during a single round of DNA synthesis catalyzed by *Taq* polymerase. These DNA fragments serve as templates for PCR amplification.

1.3.7 Real-time PCR

Real-time polymerase chain reaction, also called *quantitative real time polymerase chain reaction* (Q-PCR/qPCR) or *kinetic polymerase chain reaction*, is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample. Two common methods of quantification are the use of fluorescent dyes that intercalate with double-stranded DNA such as SYBR green and modified DNA oligonucleotide probes that are fluorescent when hybridized with a complementary DNA.

The general principle of SYBR green polymerase chain reaction is composed of the first step, denaturation: at the beginning of amplification, the unbound dye molecules are weakly fluorescent, the second step, annealing: after annealing of the primer, a few dye molecules bind to the double strand. The last step, extension: during elongation, more dye molecules bind to the newly synthesized DNA. Fluorescence measurement at the end of the elongation step of every PCR cycle is performed to monitor the increasing the amount of quantified DNA (Fig. 1.9).

Real-time PCR in the laboratory can be applied to numerous applications. It is common use for both diagnostic and research applications. Diagnostic real-time PCR is applied to rapidly detect the presence of genes involved in infection diseases, cancer and genetic abnormalities. In the research setting, real-time PCR is mainly use to provide highly sensitive quantitative measurement of gene transcription. The technology is commonly used in determining expression levels of a particular gene changes over time.

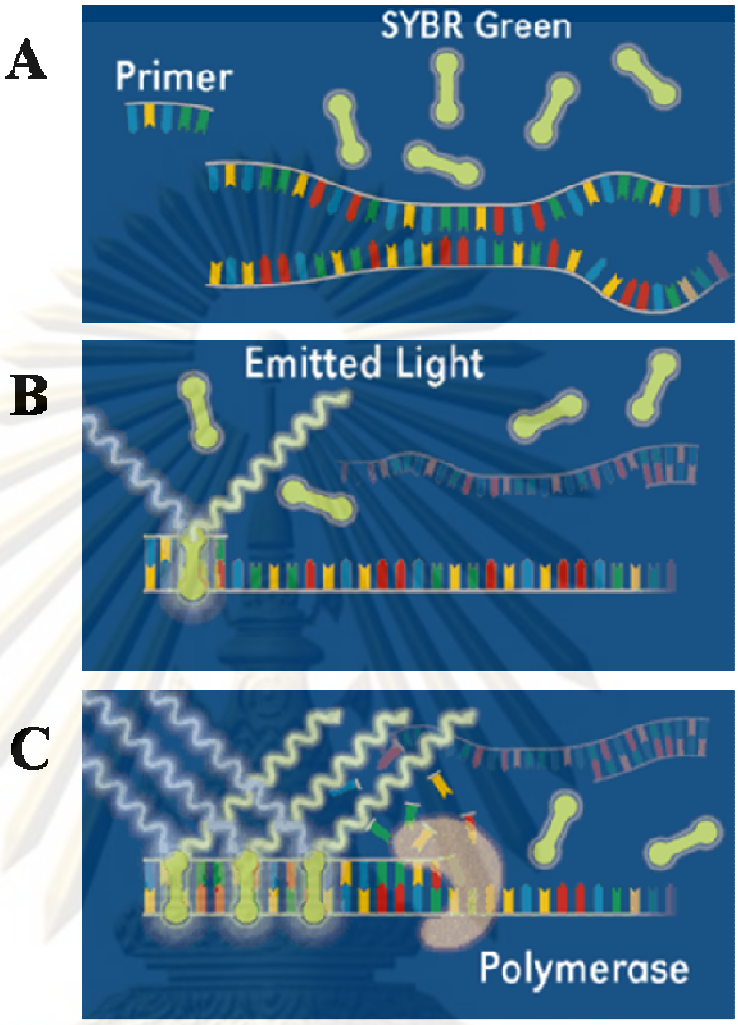

Figure 1.9 An overall concept of the Real-time PCR procedure **(www.thaiscience.com/lab_vol/p23/Real-time PCR.asp).**

1.4 Proteomic technique approaches used in this thesis

1.4.1 Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-D electrophoresis) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique separate proteins in two steps, according to two independent properties: the first-dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI); the second-

dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MW). In this way, complex mixtures consisted of thousands of different proteins can be resolved and the relative amount of each protein can be determined.

Using 2D-PAGE, hundreds to thousands of polypeptides can be analyzed in a single run. The proteins can be separated in pure form from the resultant spots. The spots can be quantified and further analyzed by mass spectrometry, depending on their resolution. Polypeptides can also be probed with antibodies and tested for post-translational modifications. 2D-PAGE is also used to study differential expression of proteins between cell types. The disadvantages of this technique include a large amount of sample handling, limited reproducibility, and a smaller dynamic range than some other separation methods. It is also not automated for high throughput analysis. Certain proteins are difficult for 2D-PAGE to separate, including those that are in low abundance, acidic, basic, hydrophobic, very large, or very small. Through advancements in this technology, scientists are, however, able to separate low abundance proteins with moderate efficiency.

1.4.2 SDS- PAGE

SDS-PAGE electrophoresis is used to determine the molecular weights of proteins. The basis of gel electrophoresis is that ions move when placed in an electric field. Sodium dodecyl sulfate (SDS) is a molecule with a negatively charged, polar sulfate head and a long hydrocarbon tail; it can act as a detergent, binding to hydrophobic regions of proteins. When mixed with proteins, SDS will denature the protein (cause it to unfold) and can uniformly coat proteins with a negative charge in a fixed ratio proportional to the molecular weight of the protein. The use of SDS is crucial so that with an approximately constant negative charge to mass ratio, the only effective variable is the molecular weight, or more correctly, the Stoke's radius, of the protein.

After denaturation by SDS, proteins are loaded onto a gel in the presence of an electric current. The gel is made by a free radical catalyzed polymerization between acrylamide and bisacrylamide. The resulting gel has cross- links and a 3D lattice

structure with pores between the acrylamide linkages through which protein molecules can pass.

Proteins are placed on a stacking gel to be concentrated, and then migrate into a resolving gel where they are separated. The driving force for the movement of these proteins is the electric field and the tendency of the negatively charged SDS molecules bound to proteins to migrate towards a positive end of the electric field. The proteins are stained and compared with a set of protein standards of known molecular weight. Their molecular weights are determined. (Ninfa and Ballou., 1998)

After electrophoresis, the separated proteins must be visualized in the gel. Most commonly, this is achieved with dyes that firmly bind protein. Individual dyes differ in sensitivity and the ability to stain all types of proteins equally. The most frequently use dye is Coomassie Blue R-250 (with a direction limit of about 1 μg of a protein). Alternatively, Coomassie Blue G-250, Amido black, and Nigrosine are also used. Silver staining is more sensitive than Coomassie Blue staining for about 10 - 20 fold. Silver staining leads to a non-stoichiometric binding of silver ions to proteins. After reduction, these complexes become visible as black to brownish bands. Unfortunately, silver stains are inconsistent as some proteins are hardly stained by silver ions. Therefore, quantity of stained proteins is not proportionally indicated from intensity of the protein spots. Fluorescent staining has been recently developed as an alternative choice for high sensitivity of staining. Dyes including SyproRuby™, deep Purple™ and 5-hexadecanoylamino-fluorescein are commercially available. However, the fluorescent staining is more expensive than conventional Coomassie Blue and silver staining and requires a specific gel documentation for visualization of electrophoresed proteins.

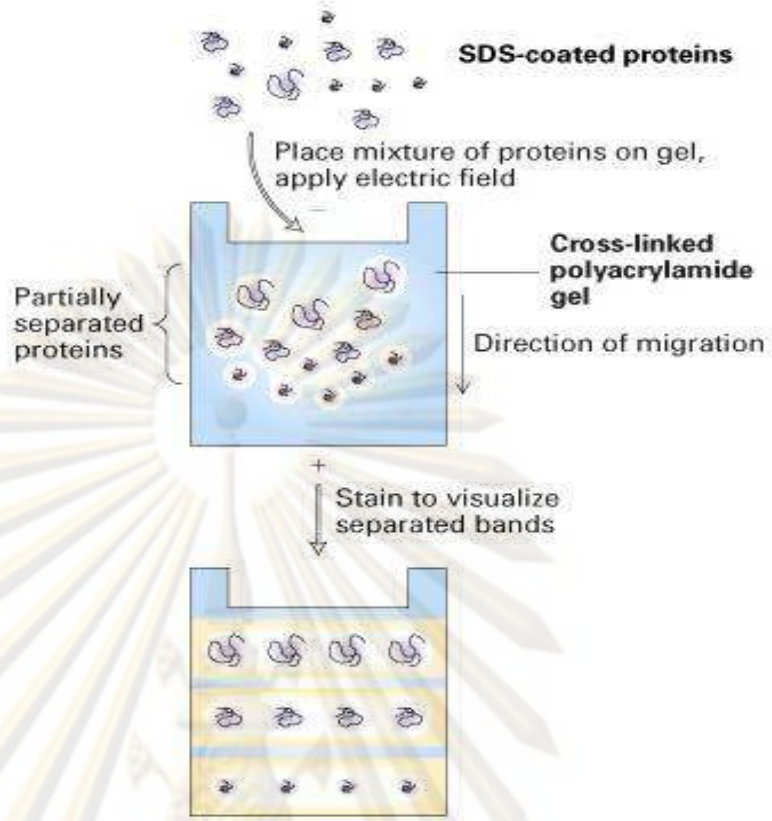

Figure 1.10 An overall concept of the SDS-PAGE procedure
(www.bio.miami.edu/~cmallery/255/...ques.htm**)**

1.4.3 Liquid chromatography-mass spectrometry (LC MS-MS)

LC-MS is also used in the study of proteomics where again components of a complex mixture must be detected and identified in some manner. The bottom-up proteomics LC-MS approach to proteomics generally involves protease digestion and denaturation (usually trypsin as a protease, urea to denature tertiary structure and iodoacetamide to cap cysteine residues) followed by LC-MS with peptide mass fingerprinting or LC-MS/MS (tandem MS) to derive sequence of individual peptides (Wysocki *et al.*, 2005). LC-MS/MS is most commonly used for proteomic analysis of complex samples where peptide masses may overlap even with a high-resolution mass spectrometer. Samples of complex biological fluids like human serum may be run in a modern LC-MS/MS system and result in over 1000 proteins being identified, provided that the sample was first separated on an SDS-PAGE gel or HPLC-SCX

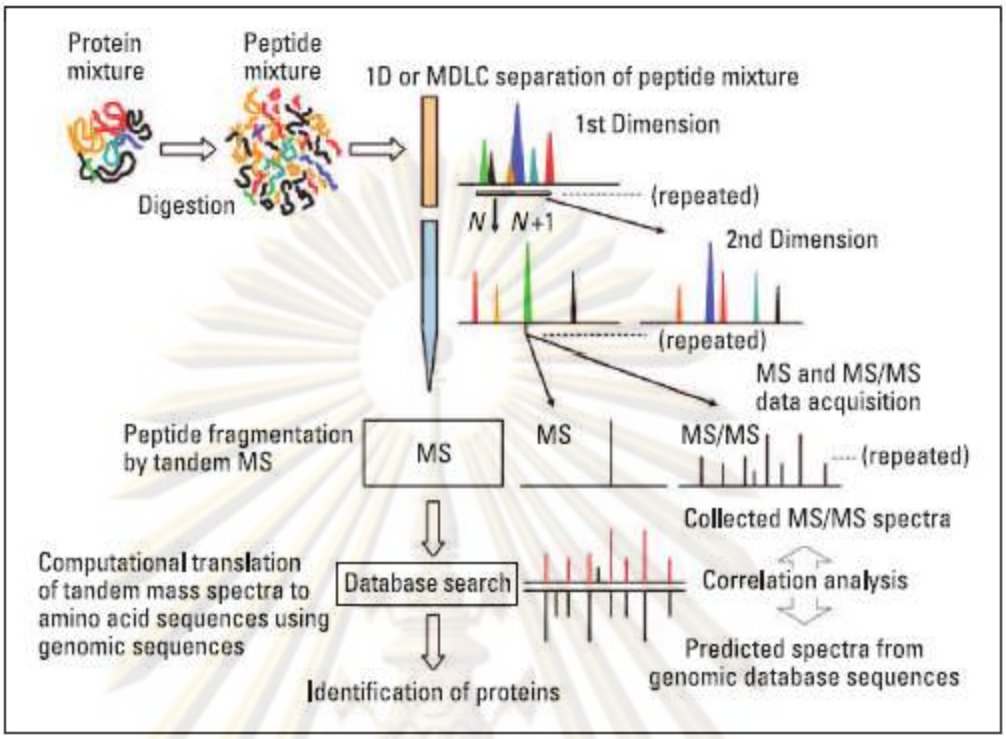

Figure 1.11. Workflow of LC/MS/MS-based shotgun proteomics strategy. *N* is the sample fraction.

1.4.4 Quantitative Mass Spectrometry in Proteomics

Mass spectrometry is increasingly used for quantitative proteomic profiling of complex biological samples. Quantitative proteomics is important to provide fundamental understanding of biological processes because the kinetics/dynamics of the cellular proteome is described in terms of changes in the concentrations of proteins in particular compartments (Ishihama *et al*., 2005). Generally, the quantification strategies can be divided into two categories: 1) quantification using stable isotope labeling, including metabolical, enzymatical labeling, labeling by chemical means or provided by spiked synthetic peptide standards and 2) label-free quantification using spectral counting or spectral feature analysis (Bantscheff *et al*., 2007).

1.4.4.1 Stable isotope labeling

One commonly used approach in bottom-up proteomics employs stable isotope labeling (^{12}C vs. ^{13}C,^{14}N vs.^{15}N,^{2}H vs.^{1}H), allowing comparison of peptides between samples. Stable isotopes labeled peptides are chemically identical to their native counterparts and therefore have similar behaviour during chromatographic and mass spectrometric analysis. Isotope labels can be introduced into amino acids 1) metabolically, 2) chemically, 3) enzymatically or, alternatively, by spiking of synthetic peptides. Although protein quantification using stable isotopic labeling has been proved as accurate, sensitive and reproducible method. It has several limitations. As first, labeling with stable isotopes is often very expensive, and some labeling procedures involve complex sample preparations. Second, labeling methods make acquired LC-MS spectra more complex due to the presence of additional isotopic peaks, which often overlap with co-eluting components of similar masses, complicating peak detection and quantification. And finally, chemical labeling approaches are prone to side reactions (e.g. thiol reactions of serine and threonine residues with iTRAQ reagent), leading to unexpected products (Bantscheff *et al*., 2007).

1.4.4.2 Label-free quantification

Label-free quantitation strategies are promising alternatives to stable isotope labeling approaches. Their advantages are simple and less expensive sample preparation, lower sample complexity, applicability to any samples, including tissues and ability to quantify and compare multiple samples. There are two fundamentally different strategies for label-free quantification: the first one measure and compare mass spectrometric signal intensities of peptide precursor ions of a given protein (Bondarenko *et al.*, 2002; Chelius and Bondarenko., 2002; Silva *et al*., 2005; Wiener *et al*., 2004) and the second one counts and compares the total number of MS/MS spectra of any peptide for a given protein (Washburn *et al*., 2001; Rappsilber *et al*., 2002; Ishihama *et al*., 2005). It should be noted, when comparing both label-free quantification methods, that spectral counts strategy more accurately quantify large changes in abundance, whereas spectral feature approach provides better estimates of smaller changes (Old *et al*., 2005; Zybailov *et al*., 2005). In addition, label-free methods provide higher dynamic range of quantification than stable isotope labeling

strategies, since the complexity of a sample significantly increases by adding of labeled internal standards (Bantscheff *et al*., 2007).

1.5 Research studies for identification of genes and proteins involving salt tolerance mechanisms in various plants

Jayaraman *et al*., (2008) identified differential gene expression in response to salt stress in foxtail millet (*Setaria italica* L.) by using cDNA-AFLP. The expression profile was compared between a salt tolerant (Prasad) and susceptible variety (Lepakshi) of foxtail millet in both control condition (L0 and P0) and after 1 h (L1 and P1) of salt stress. Nineteen transcript derived fragments (TDFs) showed differentially expression, out of which 86 TDFs were classified on the basis of their either complete presence or absence (qualitative variants) and 4 on differential expression pattern levels (quantitative variants) in the two varieties. Only 27 non redundant differentially expressed cDNAs that are unique to salt tolerant variety which represent different groups of genes involved in metabolism, cellular transport, cell signaling, transcriptional regulation, mRNA splicing, seed development and storage, etc. The expression patterns of seven out of nine such genes showed a significant increase of differential expression in tolerant variety after 1 h of salt stress in comparison to salt-sensitive variety as analyzed by qRT-PCR.

Transcript regulation in response to high salinity has been investigated in salt tolerant rice “Pokkali” with DNA microarrays (Kawasaki *et al.,* 2001). Hybridizations of RNA to microarray slides were probed for changing in transcripts from 15 min to 1 week after salt shock. The 1728 cDNAs from libraries of salt stressed roots showed that 150 mM NaCl reduced photosynthesis to one-tenth of the pre-stress value within minutes. Approximately 10% of the transcripts in Pokkali were significantly up-or down-regulated within 1 h of salt stress. The initial differences between control and stressed plants continued for hours but became less pronounced as the plants adapted over time. The interpretation of an adaptive process supported by the similar analysis of salt sensitive rice “IR29”, in which the immediate response exhibited by Pokkali delayed and later resulting in down-regulation of transcription and death. The up-regulated functions observed with Pokkali at different time points during stress adaptation changed over time. Increased protein synthesis and protein turnover were observed at early time points, followed by the induction of

known stress responsive transcripts within hours and the induction of transcripts for defense-related functions later. After 1 week, the nature of up-regulated transcripts; e.g. aquaporins indicated recovery.

Vaidyanathan *et al.*, (2003) investigated the immediate responses (enzymatic and non-enzymatic) to salinity-induced oxidative stress in two rice (*O. sativa*) cultivars, salt-sensitive Pusa Basmati 1 (PB) and salt-tolerant Pokkali (PK). Seedlings of both cultivars were subjected to NaCl stress (100–300 mM) for 42 h. Under NaCl stress, the salt-tolerant cv. PK showed higher activity of the ROS scavenging enzyme, catalase (CAT) and enhanced levels of antioxidants like ascorbate (ASC) and glutathione (GSH), than the sensitive cv. PB. Although superoxide dismutase (SOD) activity was lower in cv. PK, it showed lesser extent of membrane damage (lipid peroxidation) and lower levels of H_2O_2 than cv. PB under stress. The high levels of catalase activity indicated efficient scavenging of H_2O_2, which is produced more by non-enzymatic than via SOD in cv. PK. These data indicated that the concerted action of both enzymatic and non-enzymatic ROS scavenging machineries is vital to overcome salinity-induced oxidative stress in rice.

Lee *et al.*, (2009) identified salt-stress-induced differentially expressed genes (DEGs) in barley leaves using an annealing-control-primer (ACP)-based differentially display RTPCR method. By using 120 ACPs, a total of 11 up-regulated genes were identified and sequenced. Temporal expression patterns of some up-regulated DEGs in response to salt stress were further analyzed by Northern blot analysis. The possible roles of these identified genes are discussed within the context of their putative role in response to salt stress. Thus, the identification of some novel genes – such as SnRK1-type protein kinase; 17 kDa, class I, small heat shock protein; and RNase S-like protein precursor genes – may offer a new avenue for better understanding the salt stress response in plants, knowledge which might be helpful for developing future strategies.

Kong-ngern *et al.*, (2005) reported the salt-induced changes in protein synthesis in leaf sheaths of the Thai rice cv. Leaung Anan using one-dimensional SDS-PAGE and two-dimensional PAGE (2DE). In SDS-PAGE, two protein bands (22 and 31 kDa) whose expression was specifically increased under salt stress were identified. Enhanced expression of these protein bands was consistently observed in

plants grown under saline conditions. In 2DE, at least thirteen different proteins were induced and ten different proteins were decreased by salt. Western blot analysis of the 31 kDa of rice seedlings of three cultivars: salt-tolerant pokkali, moderately tolerant Leuang Anan, and salt-sensitive KDML 105, showed different expression patterns. The protein was strongly induced by salinity in leaf sheaths of treated tolerant pokkali and Leuang Anan, compared to control plants. In contrast, its level in the KDML 105 cultiva remained unchanged with salt treatment. Moreover, rice cv. Leaung Anan showed higher expression of the 31 kDa protein than pokkali. These results suggest that the 31 kDa polypeptide is a salt-induced protein in rice leaf sheaths.

1.6 Objectives of this thesis

The objectives of this study were identification of some of the key genes and proteins that were differentially expressed in the leaves of salt-tolerant and salt-susceptible rice in response to salinity stress using cDNA-AFLP and proteomic techniques.

CHAPTER II

MATERIALS AND METHODS

2.1. Experimental plants

Seeds of rice (*Oryza sativa* L. spp. *indica*) including both salt-tolerant cultivars, Homjan (HJ; GS.No. 4371) and Pokkali (Pok; GS.No. 17905) and salt-sensitive cultivars, IR29 (IR29; GS.No. 2818) and Pathumthani 1 (PT1), were obtained from the Pathumthani 1 Rice Research Center (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand). Homjan and Pathumthani 1 were used for analysis by RNA-arbitrarily primed (RAP) PCR but Pokkali and IR29 were used for complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) analysis.

Rice seeds were sterilized by soaking with 20% Clorox® (5.25% sodium hypochlorite) and 0.1% Tween 20® for 30 min, 5% Clorox® for 20 min and rinsed three time by sterilize distilled water. Sterilized seeds were germinated on the sugar free MS agar medium (Murashige and Skoog' 1962) for 2 weeks under the laboratory conditions: 25 ± 2°C air-temperature, 60 ± 5% relative humidity (RH), and 60 ± 10 µmol $m^{-2}s^{-1}$ photon flux (PPF) with 16 h d^{-1} photoperiod. The seedlings were aseptically transferred to 50 ml liquid sugar-free MS media (photoautotrophic system), supporting with vermiculite. The amount of air-exchange into the glass vessels through a hole over the plastic cap (∅1cm) covered the hole with a gas-permeable microporous polypropylene film (0.22 µm pore size, Nihon Millipore Ltd., Japan) was adjusted to 2.32 h^{-1} and maintained for 1 week before 513 mM NaCl was supplemented. Five plants per treatment were collected at 0 (control) 3, 6 and 12 h post treatment (hpt), immediately frozen in liquid N_2 and stored at -80°C until used.

For RAP-PCR and cDNA-AFLP, plant materials were subcultured and maintained in MS agar medium 4 -5 times before used for this experiment. But semi-quantitative RT PCR and quantitative real-time PCR analysis, the seedings of Pokkali, IR29 were prepared as described above (no subcultured) and leaves of each plant were collected at 0 (control) 3, 6, 12 and 24 h post treatment (hpt).

For RACE-PCR, leaves of geminated Pokkali (without salt-stress treatment) were collected and subjected to total RNA extraction.

2.2 Total RNA extraction

Total RNA was isolated from leave blades of each plant using TriPure® Isolation Reagent (Molecular Research Center). Approximately 100 mg of leaves were placed in a mortar containing liquid nitrogen and ground to the fine powder. The tissue powder was transferred to a 1.5 ml microcentrifuge tube containing 500 µl of TriPure® Isolation Reagent and homogenized with a glass-Teflon. Additional five hundred microliters of TriPure® Isolation Reagent were added. The homogenate was incubated at room temperature for 10 minutes and centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was transferred to new microcentrifuge tube. Phase separation was performed by the addition of 200 µl of chloroform. The mixture was briefly vortexed and incubated at room temperature for 15 minutes. Phase separation was performed by centrifugation at 12,000 rpm for 10 minutes at 4°C. The upper phase was transferred to a new microcentrifuge tube. RNA was precipitated by an addition of 500 µl of cold isopropanol. The sample tube was mixed thoroughly by inverting and incubated at -20° C for 30 minutes. Total RNA was recovered by centrifugation at 12,000 rpm for 10 minutes at 4°C. The supernatant was removed. The RNA pellet was washed with 1 ml of cold 75% ethanol and centrifuged at 12,000 rpm for 10 minutes at 4°C. The RNA pellet was air-dried for 5 - 10 minutes at room temperature. Total RNA was dissolved in 5-10 µl of DEPC-treated water for immediately used. Alternatively, the RNA pellet was kept under absolute ethanol in a -80° C freezer for long storage.

2.3 Measuring total RNA concentration using spectrophotometry and eletrophoresis

The concentration of extracted total RNA is typically estimated by measuring the optical density at 260 nanometre (OD_{260}). The value at OD_{260} allows calculation of total nucleic acid whereas the value at OD_{280} determines the amount of proteins in the RNA solution. The ratio between OD_{260}/OD_{280} provides an estimate on the purity of extracted total RNA. The ratio of approximately 2.0 indicates the good quality of the extracted RNA. An OD_{260} of 1.0 corresponds to a concentration of 40 µg/ml of RNA

(Sambrook *et al*., 1989), therefore total RNA concentration is estimated in μg/ml by the following equation;

$$[RNA] = OD_{260} \times \text{Dilution factors} \times 40$$

The quality of extracted total RNA was checked by 1% agarose gel (SeaKem ® LE agarose, USA) prepared in 1xTBE buffer (89 mM Tris-HCl, 8.91 mM boric acid and 2.5 mM EDTA, pH 8.0) at 100 V. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) for 5 minutes, destained in tap water for 10-15 minutes and visualized under a UV transilluminator.

2.4 DNase I treatment

To remove possible contamination of genomic DNA, fifteen micrograms of total RNA was digested using RNase-free DNase I (Promega, USA). Total RNA was combined with 1x DNase I buffer, DNase I (0.5 unit per μg of total RNA) and appropriate nuclease-free water in the final volume of 40 μl. The reaction mixture was incubated at 37^{o} C for 30 minutes. At the end of the incubation period, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed for 10 minutes. The solution was centrifuged at 12,000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a new microcentrifuge tube. This extraction process was repeated once with chloroform:isoamyl alcohol (24:1). The aqueous phase was transferred to a new microcentrifuge tube. One-tenth volume of 3 M DEPC-treated sodium acetated, pH 5.2 was added. RNA was precipitated by an addition of two and half volume of cold absolute ethanol and mixed thoroughly. The mixture was incubated at -80^{o} C for 30 minutes and centrifuged at 12,000 rpm for 10 minutes at 4^{o} C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 12,000 rpm for 15 minutes at 4^{o} C. Ethanol was removed and dried RNA pellet at room temperature for 5-10 minutes. The RNA pellet was dissolved in DEPC-treated water for immediately used. Alternatively, the extracted total RNA was kept under absolute ethanol in a -80^{o} C freezer for long storage.

Notably, total RNA used for RAP-PCR and cDNA-AFLP analyses did not treat with DNase I. In contrast, total RNA for RT-PCR, semi-quantitative RT-PCR

and quantitative real-time PCR was initially treated with DNase I before reverse-transcribed.

2.5 RNA-arbitrarily primed (RAP) PCR analysis

2.5.1 Synthesis of the first strand cDNA

The first strand cDNA was synthesized from 2 μg of total RNA extracted from leaves (see above) using an ImProm-II™ Reverse Transcription System Kit (Promega, USA). Total RNA was combined with 0.5 μg of oligo-dT(16)-overhang with A and appropriate DEPC-treated H2O in a final volume of 5 μl. The reaction was incubated at 70ºC for 5 minutes and immediately placed on ice for at least 5 minutes. Then 5x reaction buffer, $MgCl_2$, dNTP Mix, RNasin was added to final concentrations of 1x, 2.25 mM, 0.5 mM and 20 units, respectively. Finally, 1 μl of ImProm-II™ reverse transcriptase was added and gently mixed by pipetting. The reaction mixture was incubated at 25ºC for 5 minutes and at 42ºC for 90 minutes. The reaction was terminated by incubated at 70ºC for 15 minutes to terminate the reverse transcriptase activity. Quantity and quality of first stranded cDNA was examined by spectrophotometry (OD_{260}/OD_{280}) and electrophoresis (1.0% agarose gel). The first stranded cDNA was diluted to 500 ng/μl and kept at 20 °C until required.

2.5.2 RNA arbitrary primed (RAP)-PCR

One microgram of the first stranded cDNA was used as a template for PCR amplification in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8 at 25°C, 50 mM KCl and 0.1% Triton X - 100, 2 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP and dTTP, 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES, USA), 1 μM each of the first arbitrary primer (that used for the first strand cDNA synthesis) and the second arbitrary primers (Table 2.1) PCR was performed by predenaturation at 94 °C for 3 min, follow by 40 cycles of denaturation at 94 °C for 30 s, annealing at 36^{o} C for 60 s, and extension at 72 °C for 90 s. The final extension was carried out for 7 minutes at 72 °C. Five microlitres of the amplification product were examined with 1.5-2.0% agarose gel electrophoresis. Arbitrary primers that yielded positive amplification products were further analyzed by polyacrylamide gel electrophoresis.

Table 2.1 Sequences of arbitrary primers used for screening of gene expression marker in *Oryza sativa* using RAP-PCR analysis

Primer	Sequences
UBC 122	GTAGACGAGC
UBC 138	GCTTCCCCTT
UBC 158	TAGCCGTGGC
UBC 169	ACGACGTAGG
UBC 174	AACGGGCAGC
UBC 222	AAGCCTCCCC
UBC 228	GCTGGGCCGA
UBC 268	AGGCCGCTTA
UBC 457	CGACGCCCTG
UBC 459	GCGTCGAGGG
OPA 01	CAGGCCCTTC
OPB 02	TGCCGAGCTG
OPA-09	GGGTAACGCC

2.6 Identification of gene involved in salt tolerance mechanisms using cDNA-amplified fragment length polymorphism (cDNA-AFLP)

2.6.1 First-stand cDNA synthesis

Two microgram of total RNA from leaves of each treatment group was combined with 1 μl of 3′ SMART^{TM} CDS primer II A (15 μM final concentration), 1 μl SMART II^{TM} A Oligonucleotide (15 μM final concentration) (Table 2.2) and appropriate DEPC-treated water in the final volume of 5.5 μl. The reaction was incubated at 72^{o} C for 2 minutes and cooled on ice for 5 minutes. Then, 5x First-Strand Buffer, 20mM dithothreitol (DTT), 10 mM of each dNTP were added to the final concentration of 1x, 2mM and 1 mM, respectively. Finally, 0.5 μl of BD Powerscript^{TM} Reverse Transcriptase (Clontech, USA) was added and gently mixed by pipetting. The reaction mixture was incubated at 42^{o} C for 1.5 hours. The reaction was incubated on ice to terminate the reverse transcriptase activity and 2 μl of reverse-transcribed product were subjected to the second strand cDNA synthesis by PCR.

2.6.2 Second strand cDNA amplification by long distance PCR

The reaction components containing 10x Advantage 2 PCR Buffer, 10 mM each dNTP, 5´ PCR Primer II A (Table 2.2) and 50x BD Advantage 2 polymerase Mix (Clontech, USA) was combined to the final concentration of 1x, 0.2 mM, 0.6 μM and 1x, respectively. Appropriated amount of DEPC-treated water was added to make the final volume up to 50 μl. Then, 2 μl of the first strand cDNA were added. The reaction mixture was incubated at 95^{o} C for 1 minute followed by 3 cycles of 95^{o} C for 15 seconds; 65^{o} C for 30 seconds and 68^{o} C for 6 minutes. The resulting product was electrophoretically analyzed through a 1.0% agarose gel.

2.6.3 Agarose gel electrophoresis

An appropriate amount of agarose was weighed out and mixed with an appropriate volume of 1x TBE buffer (8.9 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.3). The gel slurry was boiled in a microwave oven to complete solubilization, and allowed to approximately 60^{o}C before poured into the gel mold. A comb was inserted. The gel was left to solidify. When needed, the comb was carefully removed. The agarose gel was submerged in a chamber containing an enough amount of 1x TBE buffer covering the gel for approximately 0.5 cm. Appropriate volumes of PCR products were mixed with the one-fifth volume of the 10x loading dye (0.25% bromophenol blue and 25% Ficoll in water) and loaded into the well. A 100 bp DNA ladder was used as the standard DNA marker. Electrophoresis was carried out at 100 volts until bromophenol blue moved to approximately one-half of the gel. The electrophoresed gel was stained with an ethidium bromide solution (0.5 μg.ml-1) for 5 - 15 minutes and destained in running tap water to remove unbound ethidium bromide from the gel. DNA fragments were visualized under a UV transilluminator and photographed using Gel Doc™ XR Imaging system (Bio-RAD, USA).

Table 2.2 Nucleotide sequences of primers used for cDNA synthesis

Primer	Sequence	Length (bp)
3'SMART™CDS Primer II A	5' AAG CAG TGG TAT CAA CGC AGA GTA CT 3'	26
SMART II™ A Oligonucleotide	5' AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG 3'	30
5' PCR Primer II A	5' AAG CAG TGG TAT CAA CGC AGA GT 3'	23

2.6.4 Purification of the synthesized second strand cDNA

The PCR product from second strand cDNA amplification was transferred to a 1.5 ml microcentrifuge tubes and the TE buffer was added to final volume of 100 μl. An equal volume of buffer-equilibrated phenol:chloroform was added and gently mixed for 2 minutes. The solution was centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a new microcentrifue tube. One-tenth volume of 3 M sodium acetate, pH 5.2 was added. DNA was precipitated by an addition of two volume of cold absolute ethanol and mixed thoroughly. The mixture was incubated at -80^{o} C for 40 minutes and centrifuged at 12,000 rpm for 10 minutes at 4^{o} C. The supernatant was removed. The cDNA pellet was washed with 1ml of 70% ethanol and centrifuged at 12,000 rpm for 10 minutes at 4^{o} C. Ethanol was removed and cDNA was dried at room temperature for 5-10 minutes. The cDNA pellet was dissolved in 10 μl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0). The DNA solution was kept at -20^{o} C until further used.

2.6.5 Restriction enzyme digestion and adaptor ligation

One and a half micrograms of cDNA from each individual was digested with 6 units of *Eco*RI and 5 units of *Mse*I in a 40 μl reaction mixture containing 1x NE Buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 1 mM Dithiothreitol) and 0.1 μl of 100x BSA. The reaction mixture was incubated at 37^{o}C for 4-5 hours. The *Eco*RI and *Mse*I adaptors (Table 2.3) were ligated to restricted cDNA in a 30 μl reaction volume composing of 20 μl of the restricted product, 1X OPA buffer, 0.25 μM of *Eco*RI and 2.5 μM of *Mse*I adaptors, 1 mM of

ATP and 3 Weiss units of T4 DNA ligase. The reaction was incubated at 4^{o}C for approximately 16 hours.

2.6.6 Preamplification

The preamplification reaction was carried out in a 25 μl reaction volume containing 10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 37.5 ng of E (5′- GACTGAGTACCAATTC-3′) and M (5′-GATGAGTCCTGAGTAA-3′) primers or, E_{+A} (5′-GACTGAGTACCAATTCA-3′) M_{+C} (5′-GATGAGTCCTGAGTAAC-3′) primers, 1.5 units of DyNazymeTMII DNA Polymerase (Finnzymes, USA) and 1 μl of the ligation product. PCR was performed by denaturation at 94^{o}C for 30 seconds, annealing at 56^{o}C for 1 minute and extension at 72^{o}C for 1 minute.

2.6.7 Selective amplification

Selective amplification was carried out in a 25 μl reaction volume containing 10 mM Tris-HCl; pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 30 ng each of a combination of E_{+2} and M_{+2} primers or E_{+3} and M_{+3} primers (Table 2.3), 1.5 units of DyNazymeTMII DNA Polymerase (Finnzymes, USA) and 1 μl of the preamplification product. PCR was performed in a PTC-200 Peltier Thermal Cycler consisting of denaturation at 94^{o}C for 30 seconds (or 45 seconds), annealing at 65^{o}C for 45 seconds (or 60 seconds) and extension at 72^{o}C for 90 seconds (or 120 seconds) for 2 cycles followed by 12 cycles of a touchdown phase with lowering of the annealing temperature for 0.7^{o}C (or 1 ^{o}C) in every cycle. The amplification consisting of 94^{o}C for 30 seconds, 56^{o}C (or 53 ^{o}C) for 45 seconds (or 60 seconds) and 72^{o}C for 90 seconds (or 120 seconds) was performed for additional 25-28 cycles. The final extension was carried out at 72^{o}C for 5 minutes

The selective amplification AFLP products were electrophoresed through 1.5-2.0% agarose gels as described previously. Primer combinations that yielded appropriate sizes of the positive amplification products were further analyzed by polyacrylamide gel electrophoresis.

Table 2.3 Primers and primer sequences used for cDNA-AFLP in this thesis

Primer	Sequences
Adaptor sequences	
Eco RI adaptor	5′-CTC GTA GAC TGC GTA CC-3′
	5′-AAT TGG TAC GCA GTC TAC-3′
Mse I adaptor	5′-GAC GAT GAG TCC TGA G-3′
	5′-TAC TCA GGA CTC AT-3′
Preamplification primers	
E	5′-GAC TGC GTA CCA ATT C-3′
M	5′-GAT GAG TCC TGA GTA A-3′
E+A	5′-GAC TGA GTA CCA ATT CA-3′
M+C	5′-GAT GAG TCC TGA GTA AC-3′
Selective amplification primers	
E-1	E+GA
E-2	E+GG
E-3	E+GT
E-4	E+TA
E-5	E+TG
E-6	E+TT
M-1	M+AA
M-2	M+AG
M-3	M+AT
M-4	M+GA
M-5	M+GG
M-6	M+GT
M-7	M+TA
M-8	M+TC
M-9	M+TG
M-10	M+TT
E_{+A}-1	E_{+A}AC
E_{+A}-2	E_{+A}AG
E_{+A}-3	E_{+A}CA
E_{+A}-4	E_{+A}CT
E_{+A}-5	E_{+A}CC

Primer	Sequences
Selective amplification primers (cont.)	
E_{+A}-6	E_{+A}CG
E_{+A}-7	E_{+A}GC
M_{+C}-1	M_{+C}AA
M_{+C}-2	M_{+C}AC
M_{+C}-3	M_{+C}AG
M_{+C}-4	M_{+C}AT
M_{+C}-5	M_{+C}TA
M_{+C}-6	M_{+C}TC
M_{+C}-7	M_{+C}TG
M_{+C}-8	M_{+C}TT
M_{+C}-9	M_{+C}GA
M_{+C}-10	M_{+C}GT

2.7 Denaturing Polyacrylamide Gel Electrophoresis

2.7.1 Preparation of Glass Plate

The long glass plate was thoroughly wiped with 2 ml of 95% commercial grade ethanol in one direction with a tissue. This process was then repeated twice. Afterwards, the long glass plate was coated with 1 ml of freshly prepared Bind silane (10 µl of Bind silane; Amersham Biosciences, 995 µl of 95% ethanol and 10 µl of 5% glacial acetic acid) and left for approximately 10-15 minutes. Excess binding solution was removed with a piece of tissue. The long glass plate was further cleaned three times with 95% ethanol. The short glass plate was treated as described above with the exception that the binding solution was replaced by the Repel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The cleaned glass plates were assembled with a pair of 0.4 mM spacer. The bottom and both sides of assembled glass plates were sealed with the plastic tape.

2.7.2 Preparation of denaturing polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels are used for the separation of single-stranded AFLP and RAP-PCR fragments. Four to six percentage denaturing polyacrylamide gels were prepared by combining 40 ml of the acrylamide solution (19:1 acrylamide: bisacrylamide with 7 M urea in 1x TBE buffer) with 240 μl of freshly prepared 10 % ammonium persulphate and 24 μl of TEMED. The acrylamide solution was gently swirled and degassed for 20 minutes. The assembled plate sandwich was hold at a 45 degree angle on the bottom corner. The acrylamide solution was then gently injected into one side of the assembled plates using a liquid bottle. The filled plate sandwich was left in the horizontal position. The flat edge of the shark-tooth comb was then inserted. The gel was left at room temperature for 1 hour. After that, the polymerized gel was covered by the water soaked tissue papers and left at room temperature for 4 hours (or overnight) for complete polymerization. When required, the spring clips and the sealing tape were carefully removed. The top of the gel was rinsed with 1x TBE.

