แอกทิวิตีของเอนไซม์ออกซิเดทีฟและข้อมูลแสดงลักษณะเฉพาะของโปรตีนของข้าว *Oryza sativa* L. ทรานสเจนิกที่มีการแสดงออกเกินปกติของยีนคัลมอดุลิน *OsCam1-1*

นายไตรเลิศ ชัยเชิดสกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

OXIDATIVE ENZYME ACTIVITIES AND PROTEIN PROFILE OF TRANSGENIC RICE *Oryza sativa* L. OVEREXPRESSING CALMODULIN GENE *OsCam1-1*

Mr. Trilert Chaicherdsakul

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctoral of Science Program in Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2011

Thesis Title	Oxidative Enzyme Activities and Protein Profile of Transgenic Rice *Oryza sativa* L. Overexpressing Calmodulin Gene *OsCam1-1*
By	Mr. Trilert Chaicherdsakul
Field of study	Biochemistry
Thesis Advisor	Associate Professor Teerapong Buaboocha, Ph.D.
Thesis Co-advisor	Associate Professor Tipaporn Limpaseni, Ph.D.
Thesis Co-advisor	Sittiruk Roytrakul, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

...…...… Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

…… Chairman

(Professor Aran Incharoensakdi, Ph.D.)

……..…… Thesis Advisor

(Associate Professor Teerapong Buaboocha, Ph.D.)

…… Thesis Co-advisor

(Associate Professor Tipaporn Limpaseni, Ph.D.)

…… Thesis Co-advisor

(Sittiruk Roytrakul, Ph.D.)

…… Examiner

(Assistant Professsor Rath Pichayangkura, Ph.D.)

…… Examiner

(Supaart Sirikantaramas, Ph.D.)

…… External Examiner

(Associate Professsor Jarunya Narangajavana, D.Agr.Sc.)

ไตรเลิศ ชัยเชิดสกุล: แอกทิวิตีของเอนไซม์ออกซิเดทีฟและข้อมูลแสดงลักษณะเฉพาะของโปรตีนของข้าว *Oryza sativa* L. ทรานสเจนิกที่มีการแสดงออกเกินปกติของยีนคัลมอดุลิน *OsCam1-1* (OXIDATIVE ENZYME ACTIVITIES AND PROTEIN PROFILE OF TRANSGENIC RICE *Oryza sativa* L. OVEREXPRESSING CALMODULIN GENE *OsCam1-1*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.ธีรพงษ์ บัวบูชา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.ทิพาพร ลิมปเสนีย์ และ ดร. สิทธิรักษ์ รอยตระกูล, 182 หน้า

ข้าว (*Oryza sativa* L.) ซึ่งพบว่ามีกลุ่มโปรตีนคัลมอดุลินและโปรตีนที่คล้ายคัลมอดุลินขนาดใหญ่เป็นธัญพืชที่สำคัญโดยเป็นอาหารหลักสำหรับประชากรโลกกว่าสามพันล้านคน ประเทศไทยเป็นแหล่งผลิตและส่งออกข้าวที่สำคัญต่อตลาดโลก อย่างไรก็ตามในการผลิตข้าวยังต้องประสบปัญหากับภาวะดินเค็มซึ่งทำให้ผลผลิตต่ำ จากการวิจัยก่อนหน้านี้พบว่าในบรรดายีน *OsCam* ทั้งห้ายีน *OsCam1-1* แสดงบทบาทที่สำคัญต่อการตอบสนองภายใต้ภาวะเครียดจากเกลือ ในการศึกษาวิจัยนี้ได้ทำการตรวจสอบถึงรูปแบบโปรตีนของข้าว *Oryza sativa* L. ทรานสเจนิกส์ที่มีการแสดงออกเกินปกติของยีนคัลมอดุลิน *OsCam1-1* โดยนำเมล็ดข้าว 3 กลุ่ม (ลักษณะปกติดั้งเดิม, ข้าวทรานสเจนิกส์ที่มีเฉพาะเวกเตอร์ และข้าวทรานสเจนิกส์ที่มียีน *OsCam1-1* มาปลูกในอาหาร 3 สัปดาห์และเหนี่ยวนำภายใต้ภาวะเครียดจากเกลือโซเดียมคลอไรด์เข้มข้น 150 มิลลิโมลาร์ จากนั้นสกัดโปรตีนจากส่วนของใบและรากข้าวภายใต้ภาวะเครียดจากเกลือแต่ละเวลา และย่อยภายในเจล แล้ววิเคราะห์เพปไทด์ที่ได้ด้วยเทคนิคแมสสเปกโทรเมทรี จากการวิเคราะห์รูปแบบการแสดงออกของโปรตีนโดยใช้โปรตีโอมิกส์ซอฟแวร์หลายชนิดพบว่ามีกลุ่มของโปรตีนที่มีหน้าที่ในกระบวนการชีวภาพหลายชนิด ได้แก่ cellular process (42%), metabolic process (14%), regulation (12%), developmental process (2%), localization (2%), และ response to stimulus (2%) ขณะที่ที่เหลือเป็นโปรตีนที่ทำหน้าที่อื่นๆ หรือไม่ทราบหน้าที่ โปรตีนที่ได้ที่ตอบสนองต่อความเครียดที่เกิดจากความเค็มที่พบจำนวนมาก localize ในนิวเคลียส (24%) และในโครโมโซม (5%) และบางโปรตีนอยู่ในไมโทคอนเดรีย (5%), ER (3%), และออร์กาเนลล์อื่นๆ (5%) ขณะที่ส่วนน้อยอยู่ในไซโทพลาสซึม นอกจากนี้ยังมีบางส่วนพบใน intracellular complex (8%) จากการเปรียบเทียบการแสดงออกของโปรตีนในพืชทั้งสามสายพันธุ์โดยใช้ Venn diagram พบโปรตีนที่ตอบสนองต่อความเค็มเฉพาะในพืชทรานสเจนิกส์ที่มีการแสดงออกของยีน *OsCam1-1* เกินปกติเมื่อเปรียบเทียบกับพืช wild type และพืชทรานสเจนิกส์ควบคุมที่ไม่มีการแสดงออกเกินปกติของยีน *OsCam1-1* นอกจากนี้ยังได้ทำการวิเคราะห์กิจกรรมของเอนไซม์ที่เกี่ยวข้องในข้าวทั้ง 3 กลุ่มด้วย ได้แก่ Peroxidase, Ascorbate peroxidase, Glutathione-S-transferase และ Adenosine Triphosphatase จากผลการทดลองทั้งหมดแสดงให้เห็นว่าโปรตีนเหล่านี้น่าจะมีหน้าที่สำคัญที่ทำให้ข้าวที่มีการแสดงออกเกินปกติของยีน *OsCam1-1* มีความทนทานต่อความเครียดที่เกิดจากความเค็มเพิ่มมากขึ้น

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2554..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

#5073920823 : MAJOR BIOCHEMISTRY

KEYWORDS: *Oryza sativa L. /*KDML 105/CALMODULIN/SALT STRESS, PROTEOMICS / LC-MS/MS

TRILERT CHAICHERDSAKUL : OXIDATIVE ENZYME ACTIVITIES AND PROTEIN PROFILE OF TRANSGENIC RICE *Oryza sativa* L. OVEREXPRESSING CALMODULIN GENE *OsCam1-1*.
ADVISOR: ASSOC. PROF. TEERAPONG BUABOOCHA, Ph.D.,
CO-ADVISORS: ASSOC. PROF. TIPAPORN LIMPASENI, Ph.D., and
SITTIRUK ROYTRAKUL, Ph.D. 182 pp.