2.7.3 Gel electrophoresis

The gel sandwich was placed in the vertical sequencing apparatus with the short glass plate inward. The gel sandwich was securely clamped with the integral gel clamps along the sides of the sequencing apparatus. The upper and lower buffer chambers were filled with 1x TBE. The shark tooth comb was reinserted into the gel until the teeth just touched the surface of the gel. Three microlitres of the loading dye (98 % formamide, 200 μl EDTA, 0.25 % bromophenol blue and 0.25 % xylene cyanol) was loaded into each well. The gel was pre-run at 35 W for 30 minutes. Six microlitres of the amplification products were mixed with 3 μl of the loading buffer and heated at 95°C for 5 minutes before snapped cooled on ice for minutes. The sample was carefully loaded into the well. Electrophoresis was carried out at 35 W for approximately 2 hours (xylene cyanol move out from the gel for approximately 10 minutes).

2.7.4 Silver staining

The gel plates were carefully separated using a plastic wedge. The long glass plate with the gel was placed in a plastic tray containing 2 liters of the fix/stop solution and agitated well for 30 minutes. The gel was soaked with shaking 3 times for 3 minutes with deionized water. The gel was lifted out from the tray between each wash and allowed the washed water draining out of the gel for 4 seconds. The gel was transferred to 0.1% silver nitrate (1.5 litres) and incubated with agitation at room temperature for 30 minutes. The gel was soaked in 1.5 litres of deionized water with shaking (10 forward and 10 backward agitation) and immediately placed in the tray containing 1.5 litres of the chilled developing solution. This step is crucial and the time taken to soak the gel in the water and transferred it to chilled developing solution should be no longer than 5 - 10 seconds. The gel was well agitated until the first bands are visible (usually 1.5 - 2 minutes). The gel was then transferred to another tray containing 1.5 litres of chilled developer and shaken until bands from every lane were observed (usually 2–3 minutes). One litre of the fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes. The stained gel was soaked in deionized water twice for 3 minutes each. The gel was dried at room temperature.

2.7.5 Elution of cDNA fragments from polyacrylamide gels

Candidate salt-stress response cDNA fragments were excised from the gel using a sterile razor blade and washed 3 times for 30 minutes each at room temperature with 200 μl of sterile deionized water. Twenty microlitres of water was then added and incubated overnight at 37°C. Reamplification of the target fragment was carried out using the same PCR recipes as those for selective amplification or RAP-PCR with the exception that 100 ng of each primer and 5 μl of the eluted gel product were used. The amplification conditions were composed of 5 cycles of 94°C for 30 seconds, 42°C for 1 minute and 72°C for 1 minute followed by additional 35 cycles at a higher stringent annealing temperature at 50°C. The final extension was performed at 72°C for 7 minutes. The reamplified product was electrophoretically analyzed through a 1.0 - 1.8 % agarose gel electrophoresis.

2.7.6 Elution of cDNA fragment from agarose gels

The reamplified product was fractionated through an agarose gel in duplication. One was run side-by-side with a 100 bp DNA markers and the other was loaded into the distal well of the gel. After electrophoresis, lanes representing the DNA standard and its proximal DNA sample were cut and stained with ethidium bromide (0.5 μg/ml) for 5 minutes. Positions of the DNA markers and the EtBr stained reamplified fragment were used to align the position of the non-stained target DNA fragment. The DNA fragment was excised from the gel with a sterile razor blade. DNA was eluted out from the agarose gels using a illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to the protocol recommended by the manufacture. The excised gel was transferred up to 200-300 mg into a microcentrifuge tube. Approximately 500 μl of Capture buffer type 3 was added to the sample and mixed thoroughly. The mixture was incubated at 60°C for 10 - 15 minutes with briefly vortexing every 2 - 3 minutes. After the gel was completely dissolved, the mixture was applied to the GFX MicroSpin column placed on a 2 ml collection tube and centrifuged at 12,000 rpm for 1 minute at room temperature. The flow-through was discarded and 0.5 ml of the wash buffer type 1 was added. The GFX MicroSpin column was centrifuged at 12,000 rpm for 1 minute at room temperature. The flow-through was discarded. The column was further centrifuged at room temperature for an additional 2 minute at 13,000 rpm to remove trace amount of the washing buffer. The column was then placed in a new microcentrifuge tube and 30 μl of the Elution buffer type 4 was added to the center of the membrane. The column was incubated at room temperature for 2 minute before centrifuged at 13,000 rpm for 2 minute. The eluted sample was stored at -20°C until further required.

2.7.7 Ligation of PCR product to vector

The ligation reaction was set up in the total volume of 5 μl containing of 1.75 μl of the gel-eluted PCR product, 25 ng of pGEM-T easy vector, 2.5 μl of 2X rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10 % PEG 8000) and 3 Weiss units of T4 DNA ligase. The ligation mixture was gently mixed by pipetting and incubated at 4°C overnight.

2.7.8 Preparation of competent cells

A single colony of *E. coli* JM109 was innoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) with vigorous shaking at 37°C overnight. The starting culture was inoculated into 50 ml of LB broth and continued culture at 37°C with vigorous shaking to the OD_{600} of 0.5 - 0.8. The cells were chilled on ice for 10 minutes before centrifuged at 3,000 g for 10 minutes at 4°C. The pellets were resuspended in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged as above. The supernatant was discarded and the pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ and divided into 100 μl aliquots. These competent cells could be used immediately or stored at –80°C for subsequent used.

2.7.9 Transformation of the ligation product to *E.coli* host cells

The competent cells were thawed on ice for 5 minutes. Two microlitres of the ligation mixture were added and gently mixed by pipetting. The mixtures were incubated on ice for 30 minutes. The reaction tube was then placed in a 42°C water bath for 45 seconds without shaking. The tube was then immediately snapped on ice for 2 – 3 minutes. One microlitre of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added to the tube. The cell suspension was incubated with shaking at 37°C for 1.5 hours. At the end of the incubation period, the cultured cell suspension was centrifuged at 6,000 rpm for 1 minute at room temperature. The pellet was gently resuspended in 100 μl of SOC and spread on a LB agar plate containing 50 μg/ml of ampicillin, 25 μg/ml of IPTG and 20 μg/ml of X-gal. The plate was left until the cell suspension was absorbed and further incubated at 37°C overnight. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.7.10 Detection of recombinant clone by colony PCR

Colony PCR was performed in a 25 μl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 μM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 0.2 μM of pUC1 (5′-TCCGGCTCGTATGT-

TGTGTGGA-3′) and pUC2 (5′ GTGGTGCAAGGCGATTAAGTTGG-3′) primers and 0.5 unit of DyNAzymeTM II DNA Polymerase. A recombinant colony was picked up by the micropipette tip and mixed well in the amplification reaction. The PCR profiles was predenatured at 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 60 seconds and 72 °C for 90 seconds. The final extension was carried out at 72°C for 7 minutes. The resulting PCR products were electrophoretically analyzed through agarose gels.

2.7.11 Isolation and digestion of recombinant plasmid DNA

A recombinant clone was inoculated into 3 ml of LB broth (1% tryptone, 0.5% yeast extract, 1.0 % NaCl) containing 50 μg/ml of ampicillin and incubated at 37°C with constant shaking at 250 rpm overnight. The culture was transferred into a 1.5-ml microcentrifuge tube and centrifuged at 12,000 rpm for 1 min. The cell pellet was collected and resuspended with 200 μl of the Lysis buffer type 7. The mixture was vortexed and treated with 200 μl of the Lysis buffer type 8 and mixed gently by inverting the tube 10 times. After that 400 μl of Lysis buffer type 9 was added and gently mixed. To separate the cell debris, the mixture was centrifuged at 13,000 rpm for 15 minutes. The supernatant was transferred into a new microcentrifuge tube and to the illustra plasmid mini column and centrifuged at 13,000 rpm for 30 seconds. The flow-through was discarded. The column was washed by adding 400 μl of the Wash buffer type 1 and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded. The spin tube was centrifuge for an additional 1 minute to remove the residual wash buffer. The column was placed in a new 1.5 ml microcentrifuge tube and 40 μl of the elution buffer was added to elute the extracted plasmid DNA. The column was left at room temperature for 2 minutes and centrifuged at 13,000 rpm for 2 minute.

The insert size of each recombinant plasmid was examined by digestion of the plasmid with *Eco R*I. The restrict digestion was carried out in a 15 μl containing 1x restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl and 50 mM $MgCl_2$), 1 μg of recombinant plasmid and 2 - 3 unit *Eco* RI and incubated at 37°C for 3 hours before elctrophoretically analyzed by agarose gel elctrophoresis.

2.7.12 DNA sequencing

The recombinant plasmid was unidirectional sequenced with the M13 reverse or M13 forward primers on an automated DNA sequencer by sending the plasmid to Macrogen (Korea).

2.8 Isolation and characterization of the full length cDNA of functionally important gene homologues of *O. sativa* using Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR)

2.8.1 Preparation of the 5' and 3' RACE template

Total RNA was extracted from leaves of each rice variety using TRI REAGENT. The quality of total RNA was determined by agarose gel electrophoresis. RACE cDNA template was prepared by combining 1.5 µg of total RNA with 1 µl of 5'-CDS primer and 1 µl of 10 µM SMART II oligonucleotide for 5' RACE-PCR or 1.5 µg of total RNA with 1 µl of 3' CDS primer A for 3' RACE-PCR (Table 2.4). The component were mixed and centrifuged briefly. The reaction was incubated at 70ºC for 2 minutes and snap-cooled on ice for 2 minutes. The reaction tube was centrifuged briefly. After that, 2 µl of 5x First-strand buffer**,** 1 µl of 20 mM DTT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript Reverse Transcriptase were added. The reaction were mixed by gently pipetting and centrifuged briefly to collect the contents at the bottom of the tube. The reaction tube was incubated at 42 ºC for 1.5 h in a thermocycler. The first strand reaction products were diluted with 125 µl of TE buffer and heated at 72 ºC for 7 min. The first strand cDNA template was kept at -20 ºC until needed.

2.8.2 Primer designed for RACE-PCR and primer walking

Gene-specific primers (GSPs) were designed from nucleotide sequences of RAP-PCR fragments. The antisense primer (and nested primer) for 5′ and/or 3′ RACE-PCR of each gene was designed (Table 2.5).

2.8.3 RACE-PCR

The master mix sufficient for 5′ and/or 3′ RACE-PCR and the control reactions was prepared (Tables 2.6 and 2.7). For each 25 µl amplification reaction, 14.0 µl sterile deionized H_2O, 2.5 µl of 10x Advantage® 2 PCR buffer, 0.5 µl of 10 uM dNTP mix and 0.5 µl of 50x Advantage® 2 polymerase mix were combined. The reaction was carried out as described in Table 2.8.

The primary 5′ and 3′ RACE-PCR product were electrophoretically analyzed through 0.8-1.0% agarose gels. If the discrete expected bands were not obtained from the primary amplification, nested PCR was performed using the recipes illustrated in Tables 2.5 and 2.6. The primary PCR product was 50-fold diluted. The secondary PCR was performed using 1 - 5 µl of the diluted first PCR product as a template using the conditions described in Table 2.8.

Table 2.4 Primer sequence for the first strand cDNA synthesis and RACE-PCR

Primers	Sequence
SMART II A Oligonucleotide	5′-AAGCAG TGG TATCAACGCAGAGTACGC GGG-3′
3′ RACE CDS Primer A	5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ N_{-1} N-3′ (N=A, C, G orT; N_{-1}= A,G or C)
5′ RACE CDS Primer	5′-$(T)_{25}$ N_{-1} N-3′ (N=A, C, G orT; N_{-1}= A,G or C)
10X Universal PrimerA Mix (UPM)	Long : 5′–CTAATACGACTCACTATAGGGCAA GCAGTGGTATCAACGCAG AGT-3′
	Short : 5′–CTAATACGACTCACTATAGGG C – 3′
Nested Universal Primer A (NUP)	5 – AAG CAG TGG TAT CAA CGC AGA GT -3′

Table 2.5 Gene-specific primers (GSPs) and nested GSP used for isolation of the full length cDNA of functionally important genes in *Oryza sativa*

Gene specific primer	Sequence	Tm (°C)
Protein kinase		
5′ RACE	R: 5' ATCGTGTACCGTGACCTGAAGCCGG 3'	69.2
3′ RACE	F: 5' GGACATCGTGTACCGTGACCTGAAG 3'	67.9
5'Nested	R: 5' CCACATCATACTCACCGACTTCGACC 3'	66.7

Table 2.6 Compositions for amplification of the 5′ end of gene homologues using 5′ RACE-PCR

Component	5′ RACE-PCR	UPM only (Control)	GSP1 only (Control)
5’ RACE–Ready cDNA template	1.5 µl	1.5 µl	1.5 µl
UPM (10x)	5.0 µl	5.0 µl	-
GSP1 (10 uM)	1.0 µl	-	1.0 µl
GSP2 (10 uM)	-	-	-
H_2O	-	1.0 µl	5.0 µl
Master Mix	17.5 µl	17.5 µl	17.5 µl
Final volume	25 µl	25 µl	25 µl

Table 2.7 Compositions for amplification of the 3′ end of gene homologues using 3′ RACE-PCR

Component	3′ RACE-PCR	UPM only (Control)	GSP1 only (Control)
5’ RACE–Ready cDNA template	1.5 µl	1.5 µl	1.5 µl
UPM (10x)	5.0 µl	5.0 µl	-
GSP1 (10 uM)	1.0 µl	-	1.0 µl
GSP2 (10 uM)	-	-	-
H_2O	-	1.0 µl	5.0 µl
Master Mix	17.5 µl	17.5 µl	17.5 µl
Final volume	25 µl	25 µl	25 µl

Table 2.8 The amplification conditions for RACE-PCR of various gene homologues of *Oryza sativa*

Gene homologue	Amplification condition
Protein kinase	
5′ RACE-PCR	2 cycles of 94 °C for 30 s and 70 °C for 2 min 2 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 2 min 25 cycles of 94 °C for 30 s, 66 °C for 30 s and 72 °C for 2 min and the final extension at 72 °C for 7 min
3′ RACE-PCR	2 cycles of 94 °C for 30 s and 70 °C for 2 min 2 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 2 min 25 cycles of 94 °C for 30 s, 66 °C for 30 s and 72 °C for 2 min and the final extension at 72 °C for 7 min
5′and 3′ semi-nested RACE-PCR	25 cycles of 94 °C for 30 s, 66 °C for 30 s, 72 °C for 2 min and the final extension at 72 °C for 7 min

2.9 Identification of proteins involving salt tolerance mechanism using proteomics

2.9.1 Protein extraction

Five leaves from each of two rice varieties were collected at 0 (control) 3, 6 and 12 h post treatment (hpt) by the salt solution. Approximately 200 mg of the leave tissue was placed in a mortar containing liquid nitrogen and ground to the fine powder. The tissue powder was transferred to a 1.5 ml microcentrifuge tube containing 500 µl of 0.2% SDS solution and mixed by vortex. The sample was incubated at 37^{o}C for 2 hours and centrifuged at 10,000 rpm for 15 minutes at 4^{o}C. The supernatant was transferred to a new microcentrifuge tube. The extracted protein was kept at -20^{o}C until used.

2.9.2 Determination of protein concentration by a Lowry method

The amount of extracted total protein was measured by Lowry–Peterson protein determination method (Peterson, 1983). The absorbance at 750 nm (OD_{750}) was measured and the protein concentration was calculated using the standard curve, plotted between OD_{750} on the Y-axis and series of BSA concentrations (µg/ml) on the X-axis.

2.9.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were size-fractionated on SDS-PAGE mini slab gel (8 x 9 x 0.1 cm, Hoefer miniVE, Amersham Biosciences, UK). The polyacrylamide gel was prepared according to the standard method described by Laemmli (1970). The separating gel used for the fractionation of soluble proteins from plant cells contained 12% acrylamide. Twenty micrograms of protein extraction was precipitated with two volume of cold acetone. The mixture was vortexed and incubated at -20^{o}C for 1 hour before centrifugation at 10,000 rpm for 30 minutes. Acetone was removed and pellet was dried for 10-15 minutes. The pellet was resuspended with 15 µl of 5x SDS-PAGE gel loading buffer (0.125M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.2M DTT, 0.02% bromophenol blue), boiled at 95^{o}C (WB-710M, Optima, Japan) for 10 min before loading onto the 12.5% SDS-PAGE. Electrophoresis was conducted at a constant voltage of 100 Volt. To estimate size of electrophoresed polypeptides, low molecular weight protein standard marker (Amersham Biosciences, UK) was used. Electrophoresis was performed in SDS electrophoresis buffer (25mM Tris-HCl pH 8.3, 192mM glycine, 0.1% SDS) until the tracking dye reached the bottom of the gel.

2.9.4 Silver staining

At the end of each run, the protein gel was fixed in the fixing solution (50% methanol, 12% acetic acid and 50 µl of 37% formaldehyde to 100 ml fixing solution) for 2 hours. The gel was removed and placed in the washing solution (35% ethanol) twice for 5 minutes each and sensitizing in 0.02% sodium thiosulfate for 2 minutes. After washing in water twice for 5 minutes each, the gel was stained with 0.2% silver nitrate for 20 minutes. The gel was shaken in the developing solution (60% $NaCO_3$ w/v, 0.04% $Na_2S_2O_3$ v/v, 37% formaldehyde) until developed protein spots were visualized and the developing reaction was terminated quickly in the stopping solution (14.6% w/v sodium EDTA $C_{10}H_{12}N_2Na_4O_8$) for 20 minutes. The gel was scanned by a Labscan (GE Healthacre). Gel image matching were carried out using Image master 2D platinum™ (GE Healthacre) and the gel was kept in 0.1% acetic acid at 4^{o}C.

2.9.5 Mass spectrometry analysis

2.9.5.1 In-gel digestion

After the gel bands were excised according to the molecular mass range of protein standard markers (Figure 2.1), the gel pieces of each group were transferred to 96 well plate (4-5 pieces per well). These gel pieces were subjected to in-gel digestion using an in-house method developed by Proteomics Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand. The gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10 mM DTT in 10mM ammonium bicarbonate at room temperature for 1 hour and alkylated at room temperature for 1 hour in the dark in the presence of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 minutes. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 minutes, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gels, and then the gels were incubated at room temperature for 10 minutes in a shaker. Peptides extracted were collected and pooled together in the new tube. The pool extracted peptides were dried by vacuum centrifuge and kept at -80°C for further mass spectrometric analysis.

2.9.5.2 HCTUltra LC-MS analysis

The protein digest was injected into the Ultimate 3000 LC System (Dionex, USA) coupled to ESI-Ion Trap MS (HCT Ultra PTM Discovery System (Bruker, Germany) with electrospray at flow rate of 300 nl/min to a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id x 150 mm). A solvent linear gradient (solvent A: 0.1% formic acid in water; solvent B: 80% 0.1% formic acid in 80% acetonitrile) was run in 40 min.

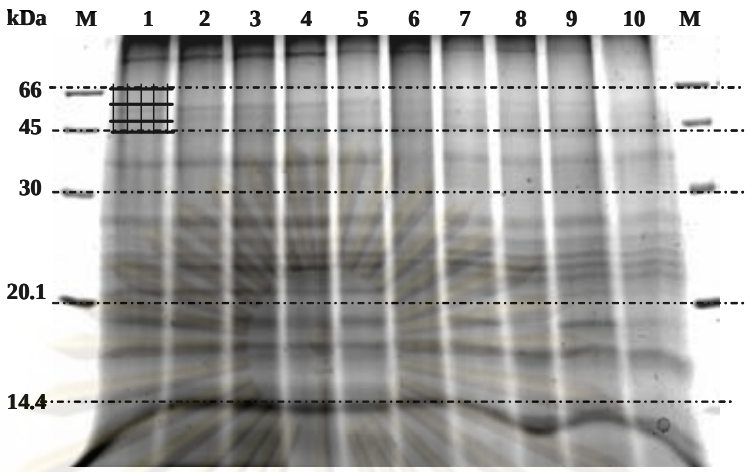

Figure 2.1 The gel bands were excised according to the molecular mass range of protein standard markers. Lanes 1- 10 are groups of sample and M is protein marker.

2.9.5.3 Protein quantitation and identification

For proteins quantitation, DeCyder MS Differential Analaysis software (DeCyderMS, GE Healthcare, Johansson *et al.*, 2006; Thorsell *et al.*, 2007) was used. Acquired LC-MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ions signal intensities in the MS mode. The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software (Matrix Science, London, UK. The data was searched against the NCBI database for protein identification. Database interrogation was; taxonomy (*Oryza sativa*); enzyme (trypsin); variable modifications (carbamidomethylation at cysteine residues, oxidation at methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (± 0.6 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages (3). Analyzed proteins considered significant match have at least one peptides with an individual mascot score corresponding to $P < 0.05$ and $P < 0.1$, respectively.

2.10 Examination of expression levels of interesting genes by Semi-quantitative RT-PCR

2.10.1 Total RNA extraction and first strand cDNA synthesis

The seeding of Pokkali and IR29 were treated with 513 mM NaCl. Leaves were collected at 0 (control) 3, 6 and 12 h post treatment (hpt), immediately frozen in liquid N_2 and stored at -80^{o}C until used. Total RNA was extracted from leaves (N = 3-4 for each group) and 1.5 μg of total RNA treated DNase was reverse-transcribed as described previously.

2.10.2 Optimization of semi-quantitative RT-PCR conditions

Initially, RT-PCR of the target genes (Table 2.9) and internal control (*hypothetical prote*$_{217}$; F:5′-TCG CTA TGA ACG CTT GGC T -3′ and R:5′- TGA AGATAA CGC AGG TGT CC -3′) were amplified in a 25 μl reaction volume following the non-quantitative RT-PCR conditions (predenaturing at 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72 °C for 30 seconds. The final extension was carried out at 72°C for 7 minutes) for the control and target gene. The reaction contained 10 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl and 0.1 % Triton X–100, 2 mM $MgCl_2$, 100 mM each of dATP, dCTP, dGTP and dTTP, 0.2 μM of each primer, 2 μl of 10-fold diluted first strand cDNA and 1 unit of DynazymeTM DNA polymerase (FINNZYMES, USA).

For semi-quantitative RT-PCR analysis, primer and $MgCl_2$ concentrations and the cycle numbers used for amplification were further optimized.

2.10.2.1 Optimization of primer concentration

The optimal primer concentration for each primer pair (between 0, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 μM) was examined using the standard PCR conditions. The resulting product was electrophoretically analyzed. The primer concentration that gave product specificity and clear results were selected for further optimization of the PCR conditions.

2.10.2.2 Optimization of $MgCl_2$ concentration

The optimal $MgCl_2$ concentration of each primer pair (0, 0.5, 1.0, 1.5 and 2.0 mM) was further examined using the standard PCR conditions with the optimized primer concentration. The concentration of $MgCl_2$ that gave the highest yields and specificity for each PCR product was chosen.

2.10.2.3 Optimization of the number of amplification cycles

The PCR amplifications were carried out at different cycles (e.g. 23, 25, 28, 30 and 35 cycles) using the optimized concentrations of primers and $MgCl_2$. The number of cycles that gave the highest yield before the product reached a plateau phase of amplification was chosen.

2.10.2.4 Gel electrophoresis, semi-quantitative RT-PCR and data analysis

Semi-quantitative RT-PCR was carried out using the optimized conditions of each gene. The amplification product of the target gene and internal control were electrophoretically analyzed by the same gel and photographed by a gel documentation machine (BioRad). The intensity of the amplified target genes and that of internal control was quantified from the photograph of the gel using the Quantity One program (BioRad). The expression level of each gene was normalized by that of internal control. Expression levels between different groups and time of IR29 and Pokkali were statistically tested using one way analysis of variance (ANOVA) followed by a Duncan's new multiple range test. Significant comparisons were considered when the *P* value was < 0.05.

2.11 Examination of expression levels of interesting genes by quantitative real-time PCR

2.11.1 Primers and construction of the standard curve

Primers for amplification of *ATP synthase subunit C family protein***,** *Cytochrome P450 monooxygenase* and *Lipin, N-terminal conserved region* (Table 2.9) were applied for quantitative real-time PCR analysis. For construction of the standard curve of each gene, the PCR product of the target gene and internal control *(hypothetical protein)*

was amplified, electrophoresed through agarose gel and eluted out. The gel-eluted product was cloned into pGEM-Teasy vector and transformed into *E. coli* JM109. Plasmid DNA were extracted and used as the template for construction of the standard curve. Templates of each gene homologues and internal control were ten fold-diluted covering 10^2–10^8 copys. Real-time RT-PCR was carried out (see below) using a Lightcycle 480 (Roche). Each standard point was run in duplicate.

2.11.2 Quantitative real-time PCR

The first strand cDNA from different treatment groups was used as the template (*N* = 3-4 for each group). The target transcript and internal control (*hypothetical protein*) of each plant were amplified in reaction volume 10 µl containing 5 µl of 2x SYBR Green Master Mix (Roach). The specific primer pairs were used at a final concentration of 0.15, 0.2 and 0.3 µM for *ATP synthase subunit C family protein***,** *Cytochrome P450 monooxygenase* and *Lipin, N-terminal conserved region*, respectively. The thermal profile for quantitative real-time RT-PCR was 95ºC for 10 minutes followed by 40 cycles of denaturation at 95 ºC for 15 seconds, annealing at 53ºC, 55ºC or 56ºC for 30 seconds and extension at 72ºC for 20 seconds. Continually, cycles for the melting curve analysis was carried out at 95ºC for 15 seconds, 65ºC for 1 minute and at 98ºC for continue and cooling 40ºC for 30 seconds. Real-time RT-PCR assay was carried out in 96 well plate and each sample was run in duplicate. Relative expression levels of different group of samples were statistically tested by one way ANOVA followed by Duncan's new multiple rang test ($P < 0.05$).

Table 2.9 Gene homologue, primer sequences and the expected amplification size of genes identified by RAP-PCR, cDNA-AFLP and proteomics of *O sativa* in this study

Gene	Primer sequence	Size (bp)
RAP-PCR		
Protein kinase domain containing protein	F: 5' GCC ACA TCA TAC TCA CCG AC 3'	178
	R: 5' AAG AGC TGG AAG CAC GAC AC 3'	
cDNA-AFLP		
ATP synthase subunit C family protein[a]	F: 5' TAT GTG GTG CTC GAG ATG AAC 3'	213
	R: 5' TGC TCT CAC CGA AGC TAT TGC 3'	
Cytochrome P450 monooxygenase[a]	F: 5' GGT GTA GGA TAA GTG GGA GCC 3'	215
	R: 5' TCA TCT TAG GAC ACC TGC GTT A 3'	
Lipin, N-terminal conserved region[a]	F: 5' GTG GAG TTG CGG TGA ATC GTC 3'	204
	R: 5' CTG CGT GCC AGA GAC CTT GTA 3'	
Hypothetical protein	F: 5' CAA CCC TTG GAA CCA CCT AC 3'	206
	R: 5' TTG ACT GCA AGA CTC ACC CG 3'	
Hypothetical protein[b]	F: 5' TCG CTA TGA ACG CTT GGC TG 3'	171
	R: 5' GGA ATT GAC GAT GAG TCC TGA G 3'	
Hypothetical protein	F: 5' CTT TCT CGC TGG TAG AGG TTC C 3'	240
	R: 5' CGA CGG TTC AGG AGG TAT GTG 3'	
Proteomics		
Cysteine protease[b]	F: 5' CAG GTA CGT CAG CGA GTT CA 3'	201
	R: 5' TGT CTC CAG TCC CAC GAA G 3'	
Sucrose transporter 4	F: 5' TGG CTT TGT GGA CCT ATT ACT G 3'	192
	R: 5' GCA GTG CTC AGT GGT ATC TCC 3'	

[a], analyzed by quantitative real-time PCR

[b,] analyzed bysemi-quantitative RT PCR

CHAPTER III

RESULTS

3.1 Identification of genes involving salt tolerance mechanisms in rice analyzed by RAP-PCR

3.1.1 Total RNA extraction

Total RNA extracted from leaves of Homjan and Pathumthani1 at different time intervals before and after salt stress revealed several discrete bands along with smear high and low molecular weight RNA (Figure 3.1). The ratio of OD_{260}/OD_{280} of the extracted total RNA was 1.9-2.1. The first strand cDNA was reverse-transcribed using oligo-dT(16)-overhang with A (oligodT_{+A}, 1 μM) as the synthesizing primer. The resulting products ranged between 100 bp - >2 kb indicating the acceptable quality of the synthesized first stand cDNA (Figure 3.2).

3.1.2 RAP-PCR

Fifteen primer combinations were screened across the first strand cDNA of IR29 and Pokkali varities. The amplification products were analyzed by agarose gel electrophoresis. Primers that did not generated the amplification products and those

Figure 3.1 A 1.0% ethidium bromide-stained agarose gel showing the quality of total RNA extracted from leaves of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hours post treatment (hpt) of Homjan (lanes 1-5) and Pathumthani1 (lanes 6-8) varieties. Lanes M and m are a λ-*Hin*d III and 100 bp DNA ladder, respectively.

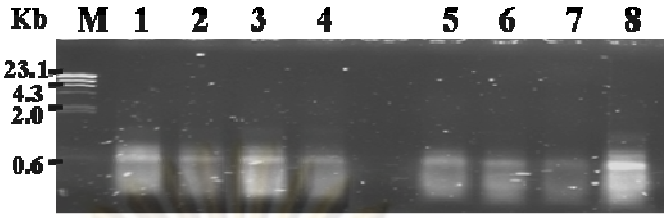

Figure 3.2 A 1.0% ethidium bromide stained agarose gel showing the first strand cDNA (using oligodT_{+A} as the synthesizing primer) of Homjan (lanes 1-4) and Pathumthani1 (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt. Lane M is a λ-*Hin*d III.

showed small amplification product (<300 bp) were not further analyzed by denaturing polyacrylamide gel electrophoresis (Figures. 3.3-3.4). The amplification products generated from appropriate primers (Figures. 3.5-3.9) were further analyzed by denaturing polyacylamid electrophoresis (Figures. 3.10 – 3.14).

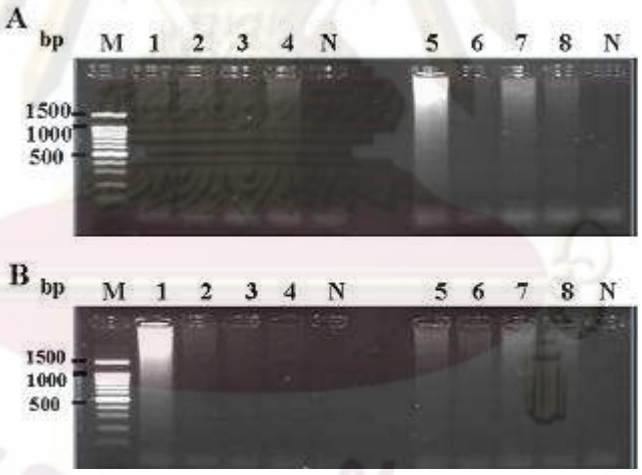

Figure 3.3 A 2.0% ethidium bromide-stained agarose gel showing RAP-PCR products generated from the first strand cDNA (using oligodT_{+A} as the synthesizing primer) of Homjan (lanes 1-4, panels A and B) and Pathumthani1 (lanes 5-8, panel A and B) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/OPA 01 (panel A), and oligodT_{+A}/OPB 03 (panel B). Lanes M and N is a 100 bp ladder and the negative control (without cDNA template), respectively.

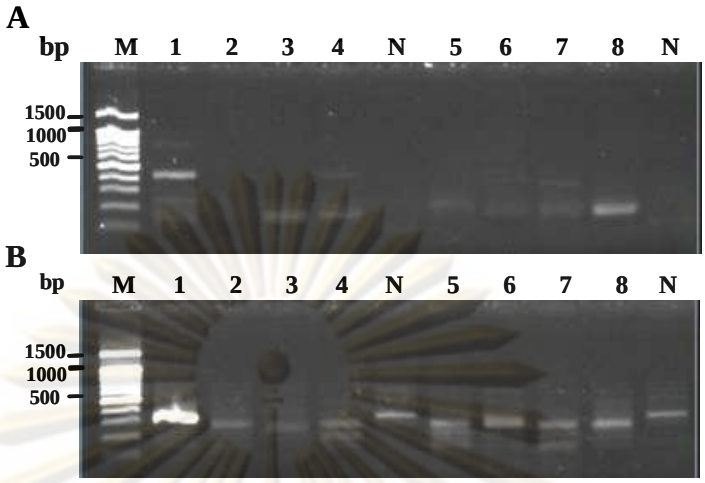

Figure 3.4 A 2.0% ethidium bromide-stained agarose gel showing RAP-PCR products generated from the first strand cDNA (using oligodT_{+A} as the synthesizing primer) of Homjan (lanes 1-4, panels A and B) and Pathumthani1 (lanes 5-8, panels A and B) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC 138 (panel A) and oligodT_{+A}/UBC 228 (panel B). Lanes M and N is a 100 bp ladder and the negative control (without cDNA template), respectively.

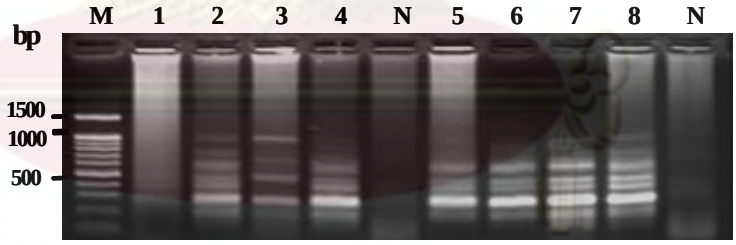

Figure 3.5 A 2.0% ethidium bromide-stained agarose gel showing RAP-PCR products generated from the first strand cDNA (using oligodT_{+A} as the synthesizing primer) of Homjan (lanes 1-4) and Pathumthani1 (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC101. Lanes M and N is a 100 bp ladder and the negative control (without cDNA template), respectively.

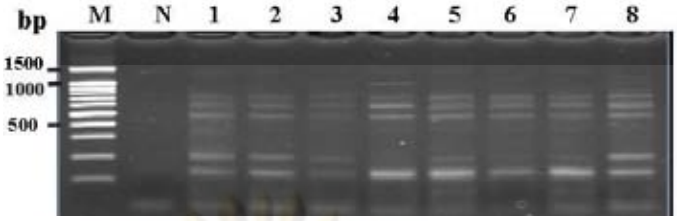

Figure 3.6 A 2.0% ethidium bromide-stained agarose gel showing RAP-PCR products generated from the first strand cDNA (using oligodT_{+A} as the synthesizing primer) of Homjan (lanes 1-4) and Pathumthani1 (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC158. Lanes M and N is a 100 bp ladder and the negative control (without cDNA template), respectively.

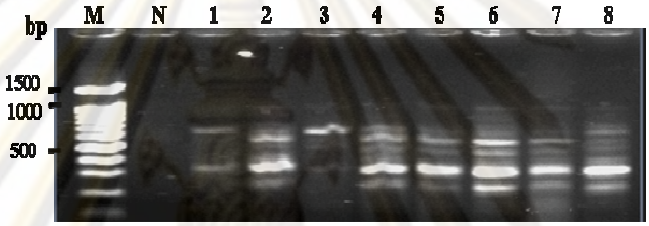

Figure 3.7 A 2.0% ethidium bromide-stained agarose gel showing RAP-PCR products generated from the first strand cDNA (using oligodT_{+A} as the synthesizing primer) of Homjan (lanes 1-4) and Pathumthani1 (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC222. Lanes M and N is a 100 bp ladder and the negative control (without cDNA template), respectively.

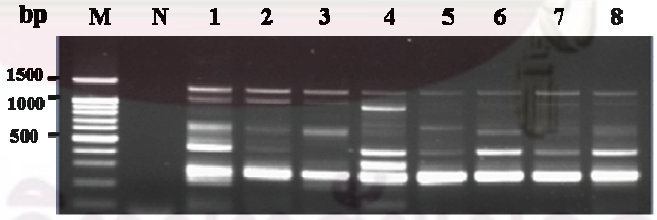

Figure 3.8 A 2.0% ethidium bromide-stained agarose gel showing RAP-PCR products generated from the first strand cDNA (using oligodT_{+A} as the synthesizing primer) of Homjan (lanes 1-4) and Pathumthani1 (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC268. Lanes M and N is a 100 bp ladder and the negative control (without cDNA template), respectively.

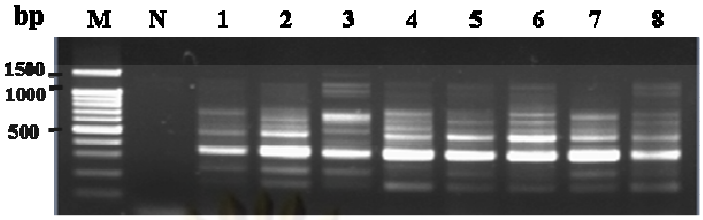

Figure 3.9 A 2.0% ethidium bromide-stained agarose gel showing RAP-PCR products generated from the first strand cDNA (using oligodT_{+A} as the synthesizing primer) of Homjan (lanes 1-4) and Pathumthani1 (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC457. Lanes M and N is a 100 bp ladder and the negative control (without cDNA template), respectively.

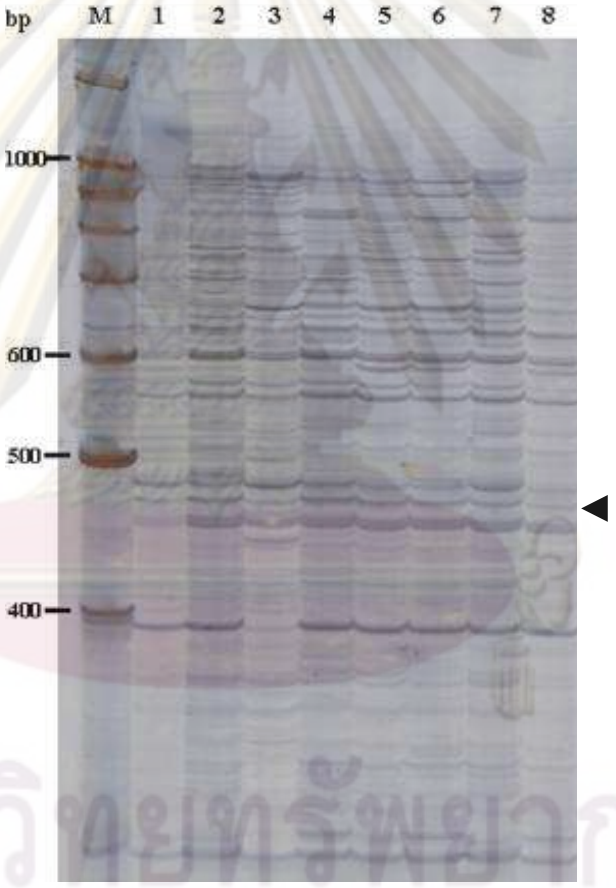

Figure 3.10 A 4.5% denaturing polyacrylamide gel showing RAP-PCR patterns of Homjan (lanes 1-4) and Pathumthani1 (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC101. An arrowhead indicates a candidate RAP-PCR fragment preferentially expressed in the Pathumthani1 variety. Lanes M is 100 bp DNA ladder.