Rice (*Oryza sativa* L.) in which a large family of CaM and CaM-related proteins has been found, is one of the most important crops in the world and is the staple food for about three billion people. Thailand is one of the most important rice-exporter and rice-producer countries in the rice-world market. However, rice production is confronting the saline soil problem and recently coping with the low yielding. Of five *OsCam* genes, *OsCam1-1* was shown to be rapidly and strongly increased in leaves under osmotic stress and is shown to play important roles in salt-stress responses. A proteomic study was performed to characterize the responses of transgenic rice *Oryza sativa* L. cv. KDML105, harboring *OsCam1-1* gene to simulated salt stress. The emphasis of the present study was to investigate the overall protein expression changes when exposed to salt. Three sets of rice seeds (wild-type, transgenic line with vector alone and transgenic line with *OsCam1-1* gene) were germinated in MS media for 3 weeks and stressed by 150 mM NaCl. Proteins in the shoot and the root of the stressed rice plants along with their controls were extracted and in-gel digested. Peptides of these samples were analysed by mass spectrometry. Each identified protein was functionally classified according to their known and putative functions by using STRAP software program. These proteins were categorized in to 7 groups based on their biological functions including cellular process (42%), metabolic process (14%), regulation (12%), developmental process (2%), localization (2%), response to stimulus (2%) while the rest are proteins in other processes or of unknown function. Many of the proteins in shoot and root responded to the induced salt stress were localized in nucleus (24%) and also in chromosome (5%). Significant fractions were also located in mitochondria (5%), ER (3%), and other intracellular organelles (5%) while only a small fraction was located in cytoplasm. Some are part of macromolecular complex (8%). Comparison of protein expression profiles among the three rice lines in both shoots and roots by using Venn diagram revealed the unique salt-responsive proteins in transgenic rice lines that constitutively over-express the *OsCam1-1* gene when compared to the wild type and the control transgenic lines without *OsCam1-1* over-expression. In addition, the complementary data on enzyme activity analysis (Peroxidase, Ascorbate peroxidase, Glutathione-S-transferase, and Adenosine Triphosphatase) in the the wild-type and the transgenic rice lines were also investigated. These results suggested that these proteins could play important roles in mediating plant response to salt stress and indicated that *OsCam1-1* overexpression probably contributes to salt resistance in rice through these proteins.

Department :…….Biochemistry…..…Student's Signature…….…….

Field of Study :….Biochemistry……Advisor's Signature……..……...….....

Academic Year :…… 2011……Co-advisor's Signature ……..…...……...…

Co-advisor's Signature ……..…...……...…

ACKNOWLEDGMENTS

First of all, I would like to express my sincere gratitude and acknowledge my debt to Associate Professor Dr. Teerapong Buaboocha, my thesis advisor for his guidance, scientific criticism, understanding, encouragement and support in the content of the text throughout in this study. I am really thankful and grateful to Associate Professor Dr. Tipaporn Limpaseni my co-advisor for her concern, suggestion, guidance and encouragement. I am extremely grateful, sincerest gratitude and deep appreciation to Dr. Sittiruk Roytrakul my co-advisor for authorizing me to use all chemical reagents and facilities at Proteomics Research Unit, Genome Institute, BIOTEC, NSTDA at Pathumthani province and for his generous advice, insight, valuable guidance, understanding, encouragement with full kindness, sharing life's experiences and skillful assistance through the period of my study. It would be impossible to mention all the things he have done for me. Generousity of them will be long to remembered.

My sincere gratitude and appreciation is extended to Professor Dr. Aran Incharoensakdi, Assistant Professor Dr. Rath Pichayangkura, Dr. Supaart Sirikantaramas and Associate Professor Dr. Jarunya Narangajavana who serve as the members of the supervisory committees for their helpful suggestion, valuable comments and criticisms.

I would like to express my sincere appreciation to Atchara Paemanee, Nuanmat Kesonchan and another members of Proteomics Research Unit for training me in laboratory, their warm assistance, sharing the great time and friendship. I wish to acknowledge the staff in the Department of Biochemistry, Faculty of Science, Chulalongkorn University for their help and assistance. In addition, the financially support of research scholarship, "The 90th Anniversary of Chulalongkorn University, Rachadaphiseksomphot Endowment Fund" is also acknowledged.

Finally, I would like to thank the warmest gratitude to members of my family and closed friends for their concern, insight, sincerity and understanding whilst I have spent many hours per day with this thesis rather than with them throughout in this study. My thanks are also extended to my patience and sedulous too.

CONTENTS

Page

Page

Page

Page

Page

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

Figure **Page**

Figure **Page**

Figure **Page**

Figure **Page**

Figure **Page**

LIST OF ABBREVIATIONS

A	Absorbance
ACN	Acetonitrile
APX	Ascorbate peroxidase
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
$CuSO_4$	Copper sulphate
Ca^{2+}	Calcium ion
CDNB	1-chloro-2,4-dinitrobenzene
C_2H_4INO	Iodoacetamide
CH_3COONa	Sodium acetate
cv.	Cultivar
cyt	Cytosol
Da	Dalton
DEPC	Diethyl pyrocarbonate
DOC	Deoxycholic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
EtOH	Ethanol

FA	Formic acid
g	Gram
GO	Gene ontology
GSH	Glutathione
GST	Glutathione-S-transferase
h	hour
H_2O_2	Hydrogen peroxide
ha	hectare
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
IAA	Iodoacetamide
KCl	Potassium chloride
KDML105	Khao Dawk Mali 105
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOH	Potassium hydroxide
L	Liter
LC-MS/MS	Liquid Chromatography Mass Spectrometry
m/z	Mass-to-charge ratio
Mg^{2+}	Magnesium ion
$MgSO_4$	Magnesium sulphate
M	Mole per liter (molar)
Mev	Multi Experiment Viewer

MS	Murashige and Skoog
mA	Milliampere
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimetre
MW	Molecular weight
ng	Nanogram
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
$Na_2S_2O_3$	Sodium thiosulfate
NCBI	National Center for Biotechnology Information
NH_4Cl	Ammonium chloride
$(NH_4)HCO_3$	Ammonium bicarbonate
NH_4OH	Ammonium hydroxide
nm	Nanometer
N-terminus	Amino terminus
OD	Optical density
OS	*OsCam1-1* overexpressing line
pmol	Picomole

PNPG	p-nitrophenyl-β-D-glucopyranoside
PPF	Proton flux
PVP	Polyvinylpyrrolidone
PX	Peroxidase
RH	Relative humidity
ROS	Reactive oxygen species
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec.	Second
SEM	Standard error of the mean
ssp.	Subspecies
STRAP	Software Tool for Rapid Annotation of Protein
STRING	Software Tool for the Retrieval of Interacting Genes
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetrametyl-ethane-1,2-diamine
Tris	Tris (hydroxyl methyl) aminomethane
TOF	Time-of-flight
U	Unit
V	Voltage
VT	Vector alone (Control of transgenic line)
w/v	Weight/volume

w/w	Weight by weight
WT	Wild-type
%	Percentage
µg	Microgram
µl	Microliter
µM	Micromolar
^{O}C	Degree Celsius

CHAPTER I
INTRODUCTION

1. Calcium signaling

Research during the last two decades has clearly established that calcium (Ca^{2+}) serves as an intracellular messenger in many cellular processes including plant responses to environmental stresses such as salinity, drought and cold. These stresses have been shown to induce transient elevation of the cytoplasmic Ca^{2+} concentration level, which is in turn detected by calcium sensor proteins. Different stimuli elicit specific calcium signatures, generated by altering the kinetics, magnitude, and cellular source of the influx (Malhó *et al.*, 1998; Allen *et al.*, 2000, 2001; Evans *et al.*, 2001; Rudd and Franklin-Tong, 2001). Calcium (Ca^{2+}) is also important in maintaining the stability of the cell wall, membrane and membrane bound protein due to its ability to bridge chemical residues among these structures (Nayyar, 2003). The concentration of Ca^{2+} in the cytoplasm of plants cells is maintained low in the nanomolar range (100-200 nM) by being actively pumped into intracellular compartments and extracellular spaces where [Ca^{2+}] is in the millimolar range (1-10 mM) as shown in **Figure 1.1** (Reddy, 2001). Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is rigorously regulated by the coordination of passive fluxes (Ca^{2+} channels) and active transport (Ca^{2+}ATPases and Ca^{2+}antiporters) across the plasma membrane and/or endomembranes, and the buffering capacity of the cytosol (Lecourieux *et al*., 2006).

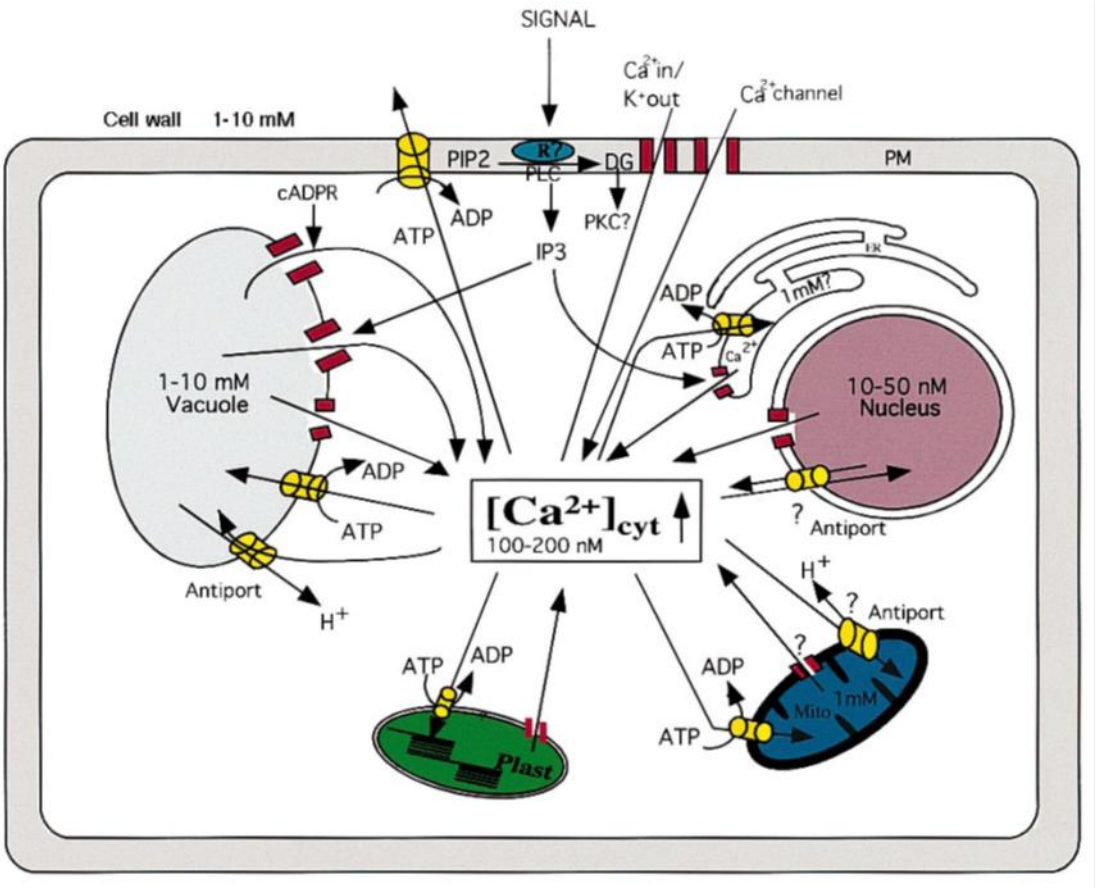


Figure 1.1 Schematic diagram illustrating the mechanisms by which plant cells elevate $[Ca^{2+}]_{cyt}$ in response to various signals and restore Ca^{2+} concentration to resting level. Ca^{2+} channels are shown in red, whereas Ca^{2+} ATPases and antiporters are indicated in yellow. Arrows indicate the direction of Ca^{2+} flow across the plasma membrane, and into and out of cellular organelles (vacuole, plastids, mitochondria, endoplasmic reticulum and nucleus). The estimated concentration of resting levels of Ca^{2+} in different organelles is indicated. Question marks indicate the lack of evidence. $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+}; PLC, phospholipase C; R, receptor, cADPR, cyclic ADP ribose, PIP_2, phosphotidyl inositol-4,5-bisphosphate, DG, diacylglycerol, PKC, protein kinase C, IP_3, inositol-1,4,5-trisphosphate; ER, endoplasmic reticulum; Mt, mitochondria; Plast, plastids; PM, plasma membrane (Reddy, 2001).

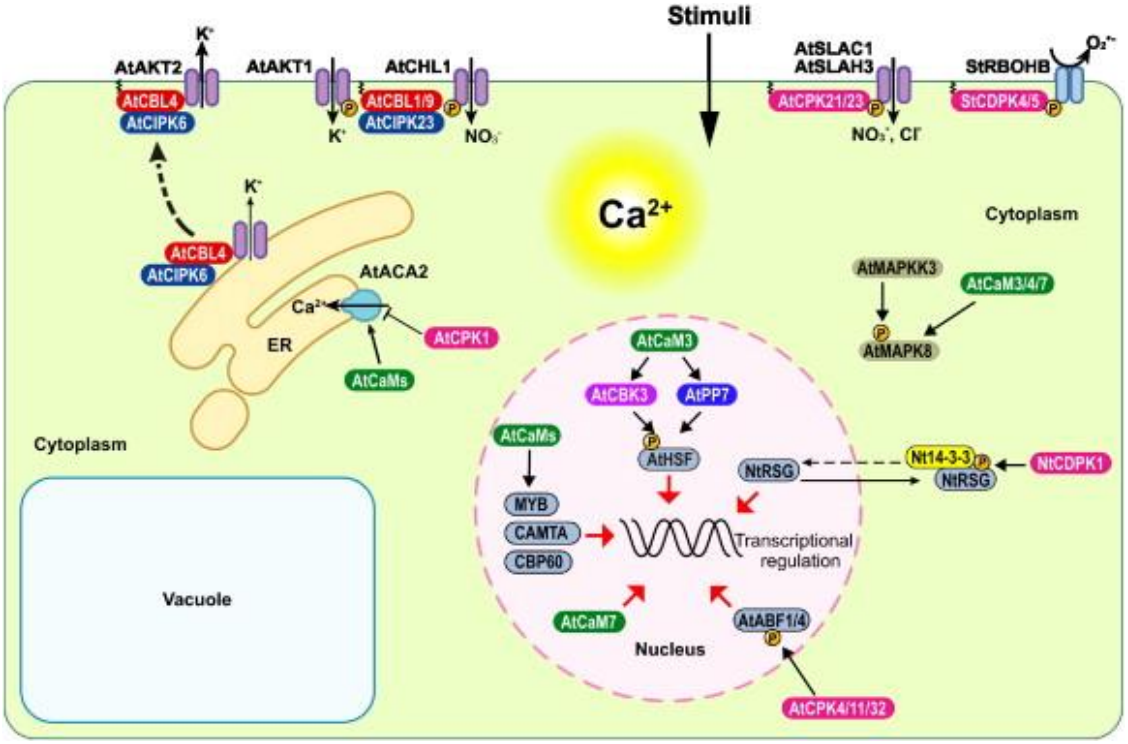


Figure 1.2 Calcium signaling pathways in plants

(Batistič O. and Kudla, J., 2012)

In response to a variety of stimuli, including hormones, light, abiotic stress and microbial elicitors, the cytosolic Ca^{2+} concentration in plants is rapidly elevated via an increased Ca^{2+} influx, and then quickly returns to the basal level by Ca^{2+} efflux. Stimuli often lead to transient increases in intracellular Ca^{2+}. Cells have elaborated exquisite ways of controlling and utilizing the gradient of ion concentration across the plasma membrane and have developed an intracellular messenger system by adopting Ca^{2+} signaling. Numerous plant signal transduction pathways have been shown to use Ca^{2+} as an integral signaling component. The universality of the Ca^{2+} ion in signaling highlights the importance of understanding how specificity can be encoded in elevations in the cytosolic concentration of this ion. The plant Ca^{2+} decoding toolkit encompasses different families of Ca^{2+} sensors, which are encoded by complex gene families and form intricate signaling networks in plants that enable specific, robust and flexible information processing as shown in **Figure 1.2** (Batistič O. and Kudla, J., 2012). A large number of Ca^{2+} sensors can be grouped into four major classes. These include (A) Ca^{2+}-dependent protein kinase (CPK) that contains CaM-like Ca^{2+} binding

domains and a kinase domain in a single protein. Each individual CPK protein is expected to detect changes in the Ca^{2+} parameters and translate these changes into the regulation of a protein kinase activity (Roberts and Harmon, 1992), (B) calmodulin (CaM) which contains four EF-hand domains but has no enzymatic activity itself, and functions by interacting with their target proteins (Zielinski, 1998), (C) other EF-hand motif-containing Ca^{2+} -binding proteins and calcineurin B-like (CBL) proteins that are similar to both the regulatory B subunit of calcineurin and the neuronal Ca^{2+} sensor (NCS) in animals (Klee *et al.*, 1998), and (D) Ca^{2+} -binding proteins without EF-hand motifs. Members of the first three classes of Ca^{2+} sensors contain helix-loop-helix motifs that bind to Ca^{2+} with high affinity (Roberts and Harmon, 1992).

2. Calmodulin

Transient elevations are sensed by several Ca^{2+} sensors or Ca^{2+}-binding proteins. One of the best-characterized calcium sensors in eukaryotes is calmodulin (CaM), a member of the EF-hand family of Ca^{2+}-binding proteins, which regulates activity of its protein targets in a calcium-dependent manner. In animals, there are only a few *Cam* genes encoding for one or a few isoforms. By contrast, each plant possesses multiple *Cam* genes that encode for several *Cam* isoforms. *Arabidopsis* has 11 *Cam* genes encoding for at least seven isoforms. The reasons that multiple *Cam* genes encode for the same or similar proteins in plants are not fully understood. Although the possibility of gene redundancy still cannot be ruled out, accumulating evidence suggests that each of those *Cam* genes could have functional significance. In all plants examined, *Cam* genes, even genes encoding the same isoform, are differentially expressed in response to stimuli. *Cam* genes are also differentially expressed in different stages, as well as in different tissue and cell types.