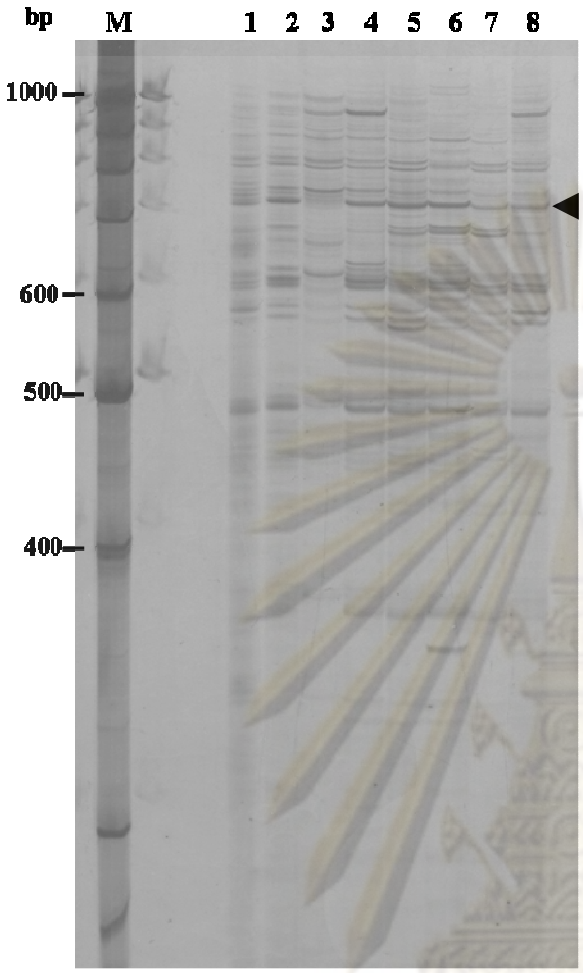

Figure 3.11 A 4.5% denaturing polyacrylamide gel showing RAP-PCR patterns of Homjan (lanes 1, 2, 3 and 4) and Pathumthani1 (lanes 5, 6, 7 and 8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC158. Lane M is 100 bp DNA ladder.

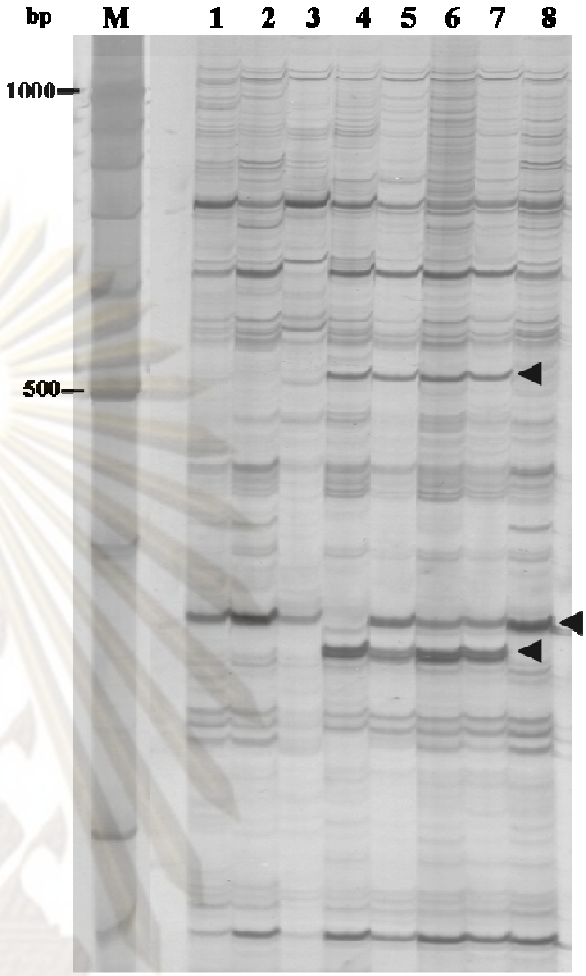

Figure 3.12 A 4.5% denaturing polyacrylamide gel showing RAP-PCR patterns of Homjan (lanes 1-4) and Pathumthani1 (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC222. An arrowhead indicates a candidate RAP-PCR fragments differentially expressed between rice varieties. Lane M is 100 bp DNA ladder.

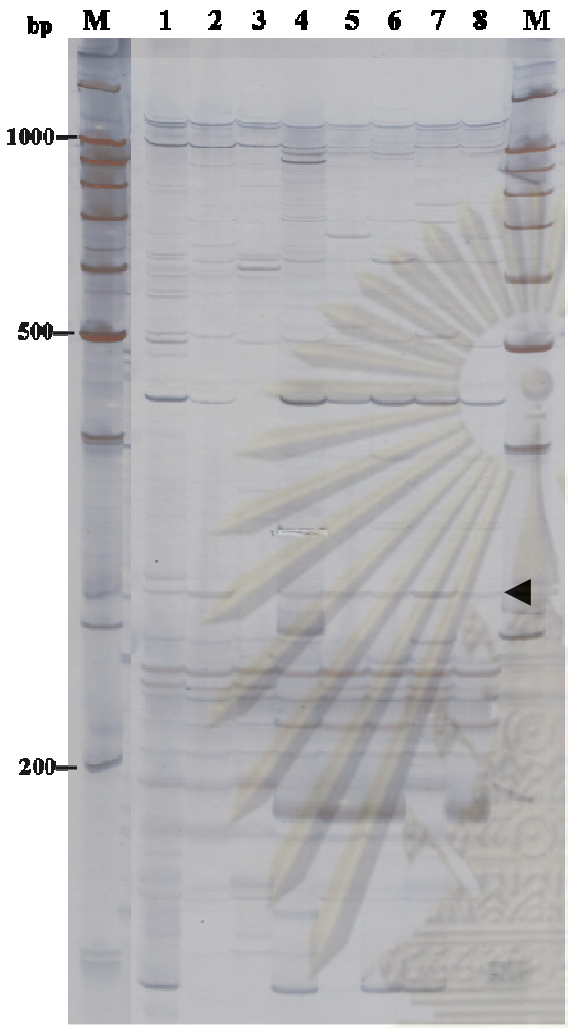

Figure 3.13 A 4.5% denaturing polyacrylamide gel showing RAP-PCR patterns of Homjan (lanes 1-4) and Pathumthani1 (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC268. An arrowhead indicates a candidate RAP-PCR fragment differentially expressed between rice varieties. Lane M is 100 bp DNA ladder.

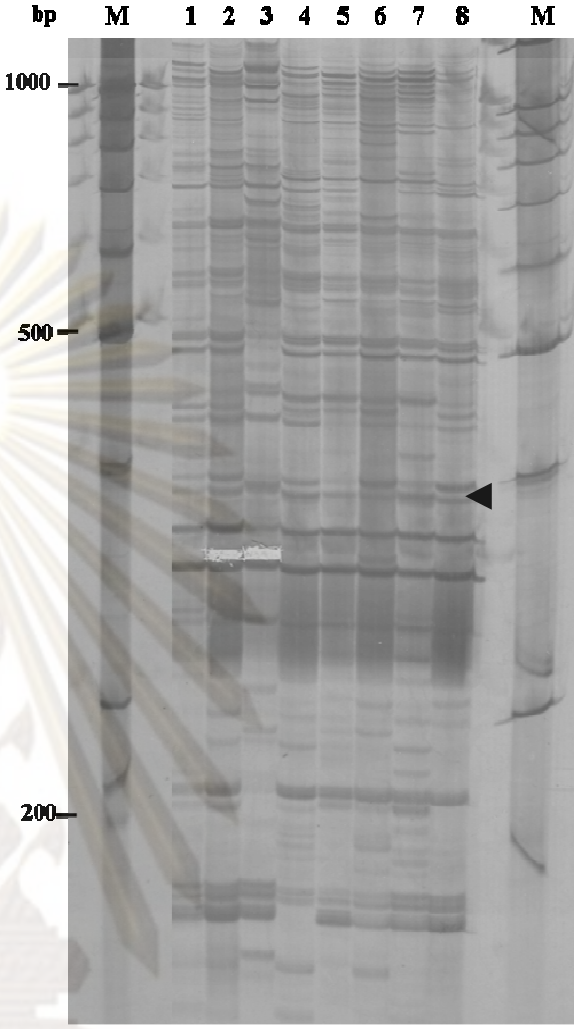

Figure 3.14 A 4.5% denaturing polyacrylamide gel showing RAP-PCR patterns of Homjan (lanes 1-4) and Pathumthani1 (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt using primers oligodT_{+A}/UBC457. An arrowhead indicates a candidate RAP-PCR fragment differentially expressed between rice varieties. Lane M is 100 bp DNA ladder.

Several RAP-PCR fragments exhibited differentially expressed profiles between two rice varieties during different time intervals of salt stress (0, 3, 6, 12 and 24 hour post treatment, hpt). In addition, 24 RAP-PCR bands showed differential expression between Homjan and Pathumthani1 and only one RAP-PCR bands were only found in Homjan variety at 12 hpt, respectively. Of these, four fragments were cloned and sequenced.

3.1.3 Cloning and characterization of RAP-PCR fragments

Fragment showing differential expression patterns between two rice varieties were reamplified by the original primers (Figure 3.15). The reamplified products of oligodT$_{+A}$/UBC101_480 contained non-specific amplification bands. This candidate RAP-PCR marker was not further characterized. On the other hand, the discrete expected band was obtained from reamplification of oligodT$_{+A}$/UBC457_370, oligodT$_{+A}$/UBC457_360, oligodT$_{+A}$/UBC268_320, oligodT$_{+A}$/UBC222_510, oligodT$_{+A}$/UBC222_380 and oligodT$_{+A}$/UBC222_350 by each original primer combination. These fragments were cloned and sequenced. Positive recombinant clones were identified by colony PCR (Figure 3.16).

Table 3.1 Examples of candidate differential expressed RAP-PCR fragments of rice

Primer	Expression of RAP-PCR fragment		
	Presence in Homjan	**Presence in** Pathumthani1	**Differential expression**
oligodT$_{+A}$/UBC101	-	-	950, 850, 750, 680, **480**
oligodT$_{+A}$/UBC158	-	-	950, 720, 490
oligodT$_{+A}$/UBC222	-	-	710, **510**, 450, **380, 350**
oligodT$_{+A}$/UBC268	300	-	1000, 630, 450, **320**, 250
oligodT$_{+A}$/UBC457	-	-	1000, 720, 460, 440, **370, 360**

Fragment that were cloned and sequenced were boldfaced. * and ** = fragments that were used for quantitative RT PCR analysis. - = not found.

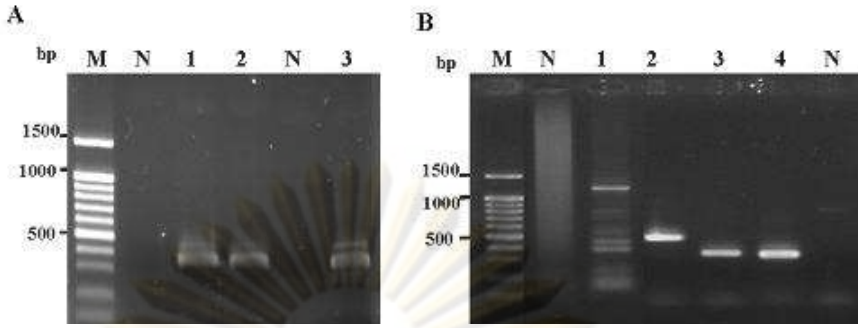

Figure 3.15 A 1.5% ethidium bromide-stained agarose gel showing the reamplified products of oligodT_{+A}/UBC457_370 and oligodT_{+A}/UBC457_360 (lanes 1 and 2 panel A), oligodT_{+A}/UBC268_320 (lanes 3 panel A), oligodT_{+A}/UBC101_490 (lane 1 panel B), oligodT_{+A}/UBC222_510, oligodT_{+A}/UBC222_380 and oligodT_{+A}/UBC222_350 (lanes 2-4 panel B). Lanes M and N are a 100 bp ladder and the negative control (without the cDNA template), respectively.

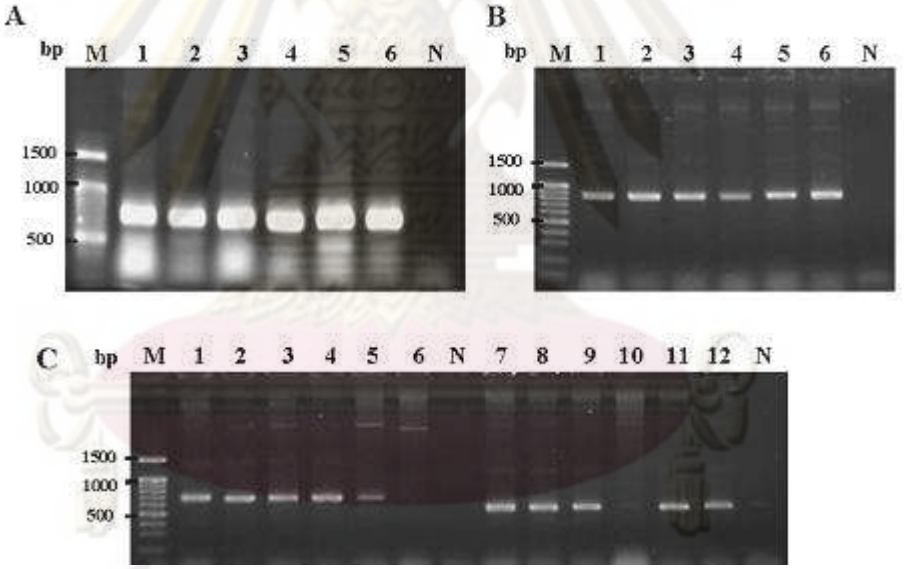

Figure 3.16 A 1.5% ethidium bromide-stained agarose gel showing colony PCR products of oligodT_{+A}/UBC457_370 (lanes 1-6, panel A), oligodT_{+A}/UBC222_510 (lanes 1-6, panel B), oligodT_{+A}/UBC222_380 (lanes 1–6, panel C).and oligodT_{+A}/UBC222_350 (lanes 7–12, panel C) Lanes M and N are a 100 bp ladder and the negative control (without the cDNA template), respectively.

Nucleotide sequences of the recombinant clones were subjected to similarity search using Blast*N* and Blast*X*. The oligodT_{+A}/UBC457_370 significantly matched *Zinc knuckle* of *O. sativa* (6e-12) whereas the oligodT_{+A}/UBC222_380 was significantly similar to *protein kinase domain containing protein* of *O. sativa* (1e-40) (Figure 3.17).

A

CGACGCCCTGAAGAGAAGATAAAGTGAAGTGACGATTAGGGTTTCGTCCTGATTCCCAGCCG
TTGCCTGTGGTGTTGGGTGTTCGTTCGTTGGTTCCGCTGCTGCTGCTGTTGTTGGTGTTTCC
TCGTCAGCGTCGTCGGTTGCATTCTCGGACTGTTCTGAGCTGCAACCTAAGTTAAGGTAAAT
AAGTCCTTTGTTTATGTTAAGGATTTGCAATGATTCAAATTTGTCACCGTGGGTACCAGCGC
TATGTCCTGGGACTGGTACTGAGATCGCGGTTTCGTAGGAAGCAATTCGCGCCGTTTTTCCT
ACGACACGCTCCAGTCAAGTGCCGTTGTACGGCGGTACCAGATTGGGGTGTGAGA

B

CCTCACCGCCGCTTCTCCGAGTCCGCCGTCAGGTTTTATGCGGCGGAGGTGGTGGCGGCGCT
GGAGTACGTCCACATGGTGGACATCGTGTACCGTGACCTGAAGCCGGAGAACGTGCTCGTCC
GCGCCGACG**GCCACATCATGCTCACCGAC**TTCGACCTCTCGCTCAAGTGCGACCCGACGGCG
CCGACGCCGGCGCACGTCATCTCGGACCCCATCGCCCTCGCCGGCGGCCAGTCTTCCTCCTC
CTCCTCCTCGTCGTGCATCATCCCGTCCTGCATCGTCCCCGCCG**TGTCGTGCTTCCAGCTCT**
TCCCAGGACGCGGGCGCCACCGCCGCCGCCGCTGGCGCGGCCGCAAGAAGCCGTCGAGCGGC
GGCGGCGGCAAT

Figure 3.17 Nucleotide sequences of oligodT_{+A}/UBC457_370 (365 bp, A) and oligodT_{+A}/UBC222_380 (384 bp, B) amplified from cDNA of *O. sativa*. The location and sequence of a forward primer (oligodT_{+A}/UBC222-380-F) and those complementary to a reverse primer (oligodT_{+A}/UBC222-380-R) are illustrated in boldface and underlined.

3.2 Identification of genes involving in salt tolerance mechanisms in rice by cDNA-AFLP analysis

3.2.1 RNA extraction

Total RNA was extracted from leaves of IR29 and Pokkali at different time intervals during the salt stress. Several discrete bands along with smear high and low molecular weight RNA were observed (Figure 3.18). The ratio of OD_{260}/OD_{280} of the extracted total RNA was 1.9-2.2. The first strand cDNA was reverse-transcribed using by BD Powerscript™ Reverse Transcriptase (Clontech). The resulting products ranged between 100 bp - >2 kb indicating the acceptable quality of the synthesized first stand cDNA (Figure 3.18). The second strand cDNA was synthesized for 3 cycles of PCR amplification (Figure 3.19).

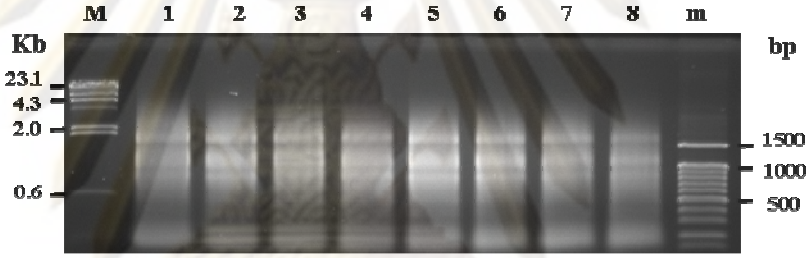

Figure 3.18 A 1.0% ethidium bromide-stained agarose gel showing the quality of total RNA extracted from leaves of IR29 (lanes 1-5) and Pokkali (lanes 5-8) varieties of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt. Lanes M and m are a λ-*Hin*d III and 100 bp DNA ladder, respectively.

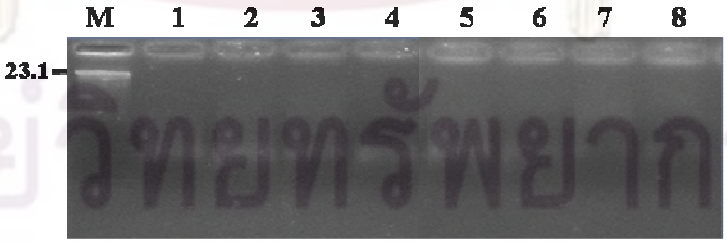

Figure 3.19 A 1.0% ethidium bromide-stained agarose gel showing the synthesized second strand cDNA from leaves of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt of IR29 (lanes 1-4) and Pokkali (lanes 5-8) varieties. Lane M is the λ-*Hin*d III DNA marker.

3.2.2 cDNA-AFLP

The preamplification using E and M primers generated the major products between 400 - 800 bp in size (Figure 3.20 A). In contrast, preamplification with E_{+A} and M_{+C} primers yielded the major product at 800 bp in size (Figure 3.20 B). This indicated successful restriction/ligation of *O. sativa* cDNA to be used as the template for cDNA-AFLP analysis.

The preamplification products were diluted and subjected to selective amplification with a pair of primers containing two or three selective nucleotides. The amplification results were preliminary analyzed by agarose gel electrophoresis.

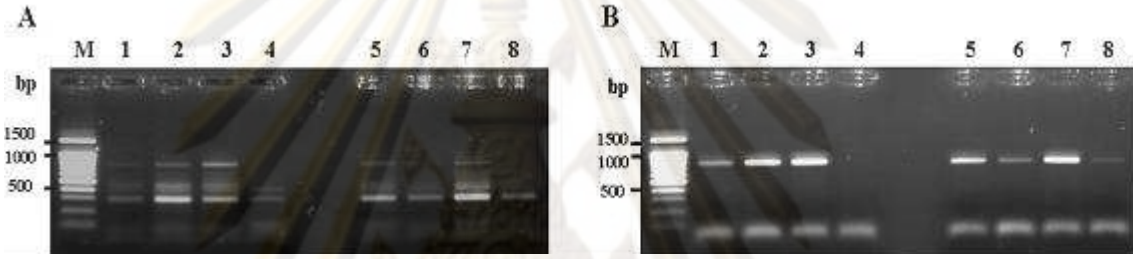

Figure 3.20 1.0% agarose gel electrophoresis showing preamplification products of E and M primers (A) and E+A and M+C primers (B) of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt of IR29 and (lanes 1-4) and Pokkali (lanes 5-8) varieties. Lane M is a 100 bp DNA ladder.

A total of 120 primer combinations were screened (Table 3.2). Seventy-six and twenty-seven combinations showed no amplification products (Figure 3.21) and small positive amplification product (<300 bp) or the single band (Figure 3.22), respectively.

Only seventeen primer combinations that yielded appropriate sizes of the positive amplification products (Figures 3.23 - 3.27) were further analyzed by denaturing polyacrylamide gel electrophoresis (Figures 3.28 -3.36).

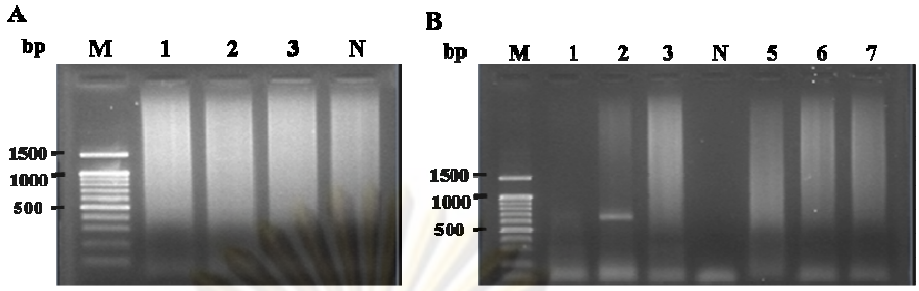

Figure 3.21 1.5% agarose gel electrophoresis showing the amplification products of E-1/M-7 (lanes 1-4, panel A), E_{+A}-6/ M_{+C}-2 (lanes 1-4, panel B) and E_{+A}-6/M_{+C}-3 (lanes 5-8, panel B). Lanes M and N are a 100 bp DNA ladder and the negative control (without the cDNA template), respectively.

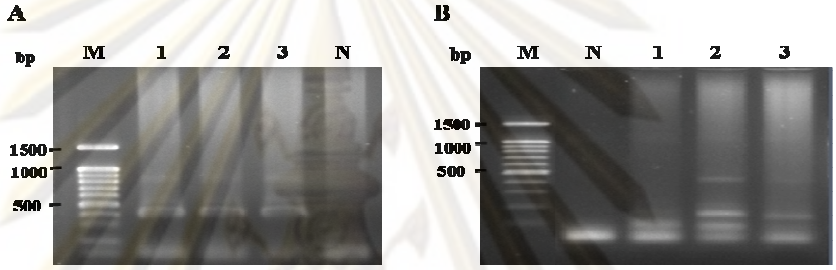

Figure 3.22 1.5% agarose gel electrophoresis showing the single amplified fragment from E-4/M-5 (panel A) or small sizes of the amplified products (< 300 bp) from E_{+A}-2/ M_{+C}-5 (panel B). Lanes M and N are a 100 bp DNA ladder and the negative control (without the cDNA template), respectively.

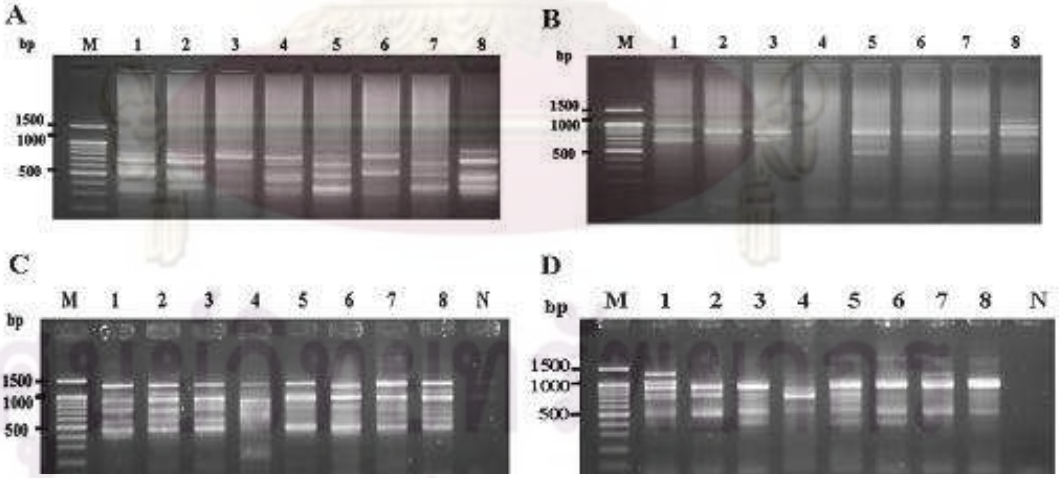

Figure 3.23 1.5% agarose gel electrophoresis showing selective amplification products of E-1/M-1 (panel A), E-1/M-4 (panel B), E-1/ M-5 (panel C) and E-1/ M-6 (panel D) of the control group (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt of IR29 (lanes 1-4) and Pokkali (lanes 5-8) varieties. Lanes M and N are a 100 bp DNA ladder and the negative control (without the cDNA template), respectively.

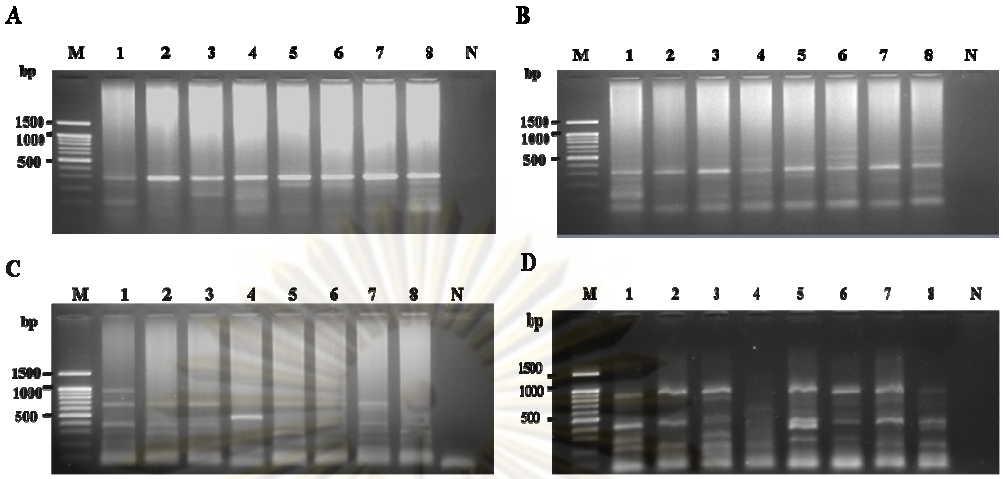

Figure 3.24 1.5% agarose gel electrophoresis showing selective amplification products of E-2/M-1 (panel A), E-2/M-5 (panel B), E-3/ M-6 (panel C) and E-4/ M-9 (panel D) of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt of IR29 (lanes 1-4) and Pokkali (lanes 5-8) varieties. Lanes M and N are a 100 bp DNA ladder and the negative control (without the cDNA template), respectively.

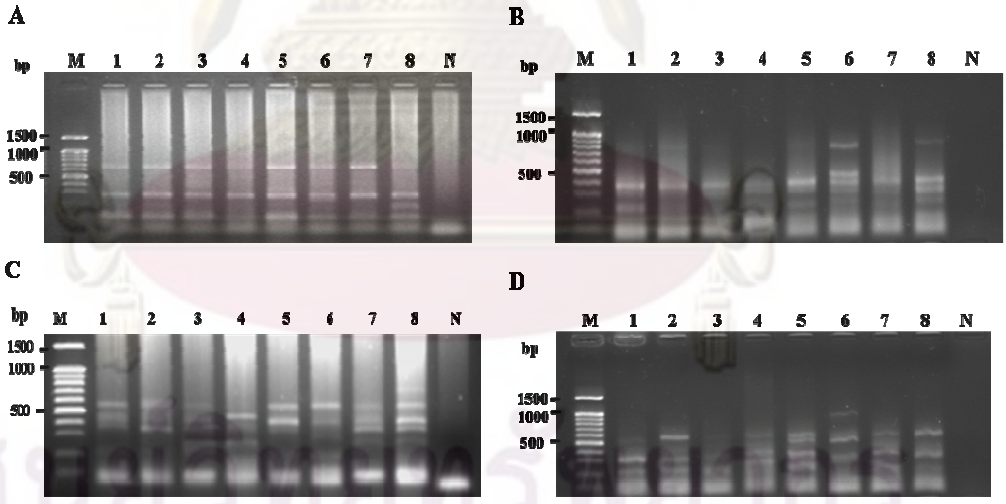

Figure 3.25 1.5% agarose gel electrophoresis showing selective amplification products of E-5/M-5 (panel A), E-3/M-1 (panel B), E-5/ M-8 (panel C) and E-6/ M-8 (panel D) of the control group (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt of IR29 (lanes 1-4) and Pokkali (lanes 5-8) varieties. Lanes M and N are a 100 bp DNA ladder and the negative control (without the cDNA template), respectively.

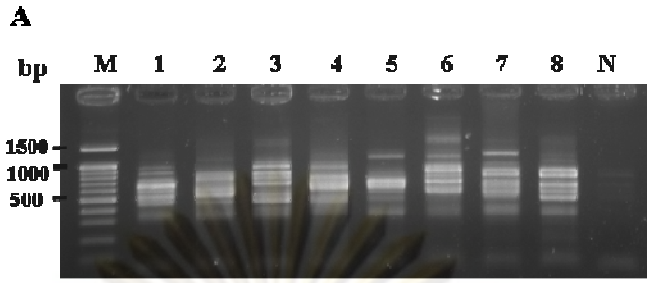

Figure 3.26 1.5% agarose gel electrophoresis showing selective amplification products of E-1/M-7 (panel A) of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt of IR29 (lanes 1-4) and Pokkali (lanes 5-8). Lanes M and N are a 100 bp DNA ladder and the negative control (without the cDNA template), respectively.

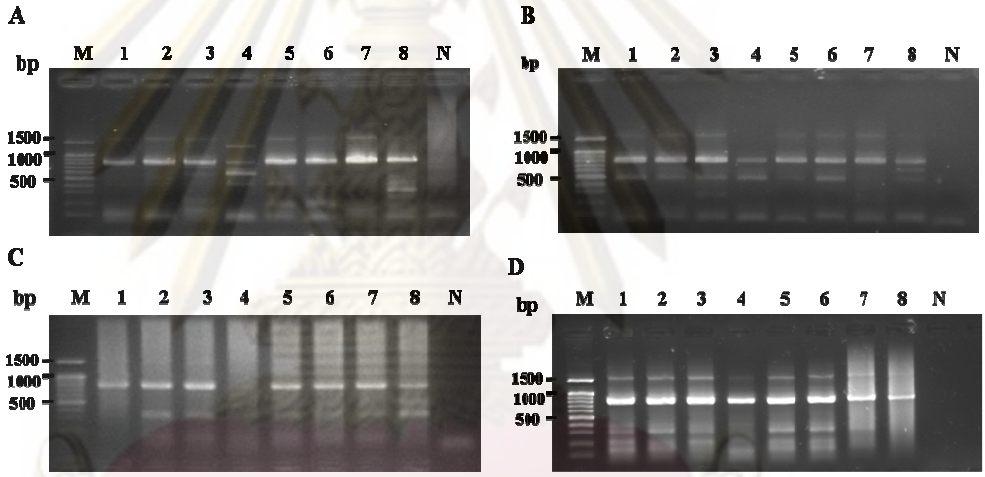

Figure 3.27 1.5% agarose gel electrophoresis showing selective amplification products of E_{+A}-1/M_{+C}-2 (panel A), E_{+A}-2/M_{+C}-1 (panel B), E_{+A}-5/M_{+C}-2 (panel C) and E_{+A}-5/M_{+C}-10 (panel D) of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt of IR29 (lanes 1-4) and Pokkali (lanes 5-8) varieties. Lanes M and N are a 100 bp DNA ladder and the negative control (without the cDNA template), respectively.

Table 3.2 Amplification results from cDNA-AFLP primer combinations screened using the cDNA template from leaves of IR29 and Pokkali varieties

Primer	Result	Primer	Result
E-1/ M-1*	++	E-4/ M-1	+
E-1/ M-2	-	E-4/ M-2	+
E-1/ M-3	-	E-4/ M-3	-
E-1/ M-4*	++	E-4/ M-4	-
E-1/ M-5*	++	E-4/ M-5	+
E-1/ M-6*	++	E-4/ M-6	+
E-1/ M-7*	++	E-4/ M-7	+
E-1/ M-8	-	E-4/ M-8	-
E-1/ M-9	-	**E-4/ M-9***	++
E-1/ M-10	-	E-4/ M-10	+
E-2/ M-1*	++	E-5/ M-1	+
E-2/ M-2	-	E-5/ M-2	-
E-2/ M-3	-	E-5/ M-3	-
E-2/ M-4	-	E-5/ M-4	+
E-2/ M-5*	++	**E-5/ M-5***	++
E-2/ M-6	-	E-5/ M-6	+
E-2/ M-7	+	E-5/ M-7	-
E-2/ M-8	-	**E-5/ M-8***	++
E-2/ M-9	+	E-5/ M-9	-
E-2/ M-10	-	E-5/ M-10	-
E-3/ M-1*	++	E-6/ M-1	-
E-3/ M-2	-	E-6/ M-2	-
E-3/ M-3	-	E-6/ M-3	-
E-3/ M-4	+	E-6/ M-4	+
E-3/ M-5	-	E-6/ M-5	+
E-3/ M-6*	++	E-6/ M-6	+
E-3/ M-7	-	E-6/ M-7	-
E-3/ M-8	+	**E-6/ M-8***	++
E-3/ M-9	-	E-6/ M-9	-
E-3/ M-10	-	E-6/ M-10	-

Table 3.2 (cont.)

Primer	Result	Primer	Result
E+A-1/ M+C-1	-	E+A-4/ M+C-1	-
E+A-1/ M+C-2*	++	E+A-4/ M+C-2	-
E+A-1/ M+C-3	-	E+A-4/ M+C-3	-
E+A-1/ M+C-4	-	E+A-4/ M+C-4	-
E+A-1/ M+C-5	+	E+A-4/ M+C-5	-
E+A-1/ M+C-6	-	E+A-4/ M+C-6	-
E+A-1/ M+C-7	-	E+A-4/ M+C-7	-
E+A-1/ M+C-8	-	E+A-4/ M+C-8	-
E+A-1/ M+C-9	-	E+A-4/ M+C-9	-
E+A-1/ M+C-10	-	E+A-4/ M+C-10	-
E+A-2/ M+C-1*	++	E+A-5/ M+C-1	+
E+A-2/ M+C-2	+	**E+A-5/ M+C-2***	++
E+A-2/ M+C-3	-	E+A-5/ M+C-3	-
E+A-2/ M+C-4	+	E+A-5/ M+C-4	+
E+A-2/ M+C-5	+	E+A-5/ M+C-5	+
E+A-2/ M+C-6	-	E+A-5/ M+C-6	-
E+A-2/ M+C-7	-	E+A-5/ M+C-7	-
E+A-2/ M+C-8	-	E+A-5/ M+C-8	+
E+A-2/ M+C-9	+	E+A-5/ M+C-9	-
E+A-2/ M+C-10	-	**E+A-5/ M+C-10***	++
E+A-3/ M+C-1	+	E+A-6/ M+C-1	-
E+A-3/ M+C-2	-	E+A-6/ M+C-2	-
E+A-3/ M+C-3	-	E+A-6/ M+C-3	-
E+A-3/ M+C-4	-	E+A-6/ M+C-4	+
E+A-3/ M+C-5	-	E+A-6/ M+C-5	+
E+A-3/ M+C-6	-	E+A-6/ M+C-6	-
E+A-3/ M+C-7	-	E+A-6/ M+C-7	-
E+A-3/ M+C-8	-	E+A-6/ M+C-8	-
E+A-3/ M+C-9	-	E+A-6/ M+C-9	-
E+A-3/ M+C-10	-	E+A-6/ M+C-10	-

*The amplification products that were further analyzed by denaturing gel electrophoresis - = no amplification products, + = positive amplification product but sizes were less than 300 bp or single band and ++ = positive amplification products exhibiting appropriate size range but the latter showed more intense amplification products

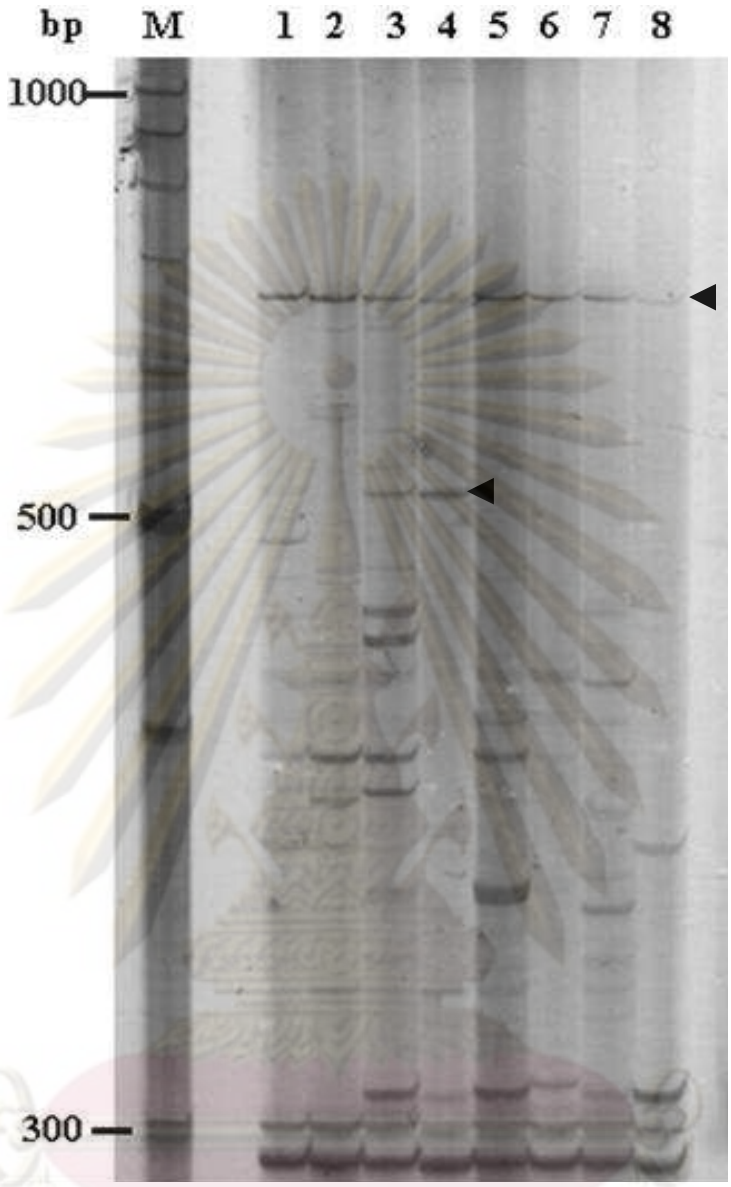

Figure 3.28 A 5.0% denaturing polyacrylamide gel showing cDNA-AFLP products of E-5/M-5 (lane 1-8) amplified using cDNA of the control (lanes 1 and 5) and 3 (lanes 2 and 6), 6 (lanes 3 and 7) and 12 (lanes 4 and 8) hpt of IR29 (lanes 1-4) and Pokkali (lanes 5-8) varieties. An arrowhead indicates a candidate cDNA-AFLP fragment differentially expressed (down regulation) between rice varieties. Lane M is a 100 bp DNA ladder.