Distinct plant CaM isoforms differ in ability to bind and activate CaM-regulated enzymes. CaM has no catalytic activity of its own. Its regulatory activities are manifested by its ability to modulate a certain set of proteins and enzymes **(Figure 1.3)**. Thus, it is crucial to isolate and to characterize the CaM-binding proteins to understand the role of the Ca^{2+}/CaM-mediated network.

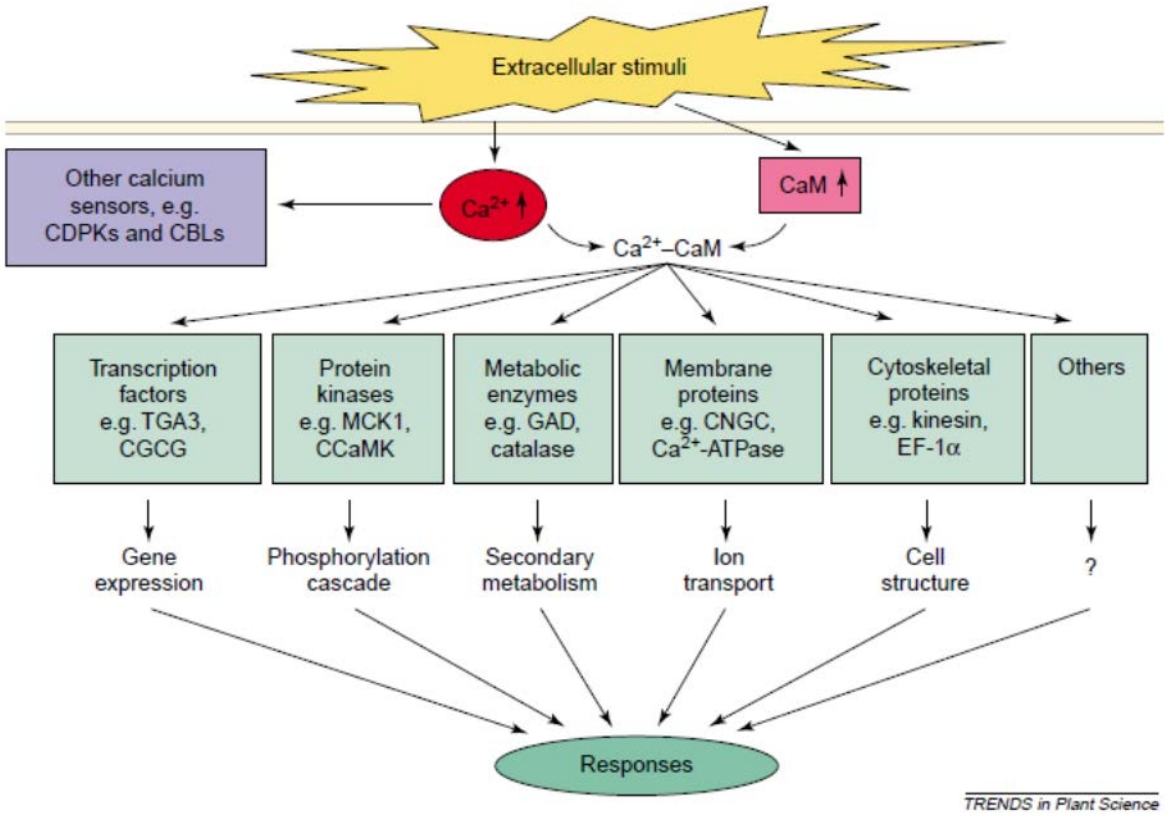


Figure 1.3 Model of Ca^{2+}/calmodulin-mediated network in plants
(Yang and Poovaiah, 2003)

Each CaM has a diverse set of target proteins that are involved in a variety of processes, including ion transport, gene regulation, cytoskeleton organization, disease resistance, metabolism and stress tolerance. Interestingly, amino acid sequences of CaMs show exceptional conservation, the CaM-binding domains of target proteins exhibit extreme sequence variability, reflecting the flexibility of CaM regulation (Yang and Poovaiah, 2003).

Increases in free Ca^{2+} concentrations originate from either extracellular pools or intracellular stores and are capable of binding to Ca^{2+}-modulated proteins including CaM and CaM-related proteins. In plants, CaM may play an important role in transducing Ca^{2+}-mediated signals from diverse environmental stresses including salt stress into appropriate adaptive cellular responses. It relays the Ca^{2+} signal by binding free Ca^{2+} ions to its C- and N-terminal EF-hand pairs, which causes a conformational

change and enables Ca^{2+}/CaM to bind to specific CaM-binding domains of its target proteins.

CaM was discovered as an activator of cyclic nucleotide phosphodiesterase in brain and heart. It was subsequently rediscovered several times, since many Ca^{2+}-dependent cellular processes were eventually shown to involve the same Ca^{2+}-binding protein. As a result, there was confusion in the nomenclature in the early literature, since the protein was referred to by several different names (e.g., Ca^{2+}-dependent regulator, modulator protein, Ca^{2+}-dependent modulator, activator protein, troponin C-like protein). However, the name 'calmodulin' was first suggested by W. Y. Cheung (Cheung, 1970).

CaM is a multifunctional protein because of its ability to interact and regulate the activity of a number of proteins. It is a small (16.7 kDa), very acidic (isoelectric point ~4), relatively stable, and heat-resistant protein. It contains four EF-hand motifs or Ca^{2+}-binding sites per molecule **(Figure 1.4a)**. One of the properties of CaM, which was discovered earlier and is by now extremely well documented, is the conformational change that takes place upon binding Ca^{2+} **(Figure 1.4b)**. It transmits the Ca^{2+} signal by binding to and activating numerous enzymes central to cellular regulation. Structural modulations of CaM proteins enable them to interact with numerous cellular targets that control a multitude of cellular functions, such as metabolism, ion balance, the cytoskeleton and protein modifications. In addition, Ca^{2+} and CaM might also regulate the expression of genes by complex signaling cascades or by direct binding to transcription factors (Luan *et al*., 2002, Phean-o-pas, *et al*., 2008).

Understanding of CaM and its relation to the wide range of Ca^{2+}-dependent enzymes and activities has provided a framework for comprehending Ca^{2+} functions in the cells at the molecular level. Further works, however, are required to unravel fully the detailed mechanisms and properties that govern the CaM-enzyme interactions and to narrow further the gaps between Ca^{2+} elicited cellular expressions and the molecular events that lead to such expression.

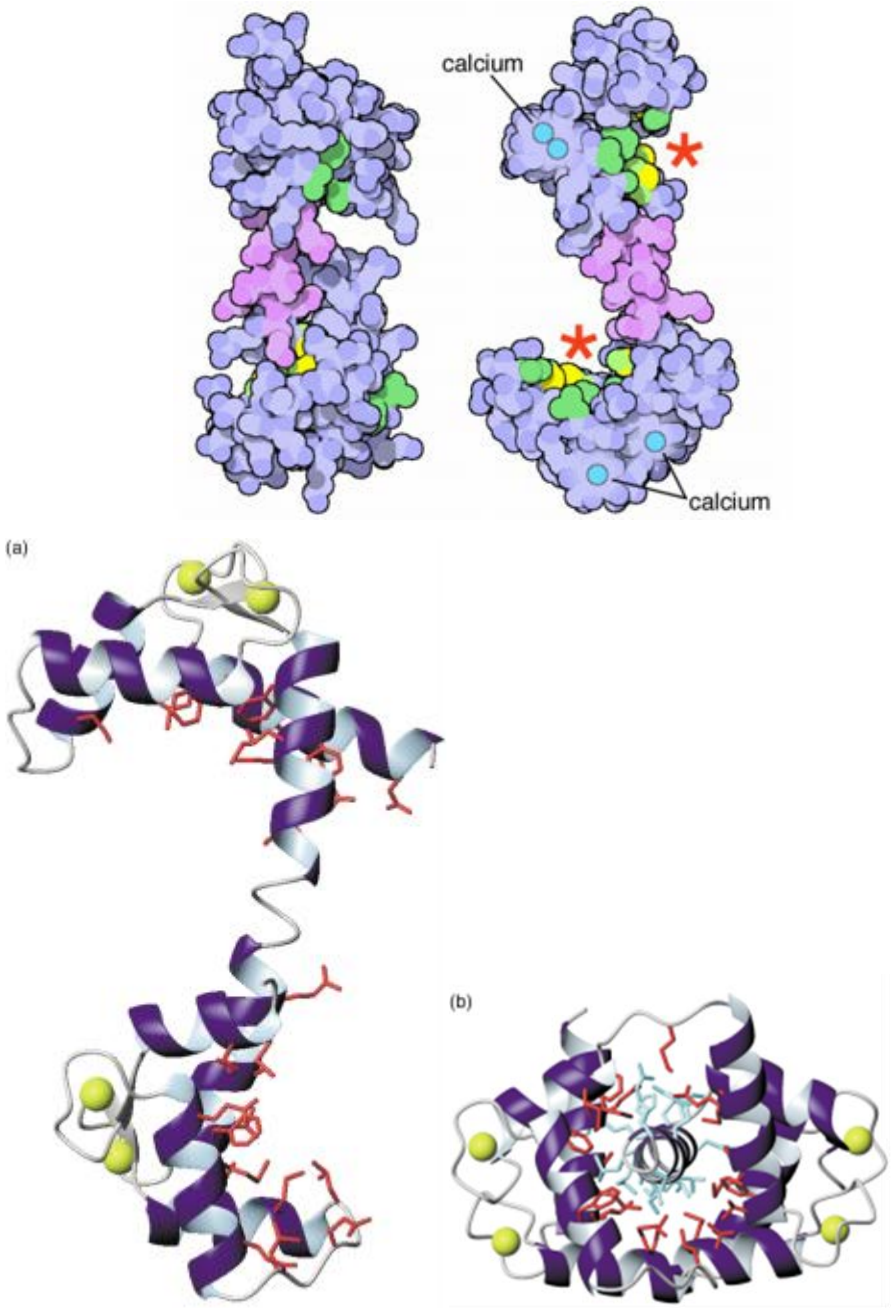


Figure 1.4 Structure of calmodulin

(Shifman and Mayo, 2002; Vetter and Leclerc, 2003)

a) Structure of Ca^{2+}-binding CaM

b) Ca^{2+}-binding CaM in complex with target peptide

Calcium ions are shown as yellow spheres. CaM side chains that interact with targets are shown in red. Peptide side chains are shown in blue.

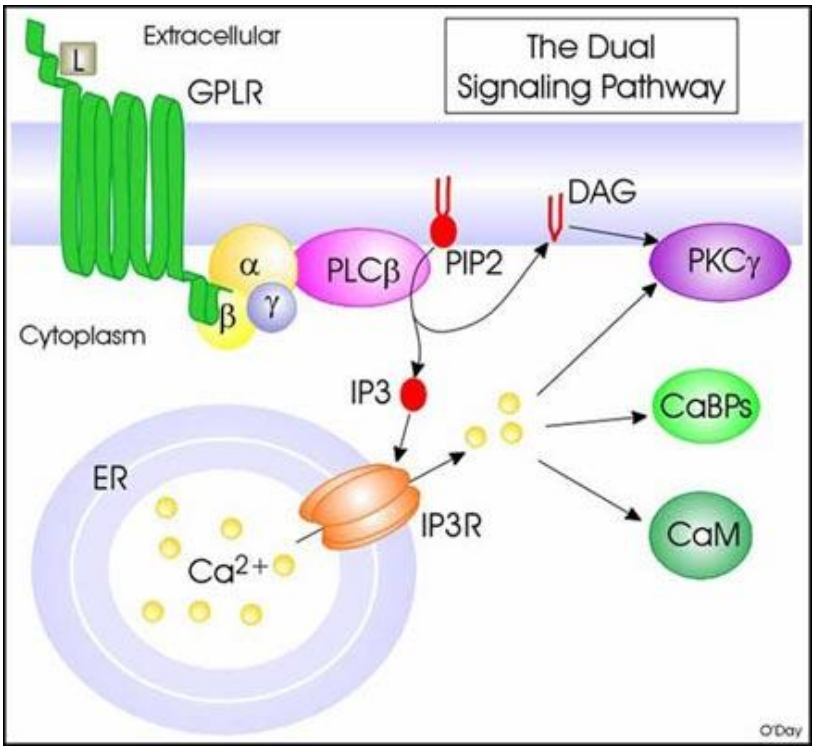


Figure 1.5 The dual signaling pathway: inositol phosphates and protein kinase C involving Ca^{2+} and CaM

PLC = Phospholipase C; (beta isoform/isoenzyme)

PIP2 = Phosphatidylinositol 1,4 bisphosphate

IP3 = Inositol 1,4,5 Trisphosphate

DAG = Diacylglycerol

PKC = Protein Kinase C (C = Kinase); (gamma isoform/isoenzyme)

CaBP = Calcium Binding Protein

CaM = Calmodulin

In plants, one striking characteristic is that numerous isoforms of CaM may occur within a single plant species. Although the broad significance of multiple CaM isoforms can be postulated to be important in distinguishing between the Ca^{2+} signals from different stimuli and thus aid in eliciting the correct response, the actual significance is, however, not clearly understood. Nevertheless, accumulating evidence suggests that each of the different *Cam* genes may have distinct and significant functions. Until now, there is no detailed information on *Cam* gene functions in response to any particular stress in rice, which is considered a model plant for

monocots (Agrawal, *et al.* 2006, Phean-o-pas, *et al.*, 2008). Of five *OsCam* genes, *OsCam1-1* was shown to be rapidly and strongly increased in leaves under osmotic stress (Phean-o-pas, *et al.,* 2008) and is thought to play important roles in salt-stress responses.

3. Salt stress problem

3.1 Saline soil

Saline soils are the soils that have high concentration of soluble salts such as NaCl, Na_2SO_4, $CaCl_2$, and $MgCl_2$. These salts are ionized by water to be toxic cations and anions. The major cations in saline soil are Na^+, $Ca^{2+,}$ Mg^{2+} and K^+, while those of anions are Cl^-, SO_4^{2-}, HCO_3^-, CO_3^- and NO_3^- (Tanji 2002). Salt concentration in the soils is measured in terms of the electrical conductivity (EC_e) of saturated paste extract (water containing ions), which is the solution extracted from a soil sample after being mixed with sufficient water to produce a saturated paste. Salinity is expressed in deciSiemens/meter (dS/m) that is the units of EC_e. Soils are classed as saline when the EC_e is $\geq$ 4 dS/m. A conductivity of 4 dS/m is equivalent to 40 mM NaCl (Munns, 2005).

Nowadays, saline soils are expanded over 800 million hectares of land throughout the world (FAO, 2005). Consequently, saline soils remain a major environmental constraint that limits crop productivity and quality and also restricts use of land previously uncultivated (Viswanathan and Zhu, 2003). These constraints are most severe in areas of the world where food distribution is problematic because of insufficient infrastructure (Shuji *et al.*, 2002).

In Thailand, the saline soil causes various problems on crop production in many regions; especially, northeastern part of Thailand. This part of Thailand covers more than one-third of the country of 16.9 million ha with 9.25 million ha of agricultural land or 44% of the arable land in the country. Moreover, the most regions are considered as an integral component of the agricultural food production and an important commodity grain, including rice producer in Thailand. More than half of the total rice area is in the northeast. Unfortunately, in the northeastern part of Thailand, the saline soil is scattered all over. There is about 2.8 million ha of saline soils or 17% of the total area of northeast Thailand. The soils are classified as severe, moderate and

slight saline areas of 240,000, 590,000 and 2,020,000 ha, respectively. The continuing impacts of salinity are reduced crop yield, environmental degradation, rural poverty and low economic growth, leading to migration of poor farmers to the city (Yuvaniyama *et al.,* 2007).

3.2 Effect of salinity on plants

Salinity in soil or water is one of the major stresses that can severely limit plant growth and productivity. Adverse effects of salinity on plant growth may be due to two main reasons which are osmotic stress and ion toxicity. Nevertheless, metabolic imbalances caused by these two impacts under saline conditions may also lead to oxidative stress (Hussain *et al*., 2008). Within a plant under salt stress, all the major processes such as, photosynthesis, protein synthesis and energy and lipid metabolisms are affected (Parvaiz and Satyawati, 2008). Resistance to environmental stress occurs when plant withstands the imposed stress that may result from either tolerance or a mechanism that permits escape from the stresses. The ability of plants to survive and maintain their growth under saline conditions is known as salt tolerance. This is a variable trait that is dependent on many factors, including the species of the plant, the length and severity of the salinity, and the age and stage of development (Bray, 1997). There is a continuous spectrum of plant tolerance to saline conditions ranging from glycophytes that are sensitive to salt, to halophytes which survive in very high concentrations of salt. Most grain crops and vegetables are highly susceptible to soil salinity even when the soil ECe is <4 dS m^{-1}. The different threshold tolerance ECe and different rate of reduction in yield beyond threshold tolerance ECe indicating variation in mechanisms of salt tolerance among crop species are shown in **Table 1** (reviewed by Zhu *et al.,* 2005).