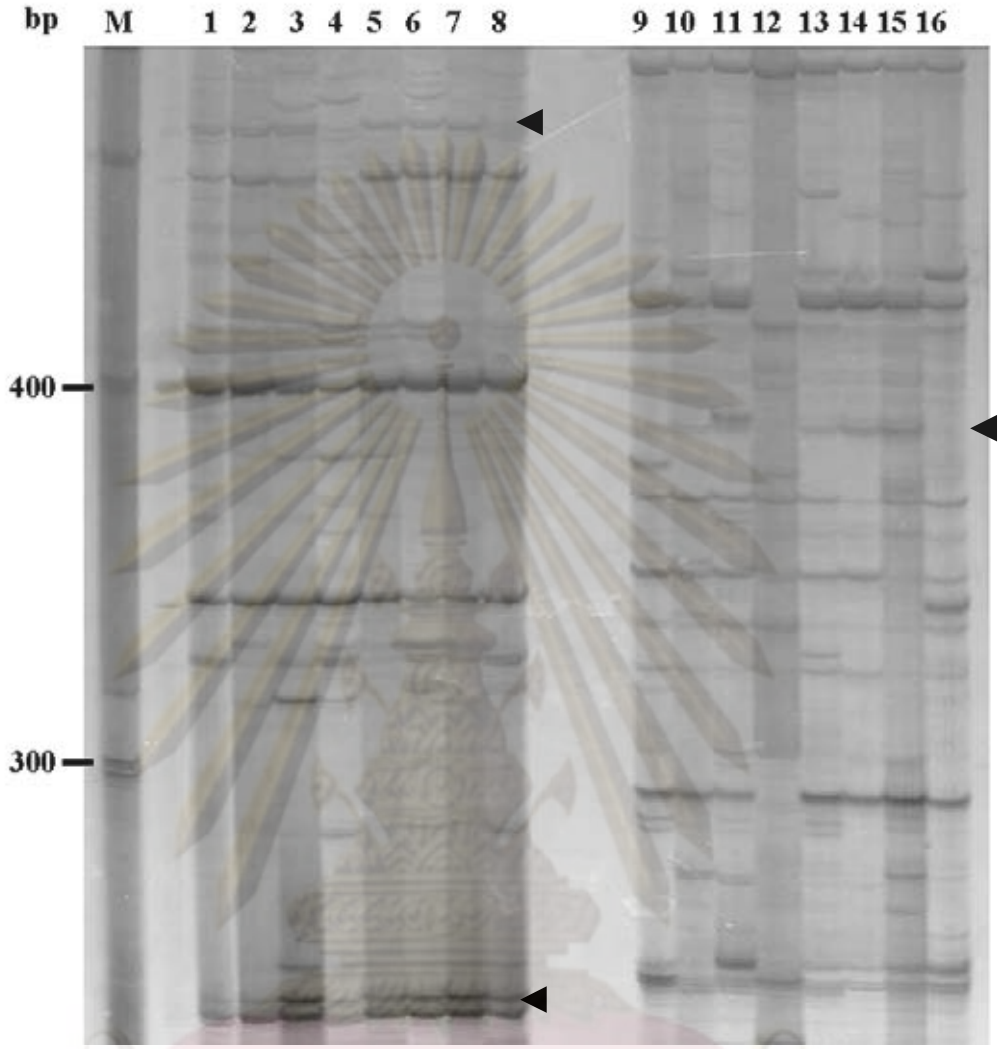

Figure 3.29 A 4.5% denaturing polyacrylamide gel showing cDNA-AFLP products of E-2/M-1 (lane 1- 8) and E-2/M-5 (lane 9 -16) amplified using cDNA of the control (lanes 1, 5, 9 and 13), and 3 (lanes 2, 6, 10 and 14), 6 (lanes 3, 7, 11 and 15) and 12 (lanes 4, 8, 12 and 16) hpt of IR29 (lanes 1-4, and 9-12) and Pokkali (lanes 5-8 and 13-16) varieties. An arrowhead indicates a candidate cDNA-AFLP fragment differentially expressed (down regulation) between rice varieties. Lane M is a 100 bp DNA ladder.

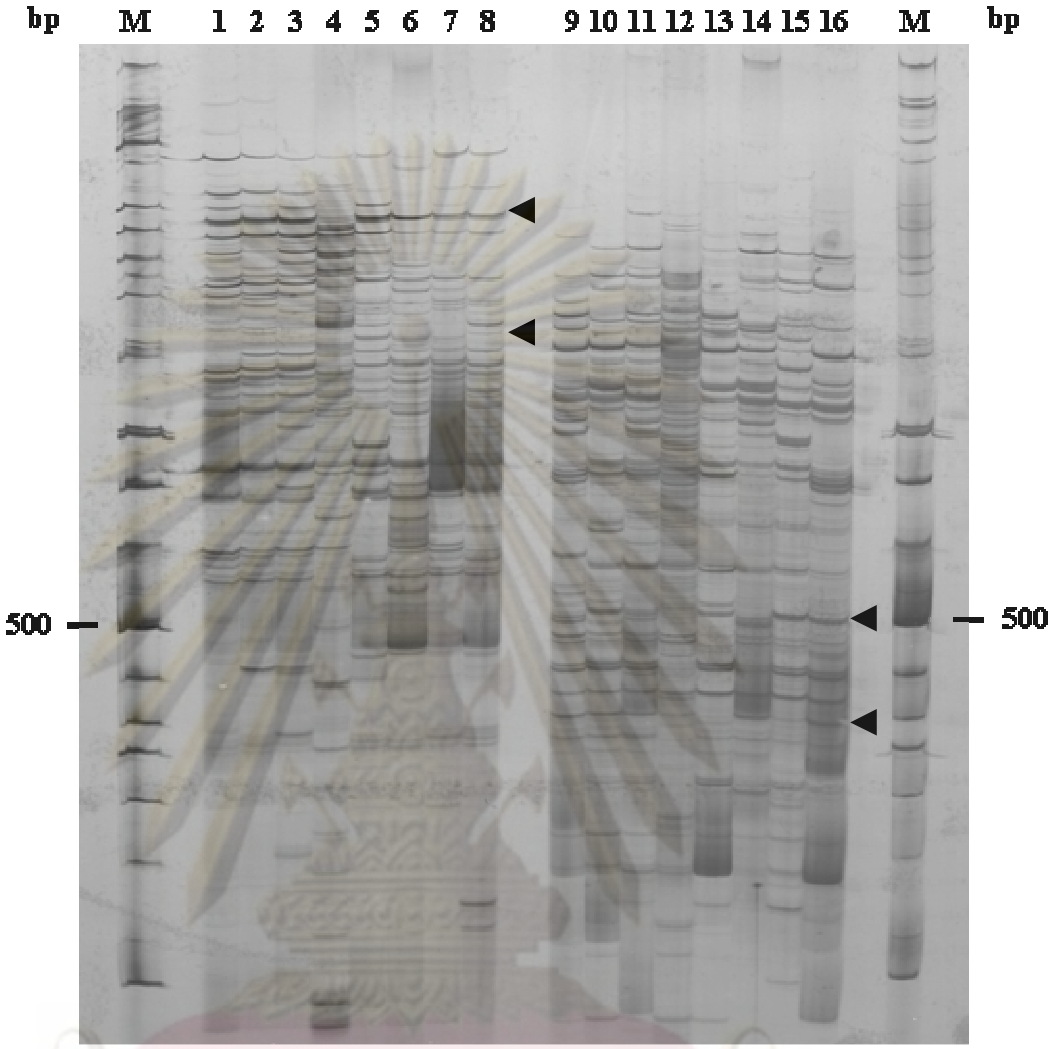

Figure 3.30 A 4.0 % denaturing polyacrylamide gel showing cDNA-AFLP products of E-1/M-6 (lane 1- 8) and E-1/M-7 (lane 9 -16) amplified using cDNA of the control (lanes 1, 5, 9 and 13) and 3 (lanes 2, 6, 10 and 14), 6 (lanes 3, 7, 11 and 15) and 12 (lanes 4, 8, 12 and 16) hpt of IR29 (lanes 1-4 and 9-12) and Pokkali (lanes 5-8, and 13-16) varieties.. An arrowhead indicates a candidate cDNA-AFLP fragment found in the Pokkali variety. Lane M is a 100 bp DNA ladder.

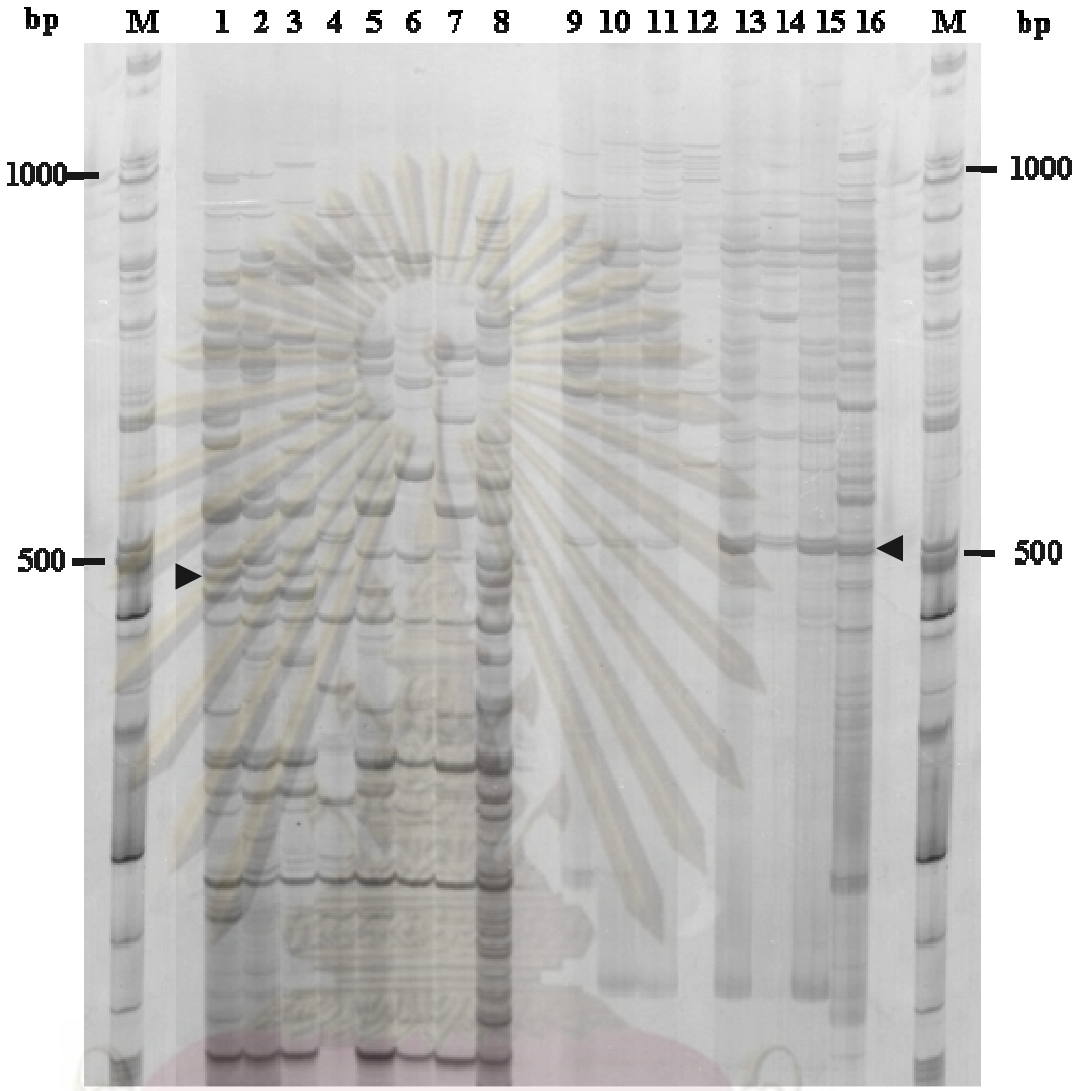

Figure 3.31 A 4.5% denaturing polyacrylamide gel showing cDNA-AFLP products of E-1/M-1 (lane 1-8) and E-1/M-4 (lane 9-16) amplified using cDNA of the control (lanes 1, 5, 9 and 13), and 3 (lanes 2, 6, 10 and 14), 6 (lanes 3, 7, 11 and 15) and 12 (lanes 4, 8, 12 and 16) hpt of IR29 (lanes 1-4 and 9-12) and Pokkali (lanes 5-8 and 13-16) varieties. An arrowhead indicates a candidate cDNA-AFLP fragment differentially expressed between rice varieties. Lane M is a 100 bp DNA ladder.

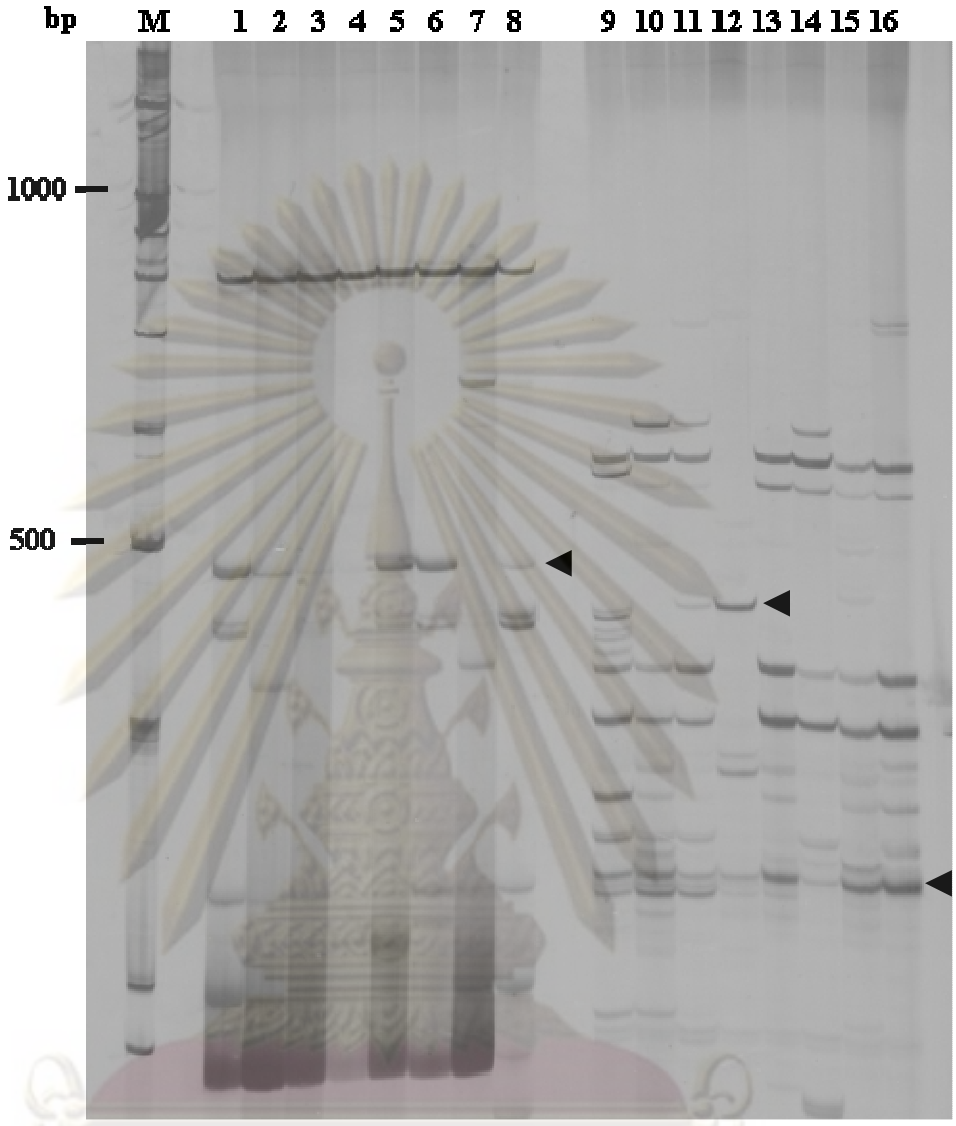

Figure 3.32 A 4.5% denaturing polyacrylamide gel showing AFLP products of E_{+A}-5/M_{+C}-2 (lane 1- 8) and E-5/M-8 (lane 9 -16) amplified using cDNA of the control (lanes 1, 5, 9 and 13), and 3 (lanes 2, 6, 10 and 14), 6 (lanes 3, 7, 11 and 15) and 12 (lanes 4, 8, 12 and 16) hpt of IR29 and (lanes 1-4 and 9-12) and Pokkali (lanes 5-8 and 13-16). An arrowhead indicates a candidate cDNA-AFLP fragment differentially expressed between rice varieties. Lane M is a 100 bp DNA ladder.

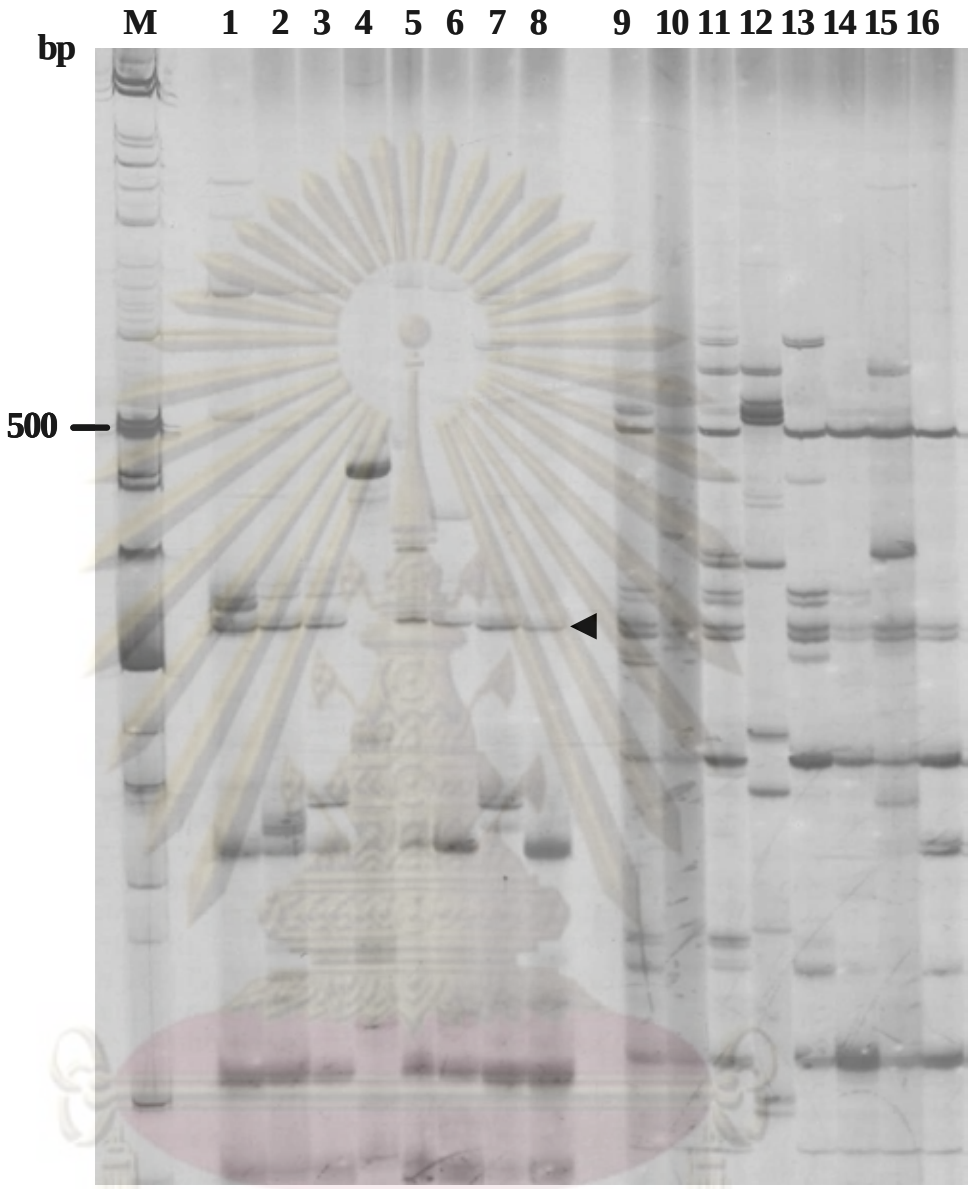

Figure 3.33 A 4.5% denaturing polyacrylamide gel showing cDNA-AFLP products of E-6/M-8 (lane 1- 8) and E-3/M-6 (lane 9 -16) amplified using cDNA of the control (lanes 1, 5, 9 and 13), and 3 (lanes 2, 6, 10 and 14), 6 (lanes 3, 7, 11 and 15) and 12 (lanes 4, 8, 12 and 16) hpt of IR29 (lanes 1-4 and 9-12) and Pokkali (lanes 5-8 and 13-16) varieties. An arrowhead indicates a candidate cDNA-AFLP fragment found in the Pokkali variety. Lane M is a 100 bp DNA ladder.

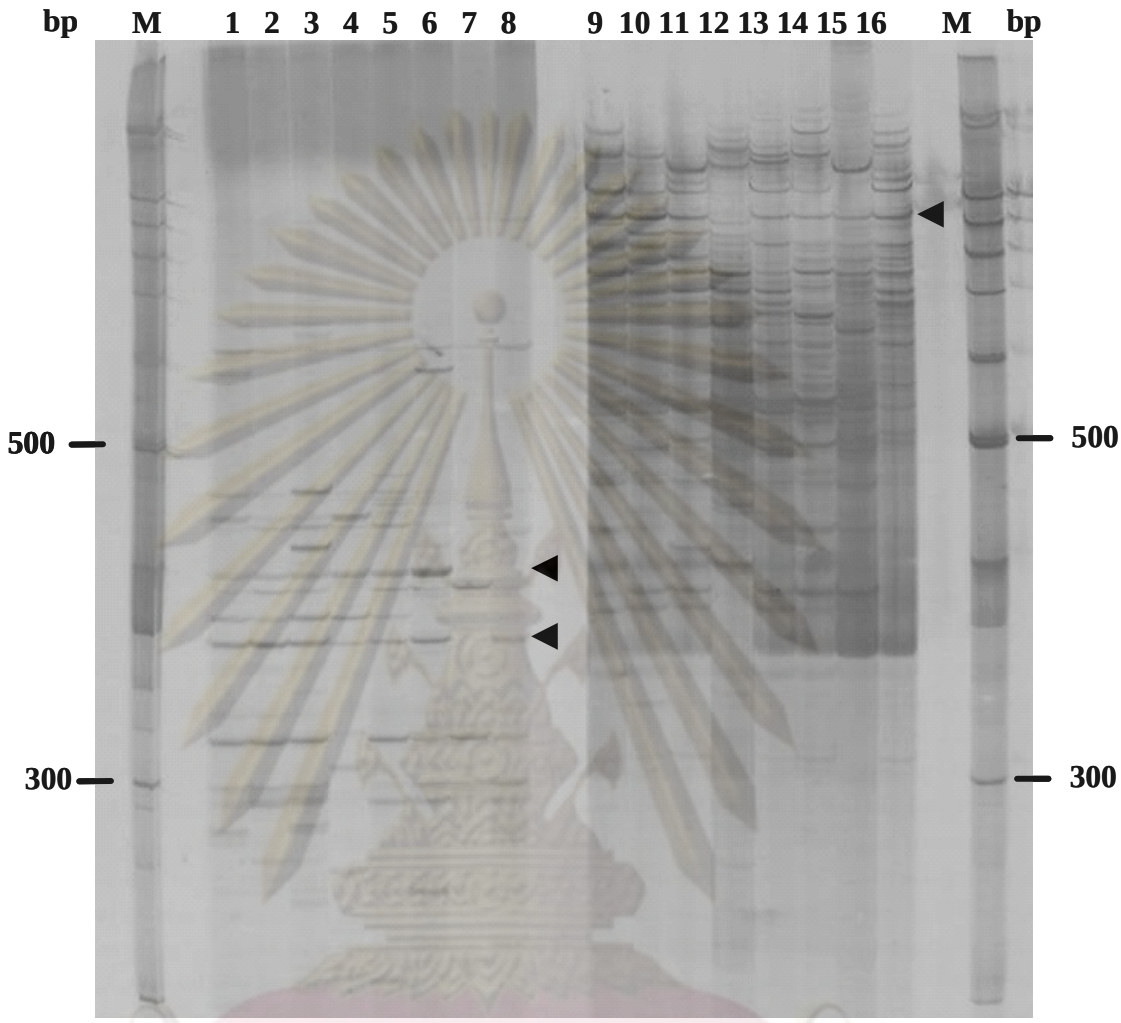

Figure 3.34 A 4.5% denaturing polyacrylamide gel showing cDNA-AFLP products of E-3/M-1 (lane 1- 8) and E-1/M-5 (lane 9 -16) amplified using cDNA of the control (lanes 1, 5, 9 and 13), and 3 (lanes 2, 6, 10 and 14), 6 (lanes 3, 7, 11 and 15) and 12 (lanes 4, 8, 12 and 16) hpt of IR29 (lanes 1-4 and 9-12) and Pokkali (lanes 5-8 and 13-16) varieties. An arrowhead indicates a candidate cDNA-AFLP fragment differentially expressed between rice varieties. Lane M is a 100 bp DNA ladder.

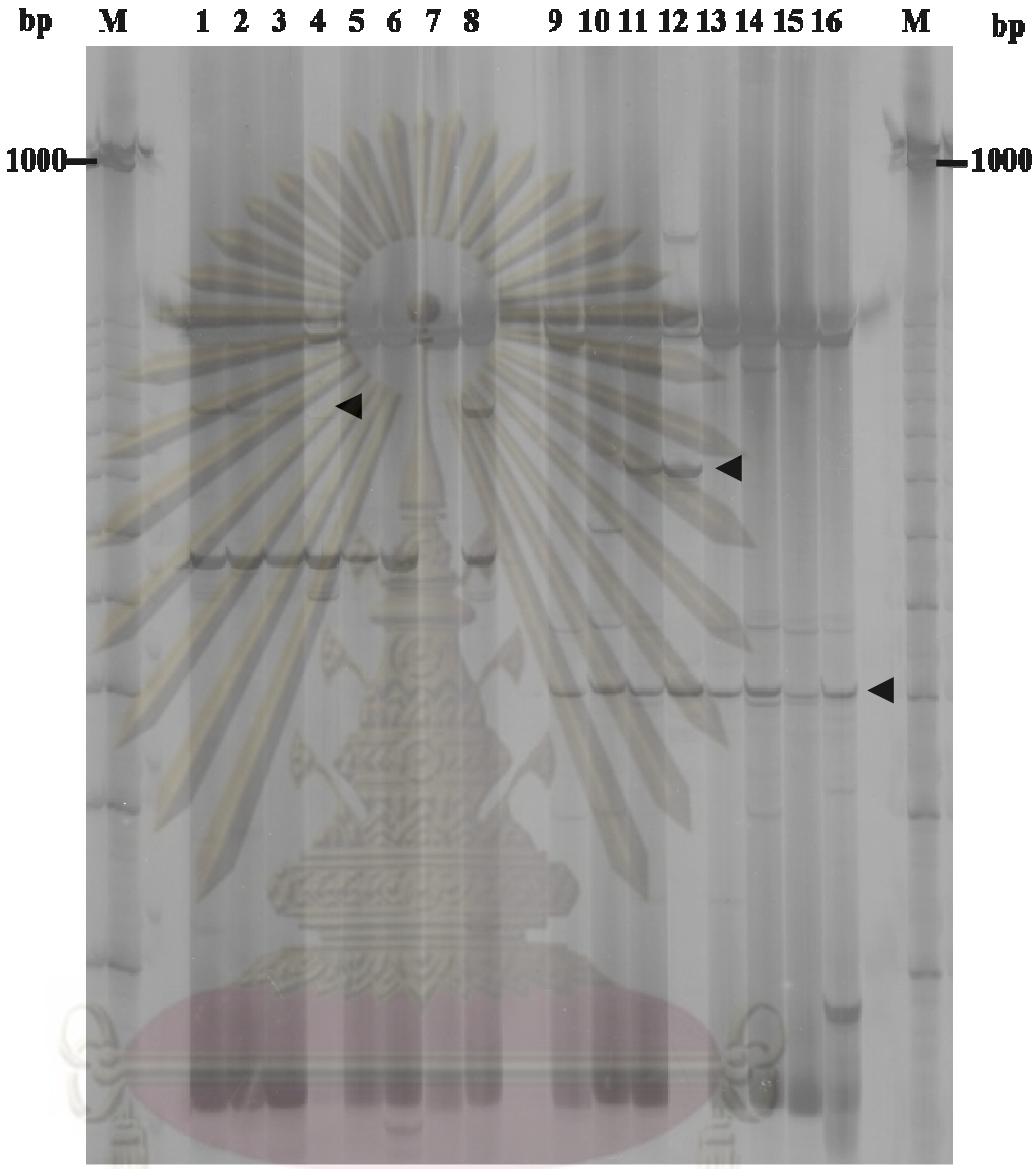

Figure 3.35 A 4.0 % denaturing polyacrylamide gel showing cDNA-AFLP products of E_{+A}-1/ M_{+C}-2 (lane 1- 8) and E_{+A}-2/ M_{+C}-1 (lane 9 -16) amplified using cDNA of the control (lanes 1, 5, 9 and 13), and 3 (lanes 2, 6, 10 and 14), 6 (lanes 3, 7, 11 and 15) and 12 (lanes 4, 8, 12 and 16) hpt of IR29 (lanes 1-4 and 9-12) and Pokkali (lanes 5-8 and 13-16) varieties. An arrowhead indicates a candidate cDNA-AFLP fragment differentially expressed between rice varieties. Lanes M is 50 bp DNA ladders.

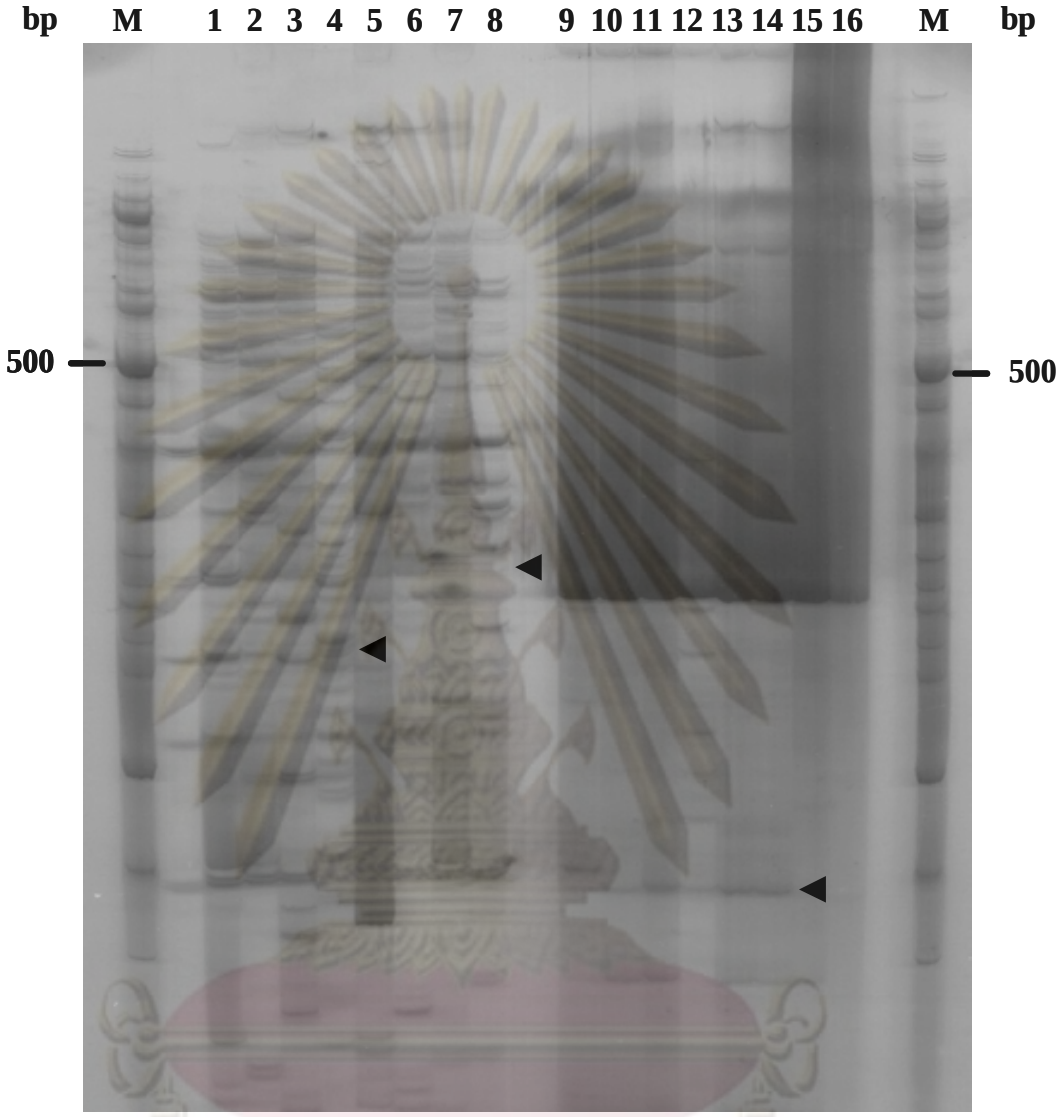

Figure 3.36 A 4.5% denaturing polyacrylamide gel showing AFLP products of E-4/M-9 (lane 1- 8) and E+A-5/ M+C-10 (lane 9 -16) amplified of the control (lanes 1, 5, 9 and 13), and 3 (lanes 2, 6, 10 and 14), 6 (lanes 3, 7, 11 and 15) and 12 (lanes 4, 8, 12 and 16) hpt of IR29 (lanes 1-4 and 9-12) and Pokkali (lanes 5-8 and 13-16) varieties. An arrowhead indicates a candidate cDNA-AFLP fragment differentially expressed between rice varieties. Lanes M is 50 bp DNA ladders.

The expression patterns of cDNA-AFLP fragments were classified to be that exhibiting differential expression between rice varieties and that presence in one but absence in the other variety.

As can be seen from Table 3.3, a large number of fragments were differentially expressed between varieties during the treatment (0, 3, 6 and 12 hour post treatment, hpt). In contrast, 2 and 7 cDNA-AFLP bands were only found in the Pokkali and IR29 groups, respectively. Twenty-four cDNA-AFLP fragments were successfully reamplified. These fragments were cloned and sequenced.

Table 3.3 Examples of candidate differentially expressed cDNA-AFLP fragments between rice varieties

Primer	Expression pattern		
	Presence in IR29	**Presence in Pokkali**	**Differential expression**
E-1/ M-1	-	-	800, 600, 500, **490**, 460, 340
E-1/ M-4	-	-	800, 680, 610, **500**
E-1/ M-5	-	-	1100, **910**, **820**, 700, 520, **380**
E-1/ M-6	450	**810**	**980**, 800, 760, 640, 600
E-1/ M-7	-	-	820, 800, 620, 530 **500**, 450, **300**
E-2/ M-1	-	-	**510**, 420, 330, **250**
E-2/ M-5	-	-	420, 380, 360, 340, 260
E-3/M-1	-	680	**600**, **460**, 430, **410**, **390**, 320
E-3/M-6	-	-	500, 440, 420, 350
E-4/M-9	-	-	680, 610, 500, **360**, **330**
E-5/M-5	510, 380	-	**680**, 390, 330
E-5/M-8	**460**, 330	-	600, 560, 430, **400**, 330
E-6/M-8	480	-	620, 420, **360**
E+A-1/ M+C-2	-	-	850, 640
E+A-2/ M+C-1	700	-	**500**
E+A-5/ M+C-2	-	-	**490**, 460, 330
E+A-5/ M+C-10	-	-	**280**

Fragment that were cloned and sequenced were boldfaced. * and ** = fragments that were used for semiquantitative RT-PCR and real-time PCR analysis, respectively. - = not found.

3.2.3 Cloning and characterization of cDNA-AFLP fragments

Fragment showing differential expression patterns were reamplified with the original primer combinations for each fragment (Figure 3.37). These fragments were cloned and sequenced. Positive recombinant clones were identified by colony PCR (Figure 3.38)

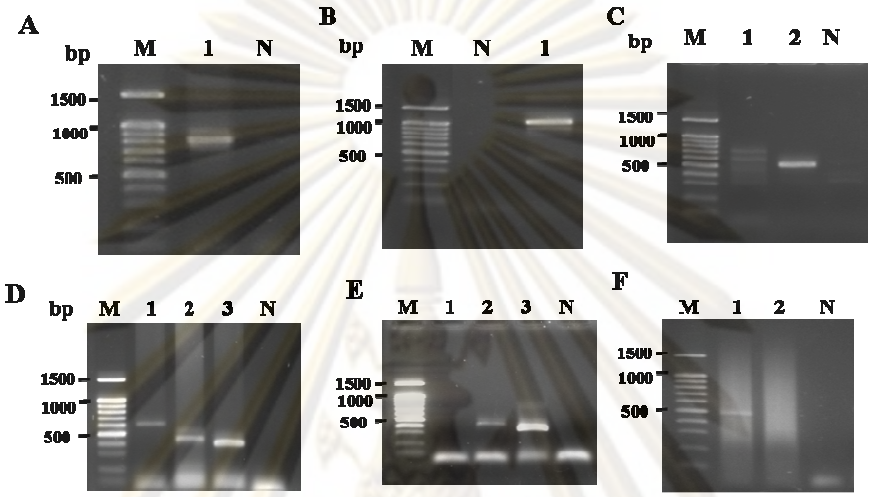

Figure 3.37 1.5% ethidium bromide-stained agarose gel showing the reamplified products generated from cDNA-AFLP fragments; E-1/M-5_820 (lane 1, panel A), E-1/M-6_980 (lane 1, panel B), E-1/M-7_500 (lane 2, panel C), E-3/M-1_600, 460 and 390 (lanes 1 2 and 3, panel D), E-5/M-8_460, 400 (lanes 2 and 3, panel E) and E_{+A}-5/M_{+C}-2_490 (lane 1, panel F). Lanes M and N is a 100 bp ladder and negative control, respectively.

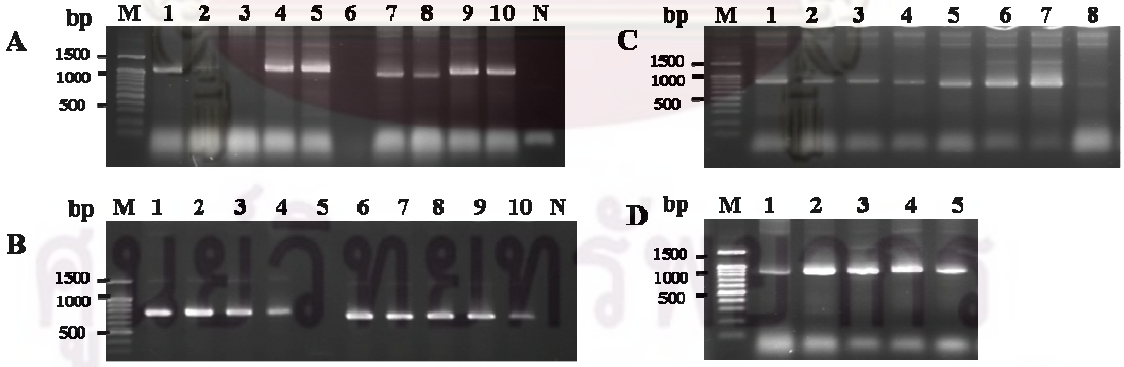

Figure 3.38 1.5% ethidium bromide-stained agarose gel showing colony PCR products of inserted cDNA-AFLP fragments; E-1/M-5_820 (lanes 1-10, panel A), E- E-5/M-8_460 (lanes 1-5, panel B), E-5/M-8_400 (lanes 6-10, panel B), E-1/M-7_500 (lanes 1-8, panel C) and E-3/M-1_600 (lanes 1-5, panel D). Lanes M and N are a 100 bp ladder and the negative control, respectively.

Similarity search was carried out using Blast*N* and Blast*X*. Nucleotides of fourteen fragments did not match any known sequences in the GenBank and were regarded as unknown transcripts. Only seven fragments significantly matched *O. sativa* genes and three sequences matched other plant genes (Figures. 3.39 – 348 and Table 3.4).

CACGAATTCAAGATCTCATGCCTAGGGGCTATCAAAGCACTATTTCCTCCTGACTCAAATCCATTCTAT
GCTGGATTTGGAAATAGAGATACAGATGAGCTTAGTTACCTCAAGGTTGGGATTCCTATGGGCAAAATT
TTTATTATAAATCCCAAGG**GTGGAGTTGCGGTGAATCGT**CGAGTAGATACAAAATCATACACATCCCTC
CATGCTCTTGTCAATGGGATGTTCCCTCCGATATCAACGTCATCTGAGCAGGAGGACTACAATACTTGG
AATTACTGGAGAATGCCGTTACCTGCTGTTGATATTAGAGGTATGGCCGCAGTGCATGCTATGCTG**TAC**
AAGGTCTCTGGCACGCAGCAGCGGCCATGAGAAATGGCTTCCATGTTGAACAAAAGCACCCTGAGACCA
ATCGCTGGTTGGTGTCATGTGGATGCTCCTGACATAAATTCTTGCGAGGCTGACACTGTACATAGGATC
ATAATAGAAAAACTTACAACCTGTATGACCAATGAGTCGGCAGAAGCAGTG

Figure 3.39 Nucleotide sequence of E_{+A}-2/ M_{+C}-1_534 (534 bp) amplified from cDNA of *O. sativa*. The location and sequence of a forward primer (E_{+A}-1/ M_{+C}-2_534-F) and those complementary to a reverse primer (E_{+A}-1/ M_{+C}-2_534-R) are illustrated in boldface and underlined.

GGTAGCCAAATGCCTCGTCATCTAATTAGTGACGCGCATGAATGGATTAACGAGATTCCCACTGTCCCT
GTCTACTATCCAGCGAAACCACAGCCAAGGGAACGGGCTTGGCGGAATCAGCGGGGAAAGAAGACCCTG
TTGAGCTTGACTCTAGTCCGACTTTGTGAAATGACTTGAGA**GGTGTAGGATAAGTGGGAGCC**CTCGGGC
GCAAGTGAAATACCACTACCTTTAACGTTATTTTACTTATTCCGTGAGTCGGAAGCGGGGCCTGGCCCC
TCCTTTTGGCTCTAAGGCCCGAGTCCCTCGGGCCGATCCGGGCGGAAGACATTGTCAGGTGGGGAGTTT
GGCTGGGGCGGCACATCTGTTAAAAGA**TAACGCAGGTGTCCTAAGATGA**GCTCAACGAGAACAGAAATC
TTGTGTGGAACAAAAGGGTAAAAGCTCGTTTGATTCTGATTTCCAGTACGAATACGAACCGTGAAAGCG
TGGCCTATCGATCCTTTAGACCTTCGGAGTTTGAAGCTAGAGGTGTCAGAAAAGTTACCACAGGGGTAA
CTGGCTTGTGGCAGCCAAGCGTTCATAGCGACGTTGCTTTTTGATCCTTCGATGTCGGCTCTTCCTATC
ATTGTGAAGCA**G**

Figure 3.40 Nucleotide sequence of E-5/ M-5_633 (633 bp) amplified from cDNA of *O. sativa*. The location and sequence of a forward primer (E-5/M-5_633-F) and those complementary to a reverse primer (E-5/M-5_633-R) are illustrated in boldface and underlined.

TGGCT**CTAATGCTGGAATCAGGGCTC**CAAAAGGACCCGCCAAGAGAAATAGCGACGCCTTTTCAGATCT
TTCTGCTATGAAGCAAAACCTACCGTCATCGACAGAGTCGGCACAAACAAAAAGCACAGGTGCTGGTTG
GGCAGCATTTTGACAAT**GAGTCTGCCATCTGCTGTTGA**TAACCTTTTCTCGGGAAAAGGAAAAGAAAAT
TGTGTAACTGAAAAAAAAAAGAAATCCCTTGGAGGAAACCTGTATCCGCTCGAATCACATGGTGATAAT
GACTTGCTAGCATCCACTTGGTACTCGAAAAAATAAAAGATTTACAGGATGATGTTGTTATTCATCGTG
CGATGCCACATGGATTCCTGGAGCTTTGGAAGTCTGAAAAATATGAGTATGGCTTTCCTTGTTTGTCAT
ATTTGTGTTGTCAGATTTTTCGCATATGAAACAAGAATCATACTGACCTCATTGTGTTGTACCC

Figure 3.41 Nucleotide sequence of E-5/ M-5_478 (478 bp) amplified from cDNA of *O. sativa*. The location and sequence of a forward primer (E-5/M-5_478-F) and those complementary to a reverse primer (E-5/M-5_478-R) are illustrated in boldface and underlined.

GAGGTGCACAGGTGCTGTTCTTCATAGCTGCTATTGGTCAACTTGCATTCTTCACTGACATACATCAAT
TTCCCAATCCTAAGTTCAATAACAAAAGAATGTCTTCTCAGAACCATAAACTCTTCCCCGCCAGCTTGC
ATCTGGAAAC**TATGTGGTGCTCGGGATGAA**CCGATCTCTAGAGCACTTTTGTGAAGCCCACGTTCCCAC
TCTTCTTTCCCCCACCAACAACTAGTAGGTTGGGGGCCTCGGTATCTCTATTTGCAAAGAGAGATATCT
ACACCAAGAAATATGTGATCGGAAAACGAATGAAATCAGAAAGGCCATCATTGGGGCAAACAAT**GCAAT**
AGCTTCGGTGAGAGCAAAGCCCAAAATGGCATAACCAAATAGTTGTTTAGCCAATGAGGGATTTCGCGC
CACGGAATGAATCAAAGAACTGAAAACGTTTCCAATACCGACAGCAGCTCCCGCTAAAGCAATTGTAGC
AGCTCCGGCACCTA

Figure 3.42 Nucleotide sequence of E-1/M-7_497 (497 bp) amplified from cDNA of *O. sativa*. The location and sequence of a forward primer (E-1/M-7_497-F) and those complementary to a reverse primer (E-1/M-7_497-R) are illustrated in boldface and underlined.

GGGCGAACAGCC**CAACCCTTGGAACCACCTAC**AGCTCCAGGTGGCGAAGAGCCGACATCGAGGTGCCAA
ACCTTCCCGTCGATGTGGACTCTTGGGGAAGATCAGCCTGTTATCCCTAGAGTAACTTTTATCCGTTGA
GCGACGGCCCTTCCACTCGGCGCCGTCGGATCACTAAGGCCGACTTTCGTCTCTGCTCGA**CGGGTGAGT**
CTTGCAGTCAAGCTCCCTTCTACCTTTGCACTCGAGGACCAATGTCCGTCTGGCCCGAGGAAAGCTTTG
CACGCCTCCGTTACCTTTTGGGAGGCCTACGCCCCATAGAAACTGTCTACCTGAGACTGTCCCTTGGCC
CGCGGGTCTGACACAAGGT

Figure 3.43 Nucleotide sequence of E-4/M-9_364 (364 bp) amplified from cDNA of *O. sativa*. The location and sequence of a forward primer (E-4/M-9_364-F) and those complementary to a reverse primer (E-4/M-9_364-R) are illustrated in boldface and underlined.

CACAACACCACCTC**CTTTCTCGCTGGTAGAGGTTCC**CTGTTACAGCTCTCTTGTCCATATACTTCCCCG
CAGAACGGCAAGGCCGAACGCATGATTCGCACCCTCAACAACTCCGTCCGTACCCTCCTCCTACAAGCC
TCCATGCCACCCTCCTATTGGGCCGAAGGTCTAGCCACCGC**CACATACCTCCTGAACCGTCG**ACCCTCG
TCCTCGGTAAACAACTCAATCCCATTCCAGCTTCTTCATCGCAAGATTCCCGATTATTCCATGTTACGA
GTGTTTGGGTGTTTATGCCATCCCATATTCACATGCTAATATGGGAGCCAGGAACAAATAATGGGGTTC
ATATAATTCATTCACACACA

Figure 3.44 Nucleotide sequence of E-5/ M-8_365 (365 bp) amplified from cDNA of *O. sativa*. The location and sequence of a forward primer (E-5/M-8_365-F) and those complementary to a reverse primer (E-5/M-8_365-R) are illustrated in boldface and underlined.

TGCTTCACAATGATAGGAAGAGCCGACATCGAAGGATCGAAAAGCAACG**TCGCTATGAACGCTTGGCTG**
CCACAAGCCAGTTATCCCTGTGGTAACTTTTCTGACACCTCTAGCTTCAAACTCCGAAGGTCTAAAGGA
TCGATAGGCCACGCTTTCACGGTTCGTATTCGTACTGTAAATCAGAATCAAACGAGCTTTTACCCTTTT
GTTCCACACGAGATTTCTGTTCTCGTTGAGCTCATCTTAGGACACCTGCGTTATCTTTTA**CTCAGGACT**
CATCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCGGAACCCAAAGACTTTGATTTCT
CATAAGGTGCCGGCGGAGTCCTATAAGCAACATCCGCCGATCCCTGGTCGGCATCGTTTATGGTTGAGA
CTAGGACGGTATCTGATCGTCTTCGAGCCCCCAACTTTCGTTCTTGA

Figure 3.45 Nucleotide sequence of E-5/M-8_461 (461 bp) amplified from cDNA of *O. sativa*. The location and sequence of a forward primer (E-5/M-8_461-F) and those complementary to a reverse primer (E-5/M-8_461-R) are illustrated in boldface and underlined.

GGCATTACGCTGGATTTAGAAGAGATTTTTACTCAGGACTCATCATGTCTGCGTACCAATTCAACCAAC
TTTGGCACATGCTAGAAGATGCTCTTGCTTTCGCATAGAGTTACTCAGGACTCATCGTGACTGCGTACC
AATTCGGCTTCACAATGATAGGAAGAGCCGACATCGAAGGATCAAAAAGCAACG**TCGCTATGAACGCTT**
GGCTGCCACAAGCCAGTTATCCCTGTGGTAACTTTTCTGACACCTCTAGCTTCAAACTCCGAAGGTCTA
AAGGATCGATAGGCCACGCTTTCACGGTTCGTATTCGTACTGGAAATCAGAATCAAACGAGCTTTTACC
CTTTTGTTCCACACGAGATTTCTGTTCTCGTTGAGCTCATCTTA**GGACACCTGCGTTATCTTCA**TGACT
GCGTACCAATTCGGGAGCCAGACAATTGCATCTGCATCAATTGTGCCCAGGTCTGCCGGCGCATCAGAC
ACGGGGTTGATGGTAGTT

Figure 3.46 Nucleotide sequence of E-2/M-1_501 (501 bp) amplified from cDNA of *oryza sativa*. The location and sequence of a forward primer (E-2/M-1_501-F) and those complementary to a reverse primer (E-2/M-1_501-R) are illustrated in boldface and underlined.

GGCTTCACAATGATAGGAAGAGCCGACATCGAAGGATCAAAAAGCAACGTCGCTACGAACGCTTGGCTG
CCACAAGCCAGTTATCCCTGTGGTAACTTTTCTGACACCTCTAGCTTCAAACTCCGAAGGTCTAAAGGA
TCGATAGGCCACGCTTTCACGGTTCGTATTCGTACTGGAAATCAGAATCAAACGAGCTTTTACCCTTTT
GTTCCACACGAGATTTCTGTTCTCGTTGAGCCCATCTTAGGACACCTGTGTTATCTT

Figure 3.47 Nucleotide sequence of E-2/M-1_264 (264 bp) amplified from cDNA of *O. sativa*.

ACCAAGTGTTGGATTGTTCACCCACCAATAGGGAACGTGAGCTGGGTCTAGACCGTCGTGAGACAGGTT
AGTTTTACCCTACTGATGACCGTGCCGCGATAGTAATTCAACCTAGCACGAGAGGAACCGTTGATTCAC
ACAATTGGTCATCGCGCTTGGTTGAAAAGCCAGTGGCGCGAAGCTACCGTGTGCTGGATTATGACTGAA
CGCCTCTAAGTCAGAATCCAAGCTAACAAGCGGCGCCTGCGCCCGCCACCCGCCCCGACCCAC

Figure 3.48 Nucleotide sequence of E+A-5/M+C-10_270 (270 bp) amplified from cDNA of *O. sativa*.

Table 3.4 Similarity comparisons of transcripts identified by cDNA-AFLP against previously deposited sequences in the database

Fragment	Homologue/Species	Accession no.	*E*-value
E+A-2/ M+C-1_534	*lipin, N-terminal conserved region family protein/Oryza sativa*	DP000010	7e-53
E-5/M-5_633	*cytochrome p450 monooxygenas Oryza sativa*	BE040811	1e-31
E-5/M-5_478	*Hypothetical protein / Oryza sativa (called hypothetical protein$_{171}$)*	NP_001064844	4e-19
E-1/M-7_497	*ATP synthase subunit C family protein/Oryza sativa*	ABA99282	7e-63
E-4/M-9_364	*Hypothetical protein Oryza sativa (called hypothetical protein$_{206}$)*	AAM08574	9e-13
E-5/M-8_365	*Hypothetical protein Oryza sativa (called hypothetical protein$_{186}$)*	CAE05417	3e-52
E-5/M-8_461	*Hypothetical protein/Oryza sativa (called hypothetical protein$_{240}$)*	EEC70730	1e-09
E-2/M-1_501	*Hypothetical protein /Zea mays(called hypothetical protein$_{217}$)*	ACR36970	3e^{-}-33
E-1/M-5_ 264	*Unknown protein/Zea mays*	ACR36970	3e-30
E+A-5/M+C-10_270	*Hypothetical protein/ Sorghum bicolor*	XP_002467304	2e-10

87

3.3. Identification of genes involving salt tolerance mechanisms in rice analyzed by proteomics

Effects of salt stress on protein expression in two varieties rice (Pokkali and IR29) were determined using proteomic techniques based on two basic methodologies: SDS-PAGE and LC-MS/MS. SDS-PAGE analysis was initially used to size-fractionated the protein patterns followed by LC-MS/MS used for protein identification and quantification.

3.3.1 SDS-PAGE

Total proteins were extracted from leaves of IR29 and Pokkali at different time intervals before (control) and after stress (3, 6, 12, 24 hpt) treatment. Twenty micrograms of total proteins were analyzed by 12.5% SDS-PAGE, and the gels visualized by silver staining are shown in Figure 3.49. The gel bands were excised according to the molecular mass range compared to protein standard markers and the in-gel trypsin digestion was performed. The extracted peptides of each molecular mass range sample were injected to LC-MS/MS.

3.3.2 Functional classification of NaCl – responsive proteins

The raw data from LC-MS/MS were analyzed using DeCyder MS Differential Analysis software. The analyzed MS/MS data from DeCyder MS were submitted to database search using the Mascot. The data was searched against the NCBI database of *O. sativa* for protein identification.

From a total of identified protein bands, rice varieties before the stress (0 hpt) treatment was selected for the control groups. A total 206 differentially expressed proteins were identified, but only 113 proteins showed high homology to known proteins existed in the NCBI database. Of these, 43, 24 and 46 proteins were found only in Pokkali or IR29 and both group, respectively (Table 3.5).

In addition, known proteins in this study were further categorized according to the biological functions of their homologues using the Gene Ontology Categorizer (GoCat software). Eighty-nine and seventy differentially expressed proteins identified in Pokkali and IR29 were involved in 13 categories (Figures 3.50, 3.51 and Table

3.5) including energy metabolism (3.37% and 4.29%), cellular metabolism (13.48% and 10.00%), biosynthetic process (5.62% and 12.86%), transferase activity (1.12% and 2.86%), oxidation reduction (2.25% and 5.71%), RNA processing (10.11% and 5.71%), hormone-related (1.12% and 1.43%), structural protein (10.11% and 10.11%) , photosynthesis (2.25% and 2.86%), signal transduction (13.48% and 14.29%), cell rescue and defence (12.36% and 10.00%), transport and binding proteins (22.47% and 17.14%) and ionic homeostasis (2.25% and 2.86%), respectively.

Several functionally important proteins were identified, for example, Na^+/H^+ antiporter, Myb-like DNA-binding domain, ATPase family and Photosystem II protein. In addition, proteins involving stress response were also identified. They were, peroxidase, salt inducible protein, heat shock protein and sedoheptulose-1,7-bisphosphatase precursor.

Two proteins including *sucrose transporter* which is involved in transport and binding proteins and *cystein protease* which is involved in protein destination represented expression decreased after salt stress in the Pokkali. These proteins related to salt stress response were selected for expression analysis by RT PCR.

3.3.3 hypothetical proteins

In addition to known proteins, the unknown proteins were abundantly expressed accounting about 45.1 % of all expressed proteins in both Pokkali and IR29 varieties (Table 3.5). It was interesting that the expression of some unknown proteins having high score were differentially expressed at almost all salt exposure times. For example, the hypothetical protein OsI_06083 (gi|218190181) represented clear differential expression profiles than any proteins as it was down-regulated at 3 to 24 hrs after salt stress in Pokkali and was up-regulated during the salt stress treatment in IR29 as compared with the untreated control.

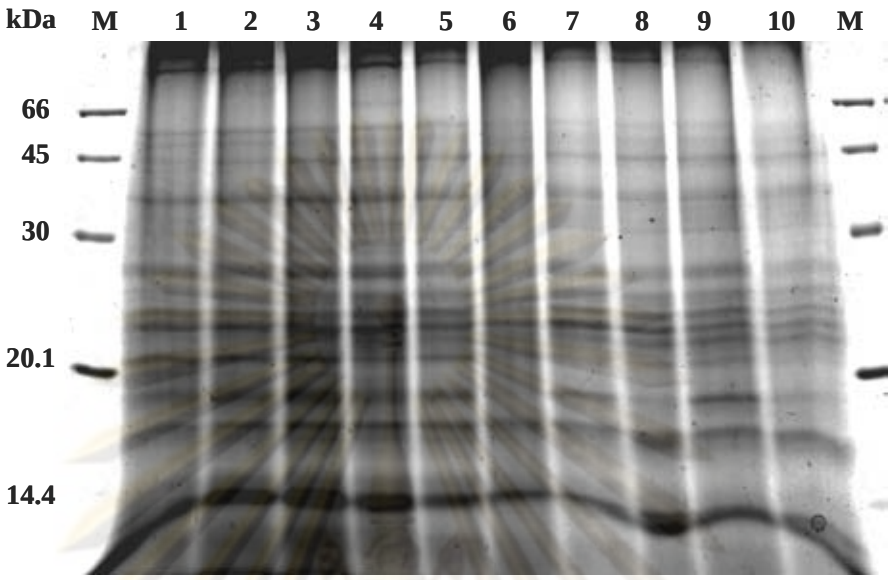

Figure 3.49 A 12 .5% SDS-PAGE showing expression patterns of IR29 (lanes 1 - 5) and Pokkali (lanes 6 -10) of the control group (lanes 1 and 6), 3 (lanes 2 and 7), 6 (lanes 3 and 8),12 (lanes 4 and 9) and 24 (lane 5 and 10) hpt. Lanes M is the protein marker.

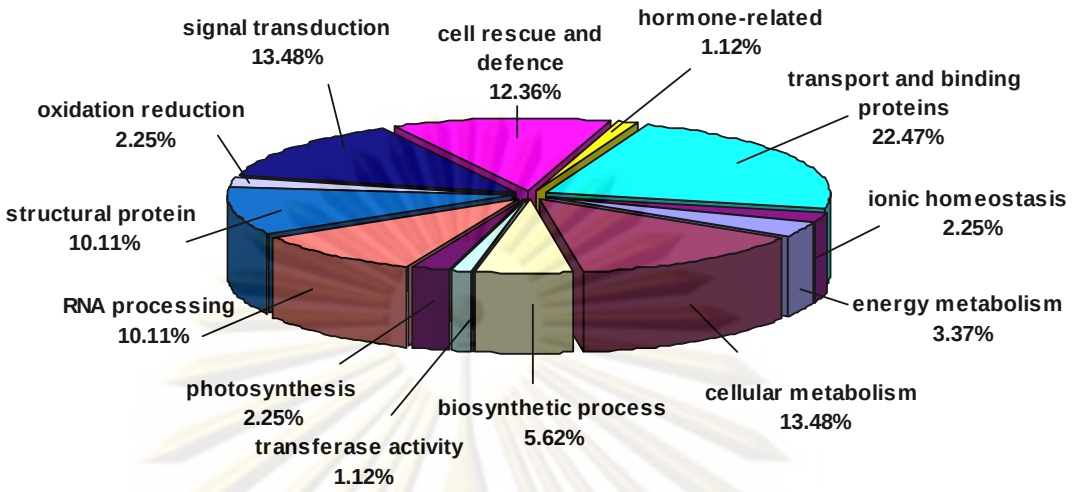

Figure 3.50. Function classification of a total of 87 known proteins identified form leaves of Pokkali.

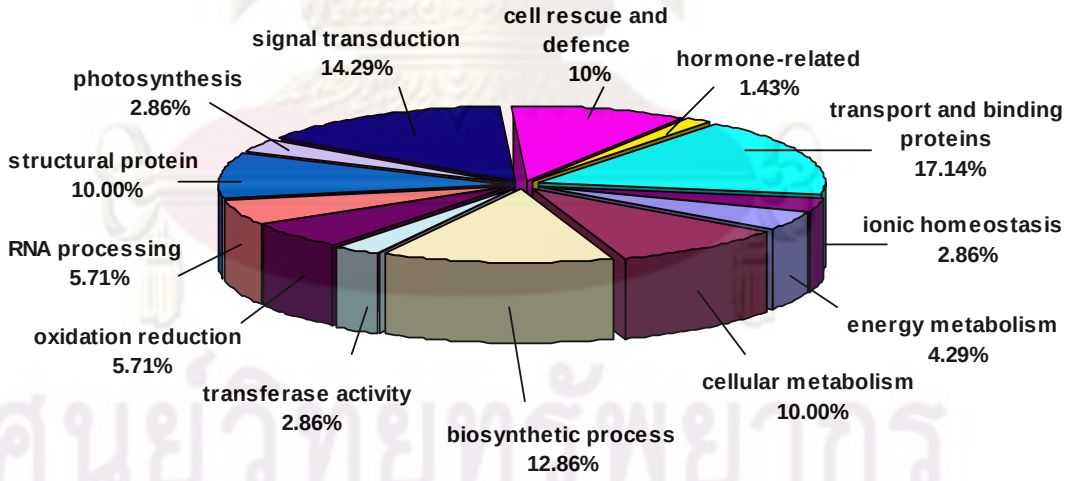

Figure 3.51. Function classification of a total of 66 proteins identified form leaves of IR 29.

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29

Aceession	Protein identity	Function	Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Energy metabolism													
gi|73918017	hexokinase 8	Glycolysis	R.LAAAGVAGILMK.L + Oxidation (M)	1	0.91	0.94	0.90	0.96	1	0.96	0.98	0.96	0.93
gi|968996	glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	K.VLPDLNGK.L	1	0.96	0.95	0.92	0.94	1	0.98	0.94	1.03	0.91
gi|108708096	ATPase family protein	ATP synthesis	R.LLTNECIDK.I	1	1.42	1.52	1.27	1.18	1	-	1.05	1.15	-
Cellular metabolism													
gi|42407620	1-phosphatidylinositol-3-phosphate 5-kinase	cellular protein metabolic process	R.DVVNK.K	1	0.92	0.92	0.85	0.82	1	0.85	0.89	0.94	0.87
gi|108708237	Phosphatidylinositol-4-phosphate 5-kinase	cellular protein metabolic process	R.ASSPHLSR.G	1	0.96	0.95	0.92	0.94	1	0.98	0.94	1.03	0.91
gi|57900311	endo-1,3-beta-glucanase	carbohydrate metabolic process	R.NVNSALAAAGIGGIKVSTAVK.S	1	0.95	0.90	-	0.89	-	-	-	-	-
gi|108708120	terpene synthase	metabolic process	K.EIEDELK.S	1	1.06	1.08	1.05	-	-	-	-	-	-
gi|52353391	flavonol glucosyltransferase	metabolic process	R.EIAVGLEK.S	1	-	0.95	0.93	0.92	-	-	-	-	-
gi|19386796	alpha-glucosidase 1	carbohydrate metabolic process	K.KVCDGAVDIIVSLMQINPK.E	-	-	-	-	-	1	-	1.30	1.12	1.19
gi|108862134	arabinoxylan arabinofuranohydrolase isoenzyme	L-arabinose metabolic process	K.VFVSEYAVNEQR.D	-	-	-	-	-	1	1.10	1.10	-	-
gi|7630193	adenylate kinase	purine nucleotide metabolic process	K.EAMDKGELVSDDLVVGIIDEAMK.K	1	-	0.76	0.77	0.72	-	-	-	-	-
gi|39653556	dihydroxyacetone kinase-like protein	glycerol metabolic process	K.YGGASAGYR.T	1	0.81	0.90	0.81	-	-	-	-	-	-

- = Protein was not detected

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity	Function	Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Cellular metabolism													
gi|51091050	metalloendopeptidase	proteolysis	K.LPGGLAASLTGER.S	1	0.92	0.81	0.90	0.87	-	-	-	-	-
gi|47497892	ulp1 protease-like	proteolysis	R.SMSPDEIGTTVAK.N + Oxidation (M)	1	0.85	-	0.87	0.71	-	-	-	-	-
gi|52076119	**cysteine protease***	**Catabolic process**	**K.LGLNKFADMTLEEFVAK.Y**	**1**	**-**	**0.94**	**0.86**	**0.79**	**1**	**0.91**	**-**	**0.88**	**0.95**
gi|41053066	alkaline/neutral invertase	catalytic activity	R.SAPVLK.R	-	-	-	-	-	1	-	0.82	0.83	0.76
gi|108863983	saccharopine dehydrogenase	Amino-acid degradation	R.VLVLGGTGR.V	1	0.88	0.92	0.93	0.84	-	1	0.95	-	1.00
gi|46404433	glutathione S-transferase	metabolic process	R.MAVMEAGFER.D	1	-	1.09	1.05	1.08	-	-	-	-	-
Biosynthetic process													
gi|2072725	Fd-glutamate synthase precursor	Amino-acid biosynthesis	K.LVAEAGIGTVASGVSK.G	-	-	-	-	-	-	1	-	1.01	0.95
gi|51090528	Phosphomannomutase	mannose biosynthetic process	R.VGYRNVIDK.G	1	0.78	-	0.87	0.96	1	1.02	1.01	1.10	1.00
gi|49387682	trehalose-phosphatase B	trehalose biosynthetic process	K.GVGILVSKHPK.E	1	1.07	-	1.10	-	1	1.04	1.03	1.01	1.02
gi|77551736	glycosyltransferase	biosynthetic process	R.VGGGGGGGGGGGGRER.E	1	0.92	0.81	0.90	0.87	1	0.85	-	-	-
gi|56784184	protoporphyrinogen oxidase	chlorophyll biosynthetic process	R.RNLGAEVFER.L	1	0.94	-	0.94	0.93	-	-	-	-	-
gi|115471705	adenylate kinase family protein	purine ribonucleotide biosynthetic process	M.ASRGGGAR.R	-	-	-	-	-	1	1.10	-	1.07	-
gi|169759	ADP-glucose pyrophosphorylase	glycogen biosynthetic process	K.FIQAHR.E	-	-	-	-	-	1	1.10	1.22	1.14	1.09

- = Protein was not detected

* = protein was selected for Quantitative RT PCR

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity	Function	Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Biosynthetic process													
gi|108862789	tryptophanyl-tRNA synthetase	Protein biosynthesis	R.KLGANLDL.-	-	-	-	-	-	1	1.05	1.08	1.06	1.03
gi|31415898	3-beta hydroxysteroid dehydrogenase	steroid biosynthetic process	K.IPQLTPSR.I	-	-	-	-	-	1	-	0.84	0.84	-
gi|19387272	chloroplastic glutamine synthetase	glutamine biosynthetic process	R.ARPAAAAAAAGGR.V	1	0.92	0.89	0.94	0.94	-	-	-	-	-
gi|47497880	molybdenum cofactor biosynthesis protein A	cofactor biosynthetic process	-.MMMPLR.C + Oxidation (M)	-	-	-	-	-	1	1.07	1.17	1.08	0.97
Transferase activity													
gi|51535072	beta-amyrin synthase	transferase activity	R.LKVAEGGAPGLR.S	1	0.97	0.92	0.91	-	-	-	-	-	-
gi|50725254	beta-1,3-galactosyltransferase	transferase activity	K.TDDDAFVR.V	-	-	-	-	-	1	0.75	0.80	0.82	0.78
gi|29569153	alanine aminotransferase	Aminotransferase	-.MFGGGGGGR.K	-	-	-	-	-	1	1.07	1.17	1.08	0.97
Oxidation reduction													
gi|38426301	cytosolic 6-phosphogluconate dehydrogenase	oxidation reduction	K.ILDKTGMK.G	-	-	-	-	-	1	0.96	-	0.95	-
gi|20341	ribulose bisphosphate carboxylase	oxidation reduction	K.AYPDAFVR.I	-	-	-	-	-	1	0.75	0.80	0.82	0.78
gi|27311240	Squalene monooxygenase	oxidation reduction	R.TMPNR.S + Oxidation (M)	-	-	-	-	-	1	1.15	1.29	-	-
gi|34393370	short-chain dehydrogenase/reductase	oxidation reduction	R.VDEIKATNPMGR.L	1	0.88	-	0.90	-	-	-	-	-	-
gi|21426123	Peroxidase	oxidation reduction	R.DGVALTGGPR.Y	1	1.16	-	1.20	1.20	1	0.89	0.94	0.97	1.03

- = Protein was not detected

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity	Function	Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
ionic homeostasis													
gi|21321024	chloride channel	Ion transport	K.LLLTGNLMLK.G + Oxidation (M)	1	-	1.26	1.13	1.15	1	0.96	0.89	0.96	-
gi|51535165	potassium channel tetramerisation	Ion transport	K.LPSAAFLR.S	1	0.90	0.92	0.91	-	-	-	-	-	-
gi|57863835	Na+/H+ antiporter	cellular potassium ion homeostasis	R.NGVPFLRPR.R	-	-	-	-	-	1	-	0.92	0.86	0.95
RNA processing													
gi|62733579	Myb-like DNA-binding domain	regulation of transcription	R.AAATAISAAAVKAK.L	1	0.92	0.81	0.90	0.87	-	-	-	-	-
gi|54290881	zinc finger	regulation of transcription	R.CGSSISMR.M	1	1.07	-	1.03	1.03	-	-	-	-	-
gi|62733549	Reverse transcriptase	transcription factor	K.LIDLGFSASK.A	1	0.95	-	0.88	-	1	1.09	-	0.96	0.98
gi|115483692	transposase	regulation of transcription	K.RFAMPNTR.I	1	1.14	1.24	1.18	1.18	-	-	-	-	-
gi|24431594	DNA cytosine methyltransferase Zmet3	gene silencing	K.YQNLGSR.T	1	0.91	0.91	0.80	0.86	-	-	-	-	-
gi|62733549	DNA-directed RNA polymerase II	gene silencing	R.GLELRAPIK.K	1	1.06	1.08	1.09	1.04	-	-	1	1.08	1.05
gi|52075837	nucleotidyltransferase	RNA processing	K.IMPSIDFDEL.-	-	-	-	-	-	1	1.10	1.12	-	1.07
gi|29150367	RNA binding ribonucleoprotein	RNA processing	K.KSSSTAQK.H	1	-	1.08	1.15	-	1	0.96	-	0.96	0.91
gi|108706011	Bromodomain containing protein	Nucleotide-binding	R.TDRFGDYSGPSK.Y	1	0.83	0.84	0.81	0.87	-	-	-	-	-
gi|37718782	U3 small nucleolar ribonucleoprotein	RNA processing	R.YPGAAR.R	1	0.90	0.86	0.83	-	-	-	-	-	-

- = Protein was not detected

Table 3.5 Differentially expressed proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity	Function	Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 Hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Signal transduction													
gi|47497193	wall-associated serine/threonine kinase	protein amino acid phosphorylation	R.TNDVVYLFLGR.E	1	0.88	-	0.89	0.94	-	0.88	0.75	0.86	0.93
gi|77553920	Leucine Rich Repeat protein	small GTPase mediated signal transduction	K.LVGNLPDSIK.E	1	1.10	1.06	1.02	-	1	0.96	-	0.99	0.95
gi|40218073	transducin	Signal transduction	R.GSLTALAAHR.H	1	1.06	1.08	1.09	1.04	-	-	1	1.02	1.03
gi|20804657	casein kinase I	protein amino acid phosphorylation	R.TSGWSSMDR.R + Oxidation (M)	1	0.96	0.93	0.90	0.93	-	-	1	1.00	1.03
gi|51535863	C2 domain-containing protein-like protein	Signal transduction	K.ETGSGAAAAAAGGK.L	1	1.05	-	-	-	-	-	1	1.01	1.02
gi|30017485	initiation factor 2 alpha kinase	Signal transduction	R.VDALIDLMER.R + Oxidation (M)	1	0.94	-	0.94	0.93	-	-	-	-	-
gi|108862067	RNA recognition motif family protein	Signal transduction	R.VPQMASNVSQR.R + Oxidation (M)	1	1.07	-	-	-	-	-	-	-	-
gi|78707680	protein kinase domain	protein amino acid phosphorylation	K.LAVLGEGR.D	1	-	0.92	0.93	-	-	-	-	-	-
gi|77552550	PAS domain containing protein	signal transduction	R.GHGNDDAGKNR.T	1	0.95	0.94	0.92	0.90	-	-	-	-	-
gi|55296243	wall-associated kinase 4	protein amino acid phosphorylation	K.NVLGRGGHGTVYR.G	-	-	-	-	-	1	0.92	-	-	0.88
gi|77551931	Jacalin-like lectin domain	protein amino acid phosphorylation	K.LQHTADTMPNDLTIDLL TR.I + Oxidation (M)	-	-	-	-	-	1	0.91	0.88	-	0.91

- = Protein was not detected

Table 3.5 Differentially expressed proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity	Function	Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Signal transduction													
gi|77549163	F-box domain containing protein	protein ubiquitination	R.TGALAR.R	1	-	0.83	0.79	0.76	1	-	0.90	0.96	0.96
gi|7243646	serine/threonine protein kinase	cell differentiation	K.TKNAPLTWR.Q	1	0.93	0.92	0.89	0.93	1	1.00	-	1.02	0.99
gi|52075918	receptor-like protein PRK1	protein amino acid phosphorylation	R.GGEGEMVKLLK.V	1	0.92	0.92	0.85	0.82	1	0.85	0.89	0.94	0.87
Photosynthesis													
gi|11466764	photosystem II protein D1	photosynthesis	-.MTAILER.R + Oxidation (M)	1	1.19	1.23	1.19	-	1	0.97	0.96	1.04	0.90
gi|18855008	chloroplast chaperonin	photosynthesis	K.VAPQSDR.V	1	1.18	-	1.18	-	1	0.90	0.88	0.95	0.99
Structural proteins													
gi|50540679	microtubule-associated protein	multicellular organismal development	R.KEIMDK.I + Oxidation (M)	1	0.76	0.92	0.91	0.86	1	0.96	-	-	1.05
gi|47847642	myosin-like protein	actin filament-based movement	K.NISDAEDLGPAGK.A	1	-	0.88	-	0.94	1	0.95	-	1.01	1.05
gi|45680447	histone H2A	nucleosome assembly	-.MDVGVGGKAAK.K + Oxidation (M)	1	0.95	-	0.88	-	1	1.09	-	0.96	0.98
gi|108862877	pentatricopeptide	Structural proteins	K.DSVSWNSIIGGFAM HGHGDK.A	1	0.87	-	-	-	-	-	-	-	-

- = Protein was not detected

Table 3.5 Differentially expressed proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity	Function	Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Structural proteins													
gi|108864642	kinesin motor protein	microtubule-based movement	R.SNGTPR.Q	1	0.90	0.86	0.83	-	-	-	-	-	-
gi|108708319	proline-rich family protein,	Structural proteins	K.REAELLQQK.A	-	-	-	-	-	1	0.91	-	-	-
gi|50540679	microtubule-associated protein	multicellular organismal development	R.KEIMDK.I + Oxidation (M)	1	0.76	0.92	0.91	0.86	1	0.96	-	-	1.05
gi|47847642	myosin-like protein	actin filament-based movement	K.NISDAEDLGPAGK.A	1	-	0.88	-	0.94	1	0.95	-	1.01	1.05
gi|45680447	histone H2A	nucleosome assembly	-.MDVGVGGKAAK.K + Oxidation (M)	1	0.95	-	0.88	-	1	1.09	-	0.96	0.98
gi|108862877	pentatricopeptide	Structural proteins	K.DSVSWNSIIGGFAM HGHGDK.A	1	0.87	-	-	-	-	-	-	-	-
Transport and binding proteins													
gi|29367367	calmodulin-related	calcium ion binding	R.AAGTGGAPRSR.R	1	1.06	1.08	1.09	1.04	-	-	1	1.08	1.05
gi|77553190	Zinc knuckle family protein	nucleic acid binding	M.ASNMSWSK.E	1	1.04	1.04	1.01	1.09	1	-	0.83	0.81	0.81
gi|52550761	phosphate transporter 1	phosphate transport	K.TMNALEELYR.I	1	0.88	0.91	0.99	0.86	1	-	0.99	0.98	-
gi|29467454	**sucrose transporter***	**sucrose transporter**	**.MDSAAGGGGLTAIR. L + Oxidation (M)**	**1**	**0.93**	**0.76**	**0.77**	**0.72**	**1**	**1.12**	**1.03**	**1.25**	**0.97**
gi|22795260	nodulin-like protein	plasma membrane	K.YSLNSWVVAK.R	1	-	0.91	0.82	0.87	-	-	1	1.01	1.06
gi|62733121	signal recognition particle protein	SRP-dependent cotranslational protein targeting to membrane	R.VVGMIPGMNK.V + Oxidation (M)	1	0.78	-	0.87	0.96	1	1.02	1.01	1.10	1.00

- = Protein was not detected

* = protein was selected for Quantitative RT PCR

Table 3.5 Differentially expressed proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity	Function	Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Transport and binding proteins													
gi|38175486	erythrocyte binding protein	binding protein	K.QSAPGGGRASGPR.V	1	-	0.95	-	-	1	0.95	1.01	0.95	0.94
gi|11034589	ABC transporter	Transport	R.LFLGGGGEER.L	1	1.10	1.09	1.07	1.04	1	-	0.96	0.97	0.95
gi|218204	GTP binding protein	binding protein	K.MVLIGDTGVGK.S	1	1.13	1.11	1.05	-	-	-	-	-	-
gi|77553880	Cytochrome P450	electron carrier	R.HWTAIHMSR.V	1	1.02	-	1.03	-	-	-	-	-	-
gi|18250696	potasium transporter	potasium transport	R.VEIGMLYKV.-	1	-	0.85	0.88	-	-	-	-	-	-
gi|20042982	calcium-transporting ATPase	calcium ion transport	R.AAPLISNAMWR.N + Oxidation (M)	-	-	-	-	-	1	0.93	0.96	0.89	0.85
gi|77552805	Ammonium Transporter	Transport	R.AGGVQVLK.Q	-	-	-	-	-	1	1.09	1.22	1.13	1.08
gi|46576038	far-red impaired response protein	zinc ion binding	K.GLGGKGNGNIPLA.-	1	0.97	0.92	0.91	-	-	-	-	-	-
gi|50582733	endoplasmic reticulum oxidoreductin	electron transport chain	.MPPQEPAPAANGAAPA PAPAAAAAAAGGK.R	1	-	0.76	0.77	0.72	-	-	-	-	-
gi|52076586	ionotropic glutamate receptor	Ion transport	R.VFVVHVNPDAGMR.I + Oxidation (M)	-	-	-	-	-	-	1	-	1.01	0.95
gi|10716600	peptide transport protein	peptide transport	K.EVAMVDTNL.- + Oxidation (M)	1	1.14	1.24	1.18	1.18	-	-	-	-	-
gi|18071340	TGF beta receptor	ATP-binding	R.LDSENSAALPIR.F	1	-	0.88	-	0.94	1	0.95	-	1.01	1.05
gi|29893633	peroxisomal Ca-dependent solute carrier protein	transmembrane transport	-.MDSGAAAAAAR.R + Oxidation (M)	1	1.18	1.16	1.25	-	-	-	-	-	-
gi|108705863	Armadillo/beta-catenin-like repeat family protein	binding protein	K.LSDMDVAGAK.K	1	1.18	1.16	1.25	-	-	-	-	-	-

- = Protein was not detected

Table 3.5 Differentially expressed proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity	Function	Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Transport and binding proteins													
gi|14488297	salt-inducible protein	intracellular protein transport	R.NILIDGFCR.E	1	0.92	0.91	0.91	0.89	-	-	-	-	-
gi|51535072	beta-amyrin synthase	Transport	R.LKVAEGGAPGLR.S	1	0.97	0.92	0.91	-	-	-	-	-	-
gi|20162451	sulphate transporter	sulphate transport	K.VQQRYSFLK.N	1	0.97	0.92	0.91	-	-	-	-	-	-
Cell rescue, Defence													
gi|2384758	GDP dissociation inhibitor protein	cell tip growth	K.LYAESLPR.F	-	-	-	-	-	1	1.02	-	-	-
gi|40714670	heat shock protein	response to stress	R.GAPTMGGGRSAA.- + Oxidation (M)	1	0.95	-	0.88	-	1	1.09	-	0.96	0.98
gi|15217337	gag-pol precursor	DNA integration	R.DPRQVQR.V	1	1.07	-	1.03	1.03	-	-	-	-	-
gi|33087081	heat stress protein	response to stress	K.QLMSQNEMRK.E	1	0.88	0.86	0.86	0.82	1	-	0.91	0.92	-
gi|54290333	NBS-LRR disease resistance protein	defense response	R.HLDSLPASLMR.L + Oxidation (M)	1	0.93	0.76	0.77	0.72	1	1.12	1.03	1.25	0.97
gi|18071411	membrane-associated protein	evasion by virus of host immune response	R.AGRQVGLWR.G	1	0.94	0.98	0.97	0.92	-	-	1	1.04	1.03
gi|13122418	glycine-rich protein	response to abscisic acid stimulus	R.GGSGAGGGAGMGGG VGAR.A	1	0.83	0.84	0.81	0.87	-	-	-	-	-
gi|52075804	zinc-induced protein	response to stress	R.SRLAMYSALR.C	1	0.97	0.92	0.91	-	-	-	-	-	-
gi|45735835	bactericidal permeability-increasing protein	defense response	R.GGREPTAAER.A	1	0.94	-	0.94	0.97	-	-	1	0.99	1.00
gi|55296378	resistance protein	resistance protein	K.IVLSLTNVNSLSGVEG LPK.L	1	0.94	-	-	0.95	-	-	-	-	-

- = Protein was not detected

Table 3.5 Differentially expressed proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity	Function	Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Cell rescue, Defence													
gi|4097102	globulin-like protein	nutrient reservoir activity	R.IYLAGMNSVLKK.L + Oxidation (M)	1	0.86	-	0.88	0.71	-	-	-	-	-
gi|27804768	sedoheptulose-1,7-bisphosphatase precursor	defense response to bacterium	R.YTGGMVPDVNQIIVK .E + Oxidation (M)	-	-	-	-	-	1	1.06		1.09	1.04
gi|55700887	peroxidase 10 precurcer	defense response	K.FAGANLGIAGLDVID AVKAK.L	1	0.95	0.90	-	0.89	-	-	-	-	-
Hormone related													
gi|51535165	auxin-independent growth promoter	Hormone related	K.LPSAAFLR.S	1	1.05	1.16	1.12	1.07	1	-	1.09	1.04	0.93

- = Protein was not detected

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29 (cont.)