As stated in the previous paragraph, the salt ions cause two main effects on plants which are osmotic and ionic stress. Firstly, plants need to maintain internal water potential to be lower than the water potential in their surroundings so as to maintain turgor pressure and water use efficiency. Nevertheless, excessive soluble salts in the soil are harmful because they lead to decreasing of external water potential until it lowers than inside plant cells. This phenomenon can inhibit water uptake or even withdraw water from the plant cells causing dehydration and eventually a

reduction in turgor and growth. (Zhu and Xiong, 2003; Shuji *et al.,* 2003) Furthermore, the excessive accumulation of Na^+ and Cl^- may be toxic to the root cell, resulting in numerous damages to plant cell such as weakening, diminishing, wilting and death of the whole plant. Secondly, excessive sodium ions are toxic to the plant cells because the excessive sodium ions at the root surface may disrupt plant potassium nutrition that is vital for the maintenance of cell turgor, membrane potential, and the activities of many enzymes (Lazof and Bernstein, 1999). Generally, salt stress causes a decrease in cellular K^+ content which may be partly responsible for reduced growth under salt stress. In addition to K^+, plant growth inhibition can be due to the effect of both Na^+ and Cl^- to limit the absorption of other ions and nutrients required for growth. While Na^+ competes with Ca^{2+}, Mg^{2+}, and Mn^{2+}. Cl^- restricts the absorption of NO_3^-, PO_4^{2-} and SO_4^{2-} (Termaat and Munns, 1986). In addition to osmotic and ionic stress, these two effects also cause the derived important stress – oxidative stress. The oxidative stress involves in the production of reactive oxygen species (ROS) in plants under salt stress, for example, hydrogen peroxide, hydroxyl radicals, and superoxide anions. These ROS can have damaging effects on cellular structures and macromolecules such as lipids, enzymes and DNA (Hong *et. al*., 2000). Therefore, this stress may be a necessary impact that reduces the crop production.

There were various studies reported about the yield of crop production under salt stress, for example, in rice *(Oryza sativa L.*), primary branches per panicle, panicle length, spikelets per pinnacle, number of filled spikelets, and seed weight per panicle were significantly reduced by salinity (Khatun *et al*., 1995). In *Triticum aestivum*, the fresh and dry weight of roots decreased after exposed to 100 mM salt. These results attributed the severity of plants to salinity related to salinity causes ionic stress, osmotic stress, and oxidative stress on plants. Therefore, it is necessary for plant to posses the defense against these three effects.

Table 1. Many important crops are susceptible to soil salinity (Zhu *et al.,* 2005)

Crop	Threshold salinity	Decrease in yield
	dS m^{-1}	Slope % per dS m^{-1}
Bean (*Phaseolus vulgaris* L.)	1.0	19.0
Eggplant (*Solanum melongena* L.)	1.1	6.9
Onion (*Allium cepa* L.)	1.2	16.0
Pepper (*Capsicum annuum* L.)	1.5	14.0
Corn (*Zea mays* L.)	1.7	12.0
Sugarcane (*Saccharum officinarum* L.)	1.7	5.9
Potato (*Solanum tuberosum* L.)	1.7	12.0
Cabbage (*Brassica oleracea* var. *capitata* L.)	1.8	9.7
Tomato (*Lycopersicon esculentum* Mill.)	2.5	9.9
Rice, paddy (*Oryza sativa* L.)	3.0	12.0
Peanut (*Arachis hypogaea* L.)	3.2	29.0
Soybean [*Glycine max* (L.) Merr.]	5.0	20.0
Wheat (*Triticum aestivum* L.)	6.0	7.1
Sugar beet (*Beta vulgaris* L.)	7.0	5.9
Cotton (*Gossypium hirsutum* L.)	7.7	5.2
Barley (*Hordeum vulgare* L.)	8.0	5.0

3.3 Cellular defense response of plant to salt stress

The defense mechanisms of plant at cellular level dealing with three main categories: ionic, osmotic, and oxidative stresses were discussed.

3.3.1 Dealing with ionic stress or ion toxicity

Restoring ion homeostasis in plants disturbed by salt stress represents an acute response. Plants employ various ways to combat ionic stress imposed by high salinity. These include restricting salt uptake and increased extrusion and compartmentalization. The restricting salt uptake is the first mechanism for plant to combat excessive sodium ions at the root surface which may disrupt plant potassium nutrition that is vital for the maintenance of cell turgor, membrane potential, and the activities of many enzymes (Lazof and Bernstein, 1999). Under sodium stress, it is necessary for plants to operate the more selective high affinity potassium uptake system in order to maintain sufficient potassium nutrition. Sodium once enters into the cytoplasm, has a strong inhibitory effect on the activity of many enzymes. Therefore, plants have to compartmentalize sodium into

the vacuole, away from cytosolic enzymes (Flowers *et al.,* 1997). Vacuolar compartmentalization of Na^+ is achieved by the action of Na^+/H^+ antiporters on the tonoplast – the vacuolar membrane. The proton gradient that drives the antiporter is generated by tonoplast ATPase (Jinglan *et al.*, 1998). The plasma membrane ATPase was stimulated in *Salicornia bigelovii* Torr. when the plant was grown in media containing 200 mM NaCl (Ayala *et al.,* 1996). Increased ATPase mRNA abundance was reported in *A. nummularia* when NaCl-adapted (342 mM) cells were re-exposed to NaCl after having been grown in media without additional NaCl. In addition to compartmentalization, extrusion of sodium out of the cell is a straightforward way to avoid Na^+ accumulation in the cytosol. This mechanism is achieved by sodium/proton antiporters on the plasma membrane (Shi *et al.*, 2000).

3.3.2 Dealing with osmotic stress

When plants are challenged with hyperosmolarity, accumulation of ions such as Na^+ in the vacuoles can serve as a means to lower osmotic potential of the cells, and this process is perhaps cost-effective with regard to the amount of energy and resources spent. A related strategy used to lower the osmotic potential of the cell cytosol is to accumulate compatible solutes. A common feature of compatible solutes is that these compounds can accumulate to high levels without disturbing intracellular biochemistry. Compatible solutes have the capacity to preserve the activity of enzymes that are in saline solutions. These compounds have minimal effect on pH or charge balance of the cytosol or lumenal compartments of organelles (Das and Parida, 2005). These osmolytes include proline, betaine, polyols, sugar alcohols, and soluble sugars. Glycine betaine and trehalose act as osmoprotectants by stabilizing quaternary structures of proteins and highly ordered states of membranes. Mannitol serves as a free-radical scavenger. Proline serves as a storage sink for carbon and nitrogen and a free-radical scavenger. It also stabilizes subcellular structures (membranes and proteins), and buffers cellular redox potential under stress. Hence, these organic osmolytes are known as osmoprotectants (Bohnert and Jensen, 1996). Genes involved in osmoprotectant biosynthesis are up-regulated under salt stress, and concentrations of accumulated osmoprotectants correlated with osmotic stress

tolerance also increase (Zhu, 2002). Salt tolerance of transgenic tobacco engineered over-accumulate mannitol was first demonstrated by Tarczynski *et al*., 1993. Genetically engineered over production of compatible osmolytes in transgenic plants such as *Arabidopsis*, rice, wheat, and *Brassica* has also shown to enhance stress tolerance as measured by germination, seedling growth, survival, recovery, photosystem II yield, and seed production under very high salt and osmotic stress (Zhu *et al.,* 2005).

Carbohydrate compounds are significant osmolytes that accumulated in plants (Galiba and Kerepesi, 2000). Their major functions are osmoprotection, osmotic adjustment, carbon storage, and radical scavenging (Das and Parida, 2005). There are two major groups of carbohydrate found to accumulate in salt-stressed plant: sugar alcohol such as sorbitol and mannitol; and water soluble carbohydrate such as; glucose, fructose, sucrose and fructan (Galiba and Kerepesi, 2000). The sugar alcohol mannitol is suggested to play an important role in osmotic adjustment and provide enhanced tolerance in response to high salinity. There are some efforts to develop transgenic salt- and drought-tolerant plants. One strategy consists in introducing a mannitol-producing chimerical gene into plant such as wheat and tobacco (Eimer, 2004). Transgenic tobacco plants transformed with *mtlD* gene showed enhanced accumulation of mannitol, leading to increased tolerance of transgenic plants in response to salinity (Tarczynski *et al.*, 1993). Transgenic wheat plants that accumulated mannitol in leaf tissues showed improved productivity under salinity as well (Eimer, 2004).