				Intensity ratio									
				Pokkali					IR29				
Aceession	Protein identity		Peptide	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Unknow function													
gi|77554591	hypothetical protein LOC_Os12g17200	unknown function	R.GLSGGTATGAVSTG RAALPAAR.I	1	0.95	0.90	-	0.89	-	-	-	-	-
gi|56785129	hypothetical protein	unknown function	R.AAEEGENR.A	1	1.05	1.16	1.12	1.07	1	-	1.09	1.04	0.93
gi|125525503	hypothetical protein OsI_01505	unknown function	K.NLQGQMEMGK.I + 2 Oxidation (M)	1	0.97	0.92	0.91	-	-	1	-	1.04	-
gi|125546498	hypothetical protein OsI_14381	unknown function	R.KPSDDQPAPPAPAA AAPRGK.H	1	0.95	0.90	-	0.89	-	-	-	-	-
gi|218191159	hypothetical protein OsI_08052	unknown function	R.EVAEVAQADR.A	1	0.93	0.92	0.89	0.93	1	1.00	-	1.02	0.99
gi|218199039	hypothetical protein OsI_24781	unknown function	K.ENDGSAGEEQGKGR .G	-	-	-	-	-	-	1	-	1.02	1.06
gi|218184372	hypothetical protein OsI_33203	unknown function	R.GGVGGGGGHSSAR. L	1	-	1.08	1.17	1.04	-	-	-	-	-
gi|52077144	hypothetical protein	unknown function	R.SSMLSPATVVDAVL.	1	1.10	1.06	1.02	-	1	0.96	-	0.99	0.95
gi|125524714	hypothetical protein OsI_00694	unknown function	M.EDAAVLSGVGQAK. S	1	0.92	0.92	0.94	-	-	-	-	-	-
gi|218201917	hypothetical protein OsI_30860	unknown function	M.TGDDADAIAAAAA AK.A	1	0.83	0.84	0.81	0.87	-	-	-	-	-
gi|108862762	hypothetical protein LOC_Os12g34419	unknown function	R.LGSGLAGPAR.L	1	1.07	-	1.03	1.03	-	-	-	-	-
gi|222631747	hypothetical protein OsJ_18703	unknown function	R.ERGDVVGGGGR.E	1	1.10	1.06	1.02	-	1	0.96	-	0.99	0.95
gi|50725097	hypothetical protein	unknown function	R.VGGEGLAVVGAR.H	1	-	-	0.93	0.86	-	1	-	1.02	1.04

- = Protein was not detected

102

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity		Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Unknow function													
gi|115456459	hypothetical protein	unknown function	R.NVVFFDINMK.G + Oxidation (M)	-	-	-	-	-	1	0.92	0.81	0.90	0.87
gi|115458502	hypothetical protein	unknown function	K.EVVGPASASLR.Y	1	0.94	-	1.02	1.00	1	0.91	0.95	0.97	0.94
gi|115472971	hypothetical protein	unknown function	K.LMPIVTDEMAR.R	1	-	1.07	1.06	1.10	1	1.04	1.14	1.11	1.05
gi|115489380	hypothetical protein	unknown function	R.EVNYFDLFVK.K	1	-	1.07	1.06	1.10	1	1.04	1.14	1.11	1.05
gi|115489378	hypothetical protein	unknown function	R.VSSTPDAAAAR.T	-	-	1	1.01	1.01	1	0.94	0.98	0.97	0.92
gi|115452639	hypothetical protein	unknown function	-.PPPSDVAEDR.H	-	1	-	1.02	1.03	1	-	-	0.93	0.86
gi|115474715	hypothetical protein	unknown function	K.NGAKPMDVDKAK.T	-	1	-	1.02	1.03	1	-	-	0.93	0.86
gi|115481730	hypothetical protein	unknown function	R.VGGLQGEYQICNGYGK.A	-	1	0.94	-	0.97	1	0.88	0.92	0.93	0.84
gi|115486013	hypothetical protein	unknown function	R.GGGGGRFGGGGGGR.F	1	1.08	1.17	1.12	1.14	1	0.83	0.93	0.93	0.82
gi|115470313	hypothetical protein	unknown function	R.MAAAAMLVLVAMAGGA TVCAAQLR.R + Oxidation (M)	1	0.92	0.90	-	0.94	-	-	-	-	-
gi|115454055	hypothetical protein	unknown function	R.GSNGEIPEETTAAYLR.A	1	0.98	0.93	1.03	0.91	1	0.96	0.95	0.92	0.94
gi|218187575	hypothetical protein OsI_00539	unknown function	R.GGNKSTPITLAAR.L	1	-	0.88	-	0.94	1	0.95	-	1.01	1.05
gi|34394082	hypothetical protein	unknown function	R.MDLLLGVLR.R + Oxidation (M)	1	0.94	0.98	0.97	0.92	-	-	1	1.01	1.01
gi|115477399	hypothetical protein	unknown function	-.MMPHSGVVDAAAK.G + Oxidation (M)	-	-	-	-	-	1	0.83	0.84	0.81	0.87
gi|115445505	hypothetical protein	unknown function	R.DPGQAKDVVK.S	1	0.97	-	0.99	0.95	1	1.10	1.06	1.02	

- = Protein was not detected

103

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29 (cont.)

Aceession	Protein identity		Peptide	Intensity ratio									
				Pokkali					IR29				
				0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
unknown function													
gi|125603973	hypothetical protein OsJ_27895	unknown function	R.QKGGSGVALR.C	1	-	0.89	-	0.86	-	-	-	-	-
gi|222613068	hypothetical protein OsJ_32013	unknown function	R.DLTVTVVLGGRR.R	1	-	0.88	-	0.94	1	0.95	-	1.01	1.05
gi|222641083	hypothetical protein OsJ_28433	unknown function	K.LGIANMVEAK.E	1	0.94	0.98	0.97	0.92	-	-	1.00	1.01	1.01
gi|222616376	hypothetical protein	unknown function	K.RNVADTVTTGR.K	1	0.94	-	0.94	0.93	-	-	-	-	-
gi|125606578	hypothetical protein OsJ_30281	unknown function	K.GVMDTVPDANLK.A + Oxidation (M)	1	1.04	1.14	1.11	1.05	1	-	1.07	1.06	1.09
gi|125606644	hypothetical protein OsJ_30351	unknown function	R.RAEASGSSSTR.T	1	0.92	0.91	0.91	0.89	1	-	-	1.03	1.04
gi|218193996	hypothetical protein OsI_14098	unknown function	R.SAAVTMSPSR.S + Oxidation (M)	1	-	0.94	0.92	0.90	-	-	-	-	-
gi|62732708	hypothetical protein LOC_Os11g23860	unknown function	K.ESFDVMEKLK.V + Oxidation (M)	1	0.92	0.92	0.94	-	-	-	-	-	-
gi|125538960	hypothetical protein	unknown function	.MASSSLASACTR.R	1	0.92	0.81	0.90	0.87	1	-	-	1.01	1.02
gi|125575295	hypothetical protein OsJ_32051	unknown function	R.GYEIIGAMK.- + Oxidation (M)	1	1.06	1.08	1.09	1.04	-	-	1	1.08	1.05
gi|56784673	hypothetical protein	unknown function	R.CCSTTAAK.A	1	1.07	-	1.03	1.03	-	-	-	-	-
gi|125531689	hypothetical protein OsI_33299	unknown function	R.GGGGGATAAPGPR.G	1	-	0.94	0.92	0.90	-	-	-	-	-
gi|34393980	hypothetical protein	unknown function	R.AAGGDAVLEAK.R	1	1.06	1.08	1.09	1.04	-	-	1	1.08	1.05
gi|125577348	hypothetical protein OsJ_34099	unknown function	R.STAPPPPPPPAATSEMGEIEGWR.S	-	-	-	-	-	1	0.95	1.01	0.95	0.94

- = Protein was not detected

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29 (cont.)

				Intensity ratio									
				Pokkali					IR29				
Aceession	Protein identity		Peptide	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
unknown function													
gi|125553200	hypothetical protein OsI_20864	unknown function	K.AFFSLVVAMLGVSSTAAMASDSSK.A	-	-	-	-	-	1	0.95	1.01	0.95	0.94
gi|218191703	hypothetical protein OsI_09195	unknown function	R.LDGNRSAGASGPLR.I	1	0.99	-	-	-	1	-	0.98	1.01	0.99
gi|218190181	hypothetical protein OsI_06083	unknown function	K.GSGGGGGGLARK.W	1	0.89	0.76	0.78	0.76	1	1.04	-	1.17	1.18
gi|48475129	hypothetical protein	unknown function	K.TGGDSTGSGK.G	-	-	-	-	-	1	1.09	0.96	1.08	0.93
gi|13486904	hypothetical protein	unknown function	R.DLGAASPPHR.L	-	-	-	-	-	1	1.08	1.07	1.06	0.97
gi|41052701	hypothetical protein	unknown function	R.GEMRPAGGGK.E + Oxidation (M)	-	-	-	-	-	-	1	0.99	1.02	0.98
gi|222635077	hypothetical protein OsJ_20350	unknown function	R.AGCGCTGR.T	1	-	1.08	1.15	-	1	0.96	-	0.96	0.91
gi|222624526	hypothetical protein	unknown function	R.TAPSAVDRV.-	1	0.93	0.85	-	0.79	1	0.96	0.98	0.97	0.90
gi|41393231	hypothetical protein	unknown function	R.ARVHESGGEGR.D	1	1.02	-	1.03	-	-	-	-	-	-
gi|218189891	hypothetical protein OsI_05511	unknown function	R.AEIVSTHP.-	1	1.08	-	1.16	1.11	-	-	-	-	-
gi|218199855	hypothetical protein OsI_26516	unknown function	-.MYDLPAK.F + Oxidation (M)	1	1.08	-	1.16	1.11	-	-	-	-	-
gi|115467954	hypothetical protein	unknown function	-.MATFPSSMSLR.N + Oxidation (M)	1	-	0.91	0.97	-	1	0.92	0.91	0.91	0.97
gi|222619020	hypothetical protein	unknown function	R.KTPTGTIEICWK.C	1	1.09	-	-	1.14	1	1.05	1.00	1.09	0.96
gi|218190937	hypothetical protein OsI_07592	unknown function	K.VNLTGYQIGK.T	1	0.90	0.91	-	-	1	1.13	1.20	-	1.11

- = Protein was not detected

105

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29 (cont.)

				Intensity ratio									
				Pokkali					IR29				
Aceession	Protein identity		Peptide	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
unknown function													
gi|222642019	hypothetical protein OsJ_30202	unknown function	K.DPGAAAGFR.R	1	0.91	-	-	-	-	1	-	0.90	0.98
gi|218195941	hypothetical protein OsI_18132	unknown function	K.IDASPCSQLR.T	1	0.96	-	-	-	1	0.97	-	1.03	0.99
gi|218190213	hypothetical protein OsI_06154	unknown function	R.AGPAVAACR.H	1	0.88	-	-	-	-	-	1.00	1.05	-
gi|22773240	Hypothetical protein	unknown function	R.AEGLGNK.E	1	0.92	-	-	-	1	1.01	-	1.01	-
gi|125556054	hypothetical protein OsI_23695	unknown function	-.MALPGASAR.F + Oxidation (M)	1	0.92	-	0.95	-	1	1.14	1.13	1.09	1.04
gi|49328177	hypothetical protein	unknown function	R.RIPMFCLFR.R + Oxidation (M)	1	0.93	0.76	0.77	0.72	1	1.12	1.03	1.25	0.97
gi|218186099	hypothetical protein OsI_36815	unknown function	R.SLPQSDVFR.S	1	-	0.97		0.94	1	1.02	1.07	1.09	1.03
gi|15528633	hypothetical protein	unknown function	R.AMRSITDAMGHYAAAK.D	1	0.96	0.95	0.92	0.94	1	0.98	0.94	1.03	0.91
gi|222635730	hypothetical protein OsJ_21653	unknown function	R.EAGRTATAQR.A	1	0.85	0.98	-	0.94	1	0.99	0.96	0.98	1.03
gi|41052656	hypothetical protein	unknown function	R.SDAAAAAAR.S	1	0.94	0.91	0.97		1	-	1.04	0.99	0.95
gi|125590188	hypothetical protein OsJ_14585	unknown function	R.STAAAAAGGGGDAR.L	1	0.91	0.94	0.90	0.96	1	0.96	0.98	0.96	0.93
gi|218187716	hypothetical protein OsI_00841	unknown function	R.EAGRTATAQR.A	1	0.92	0.96	0.94	0.91	1	1.11	1.07	1.01	1.10
gi|125552754	hypothetical protein OsI_20377	unknown function	R.VIGAAAERR.Y	1	1.16	-	1.20	1.20	1	0.89	0.94	0.97	1.03

- = Protein was not detected

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29 (cont.)

				Intensity ratio									
				Pokkali					IR29				
Aceession	Protein identity		Peptide	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
unknown function													
gi|218197392	hypothetical protein	unknown function	K.KASSPSPTSTAR.E	1	1.06	1.07	1.11	1.12	1	-	1.12	1.17	1.04
gi|222631309	hypothetical protein OsJ_18254	unknown function	.MGGNGNGADDK.A + Oxidation (M)	1	0.97	0.94	0.94	0.95	1	-	1.02	1.04	1.02
gi|222639834	hypothetical protein	unknown function	R.GPGIVGVVR.N	1	0.96	0.95	0.92	0.94	1	0.98	0.94	1.03	0.91
gi|57899086	hypothetical protein	unknown function	R.GGSGADDGMEEK.Y	1	0.95	0.87	0.98	0.91	1	0.91	1.01	1.04	1.02
gi|125555540	hypothetical protein	unknown function	K.AQAGPTLALHK.A	1	0.83	0.93	0.93	0.82	1	1.09	1.17	1.12	1.14
gi|218190262	hypothetical protein OsI_06262	unknown function	R.HLIESAPK.N	1	0.97	0.98	0.94	-	1	-	1.07	1.06	1.03
gi|218191902	hypothetical protein OsI_09617	unknown function	R.RYQLGVNVLLADHG QMGLGK.T	1	1.14	1.16	0.99	-	1	0.93	0.95	0.95	0.90
gi|125551887	hypothetical protein	unknown function	R.LVLVGDR.G	-	-	-	-	-	1	-	-	1.08	1.03
gi|125556256	hypothetical protein OsI_23884	unknown function	R.CATEPMATSDPAAYH LKPR.F	1	0.87	-	-	-	-	-	-	-	-
gi|218187850	hypothetical protein OsI_01097	unknown function	R.NANDGISLAQTAEGA LK.S	1	0.91	0.91	0.80	0.86	-	-	-	-	-
gi|54291774	hypothetical protein	unknown function	K.STSSTSSAIAPLSSLSG NSRSVIR.S	1	0.92	0.90	-	0.94	-	-	-	-	-
gi|218185801	hypothetical protein OsI_36227	unknown function	R.ANAVPMVR.H + Oxidation (M)	1	0.88	-	-	-	-	-	-	-	-
gi|115452015	Hypothetical protein	unknown function	R.RPARMP.-	1	0.96	0.95	0.92	0.88	-	-	-	-	-
gi|125558601	hypothetical protein OsI_26280	unknown function	K.ASEMKAINK.R + Oxidation (M)	1	1.14	1.24	1.18	1.18	-	-	-	-	-
gi|22711556	Hypothetical protein	unknown function	K.IITRAAK.A	1	1.18	-	1.18	-	1	0.9	0.88	0.95	0.99

- = Protein was not detected

Table 3.5 Differentially expressed and characterized proteins from leaves of Pokkali and IR29 (cont.)

				Intensity ratio									
				Pokkali					IR29				
Aceession	Protein identity		Peptide	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt	0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
unknown function													
gi|218187627	hypothetical protein OsI_00653	unknown function	K.RARPPASSPVDPPASP PAPEDPR.V	1	0.92	0.90	-	0.94	-	-	-	-	-
gi|46576031	hypothetical protein	unknown function	R.DYGGCFAVR.Y	1	1.06	1.09	1.07	1.01	1	0.96	1.02	1.03	-
gi|125555711	hypothetical protein OsI_23348	unknown function	R.QAGAGGGGGIAR.S	1	0.90	0.86	0.80	0.77	-	-	-	-	-
gi|20219037	hypothetical protein	unknown function	K.ADADDGNVTNR.K	1	1.09	1.04	1.12	1.04	-	-	-	-	-
gi|14091856	hypothetical protein	unknown function	R.GHHKGLQLPK.K	-	-	-	-	-	1	1.11	1.18	1.16	1.10
gi|218190596	hypothetical protein	unknown function	R.IDMVGIELPK.I	-	-	-	-	-	1	1.11	1.18	1.16	1.10
gi|50508823	hypothetical protein	unknown function	R.GNGGAAARER.R	-	-	-	-	-	1	1.15	1.18	1.24	-
gi|218198196	hypothetical protein	unknown function	K.KPLSPASMK.M	-	-	-	-	-	1	1.15	1.18	1.24	-
gi|46805635	hypothetical protein	unknown function	M.ADMGGQR.R + Oxidation (M)	-	-	-	-	-	1	0.87	0.86	0.85	-

- = Protein was not detected

108

3.4 End point RT-PCR

OligodT$_{+A}$/UBC222_433-F/R *(protein kinase domain containing protein*) primers were designed from the nucleotide sequences of a RAP-PCR fragment. The amplification success of this primer pair was examined by RT-PCR. The expected amplification product of 178 bp in size was obtained. However, the non-specific amplification products were also obtained. Therefore, primers for amplification of the oligodT$_{+A}$/UBC222-433 transcript are not suitable for further quantitative analysis (Figure 3.52)

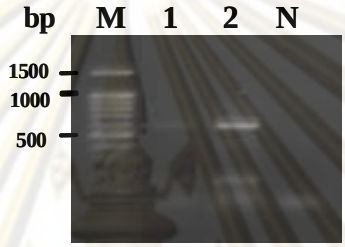

Figure 3.52 A 1.6% ethidium bromide stained agarose gel showing the amplification results of oligodT$_{+A}$/UBC222-433-F/R using the first strand cDNA template of untreated IR29 (lanes 1) and Pokkali (lanes 2). Lanes M and N are a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

For candidate salt responsive fragments isolated by cDNA-AFLP, six primer pairs were designed. Conventional RT-PCR was carried out and E-4/M-9_364-F/R and E-5/M-8_365-F/R did not generate the amplification product (Figures 3.53C and 3.53E).

E-5/M-5_478-F/R for amplification of *hypothetical protein$_{171}$* generated low yield of the amplification products (Figure 3.53F) and difficult to be used for quantitative real-time PCR analysis. Therefore, the expression profile of this transcript during salt stress treatment was analyzed using semi-quantitative analysis.

E-1/M-7_497-F/R, E+A-2/M+C-1_534-F/R and E-5/M-5_633-F/R for amplification of *ATP synthase subunit C family protein*, *Lipin, N-terminal conserved region* and *Cytochrome P450 monooxygenase* yielded the intense amplified products. The expression of these transcripts are suitable to be analyzed by quantitative real-time PCR analysis (Figures 3.53 A, B and D).

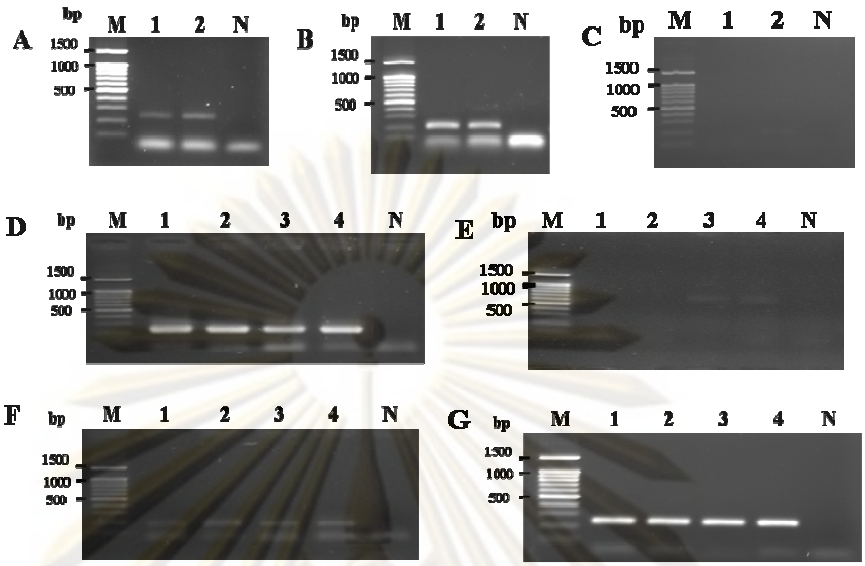

Figure 3.53 A 1.5-1.8 % ethidium bromide stained agarose gel showing the amplification results of E-1/M-7_497-F/R (213 bp; panel A), E_{+A}-2/ M_{+C}-1_534-F/R (204 bp; panel B), E-4/M-9_364-F/R (206 bp; panel C), E-5/M-5_633-F/R (215 bp; panel D), E-5/M-8_461-F/R (240 bp; panel E), E-5/ M-5_478-F/R (171 bp, panel F) and the internal control (217 bp, panel G) against the first strand cDNA of IR29 (lanes 1 of panel A, B and C and lane 1 and 2 of panel D, E, F and G) and Pokkali (lanes 2 of panel A, B and C and lane 3 and 4 of panel D, E, F and G). Lanes M and N are a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

From proteomics analysis, two proteins including cysteine protease (CTP) and sucrose transporter 4 (SUT4) that are functionally related to salt stress and showed clearly differential expression during the salt stress treatment, were selected for quantitative analysis. Nevertheless, low amplification yields were observed from RT-PCR of CTP_{201} (Figure 3.54 A). Therefore, the expression levels of CTP_{201} in IR29 and Pokkali were further examined using semi-quantitative RT-PCR analysis. SUT1 primer pairs did not generate the amplification product (Figure 3.54 B).

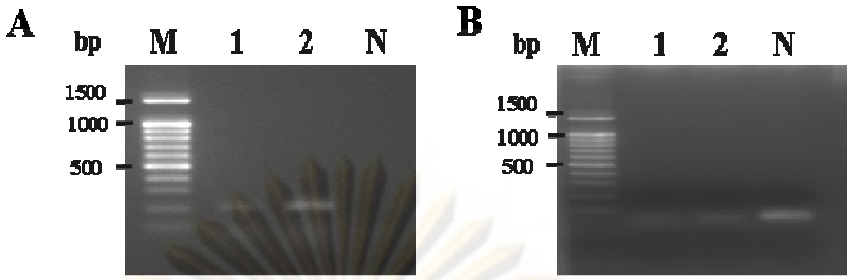

Figure 3.54 A 1.5% ethidium bromide stained agarose gel showing the amplification results of CTP-F/R (panel A) and SUT4-F/R (panel B) between the cDNA template of the untreated IR29 (lanes 1) and Pokkali (lanes 2). Lanes M and N are a 100 bp DNA ladder and the negative control (without cDNA template), respectively.

3.5 Isolation and characterization of the full length cDNA of stress response gene homologues of *O. sativa*

Initially, the primary 5' and 3' RACE-PCR of *protein kinase domain containing protein* generated smear products (Figures 3.55 A and C). Subsequently, 5' nested RACE-PCR and semi-nested 3' RACE-PCR were carried out using the diluted primary RACE-PCR product as the template. For semi-nested 3' RACE-PCR, the amplification product was generated with the same gene-specific primer combined with Nested Universal Primer (NUP). For nested 5' RACE-PCR, product was generated with nested 5' RACE-PCR and NUP primers.

After electrophoresis, the nested 5' RACE-PCR product showed the smear pattern (Figure 3.55, B). While discrete band of approximately 1500 bp in size was obtained from semi-nested 3' RACE-PCR (Figure 3.55 D). This fragment was cloned and sequenced. Nucleotide sequences of 3′ RACE-PCR of this gene were then assembled (Figure 3.56).

The assembled sequence of *protein kinase domain containing protein* was 1353 bp containing the open reading frame of 969 bp deducing to 323 amino acids with the 3' UTRs of 538 bp long (excluding the poly A tail). This transcript was significantly similar to *protein kinase domain containing protein* of *O. sativa* L (*E*-value = 2e-135 (Figure 3.57).

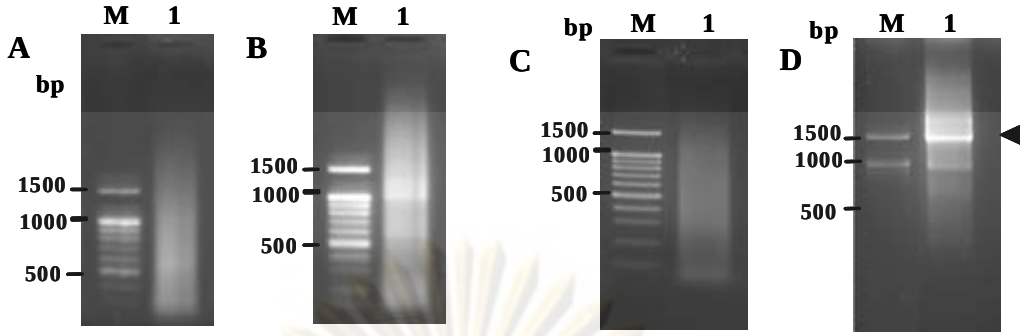

Figure 3.55 The primary 5′(lane 1, A), nested 5′ (lane 1, B), primary 3′ (lane 1, C) and semi-nested 3′ (lane 1, D) RACE-PCR products of *protein kinase domain containing protein*. Arrowheads indicate RACE-PCR products that were cloned and sequenced. Lanes M are 100 bp DNA ladder.

A.

CACCGAGTTCTGCCCCGGCGGCGACCTCCACGTCCTCCGCCAGCGCCAGCCTCACCGCCGCTTCTCCGA

3′ RACE-PCR

GTCCGCCGTCAGGTTTTATGCGGCGGAGGTGGTGGCGGCGCTGGAGTACGTCCACATGGTGGACATCGT
GTACCGTGACCTGAAGCCGGAGAACGTGCTCGTCCGCGCCGACGGCCACATCATACTCACCGACTTCGA
CCTCTCGCTCAAGTGCGACCCGACGGCGCCGACGCCGGCGCACGTCATCTCGGACCCCATCGCCCTCGC
CGGCGGCCAGTCTTCCTCCTCCTCCTCCTCGTCGTGCATCATCCCGTCCTGCATCGTCTCCGCCGTGTC
GTGCTTCCAGCTCTTCCCAGGACGCGGGCGCCACCGCCGCCGCCGCTGGCGCGGCCGCAAGAAGCCGTC
GAGCGGCGGCGGCGGCAAT

B.