In the case of water soluble carbohydrate, the accumulation of soluble sugars in plants has been widely reported as a response to salinity. Most studies found that tolerant genotypes accumulated more soluble carbohydrate than did sensitive ones. In 2001, Kerepesi and Galiba determined the content of water soluble sugar in stem of wheat (*Triticum aestivum* L.) and found that salt stress increased water soluble carbohydrate. In 2001, Kawasaki *et al*. studied the gene expression profiles during the initial phase of salt stress in rice root by microarrays. Rice sucrose synthase-2 (*sus2*) gene was down regulated after 3 hours of salt stress. Sucrose synthase, a key enzyme in carbohydrate metabolism, catalyzes the reversible conversion of sucrose uridine-diphosphate to fructose and UDP-glucose (Koch, 1996). The transcription of this gene was modulated by glucose

and sucrose (Wang *et al.,* 1999). For example, maize *Sh1*, an ortholog of rice *sus2*, is down regulated by increased glucose (Koch, 1996), suggesting that root carbohydrate metabolism may be altered by salt stress.

3.3.3 Dealing with oxidative stress

Although the exact mechanisms of salt stress are still uncertain, it is clear that excessive accumulation of salt ions, mainly sodium (Na^+) and chloride (Cl^-) is a major contributory factor. Excessive accumulation of Na^+ causes ion imbalance and many metabolic disturbances to the plants. There are some evidences showing that salt stress induces the production of reactive oxygen species (ROS) (Shalata and Tal, 1998). Reactive oxygen species include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical. These ROS can seriously disrupt normal metabolism through oxidative damage to membrane lipids, proteins, and nucleic acids. Plants employ antioxidants (e.g., ascorbate, glutathione, α-tocopherol, and carotenoids) and detoxifying enzymes, such as superoxide dismutase, catalase, and enzymes of ascorbate-glutathione cycle to combat oxidative stresses. The activity and expression levels of the genes encoding detoxifying enzymes are probably enhanced by ROS under abiotic stresses. Transgenic plants overexpressing ROS scavenging enzymes, such as superoxide dismutase (reviewed by Alscher*et al.*, 2002), ascorbate peroxidase (Wang *et al.,* 1999), and glutathione *S*-transferase/ glutathione peroxidase (Roxas *et al.* 2000) showed increased tolerance to osmotic, temperature, and oxidative stresses.

3.4 Rice and salt stress

Rice (*Oryza sativa* L.) in which a large family of CaM and CaM-related proteins has been found, is one of the most important crops in the world and is the staple food for more than two-third of the world's population. Rice as a food source provides more than 60% of the calories for about 2 billion people in Asia and more than 30% of the calories for 1 billion people in Africa and Latin America. *Oryza sativa* is an annual grass growing best when submerged in water as shown in Figure 1.6. It grows in upland areas, irrigated areas, rainfed lowland areas, and flood-prone areas. Rice is highly adaptable and can be

grown in diverse environments. Rice is constantly bombarded with environmental signals, both biotic and abiotic such as soil salinity, drought, cold, disease, and pathogenic microorganism, some of which cause stress and limit the growth and development and affect the yield and quality.

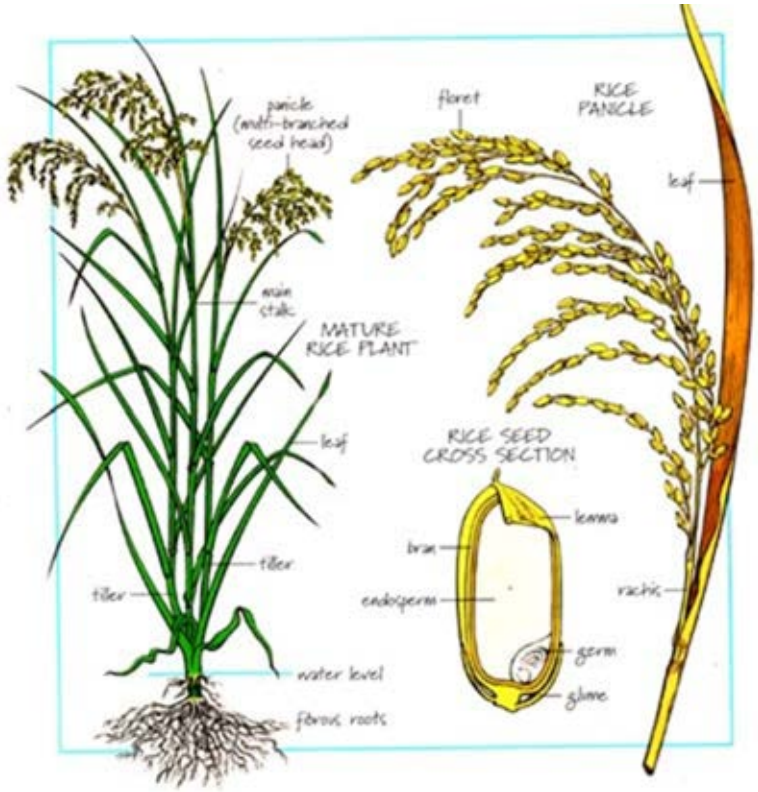


Figure 1.6: *Oryza sativa* L. (http://en.wikipedia.org)

Oryza sativa was a cereal selected to be sequenced as a priority and has the status of a "model organism". Rice with its relatively small genome size (~430 Mb), ease of transformation, well developed genetics, availability of a dense physical map and molecular markers (Chen *et al.*, 2002; Wu *et al.*, 2002a), high degree of chromosomal co-linearity with other major cereal such as maize, wheat, barley and sorghum (Ohyanagi *et al.*, 2006) and together with its complete genome sequence (Sasaki *et al.*, 2005) is considered a model monocot system. It is being used to understand several fundamental

problems of plant physiology, growth and developmental processes ranging from elucidation of a single gene function to whole metabolic pathway engineering. In addition, rice shares extensive synteny among other cereals thereby increasing the utility of this system (Devos and Gale, 2000). These, together with availability of ~28,000 full length cDNAs, a large number of expressed sequence tags, yeast artificial chromosomes, bacterial artificial chromosomes, P1-derived artificial chromosomes, libraries and rich forward and reverse genetics resources (Hirochika *et al.*,2004) have made rice a worthy forerunner among the plants especially among the cereals.

Nowadays, the world populations were significantly increased, so yield enhancement to increase rice production is one of the essential strategies to meet the demand for food of the growing population. Both abiotic and biotic features, especially salinity problem, limit adversely the productivity of rice growing areas (Giri and Laxmi, 2000). Irrigated plantation area where the saline soil is a problem accounts for 55% of total rice area and contributes 75% of global rice production. Thailand is one of the most important rice-exporter and rice-producer countries to rice-world market, particularly in the northeastern part, which rice production is confronting against the saline soil problem.

Aromatic rice 'Khao Dawk Mali 105' ('KDML 105') is extensively grown in this region and recently coping with the low yielding (Ahloowalia *et al.*, 2004). It is a famous aromatic rice variety of Thailand and worldwide because of its aromatic, solf and tender cooked rice. KDML 105 is popularly grown under rainfed lowland in the North and Northeast of Thailand which frequently experience the problems about drought and soil salinity. However, KDML105 itself is a tall variety and cannot produce high enough grain yield. KDML 105 is also photoperiod sensitive which restricts its multiple cropping per year. Moreover, KDML 105 is susceptible to many insect pets and disease, although it can resist several adversed planting conditions such as modurate degree of drought, salted soil or acid soil (Tassongchant, 1987). The research found that primary branches per panicle, panicle length, spikelets per pinnacle, number of filled spikelets, and seed weight per panicle of rice (*Oryza sativa* L.) were significantly reduced by salinity (Khatun *et al.*, 1995). It is because many physiological and biochemical processes such as, stomatal closure, chlorophyll and protein degradation, photosynthetic efficiency, ATP synthesis,

membrane permeability and carbohydrate metabolisms are interfered (Cha-um *et al*., 2004; Pieters and Souki, 2005). As a result, rice possesses three main mechanisms as discussed previously in order to combat with salt stress. Therefore, considerable efforts have been made to unravel plant salt tolerant mechanisms with the ultimate goal of improving the rice productivity in saline soils.

4. Study of Proteomics

4.1 Proteomics

"Proteome" refers to the total set of proteins encoded by the genome of an organism. (Wilkins *et al.,* 1996) Proteomics is the study of all proteins synthesized in a cell or organism. It is the newly developed science for the study of comprising the proteome, including the changes in structure and abundance in response to developmental and environmental cues (Wilkins *et al.*, 1996 Seyed *et al,* 2009)). In recent years, the term proteomics has also been applied to all the protein expressed in a particular organelle or tissue or in response to a particular abiotic stress such as drought and salt stress with the emphasis being on a global analysis of how cell and organisms respond to these stress at the protein level. (Salekdeh *et al*., 2002) Protein Expression profiling by gel electrophoresis is a primary analysis tool used to characterize the expression of proteins. Large numbers of proteins, mostly protein variants, are identified with these methods, and highly expressed proteins are easily located. The resulting differences in protein expression due to treatment with various stimulating factors are the basis for comparative gel electrophoresis maps. Proteins or peptides after separation by electrophoresis are identified by determining the sequence of amino acid comprising them. Traditionally, this was done by Edman degradation, which determined one amino acid at a time from N-terminus of the proteins or peptides. However, the process of the identification of proteins was revolution with the advances in genomics and bioinformatics, which made the gene and protein data available for the assignment of a particular peptide sequence to a protein and to the encode gene.