GGACATCGTGTACCGTGACCTGAAGCCGGAGAACGTGCTCGTCCGCGCCGACGGCCACATCATGCTCAC
CGACTTCGACCTCTCGCTCAAGTGCGACCCGACGGCGCCGACGCCGGCGCACGTCATCTCGGACCCCAT
CGCCCTCGCCGGCGGCCAGTCTTCCTCCTCCTCCTTCTCGTCGTGCATCATCCCGTCCTGCATCGTCCC
CGCCGTGTCGTGCTTCCAGCTCTTCCCAGGACGCGGGCGCCACCGCCGCCGCCGCTGGCGCGGCCGCAA
GAAGCCGTCGAGCGGCGGCGGCGGCAATGGCGGCAGCAGCTTCTCCTCCGGTGGGCTGGAGCTGAAGTT
CGTGGCGGAGCCGGTGGAGCTCCGGTCAATGTCGTTCGTCGGGACGCACGAGCACCTCGCGCCGGAGAT
CGTCTCCGGCGAGGGGCACGGCAGCTCCGTCGACTGGTGGACGCTGGGGGTGTTCGTCTTCGAGCTGCT
CTACGGCGTGACGCCGTTCAAGGGCCACGACAACGAGATGACCCTGGCGAACATCGTGGCGCGCGCGCT
CGAGTTCCCCAGGGAGCCGCCCGTCTCCGCCGCCGCCAAGGACCTCGTCACGTCGCTCCTCGCCAAGGA
CCCGGCGCGCCGCCTCGGCGCCACCGTCGGCGCCGCGGTGATCAAGCGGCACCCCTTCTTCAGCGGCGT
CAACTGGGCGCTCCTCCGCTGCGCCACGCCTCCGTACGTTCCCCCGCCGTTCAGCGTCGCCACCGCCAC
CGCCGCGAACGCCGCCGCCGCGAACGCTGACATGTCATACGACGACGACAGCTGCCCCGGCACACCCGT
GGAGTACTACTAGGACAAGACAGGCTATCCCAATGGAGGAAGCTGCCATGGATGAACGTGCAAAGCTAG
GGAGGAAGCCGCCATTGCCGGCGCACGTGGTGGGGGACGCGGGACGGCGGCGAACCGAACTGGCATGGC
GCGCACGCGGGCGCCGCGCTGTCGCGCCAAATAGTTGCCGGGTGCGTGTCCCCCCGGACGGGAGTAAAT
TATTGCTGCGCGTATCTGACTTTGTCTGAACGTGGTCACTTGCCTGGTTGGTGACTGTCTCACTAAAAA
AGAACTTATTAACGAGTATCTCCATAGTGCTATACTAGTATATACAAATATTTTAGGCCCATTTGGACT
TTTCGAGCATGTAACCCCGCAAAATTCCTATGAACTTTGAAACATGTTGCTCAGTGGTGAGTTGTATTT
ATGAATTCTTTTTACTAGTAAGATTTGTTCTTCCGGTCTACCGCTGACAGGCCGTAAAACTGCATATAA
GCAATAAAATAATAATAATTTGTGGATAAAACTTAAAAATATTCTTCAGTCTACAACAGATAAACTGTA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.56 Nucleotide sequence from RAP-PCR (A) and 3' RACE-PCR (B) of *protein kinase domain containing protein* of *O. sativa* L. A gene-specific primer was used for 3' RACE-PCR of this gene.

```
CACCGAGTTCTGCCCCGGCGGCGACCTCCACGTCCTCCGCCAGCGCCAGCCTCACCGCCG 60
  T  E  F  C  P  G  G  D  L  H  V  L  R  Q  R  Q  P  H  R  R 156
CTTCTCCGAGTCCGCCGTCAGGTTTTATGCGGCGGAGGTGGTGGCGGCGCTGGAGTACGT 120
  F  S  E  S  A  V  R  F  Y  A  A  E  V  V  A  A  L  E  Y  V 176
CCACATGGTGGACATCGTGTACCGTGACCTGAAGCCGGAGAACGTGCTCGTCCGCGCCGA 180
  H  M  V  D  I  V  Y  R  D  L  K  P  E  N  V  L  V  R  A  D 196
CGGCCACATCATGCTCACCGACTTCGACCTCTCGCTCAAGTGCGACCCGACGGCGCCGAC 240
  G  H  I  M  L  T  D  F  D  L  S  L  K  C  D  P  T  A  P  T 216
GCCGGCGCACGTCATCTCGGACCCCATCGCCCTCGCCGGCGGCCAGTCTTCCTCCTCCTC 300
  P  A  H  V  I  S  D  P  I  A  L  A  G  G  Q  S  S  S  S  S 236
CTTCTCGTCGTGCATCATCCCGTCCTGCATCGTCCCCGCCGTGTCGTGCTTCCAGCTCTT 360
  F  S  S  C  I  I  P  S  C  I  V  P  A  V  S  C  F  Q  L  F 256
CCCAGGACGCGGGCGCCACCGCCGCCGCCGCTGGCGCGGCCGCAAGAAGCCGTCGAGCGG 420
  P  G  R  G  R  H  R  R  R  R  W  R  G  R  K  K  P  S  S  G 276
CGGCGGCGGCAATGGCGGCAGCAGCTTCTCCTCCGGTGGGCTGGAGCTGAAGTTCGTGGC 480
  G  G  G  N  G  G  S  S  F  S  S  G  G  L  E  L  K  F  V  A 296
GGAGCCGGTGGAGCTCCGGTCAATGTCGTTCGTCGGGACGCACGAGCACCTCGCGCCGGA 540
  E  P  V  E  L  R  S  M  S  F  V  G  T  H  E  H  L  A  P  E 316
GATCGTCTCCGGCGAGGGGCACGGCAGCTCCGTCGACTGGTGGACGCTGGGGGTGTTCGT 600
  I  V  S  G  E  G  H  G  S  S  V  D  W  W  T  L  G  V  F  V 336
CTTCGAGCTGCTCTACGGCGTGACGCCGTTCAAGGGCCACGACAACGAGATGACCCTGGC 660
  F  E  L  L  Y  G  V  T  P  F  K  G  H  D  N  E  M  T  L  A 356
GAACATCGTGGCGCGCGCGCTCGAGTTCCCCAGGGAGCCGCCCGTCTCCGCCGCCGCCAA 720
  N  I  V  A  R  A  L  E  F  P  R  E  P  P  V  S  A  A  A  K 376
GGACCTCGTCACGTCGCTCCTCGCCAAGGACCCGGCGCGCCGCCTCGGCGCCACCGTCGG 780
  D  L  V  T  S  L  L  A  K  D  P  A  R  R  L  G  A  T  V  G 396
CGCCGCGGTGATCAAGCGGCACCCCTTCTTCAGCGGCGTCAACTGGGCGCTCCTCCGCTG 840
  A  A  V  I  K  R  H  P  F  F  S  G  V  N  W  A  L  L  R  C 416
CGCCACGCCTCCGTACGTTCCCCCGCCGTTCAGCGTCGCCACCGCCACCGCCGCGAACGC 900
  A  T  P  P  Y  V  P  P  P  F  S  V  A  T  A  T  A  A  N  A 436
CGCCGCCGCGAACGCTGACATGTCATACGACGACGACAGCTGCCCCGGCACACCCGTGGA 960
  A  A  A  N  A  D  M  S  Y  D  D  D  S  C  P  G  T  P  V  E 456
GTACTACTAGGACAAGACAGGCTATCCCAATGGAGGAAGCTGCCATGGATGAACGTGCAA 1020
  Y  Y  *                                                    458
AGCTAGGGAGGAAGCCGCCATTGCCGGCGCACGTGGTGGGGGACGCGGGACGGCGGCGAA 1080
CCGAACTGGCATGGCGCGCACGCGGGCGCCGCGCTGTCGCGCCAAATAGTTGCCGGGTGC 1140
GTGTCCCCCCGGACGGGAGTAAATTATTGCTGCGCGTATCTGACTTTGTCTGAACGTGGT 1200
CACTTGCCTGGTTGGTGACTGTCTCACTAAAAAAGAACTTATTAACGAGTATCTCCATAG 1260
TGCTATACTAGTATATACAAATATTTTAGGCCCATTTGGACTTTTCGAGCATGTAACCCC 1320
GCAAAATTCCTATGAACTTTGAAACATGTTGCTCAGTGGTGAGTTGTATTTATGAATTCT 1380
TTTTACTAGTAAGATTTGTTCTTCCGGTCTACCGCTGACAGGCCGTAAAACTGCATATAA 1440
GCAATAAAATAATAATAATTTGTGGATAAAACTTAAAAATATTCTTCAGTCTACAACAGA 1500
TAAACTGTAAAAAAAAAAAAAAAAAAAAAAAAAAA                         1535
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Figure 3.57 Partial nucleotide sequence and deduced amino sequences of *protein kinase domain containing protein* of *O. sativa* L. The stop codon and 3' RACE-PCR primer position are illustrated in boldfaced and underlined.

3.6 Examination of expression levels of salt-stress response transcripts by semi-quantitative RT-PCR

Total RNA was extracted from leaves of IR29 and Pokkali varieties collected from different time intervals (*N* = 3 - 4 for each group of the control and 0, 3, 6, 12 and 24 hpt) of the salt treatment. After DNase I treatment, DNA-free total RNA of each plants was reverse transcribed. The first strand cDNA was used as the template for semi-quantitative RT-PCR analysis.

3.61 Optimization of semi-quantitative RT-PCR conditions

Expression levels of *cysteine protease* (*CTP_{201}*, the amplification size = 201 bp) and a *hypothetical protein$_{171}$* (from a E-5/M-5_478 cDNA-AFLP fragment; the amplification size = 171 bp) were examined using semi-quantitative RT-PCR. This technique requires optimization of several parameters including concentrations of $MgCl_2$, and primers and the number of PCR cycles. The *hypothetical protein$_{240}$* (from primers F:5′-TCG CTA TGA ACG CTT GGC T -3′, R:5′- TGA AGATAA CGC AGG TGT CC -3′ hereafter called *HTP_{240}*) was used as the internal control. The preliminary RT-PCR was carried out using the annealing temperature of 53°C as described previously.

3.62 Optimization of the primer concentration

The optimal concentration of each primer (between 0, 0.10, 0.15, 0.20, 0.25 and 0.30 μM) was carefully examined using the optimized $MgCl_2$ concentration of each transcript. Lower concentrations may result in non-quantitative amplification whereas higher concentrations of primer may leave a large amount of unused primers which could give rise to non-specific amplification products. The suitable concentration of primers for each gene was shown by Figures 3.58 A-3.59 A and Table 3.6.

3.63 Optimization of the $MgCl_2$ concentration

RT-PCR of each gene was further carried out with optimized primer concentration and other fixed components as for the typical RT-PCR with the exception that $MgCl_2$ concentrations was varied (0, 1.0, 1.5, 2.0, 2.5 and 3.0 mM).

The concentration of $MgCl_2$ that gave the highest yield and specificity for each PCR product was chosen (Figures 3.58 B-3.59 B and Table 3.6).

3.64 Optimization of the cycle numbers

The number of amplification cycles was important because the PCR product reflecting the expression level should be measured quantitatively before reaching a plateau amplification phase. At the plateau stage, transcripts initially present at different level may give equal intensity of the amplification products.

In this experiment, RT-PCR of each gene was performed using the conditions that $MgCl_2$ and primer concentrations were optimized for 25, 28 and 30 cycles for control and 28, 32 and 35 cycles for the target gene. The number of cycles that gave the highest yield before the product of each transcript reached a plateau phase of amplification was chosen (Figures 3.58 C-3.59 C and Table 3.6).

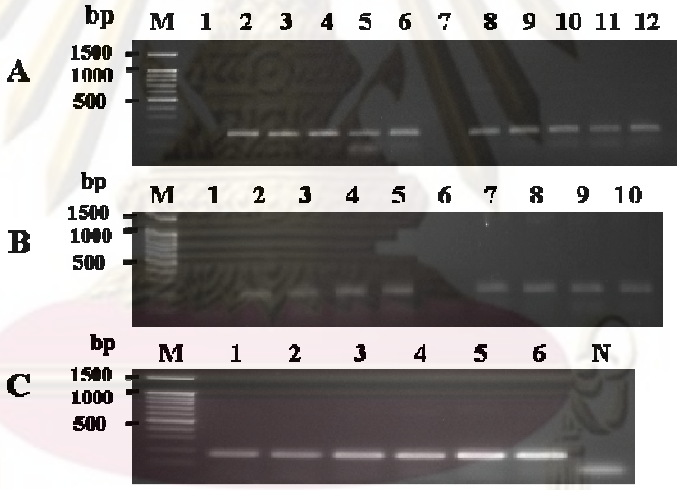

Figure 3.58 Optimization of primer concentration (A), $MgCl_2$ concentration (B) and number of cycles (C) of hypothetical protein $_{171}$. Lanes 1-6, A; 1-5, B and 1, 3 and 5, C = IR29. Lanes 7-12, A; 6-10, B and 2, 4 and 6, C = Pokkali. (A). Lanes 1 & 7 = 0 µM, lanes 2 & 8 = 0.1 µM, lanes 3 & 10 = 0.15 µM, lanes 4 & 11 = 0.2 µM, lane 5 & 11 = 0.25 µM and lane 6 & 12 = 0.3 µM primer concentration. (B) Lanes 1 & 6 = 0 mM, lanes 2 &7 = 0.5mM, lanes 3 & 8 = 1.0 mM, lanes 4 & 9 = 1.5 mM and lanes 5 & 10 = 2.0 mM $MgCl_2$ concentration. (C) Lane 1 & 2 = 28 cycles, lanes 3 & 4 = 32 cycles and lanes 5 & 6 = 35 cycles. Lanes M and N are a 100 bp DNA ladder and the negative control, respectively.

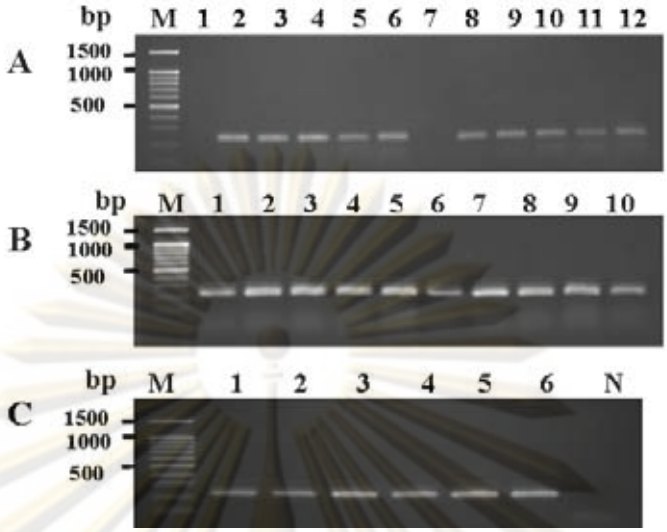

Figure 3.59 Optimization of primer concentration (A), $MgCl_2$ concentration (B) and number of cycles (C) of hypothetical $protein_{217}$ (the internal control). Lanes 1-6, A; 1-5, B and 1, 3 and 5, C = IR29. Lanes 7-12, A; 6-10, B and 2, 4 and 6, C = Pokkali. (A). Lanes 1 & 7 = 0 µM, lanes 2 & 8 = 0.1 µM, lanes 3 & 10 = 0.15 µM, lanes 4 & 11 = 0.2 µM, lane 5 & 11 = 0.25 µM and lane 6 & 12 = 0.3 µM primer concentration. (B) Lanes 1 & 6 = 0 mM, lanes 2 &7 = 0.5mM, lanes 3 & 8 = 1.0 mM, lanes 4 & 9 = 1.5 mM and lanes 5 & 10 = 2.0 mM $MgCl_2$ concentration. (C) Lane 1 & 2 = 23 cycles, lanes 3 & 4 = 25 cycles and lanes 5 & 6 = 28 cycles. Lanes M and N are a 100 bp DNA ladder and the negative control, respectively.

Table 3.6 Optimized $MgCl_2$ and primer concentrations and the number of amplification cycles for semi-quantitative RT-PCR of candidate thermal-stress response genes resulted from cDNA-AFLP analysis of rice and the internal control (*hypothetical protein*)

Transcript	Expected amplicon (bp)	$MgCl_2$ concentration (mM)	Primer concentration (µM)	PCR cycles
Hypothetical $protein_{171}$	171	1.0	0.15	32
CTP_{201}	201	1.5	0.2	35
internal control (HTP_{217})	217	2.0	0.2	25

3.7 Semi-quantitative RT-PCR analysis

3.71 *Hypothetical protein*$_{171}$ (E-5/M-5_478)

The expression patterns of *hypothetical protein*$_{171}$ in leaves of IR29 and Pokkali at 0 (control), 3, 6, 12 and 24.hpt examined by semi-quantitative RT-PCR (Figure. 3.60). Expression level of this gene in leaves of IR29 was not significantly different (0.0254 ± 0.0127, 0.7858 ± 0.07371, 0.7751 ± 0.0641, 0.7082 ± 0.0415 and 0.7092 ± 0.0219) during the salt stress (Figure. 3.61 A).

The expression level of *hypothetical protein*$_{171}$ in leaves of Pokkali at 0 hpt (0.6641 ± 0.0450) was significantly lower than that at 3 hpt (0.7420 ± 0.0342, $P < 0.05$). Its expression was further increased at 6 and 12 hpt (0.810 ±.0.0322 and 0.8453 ± 0.0278, respectively; $P < 0.05$) and decreased to the basal level at 24 hpt (0.7010 ± 0.0325, $P < 0.05$) (Figure. 3.61 B)

When data from both rice varieties were analyzed together, the expression level of this transcript in leaves of IR29 was significantly higher than that of Pokkali at 0 hpt (0.7487 ± 0.0254 and 0.6641 ± 0.0450, respectively). After the NaCl stress, the expression level of Pokkali was up-regulated and significantly higher than that of IR29 at 12 hpt (0.8453 ± 0.0278 compared to 0.7082 ± 0.0415) (Figure 3.61 C).

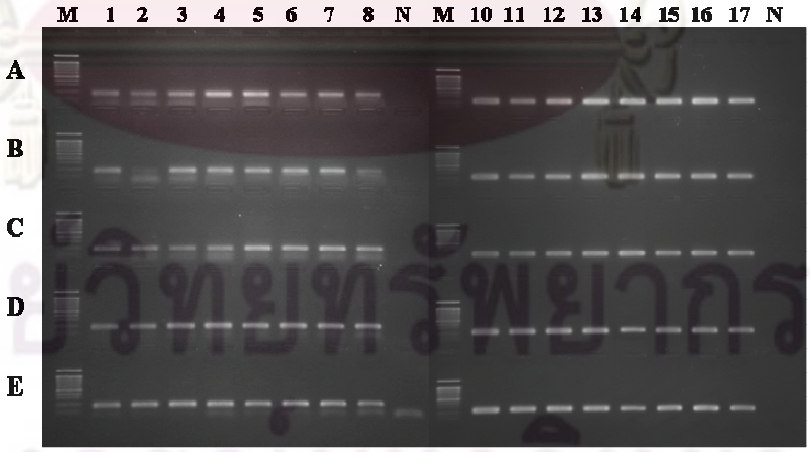

Figure 3.60 A 1.8% ethidium bromide-stained agarose gel showing the expression level of *hypothetical protein*$_{171}$ (lanes 1-8; A-E) and the internal control (lanes 10-17; A-E) in leaves of IR29 (lanes 1-4, 10-14, A-E) and Pokkali (lanes 5-8, 14-17, A-E) at the normal condition (0 hr, A) and after salt treatment for 3 (B), 6 (C), 12 (D) and 24 (E) hours. Lanes M and N are a 100 bp DNA ladder and the negative control (without DNA template), respectively.

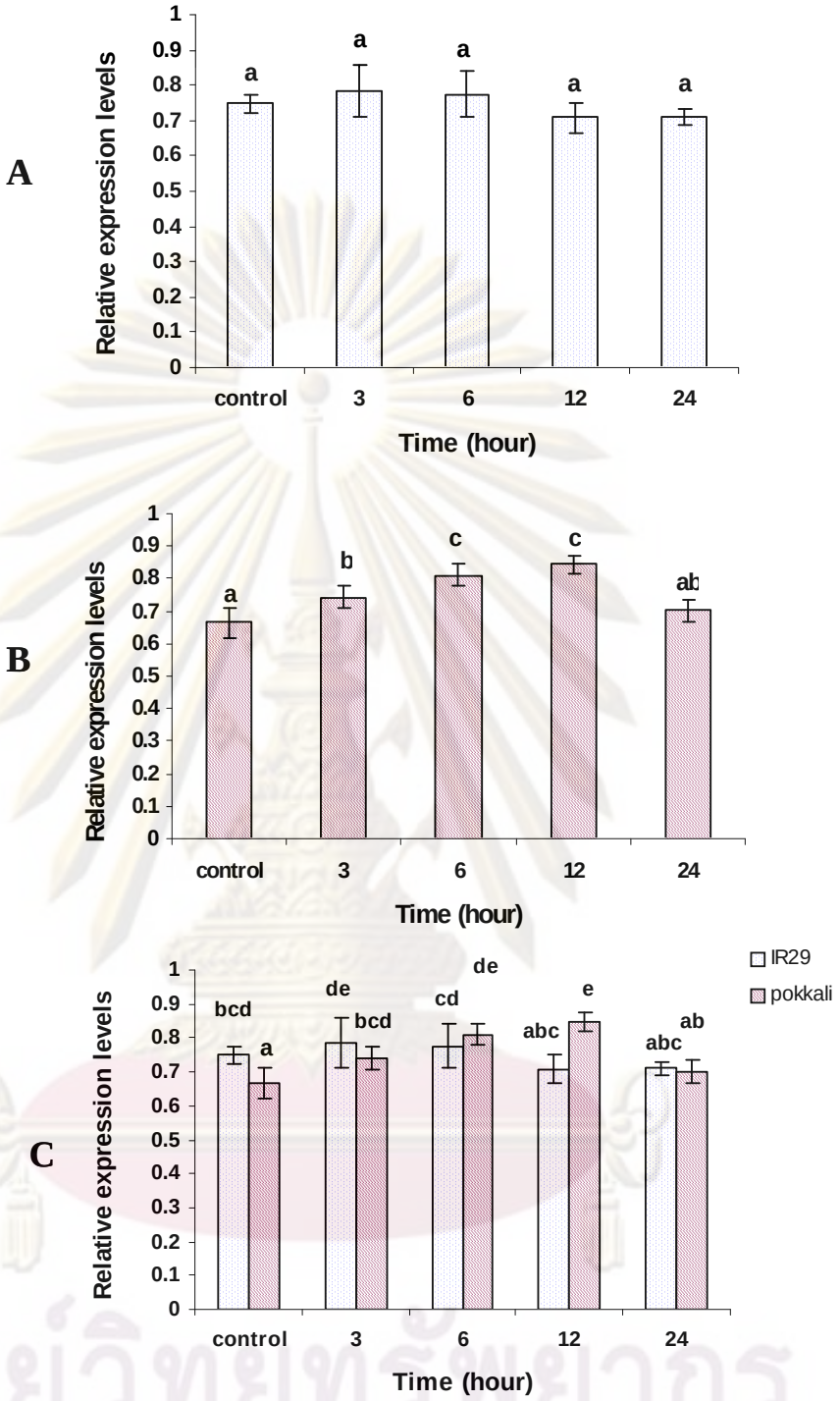

Figure 3.61 Histograms showing the time-course relative expression levels of *hypothetical protein*$_{171}$ in leaves of IR29 (A), Pokkali (B) varieties at 0, 3, 6 12 and 24 hour post treatment (hpt). The relative expression levels of this transcript in both rice varieties were also analyzed simultaneously (C). The same letters indicate non-significant differences between relative expression levels of different groups of samples.

3.72 *Cyteine protease* (*CTP_{201}*)

The expression patterns of *CTP_{201}* in leaves of IR29 and Pokkali at 0 (control), 3, 6, 12 and 24 hpt were represented by Figure 3.62. The expression level of IR29 was significantly down-regulated at 3 hpt (0.9209 ± 0.1014, $P < 0.05$), returned to the basal level at 6 hpt (1.1140 ± 0.0999, $P > 0.05$) and down-regulated at 12 and 24 hpt (0.9381 ± 0.0845, $P < 0.05$ and 0.7839 ± 0.0497, $P < 0.05$) (Figure. 3.63 A).

The expression patterns of *CTP_{201}* in leaves of Pokkali were comparable to those of IR29. Its expression level was significantly increased at 6 (0.9146 ± 0.0527, $P < 0.05$) and 12 hpt (0.8724 ± 0.0821, $P < 0.05$) before returned to the normal level at 24 hpt (0.7338 ± 0.0443, $P < 0.05$; Figure. 3.63 B).

When data from both rice varieties were analyzed together, the expression levels of *CTP_{201}* in leaves of both rice varieties were significantly higher than that of Pokkali at 0, 3 and 6 hpt ($P < 0.05$). After the NaCl treatment for 12 and 24 hpt. the expression levels of both rice varieties ($P > 0.05$) were not significant owing to large standard errors between treatment groups (Figure. 3.63 C).

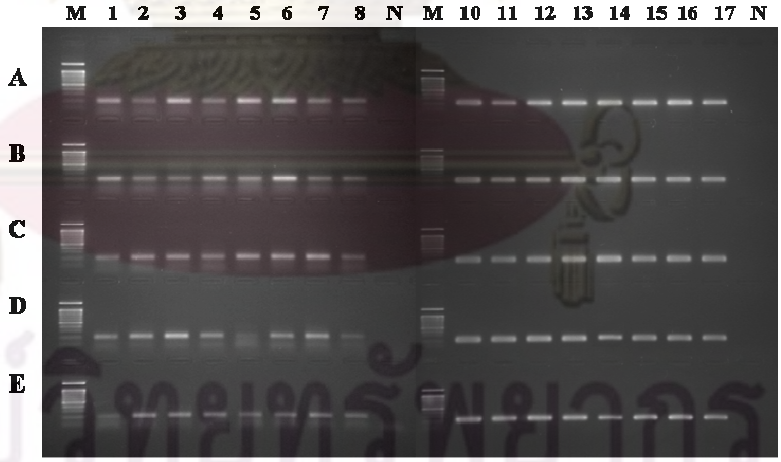

Figure 3.62 A 1.8% ethidium bromide-stained agarose gel showing the expression level of *CPT_{201}* (lanes 1-8; A - E) and the internal control (lanes 10-17; A - E) in leaves of IR29 (lanes 1-4, 10-14, A - E) and Pokkali (lanes 5-8, 14-17, A - E) varieties at the normal condition (0 hr, A) and after salt treatment for 3 (B), 6 (C), 12 (D) and 24 (E) hours. Lanes M and N are a 100 bp DNA ladder and the negative control (without DNA template), respectively.

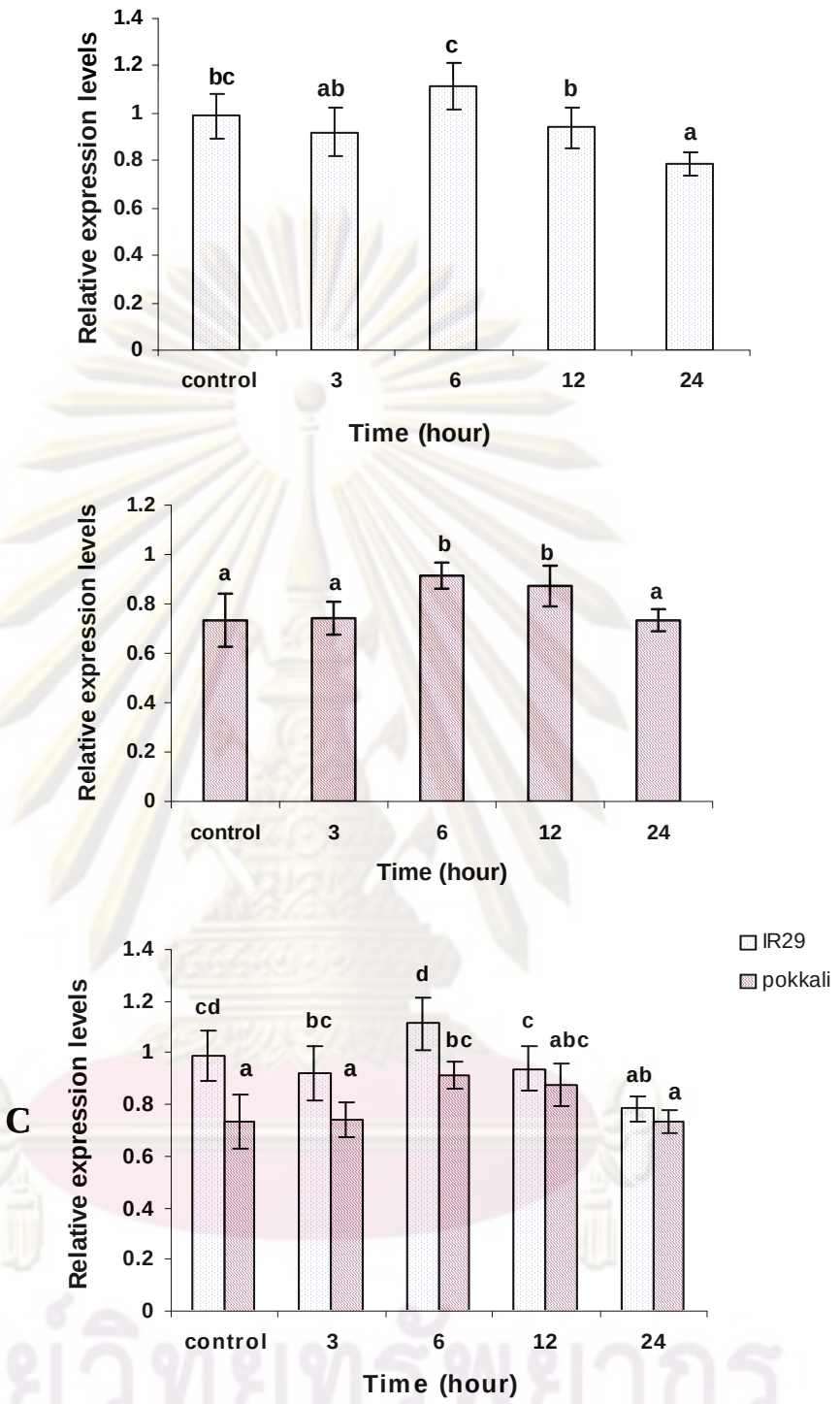

Figure 3.63 Histograms showing the time-course relative expression levels of *CPT_{201}* in leaves of the IR29 (A) and Pokkali (B) at 0, 3, 6 12 and 24 hpt. The relative expression levels of this transcript in both rice varieties were also analyzed simultaneously (C). The same letters indicate non- significant difference between relative expression levels of different groups of samples.

Table 3.7 A time-course semi-quantitative RT-PCR of candidate salt-stress response genes (E-5/M-5_478$_{171}$, and CTP$_{201}$) in leaves of IR29 and Pokkali varieties

Transcript	Sample	Mean relative expression level*				
		0 hpt	3 hpt	6 hpt	12 hpt	24 hpt
Hypothetical protein$_{171}$	IR29	0.7488 ± 0.0255^{a}	0.7859 ± 0.0737^{a}	0.7751 ± 0.0641^{a}	0.7082 ± 0.0416^{a}	0.7093 ± 0.0219^{a}
	Pokkali	0.6642 ± 0.0451^{a}	0.7420 ± 0.0343^{b}	0.8104 ± 0.0322^{c}	0.8453 ± 0.0278^{c}	0.7010 ± 0.0326^{ab}
CTP$_{201}$	IR29	0.9875 ± 0.0969^{bc}	0.9209 ± 0.1014^{a}	1.11400 ± 0999^{c}	0.9381 ± 0.0845^{b}	0.7839 ± 0.0497^{a}
	Pokkali	0.7337 ± 0.1079^{a}	0.7423 ± 0.0666^{a}	0.9146 ± 0.0527^{b}	0.8724 ± 0.0821^{b}	0.7338 ± 0.0443^{a}

*The same superscripts between different time interval data are not significantly different ($P > 0.05$).

121

3.8 Quantitative real-time PCR analysis of *lipin N-terminal conserved region family protein, cytochrome p450 monooxygenase* and *ATP synthase subunit C family protein* in leaves of IR29 and Pokkali

3.8.1 *Internal control HTP_{217}*

The standard curve of the internal control, HTP_{217} was constructed with the amplication efficiency of 93.1% (log_2 = 1.969; Figure 3.64). The expression levels of HTP_{217} in all specimens were quantified and used to normalize the expression levels of the target genes in different groups of IR29 and Pokkali varieties.

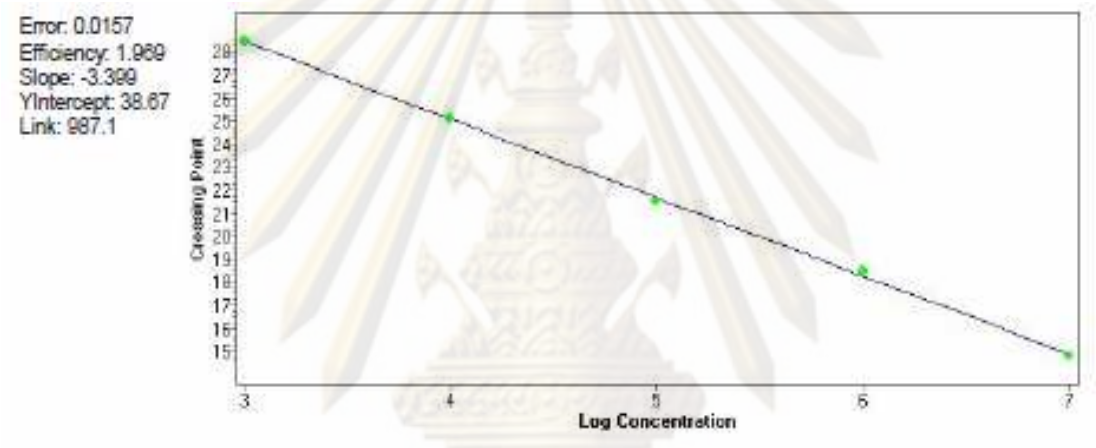

Figure 3.64 The standard curve of internal control HTP_{217} (error = 0.0157, amplification efficiency log_2 = 1.969 and equation; Y = -3.399 * log(X) + 38.67) using 10-fold dilution of plasmid DNA between 10^3-10^7 copies of as the template.

3.8.2 *Lipin, N-terminal conserved region family protein* (E+A-2/ M+C-1_{205})

The standard curve of *lipin, N-terminal conserved region family protein* was constructed with the amplification efficiency of 89.53% (log_2 = 1.952; Figure 3.65). Results from quantitative real-time PCR revealed that the relative expression level in leaves of IR29 at 6 hpt seems to be lower than that of 0, 3 and 12 hpt but the expression levels were not significant owing to large standard errors between treatment groups. The expression level of *lipin* was up-regulated from 6 hpt to at 24 hpt (Figure 3.68A).

Likewise, the relative expression levels of *lipin* in Pokkali seem to be increased at 12 hpt and clearly significantly increased at 24 hpt ($P < 0.05$) (Figure 3.68B).

When data from both rice varieties were analyzed together, the relative expression levels of *lipin* in leaves of IR29 was significantly lower than that in Pokkali at 6 hpt ($P < 0.05$) (Figure 3.68C).

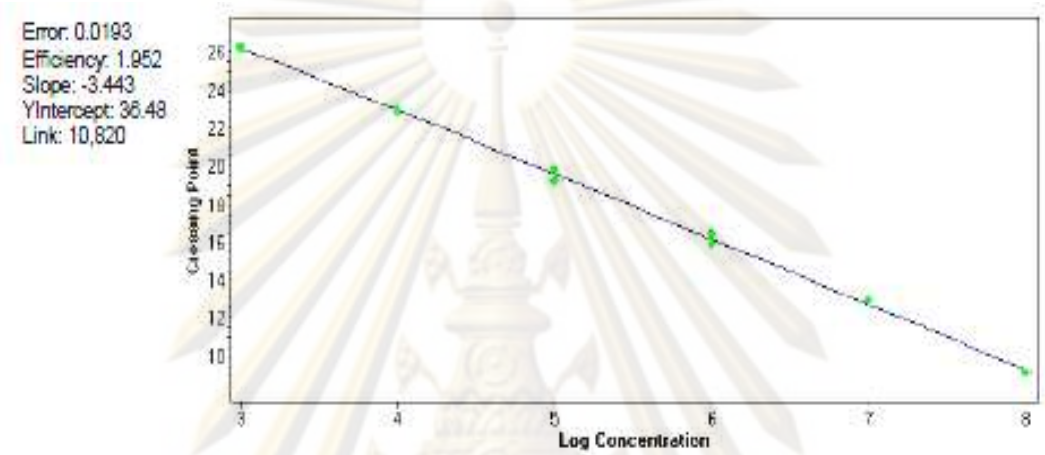

Figure 3.65 The standard curve of *lipin, N-terminal conserved region family protein* (error = 0.0193, amplification efficiency $\log_2$ = 1.952 and equation; Y = -3.443 * log(X) + 36.48) using 10-fold dilution of plasmid DNA between 10^2-10^7 copies of *lipin, N-terminal conserved region family protein* as the template.

3.8.3 *ATP synthase subunit C family protein* (E-1/M-7_{213})

The standard curve of *ATP synthase subunit C family protein* was constructed with the amplification efficiency of 86.69% ($\log_2$ = 2.062, Figure 3.68). The expression level of this transcript in leaves of IR29 at 3 hpt was up-regulated and significantly different from that of the control ($P < 0.05$) (Figure 3.69A).

In contrast, the expression level of this gene in Pokkali was comparable during the stress treatment period (Figure 3. 69B).

When data from both rice varieties were analyzed together, the expression levels of *ATP synthase subunit C family protein* in leaves of IR29 was significantly greater than that in Pokkali at 3 hpt ($P < 0.05$) (Figure 3.69 C).

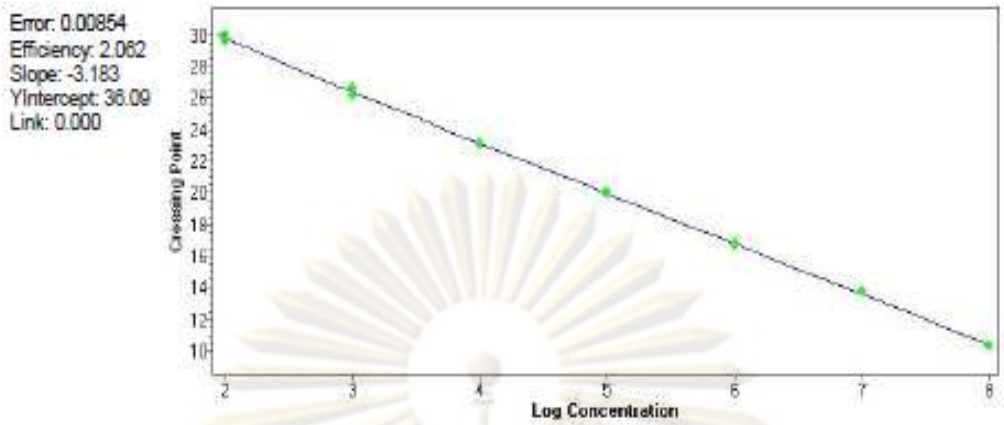

Figure 3.66 The standard curve of *ATP synthase subunit C family protein* (error = 0.0085, amplification efficiency $\log_2$ = 2.062 and equation; Y = -3.183 * log(X) + 36.09) using 10-fold dilution of plasmid DNA between 10^2-10^8 copies of *ATP synthase subunit C family protein* as the template.

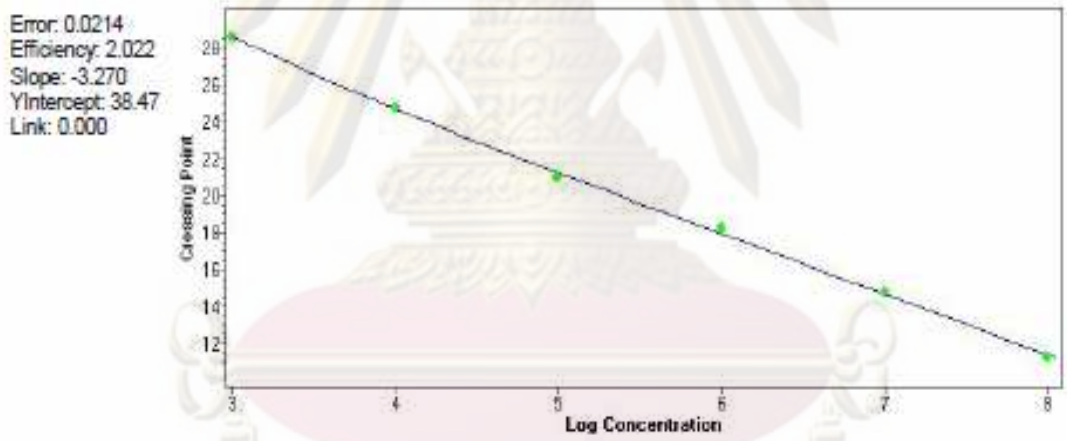

Figure 3.67 The standard curve of *cytochrome p450 monooxygenase* (error = 0.0214, amplification efficiency $\log_2$ = 2.022 and equation; Y = -3.270 * log(X) + 38.47) using 10-fold dilution of plasmid DNA between 10^3-10^8 copies of *cytochrome p450 monooxygenase* as the template.

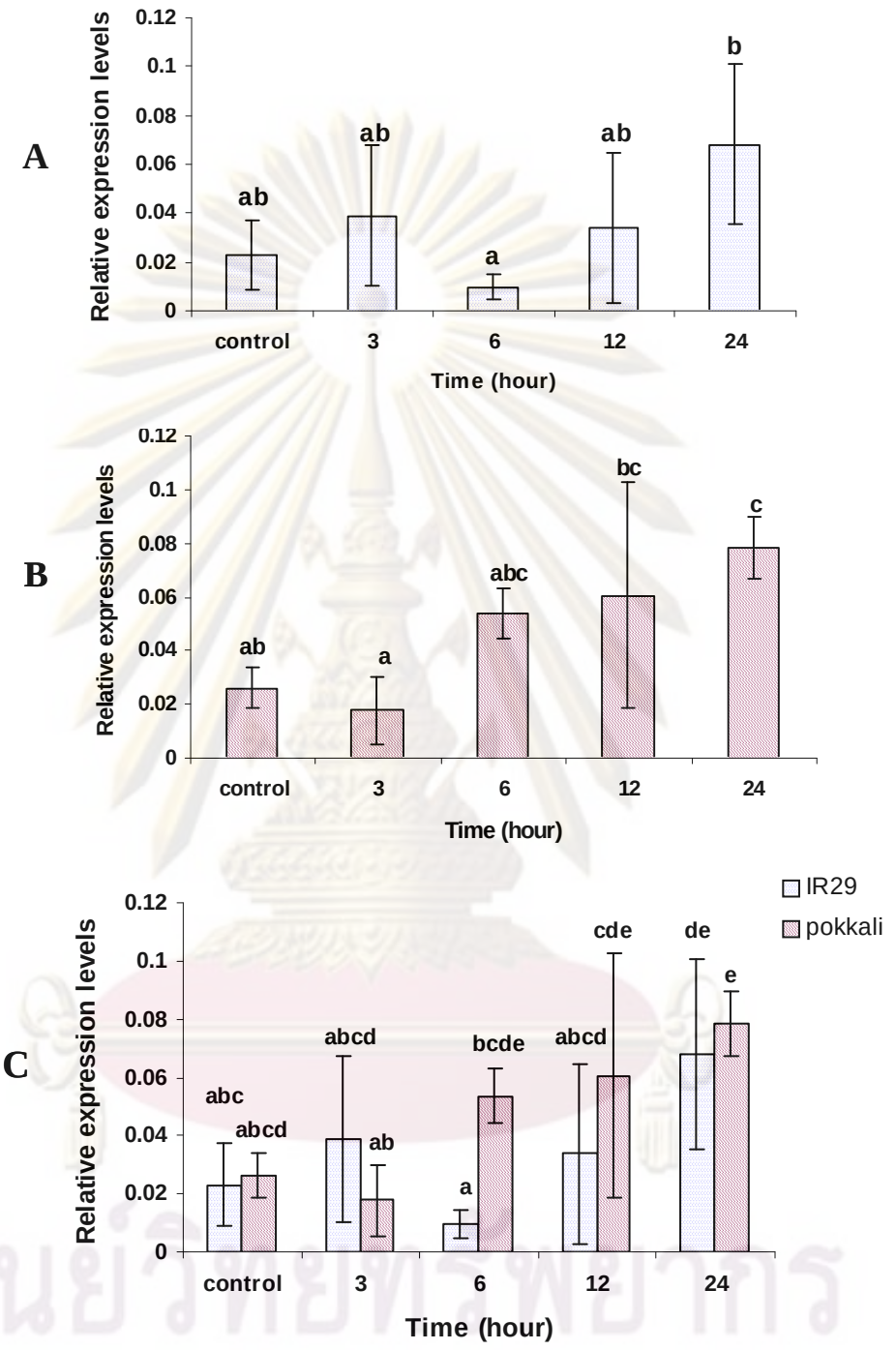

Figure 3.68 Histograms showing relative expression levels of *lipin, N-terminal conserved region family protein* in leaves of IR29 (A) and Pokkali (B) varieties at 0, 3, 6 12 and 24 hpt. The relative expression levels of this transcript in both rice varieties were also analyzed simultaneously (C). The same letters indicate non- significant difference between relative expression levels of different groups of samples.

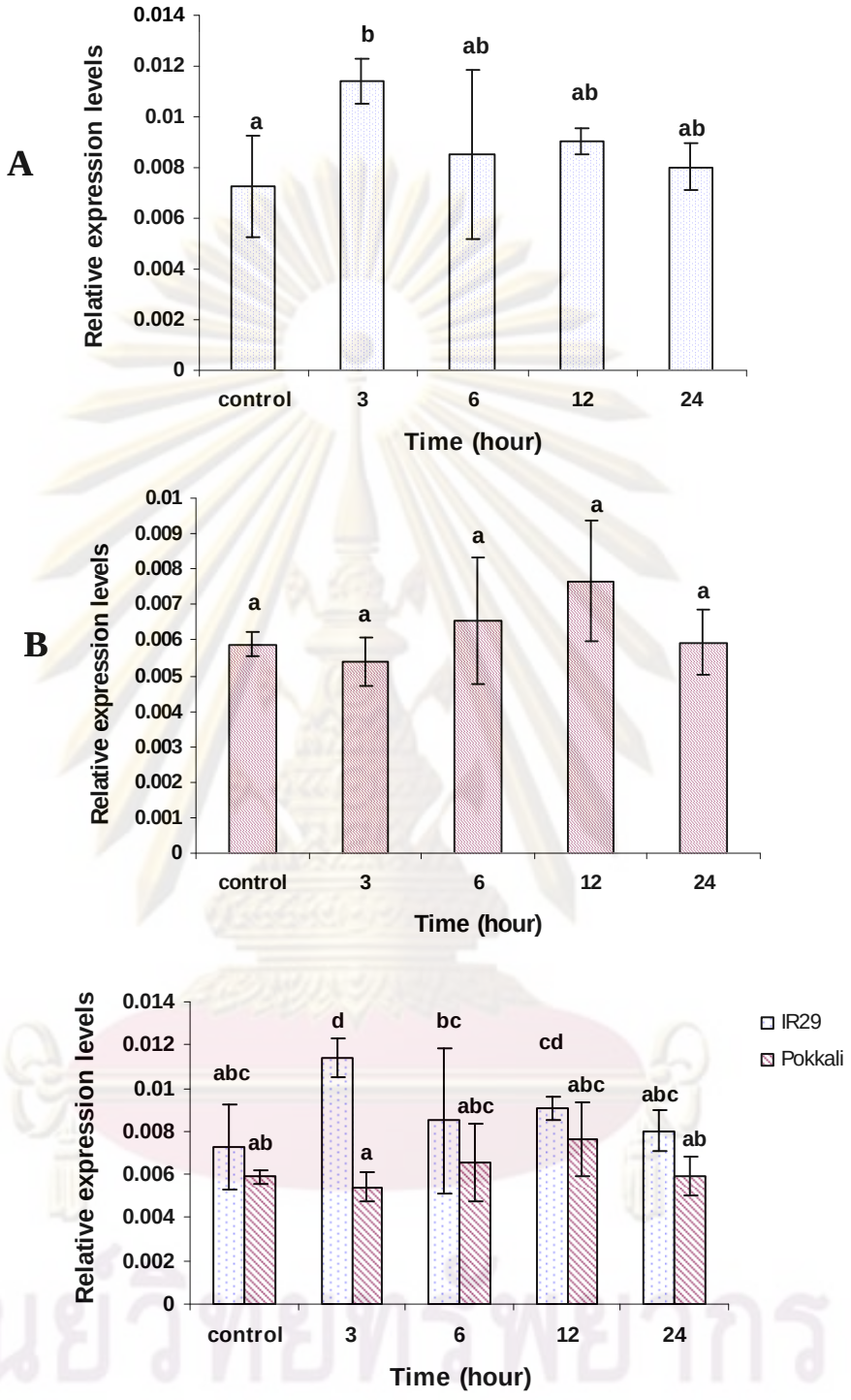

Figure 3.69 Histograms showing relative expression levels of *ATP synthase subunit C family protein* in leaves of IR29 (A) and Pokkali (B) varieties at 0, 3, 6 12 and 24 hpt. The relative expression levels of this transcript in both rice varieties were also analyzed simultaneously (C). The same letters indicate non- significant difference between relative expression levels of different groups of samples.

3.8.3 *Cytochrome p450 monooxygenase* (E-5/M-5_{215})

The standard curve of *cytochrome p450 monooxygenase* was constructed with the amplification efficiency of 95.06% ($\log_2$ = 2.022; Figure 3.67). Results from quantitative real-time PCR analysis revealed that the expression level of this gene in IR29 at 6 hpt was significantly lower than at 24 hpt ($P < 0.05$) (Figure 3.70 A). In Pokkali, expression level at 12 hpt was significantly higher than that of the control ($P < 0.05$) (Figure 3.70 B). Considering expression level between IR29 and Pokkali, the expression level of this gene in IR29 at 24 hpt was only significantly greater than that in Pokkali ($P < 0.05$) (Figure 3.70 C).

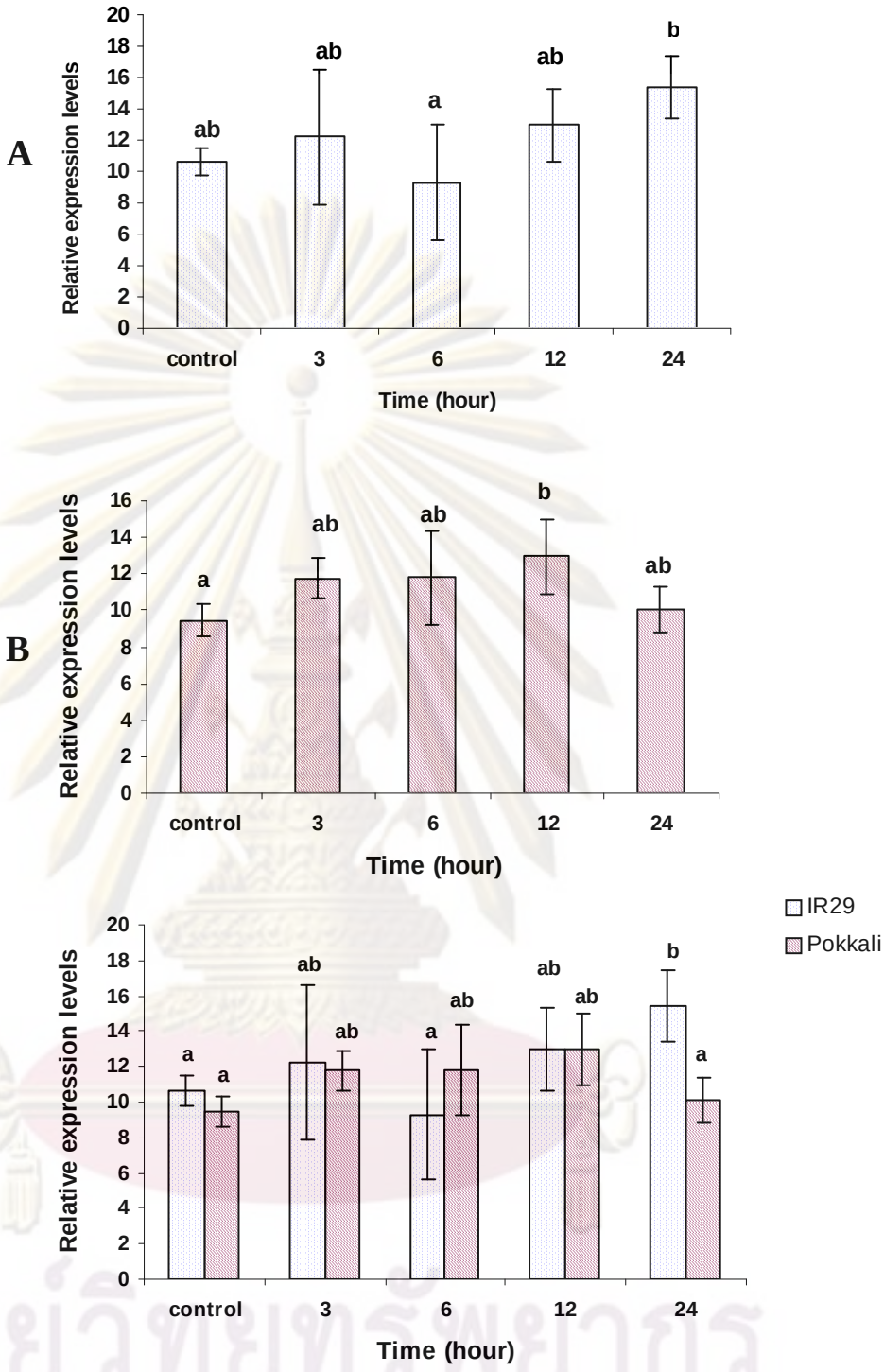

Figure 3.70 Histograms showing relative expression levels of *cytochrome p450 mono-oxygenase* in leaves of IR29 (A) and Pokkali (B) varieties at 0, 3, 6 12 and 24 hpt. The relative expression levels of this transcript in both rice varieties were also analyzed simultaneously (C). The same letters indicate non- significant difference between relative expression levels of different groups of samples.

CHAPTER IV

DISCUSSION

Identification of genes responded to salt stress in leaves of *O. sativa*

Salt stress is a critical abiotic factor affecting plant growth and productivity. When plants are exposed to salinity, the ion homeostasis in cells is disturbed. To adapt to the osmotic and ionic imbalance, plants develop numerous responses. These include the induction of many genes whose products are classified into two groups. One group involves genes/proteins in the regulation of signal transduction and gene expression in stress responses. The other group involves proteins that possibly function in stress tolerance, e.g. transport proteins, key enzymes required for the biosynthesis of different osmoprotectants, and proteins protecting macromolecules and membrane structures (Shinozaki and Yamaguchi-Shinozaki., 1997).

The mechanism of salt tolerance is one of the most important subjects in plant science as salt stress decreases worldwide agricultural production. Salinity (salt stress) causes a significant loss of rice yield. The identification of novel genes, determination of their expression patterns, and the understanding of their functions in stress adaptation is essential to improve stress tolerance in rice.

Several techniques could be used for identification of molecular markers linked to salt stress in rice. Chen et al. (1991) reported the presence of allelic differences in a salt-tolerant mutant (M-20) M-20 at two linked loci, RG711 and RG4, on chromosome 7. A RAPD marker was also identified to link to the salt tolerance gene using the F2 population from a cross between M-20 and its original variety 77–170 (Ding et al., 1998). In addition, several salt-inducible genes have been isolated and characterized and their expression during the salt stress was examined (Chen *et al.*, 1991; Kawasaki *et al.*, 2001).

Kawasaki *et al,* (2001) used microarrays (1728 cDNAs from libraries of salt-stressed roots) for identification of genes responded to initial salt stress in Pokkali and IR29 rice varieties. Salt stress at 150 mM NaCl reduced photosynthesis to one tenth of

the pre-stress value within minutes. Changes in expression of transcripts were observed from 15 min to 1 week after salt stress. Beginning 15 min after the treatment, Pokkali showed up-regulation of transcripts. Approximately 10% of the transcripts in Pokkali were significantly up-regulated or down-regulated within 1 hr of salt stress. The initial differences between control and stressed plants continued for hours but became less pronounced as the plants adapted over time. The interpretation of an adaptive process was supported by the similar analysis of salinity-sensitive rice (IR29), in which the immediate response exhibited by Pokkali was delayed and later resulted in down-regulation of transcription and death. The up-regulated functions observed with Pokkali at different time points during stress adaptation changed over time. Increased protein synthesis and protein turnover were observed at early time points, followed by the induction of known stress-responsive transcripts within hours, and the induction of transcripts for defense-related functions later. After 1 week, the nature of up-regulated transcripts (e.g., aquaporins) indicated recovery from salt stress.

In this study, RAP-PCR and cDNA-AFLP were used to identify candidate differentially expressed transcripts between salt-tolerant (Pokkali) and salt-sensitive (IR29) rice varieties during the exposure to 513 mM NaCl. Additionally, proteomic techniques were also applied for isolation of proteins responded to salt stress in these varieties. The expression profiles of potential genes were further examined using semi-quantitative and quantitative real-time PCR.

Isolation of transcripts related with salt stress response using RAP-PCR

Generally, RNA fingerprinting using arbitrarily primed PCR (RAP-PCR) (Welsh *et al*, 1992) and differential display reverse transcription PCR (DDRT-PCR) (Liang and Pardee., 1992) and cDNA-AFLP (Bachem *et al*., 1996) allow a semi-quantitative simultaneous comparison of differentially expressed transcripts in a large number of RNA samples.

In this study, RAP-PCR was initially used for identifying differential gene expression in response to salt stress between Homjan (salt tolerant variety) and Pathumthani 1 (salt-sensitive variety). *Protein kinase domain containing protein* which is involved in phosphorylation of an amino acid residue in a protein was

identified. RACE-PCR was applied for further characterized of the cDNA sequences of *protein kinase domain containing protein*. Nevertheless, only the complete 3´ end of this transcript was obtained.

Although RAP-PCR is a rapid and convenient method for identifying differentially expressed genes, it generates a high level of false positives and is biased for high copy-number mRNA (Bertioli *et al*., 1995). Therefore differential expression of RAP-PCR should be further confirmed by semiquantitative RT-PCR or real-time PCR.

However, the amplification product of *protein kinase domain containing protein* contained the target along with non-specific RT-PCR products. Further optimization of the amplification conditions did not eliminate the non-specific products. Accordingly, expression analysis of this transcript was not carried out further.

Isolation and characterization of genes responded to salt stress using cDNA-AFLP

Results from RAP-PCR analysis provided only one candidate genes during salt stress response in local rice varieties (Homjan and Pathumthani 1). To provide wider applications of the identified salt stress-related genes in rice, those local salt-tolerant and salt-sensitive varieties were replaced by Pokkali and IR29 varieties. These varieties are often used as a model for a comparative widely expression analysis between salt-tolerant and salt-sensitive rice, respectively. In addition, cDNA-AFLP was used for identification of candidate differential display transcripts during the salt stress treatment of Pokkali and IR29 varieties.

Generally, cDNA-AFLP has a major advantage over RAP-PCR in that a large number of primer combinations (with the selection bases) could be used in cDNA-AFLP whereas the primer used for synthesizing the first strand cDNA and an oligonucleotide with the random sequences are used for the amplification reaction of RAP-PCR. Accordingly, cDNA-AFLP should provide more information on differentially expressed transcripts than RAP-PCR. In addition, much lower amount of the template is required for the cDNA-approach.

The disadvantage of cDNA-AFLP is that samples should be collected with caution because contamination of nucleic acids (both DNA and RNA) from other organisms may interfere the results by generating additional false positive of the desired fragments.

Recently, cDNA-AFLP analysis was used to examine gene expression in seminal root tips at four time points (4, 16, 48 and 72 h) during the water deficit as the seminal roots of an upland rice variety, Azucena, showed accelerated elongation in response to a water deficit. One hundred and six unique genes (60 genes of known function, 28 genes of unknown function and 18 novel genes) induced by the water deficit were obtained. Expression of 22 genes revealed by Northern blot analysis reached a maximum within 16 h of water deficit treatment. They were, for example, *aquaporin* (*PIP2a*), *9-cis-epoxycarotenoid dioxygenase* (*NCED1*) and a negative regulator of gibberellin signal transduction (*SPY*); eight other genes participated in cell wall loosening or vesicle traffic (Yang *et al.*, 2003).

Jayaraman *et al.,* (2008) used cDNA-AFLP analysis to identify differential gene expression in response to salt stress in salt tolerant (Prasad) and susceptible variety (Lepakshi) of the foxtail millet (*Setaria italica* L.) at the normal and salt stress for 1 hour. The expression profiles of identified transcripts were further validated by quantitative real-time PCR. In total, 27 non-redundant differentially expressed cDNAs that are unique to the salt tolerant variety which represent different groups of genes involved in for example, metabolism, cellular transport, cell signaling, transcriptional regulation, etc. The expression patterns of seven out of nine such genes showed a significant increase of differential expression in tolerant variety after 1 hr of salt stress in comparison to salt-sensitive variety as analyzed by quantitative real-time PCR.

In this study, 120 primer combinations were screened and 66 primer combinations yielded positive amplification products. On the basis of preliminary screening, several fragments exhibited up- and down-regulated expression patterns were identified. Twenty-four fragments showing differential expression in IR29 and pokali were cloned and sequenced. Only 7 transcripts showed significant similar to rice genes in the database. They were *lipin, N-terminal conserved region family protein, ATP synthase subunit C family protein, cytochrome P450 monooxygenase,*

hypothetical protein$_{186}$*, hypothetical protein*$_{171}$*, hypothetical protein*$_{217}$ and *hypothetical protein*$_{240}$*.*

Plants appear to have two lipin-related genes. Lipin proteins in nearly all species possess nuclear localization signals and several putative serine and threonine phosphorylation sites. In addition, extended regions at the amino- and carboxy-terminal ends of the lipin proteins (the amino-terminal lipin (N-LIP) and carboxy-terminal lipin (C-LIP) domains) are highly conserved among all lipin proteins in all organisms, suggesting an important functional role for these domains (Reue K and Zhang P., 2008).

ATP synthase subunit C, a member of F_1F_0 ATP synthase produces ATP from ADP in the presence of a proton or sodium gradient. F-type ATPases consist of two structural domains, F_1 containing the extra-membrane catalytic core and F_0 containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F_1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation.

Zhang *et al.*, (2006) identified a *mitochondrial ATP synthase small subunit 6* gene (*RMtATP6*) expressed in response to salts and osmotic stresses in *O. sativa*. Among genes for F1F0-ATPase of rice, some genes were found to be up-regulated by environmental stresses and others were not. Their data suggest that the RMtATP6 protein acts as a subunit of ATP synthase, and is expressed in response to stress from several salts.

The cytochrome P450 family (CYP) is a large and diverse group of enzymes. The function of most CYP enzymes is to catalyze the oxidation of organic substances. The substrates of CYP enzymes include metabolic intermediates such as lipids, steroidal hormones as well as xenobiotic substances such as drugs. Plant cytochrome P450 monooxygenases participate in myriad biochemical pathways, including biosynthesis of phenylpropanoids, alkaloids, terpenoids, lipids, and plant growth regulators, such as gibberellins, JA, and brassinosteroids (Chapple., 1998).

Isolation of salt stress response proteins using proteomic analysis

Proteomics is a powerful tool for separating complex protein mixtures, and has been employed to analyze protein changes in response to environmental changes of various plant species.

Abbasi and Komatsu., (2004) examined rice proteins induced by salt stress using proteomic approaches. The expression of superoxide dismutase was a common response to cold, drought, salt, and ABA stresses. Fructose bisphosphate aldolases, photosystem II oxygen evolving complex protein, and oxygen evolving enhancer protein 2 were expressed in leaf sheaths and leaf blades but not in roots. This result indicated that specific proteins, expressed in specific regions of rice, showed a coordinated response to salt stress.

More recently, Parker *et al.,* (2006) studied an accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina and they found an increase in eight proteins, including RuBisCo activase and ferritin occurred by 24 h of the exposure period to 50 mM NaCl and the expression continued to increase during the following treatment for 6 days.

Yan *et al.,* (2005) examined salt stress-responsive proteins in *O.sativa* (cv. Nipponbare). Three-week-old seedlings were treated with 150 mM NaCl for 24, 48 and 72 h. Total proteins of roots were extracted and separated by two-dimensional gel electrophoresis. More than 1100 protein spots were reproducibly detected, including 34 that were up-regulated and 20 that were down-regulated. Mass spectrometry analysis and database searching revealed identify 12 spots representing 10 different proteins. Three spots were identified as the same protein, enolase. While four of them were previously confirmed as salt stress-responsive proteins, six are novel including UDP-glucose pyrophosphorylase, cytochrome *c* oxidase subunit 6b-1, glutamine synthetase root isozyme, putative nascent polypeptide-associated complex alpha chain, putative splicing factor-like protein and putative actin-binding protein. Results indicated that these proteins should also play the important roles in salt stress response in rice roots apart from their main functions.

There have been several reports about systematic proteomic analysis of rice proteins under abiotic stresses. Typically, two dimensional gel electrophoresis (2-DE) and mass spectrometry were used for identification and characterization of examined protein spots. However, the disadvantages of 2-DE are found including a large amount of sample handling, limited reproducibility, and a smaller dynamic range than some other separation methods. It is also not automated for high throughput analysis. Certain proteins are difficult for 2-DE to separate, including those that are in low abundance levels, acidic, basic, hydrophobic, very large, or very small in size.

Here, one dimension polyacrylamide gel electrophoresis (SDS-PAGE) was used for separation of examined proteins. Electrophoresed proteins were analyzed by LC-MS/MS. With this simple approach, very large or small proteins could be effectively analyzed.

In this study, 206 differentially expressed proteins were analyzed by DeCyder MS Differential Analaysis software. These proteins significantly matched to plant proteins in the NCBI database and 113 of which significantly similar to proteins with known functions. Of these 43 proteins were found in Pokkali, 24 proteins were observed in IR29 and 46 proteins were found in both varieties. These proteins were functionally classified according to the functions of their homologues. Of the identified proteins, cysteine protease showed high differential expression in leaves of Pokkali was selected for expression analysis using semi-quantitative RT PCR.

Cysteine proteases are enzymes that degrade polypeptides. It plays multi-faceted roles, virtually in every aspect of physiology and development, in plants such as in the growth and development, in senescence and programmed cell death, in accumulation and mobilization of storage proteins such as in seeds. In addition, they are involved in signaling pathways and in the response to biotic and abiotic stresses (Grudkowska and Zagdańska., 2004.)

Expression analysis of salt stress response genes identified by cDNA-AFLP and proteomic analyses

Time-course expression analysis of salt stress-related genes in Pokkali and IR29 may provide the progression of regulated functions such that different categories of transcripts show regulation at different time points. A difference between the two

varieties existed with respect to the onset of the initial response may respond to salt stress differently between rice varieties. Also, after the salt stress period, transcript that are up-regulated or down-regulated began to converge on the pre-stress level more rapidly in Pokkali than in IR29 may reveal the recover of salt-tolerance rice varieties during the salt stress.

The expression *hypothetical protein*$_{171}$ upon salt-stress was not significantly different in leaves of IR29 ($P > 0.05$). In contrast, this transcript was up-regulated at 3, 6 and 12 hpt before returned to the pre-stress level at 24 hpt in Pokkali ($P > 0.05$). The expression profiles of *hypothetical protein*$_{171}$ implied that functional contribution of this gene in the stress response mechanism of IR29 and Pokkali may be different.

The expression profiles of CPT_{201} in leaves of IR29 and Pokkali were similar as the expression level was up-regulated at 6 hpt in the former and at 6 and 12 hpt in the latter. Accordingly, this gene may not suitable to be used as the biomarker for stress response.

The expression level of *lipin, N-terminal conserved region family protein* in leaves of IR29 was up-regulated from 6 hpt and 24 hpt ($P > 0.05$). In contrast, this transcript in Pokkali was earlier up-regulated from 3, 12 and 24 hpt ($P > 0.05$). The expression profiles of *lipin, N-terminal conserved region family protein* are related with the recovery on salt stress responses in rice.

The expression level of *ATP synthase subunit C family protein* in leaves of IR29 was up-regulated at 3 hpt and immediately returned to the basal level at 6 hpt ($P < 0.05$). In contrast, this transcript in Pokkali was comparably expressed ($P > 0.05$). Like *hypothetical protein*$_{171}$, the expression profiles of *ATP synthase subunit C family protein* implied that functional contribution of this gene in the stress response mechanism of IR29 and Pokkali may also be different.

The expression level of *cytochrome p450 monooxygenase* was up-regulated from 6 hpt and 24 hpt in leaves of IR29 ($P < 0.05$). An earlier up-regulation of this gene was observed at 12 hpt ($P < 0.05$) and the expression level was decreased to the pre-stress level at 24 hpt in Pokkali ($P > 0.05$). Therefore, the expression profiles of *cytochrome p450 monooxygenase* revealed salt stress expression profiles in a time-dependent manner.

In this study, candidate differentially expressed genes and proteins are identified. Semi-quantitative RT PCR and quantitative real-time PCR further confirmed the sensitive response of various genes in response to salt stress. The ability to detect the early signs of serious salt stress is crucial for cultivation of rice. Results from the present study showed the response of *cytochrome p450 monooxygenase*, *lipin, N-terminal conserved region family protein*, *ATP synthase subunit C family protein* and *hypothetical protein*$_{171}$ to salt stress. The expression levels of these genes may be used as bioindicators for monitoring effects of salt stress inrice.

CHAPTER V

CONCLUSIONS

1. RAP-PCR was used for isolation of differentially expressed transcripts in Homjan and Pathumthani 1 varieties. A RAP-PCR fragment significantly matched *protein kinase domain containing protein* of *O. sativa* previously deposited in the NCBI database was successfully identified.

2. cDNA-AFLP analysis was further carried out. A total of 120 primer combinations were screened. Twenty-four fragments showing differential expression in IR29 and Pokkali were cloned and sequenced. Three cDNA-AFLP fragments significantly similar to *lipin, N-terminal conserved region family protein*, *ATP synthase subunit C family protein* and *cytochrome P450 monooxygenase* of *O. sativa*. Other four fragments significant hypothetical proteins of *O. sativa* (called *hypothetical protein*$_{186}$, *hypothetical protein*$_{171}$, *hypothetical protein*$_{217}$ and *hypothetical protein*$_{240}$, respectively)

3. Proteomic analysis of proteins in leaves of Pokkali and IR29 was carried out. In total, 206 differential expressed proteins matched plant proteins in the NCBI database and 113 proteins matched proteins with known functions. Of these, 43, 24 and 46 proteins were found in Pokkali, IR29 or both varieties, respectively. There were several proteins that showed differential expression after salt stress such as cysteine protease, ATPase family, Photosystem II protein, peroxidase, salt inducible protein, heat shock protein and hypothetical proteins. These protein data may provide valuable insight for studying protein responses to salt stress in rice leaves.

4. Semi-quantitative RT-PCR revealed that the expression level of *hypothetical protein*$_{171}$ (from cDNA-AFLP) was not significantly different in leaves of IR29 ($P > 0.05$) but that of this gene in Pokkali was significantly up-regulated at 3 to 12 hpt ($P < 0.05$). Cysteine protease (*CTP*$_{201}$ from proteomics) was significantly down-regulated at 3 to 24 hpt in IR29 but that in Pokkali was significantly higher than the control at 6 and 12 hpt ($P < 0.05$).

5. Quantitative real-time PCR indicated that the expression of *lipin N-terminal conserved region family protein* in IR29 at 6 hpt was significantly lower than that at 24 hpt ($P < 0.05$) but that in Pokkali at 0 and 3 hpt were significantly lower than that at 24 hpt ($P < 0.05$). *ATP synthase subunit C family protein* in leaves of IR29 at 3 hpt was significantly greater than that of the control ($P < 0.05$) but its expression in Pokkali was comparable during the stress treatment ($P > 0.05$). *Cytochrome p450 monooxygenase* was less abundantly expressed at 6 hpt than that at 24 hpt in IR29 ($P < 0.05$) but its expression level at 12 hpt in Pokkali was significantly higher than that of the control ($P < 0.05$).

6. Gene expression profiles of *cytochrome p450 monooxygenase*, *lipin, N-terminal conserved region family protein*, *ATP synthase subunit C family protein* and *hypothetical protein*$_{171}$ clearly responded to salt stress. Therefore, the expression levels of these genes may be used as bioindicators for monitoring effects of salt stress in rice.

7. Further, characterization and functional analysis of the genes that are identified in this study can lead to a more comprehensive understanding of stress tolerance to salt in rice.

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APPENDICES

Appendix A

Table. A1 Raw materials and relative expression level data of *hypothetical protein* from leaves of IR29 and pokkali using semi-quantitative RT-PCR.

Sample group	Densities of band		Ratio of Gene/internal control	average	STD
	Hypothetical protein	**Internal control**			
IR29 (t=0)	72059.58	95372.76	0.755557	0.748793	0.025488
	77105.98	101354.3	0.760757		
	90763.63	127605.9	0.711281		
	108053.1	140771.9	0.767575		
IR29 (t=3)	73225.98	87622.71	0.835696	0.785882	0.073719
	90064.73	109734.3	0.820753		
	93447.89	133269	0.701198		
IR29 (t=6)	65325.59	80444.09	0.812062	0.775129	0.064113
	73119.1	86399.85	0.846287		
	75599.49	104037	0.72666		
	85104.06	118942.1	0.715508		
IR29 (t=12)	65116.41	94852.36	0.686503	0.708214	0.041558
	69769.13	105133.5	0.663624		
	81401.06	112249.3	0.725181		
	88208.21	116439.2	0.757547		
IR29 (t=24)	67247.5	97935.41	0.686651	0.709289	0.021912
	76015.43	108455.8	0.700889		
	83165.11	116952.6	0.711101		
	86589.66	117248.7	0.738513		
Pokkali (t=0)	103508.3	142300.3	0.727393	0.6642	0.0451
	93394.71	140597.4	0.664271		
	90400.66	141468.9	0.639014		
	77578.79	123938.2	0.625947		
Pokkali (t=3)	93682.39	133359.6	0.70248	0.7420	0.0343
	98092.06	128693.8	0.762213		
	95225.63	125061.5	0.761431		
Pokkali (t=6)	97314.5	125668.7	0.774373	0.8104	0.0322
	91822.02	111363.8	0.824523		
	91118.91	114596.3	0.795129		
	88531.27	104446.1	0.847626		
Pokkali (t=12)	86796.79	105134.5	0.825579	0.8453	0.0278
	86095.99	102869	0.836948		
	86214.54	103584.6	0.832311		
	82443.27	93006.06	0.886429		
Pokkali (t=24)	84901.23	117615.6	0.721854	0.7010	0.0326
	82318.98	124069.1	0.663493		
	72071.73	100411	0.717767		

Table. A2 Raw materials and relative expression level data of *cystein protease* from leaves of IR29 and pokkali using semi-quantitative RT-PCR.

Sample group	Densities of band		Ratio of Gene/internal control	average	STD
	Cystein protease	Internal control			
IR29 (t=0)	104677.1	95372.76	1.097558		
	92695.71	101354.3	0.914571	0.987556	0.096947
	121294.5	127605.9	0.950539		
IR29 (t=3)	98207.44	94640.72	1.037687		
	95536.01	109734.3	0.870612	0.92095	0.101415
	113885.3	133269	0.854552		
IR29 (t=6)	92156.72	80444.09	1.1456		
	103196.9	86399.85	1.194411	1.11407	0.099909
	104266	104037	1.002201		
IR29 (t=12)	90342.83	94852.36	0.952457		
	98038.7	105133.5	0.932516	0.938149	0.084508
	116363.1	112249.3	1.036649		
	96758.15	116439.2	0.830975		
IR29 (t=24)	75141.43	97935.41	0.767255		
	92839.3	108455.8	0.85601	0.783993	0.049783
	90192.98	116952.6	0.771193		
	86941.49	117248.7	0.741514		
Pokkali (t=0)	118819.3	142300.3	0.83499		
	115127.1	140597.4	0.818842	0.73371	0.107915
	91366.21	141468.9	0.64584		
	78721.64	123938.2	0.635168		
Pokkali (t=3)	109113.2	133359.6	0.818188		
	89554.59	125061.5	0.716085	0.742362	0.06669
	79419.79	114633.8	0.692813		
Pokkali (t=6)	108942.1	125668.7	0.866899		
	108169.4	111363.8	0.971316	0.914639	0.052779
	103790.3	114596.3	0.905703		
Pokkali (t=12)	94010.27	102869	0.913883		
	95895.69	103584.6	0.925772	0.872495	0.082198
	72342.88	93006.06	0.77783		
Pokkali (t=24)	82679.09	105066.7	0.78692		
	84087.45	117615.6	0.714935	0.733839	0.0443
	84874.89	124069.1	0.684094		
	75248.7	100411	0.749407		

Table. A3 Raw materials and relative expression level data of *lipin-N terminal conserve region* from leaves of IR29 and pokkali using quantitative real-time PCR.

Sample group	Mean concentration		Ratio of Gene/internal control	average	STD
	lipin protein	Internal control			
IR29 (t=0)	2159.97891	152017.9316	0.014208711		
	2008.482353	129363.5027	0.015525881	0.0230476	0.0141840
	1340.402232	34013.22983	0.039408261		
IR29 (t=3)	5341.476383	202895.2829	0.026326272		
	328.559253	17247.02191	0.019050202	0.0390258	0.0141840
	1621.551314	22615.48429	0.071700933		
IR29 (t=6)	966.0896681	123762.5578	0.007805993		
	3015.656466	194000.000	0.015544621	0.0097918	0.0050609
	759.4447195	126049.5379	0.00602497		
IR29 (t=12)	2436.371158	103115.3748	0.023627623		
	752.5676405	81420.81531	0.009242939	0.0337972	0.0309199
	2171.524156	31691.29391	0.068521158		
IR29 (t=24)	6465.431911	128673.4677	0.050246815		
	7992.157016	75442.5048	0.105937058	0.0681655	0.0327253
	2692.423679	55729.04941	0.048312751		
Pokkali (t=0)	1208.386812	65515.70042	0.018444233		
	628.827118	24229.78326	0.025952651	0.0260478	0.0076516
	991.013125	29366.20835	0.033746717		
Pokkali (t=3)	695.00	49717.61923	0.013978948		
	1916.508187	60689.75992	0.031578774	0.0177419	0.0123914
	254.9603906	33248.96051	0.007668221		
Pokkali (t=6)	5528.640539	84046.37882	0.06578083		
	2964.474996	60438.73772	0.049049254	0.0537549	0.0092007
	4631.365337	103844.2307	0.044599159		
	4526.710811	81429.2731	0.055590706		
Pokkali (t=12)	2495.316443	103115.3748	0.024199267		
	4161.001986	81420.81531	0.051104892	0.0606200	0.0419947
	3376.894715	31691.29391	0.106555912		
Pokkali (t=24)	8042.320628	93393.38494	0.086112315		
	8322.762266	99451.99472	0.083686228	0.0783603	0.0113904
	2745.00241	42048.00106	0.06528259		

Table. A4 Raw materials and relative expression level data of *ATP synthase subunit C* from leaves of IR29 and pokkali using quantitative real-time PCR.

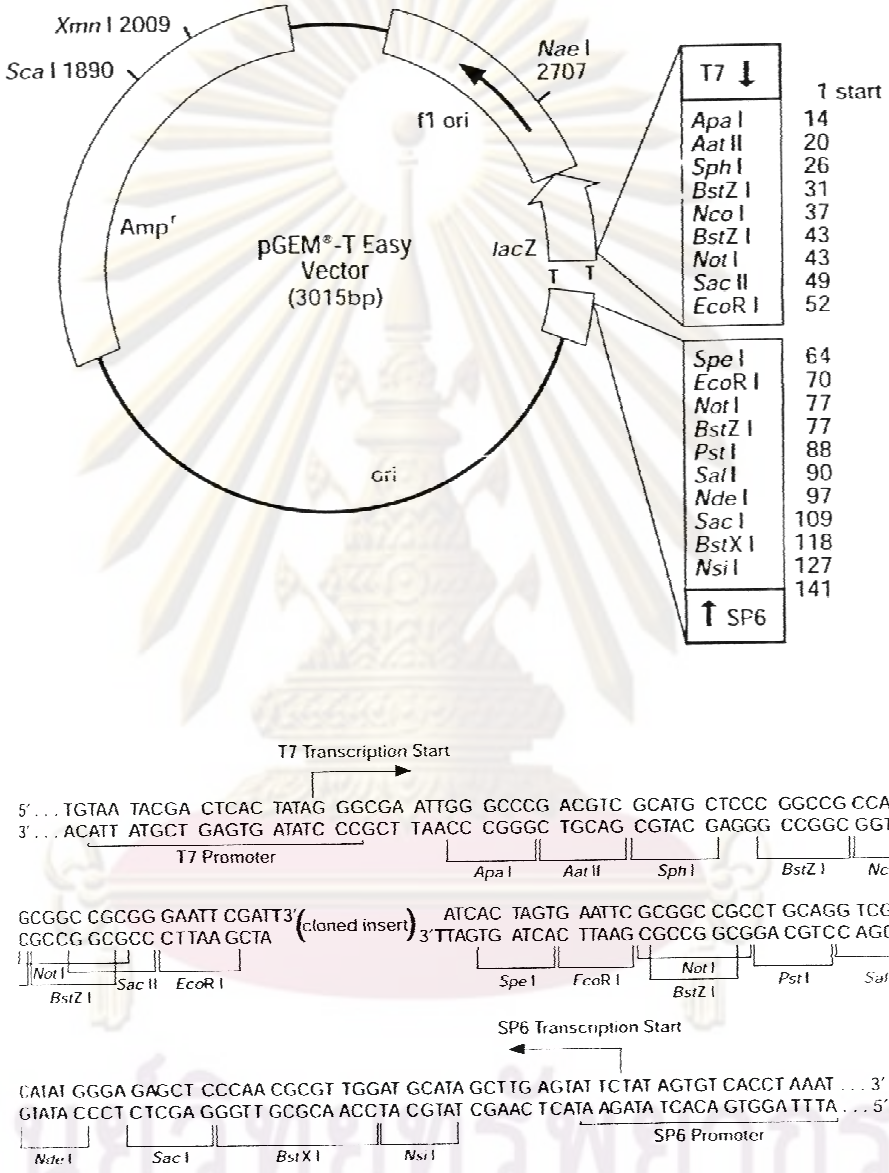
Sample group	Mean concentration		Ratio of Gene/internal control	average	STD
	ATP synthase subunit C	Internal control			
IR29 (t=0)	650.165624	129363.5027	0.005025881		
	98.42039278	12410.33548	0.007930518	0.0072676	0.0019947
	300.9017048	34013.22983	0.008846608		
IR29 (t=3)	179.3585928	17247.02191	0.010399395		
	266.0407554	22615.48429	0.011763655	0.0114235	0.0009034
	385.8746592	31870.80816	0.012107464		
IR29 (t=6)	637.4872923	123762.5578	0.00515089		
	340.3857382	28651.97752	0.011880009	0.0085199	0.0033645
	1075.087737	126049.5379	0.008529089		
IR29 (t=12)	870.5615806	103115.3748	0.008442597		
	484.3061931	51056.38409	0.009485713	0.0090495	0.0005421
	292.207923	31691.29391	0.009220448		
IR29 (t=24)	531.8195651	75442.5048	0.007049336		
	343.4285656	38419.83406	0.008938835	0.0080214	0.0009459
	450.0760584	55729.04941	0.008076148		
Pokkali (t=0)	374.419271	65515.70042	0.005714955		
	336.4028296	59360.30092	0.005667135	0.0058835	0.0003343
	151.8889418	24229.78326	0.006268688		
Pokkali (t=3)	298.1578692	49717.61923	0.005997026		
	282.0630385	60689.75992	0.004647622	0.0054024	0.0006888
	138.2914652	24860.97546	0.005562592		
Pokkali (t=6)	445.0853743	84046.37882	0.005295711		
	346.3740655	60438.73772	0.005730994	0.0065308	0.0017757
	889.5117362	103844.2307	0.008565827		
Pokkali (t=12)	990.5967485	103115.3748	0.009606683		
	531.1626172	81420.81531	0.006523671	0.0076588	0.0016945
	349.5371866	51056.38409	0.006846101		
Pokkali (t=24)	540.8126362	99451.99472	0.005437926		
	358.5962151	51342.80895	0.006984351	0.0059341	0.0009099
	226.2260647	42048.00106	0.005380186		

Table. A5 Raw materials and relative expression level data of *cytochrome P450 monooxygenase* from leaves of IR29 and pokkali using quantitative real-time PCR.

Sample group	Mean concentration		Ratio of Gene/internal control	average	STD
	Cytochrome P450 monooxygenase	**Internal control**			
IR29 (t=0)	1605721.651	152017.9316	10.56271214		
	1449645.238	129363.5027	11.2059832	10.605035	0.86822083
	116561.9105	12410.33548	9.392325509		
	382959.0572	34013.22983	11.25912062		
IR29 (t=3)	2407367.296	202895.2829	11.86507277		
	288496.6758	17247.02191	16.72733283	12.227191	4.33045219
	182940.4736	22615.48429	8.089168964		
IR29 (t=6)	1587378.417	123762.5578	12.82599879		
	1064031.478	194000.000	5.484698338	9.2886588	3.67790537
	273777.6488	28651.97752	9.555279337		
IR29 (t=12)	1501883.205	103115.3748	14.56507536		
	837922.8258	81420.81531	10.29126057	12.961050	2.32771653
	444527.9248	31691.29391	14.02681525		
IR29 (t=24)	2245521.712	128673.4677	17.45131885		
	1013728.433	75442.5048	13.43709936	15.399394	2.00861013
	853198.6155	55729.04941	15.30976438		
Pokkali (t=0)	591289.7231	59360.30092	9.961029744		
	204675.3259	24229.78326	8.447261937	9.4438928	0.86331291
	291412.2501	29366.20835	9.923386998		
Pokkali (t=3)	623986.9328	49717.61923	12.55061973		
	303507.8867	24860.97546	12.20820507	11.764322	1.0790353
	350249.336	33248.96051	10.53414394		
Pokkali (t=6)	1240203.419	84046.37882	14.7561791		
	614649.3968	60438.73772	10.16979209	11.784123	2.57707131
	1082721.501	103844.2307	10.42640014		
Pokkali (t=12)	2725197.742	183882.6451	14.82030967		
	1321040.216	122752.3083	10.76183605	12.953198	2.04857406
	485065.1721	36533.0116	13.27744828		
Pokkali (t=24)	1030083.086	93393.38494	11.029508		
	444461.9423	51342.80895	8.656751577	10.078371	1.25439579
	443558.2238	42048.00106	10.54885399		

Appendix B

Restriction mapping of pGEM® T-easy Vector



BIOGRAPHY

Miss. Rattanawadee Wichajarn was born on April 4, 1979 in Prachuap Khiri Khan. She graduated with the degree of Bachelor of Science from the Department of Science (Biotechnology), Silpakorn University in 2001. She has enrolled a Master degree program at the Program in Biotechnology, Chulalongkorn University since 2009.

Publications related with this thesis

1. **Wichajarn, R.**, Roytrakul, S., Klinbunga, S., Khamnamtong, B and Chulalaksananukul, W. (2009). Identification of genes involving salt tolerance mechanisms in rice (*Oryza sativa*) analyzed by cDNA-AFLP. The 21th Annual Meeting and International conference of the Thai Society for Biotechnology, 24 - 25 september 2009, Queen Sirikit National Convention Hall, Bangkok, Thailand (poster presentation).