4.2 Mass spectrometry

Mass spectrometry is unsurpassed capacity for accurate protein identification and quantitation. Proteomics applications that involve LC/MS are at similar stages of growth as biotechnology in early 1990s To date, the predominant application involved the qualitative analysis of proteins via automated database searching such as protein expression profiling. Mass spectrometry-based formats and industry preferences are still evolving. Sensitive and accurate mass spectrometry approaches for quantitation of proteins appear to be destined for major advances. The principles of mass spectrometer originated that molecules can be ionized, and the ionized molecules can be separated based on their mass-to-charge ratio be applying a magnetic force. The positively charged particles are the ionized molecules, whereas the negatively charged particles are the electrons. The results yield information about their molecular weights and structure.

Mass spectrophotometry (MS) is an analytical technique that measures the molecular masses of individual compounds and atoms precise by converting them into charged ions. Quite often, the structure of a molecule can also be deduced. Mass spectrophotometry is also uniquely qualified to provide quantitative information of an analyte at levels of structures specificity and sensitivity that are beyond imagination (e.g. in the zeptomole range). In addition, mass spectrophotometry allows one to study reaction dynamics and chemistry of ions, to provide data on physical properties such as ionization energy, appearance energy, enthalpy of a reaction, proton and ion affinities, and so on, and to verify molecular orbital calculations-based theoretical predictions. Thus, mass spectrophotometer probably is the most versatile and comprehensive analytical technique currently at the disposal of chemists and biochemists. Several areas of physics, chemistry, medical chemistry, pharmaceutical science, geology, cosmochemistry, nuclear science, material science, archeology, petroleum industry, forensic science, and environmental science have been benefited from this hightly precise and sensitive instrumental technique (Dass, 2007).

The power of mass spectrometry lies in the fact that the mass spectra of many compounds are sufficiently specific to allow their identification with a high degree of confidence, if not with complete certainty. If the analyte of interest is encountered as part

of a mixture, however, the mass spectrum obtained will contain ions from all of the compounds present and, particularly if the analyte of interest is a minor component of that mixture, identification with any degree of certainty is made much more difficult, if not impossible. The combination of the separation capability of chromatography to allow 'pure' compounds to be introduced into the mass spectrometer with the identification capability of the mass spectrometer is clearly. Therefore advantageous, particularly as many compounds with similar or identical retention characteristics have quite different mass spectra and can therefore be differentiated. This extra specificity allows quantitation to be carried out which, with chromatography alone, would not bepossible. The combination of HPLC with mass spectrometry therefore allows more definitive identification and the quantitative determination of compounds that are not only fully resolved chromatographically (Ardrey, 2003)

A spectrometer consists of the following five major components: a port or device for the introduction of sample into the machine, a device for ionization of molecules, an analyzer for the separation of ionized molecules on the basis of their mass to charge (m/z) ratio, a detector that monitors the presence of the separated ions and records them, and a high vacuum system to allow free movement for ions within the spectrometer. In a mass spectrometer the role of the ion source is to create gas phase ions. Analyte atoms, molecules or clusters are transferred into gas phase and ionized either concurrently (as in electrospray ionization) or through separate processes (as in the glow discharge). The choice of ion source depends heavily on the application. So called soft ion sources can produce intact ions of large fragile molecules.

Electrospray ionization (ESI) was first introduced by Dole and colleagues (1968) and coupled to MS by Yamashita and Fenn (1984). In ESI, a sample is vaporized by high voltage and then ions are generated as the solution of proteins or peptides is forced through a fine syringe. The sample is dissolved in a polar and transported through a needle placed at high positive or negative potential (Yamashita, *et al*., 1984; Aleksandrov, *et al*., Fenn, *et al*., 1989). The high electric potential (1 to 4 kV) between the needle and nozzle causes the fluid to form a Taylor cone, which is enriched with positive or negative ions at the tip. A spray of charged droplets is ejected from the Taylor cone by the electric

field. The droplets shrink through evaporation, assisted by a warm flow of nitrogen gas passing across the front of the ionization source **(Figure 1.7: Schematic of ESI-MS)**. Ions are formed at atmospheric pressure and pass through a cone shaped orifice, into and intermediate vacuum region, and from there through a small aperture into the high vacuum of the mass analyzer. ESI has been used in conjuction with all common mass analyzers. The exact mechanism of ion formation from charged droplets has still not been fully elucidated and there are different theories proposed (Mora, *et al*., 2000; Iribarne, *et al.*, 1976).

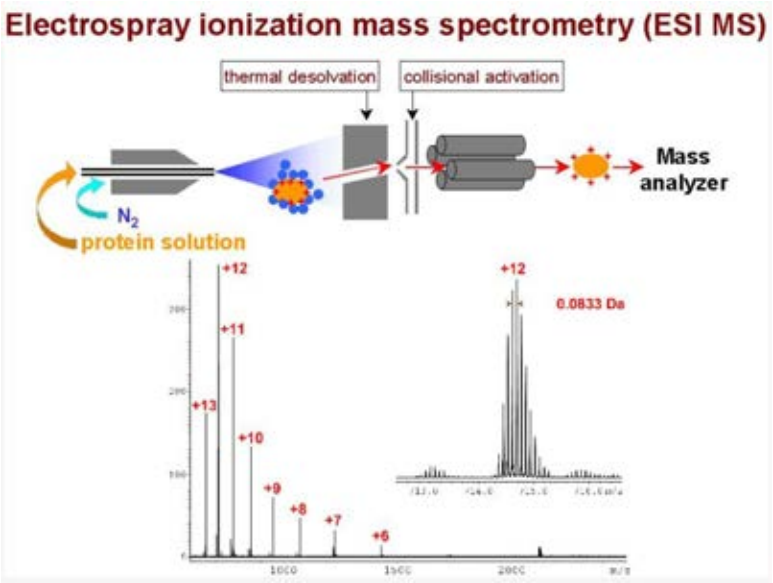


Figure 1.7: Schematic of electrospray ionization mass spectrometry (ESI-MS)
(Westman and Brinkmalm, 2002)

Sample preparation requires only dissolution of the sample to a suitable concentration in a mixture of water and organic solvent, commonly methanol, isopropanol, or acetonitrile. A trace of formic acid or acetic acid is often added to aid protonation of the analyte molecules in the positive ionization mode. In negative ionization mode, ammonia solution or a volatile amine is added to aid deprotonation of the analyte molecules. The sensitivity of ESI-MS is good, with low femtomole or

attomole detection levels for many peptides. However, the sensitivity of ESI is a function of the concentration of the injected sample. High flow rates, that is 1 to 1,000 mL/min in conventional ESI-MS, result in high sample consumption. It is therefore advantageous to use the lowest possible flow rate. A recent version of electrospray ionization called "nanospray ionization" has become more popular. In nanospray ionization, a much smaller volume of liquid as little as 1 nL/min is passed through the charged capillary needle. This results in generation of a finer spray with much reduced size of the ionized droplets and considerably higher sensitivity (Wilm, *et al*., 1996)

When the process of ionization is completed, the ionized molecules of proteins or peptides enter the section of the mass spectrometer called "Mass Analyzer", where they are separated based on their mass-to-charge ratio by electric and/or magnetic fields or by measuring the time taken by an ion to reach a fixed distance from the point of ionization to the detector. Different kinds of analyzers are available for the separation of ionized molecules. Among the different kinds of analyzers, two particular kinds, called the quadrupole and the time-of-flight (TOF) analyzers, are the most important from the point of proteomics for their use in mass spectrometers. A particular spectrometer may use one or the other or at times a combination of both quadrupole and TOF analyzers. The separation should also be independent of the chemical conformation of the species. All mass analyzers presently in use are based on electromagnetism so ions are required to obtain separation. Therefore, an ion source has to be coupled to the analyzer. The analyzer will then separate ions coming from the source according to their m/z. There are several types of mass analyzers used in mass spectrometric research and they can be divided into different categories, such as magnetic or pure electric, scanning or non-scanning (pulse based), and trapping or non-trapping analyzers (Blaum, *et al*, 2006). Ion trap analysers use a similar principle to quadrupole mass analysers but employ a system of entrance, exit and end-cap electrodes together with a ring electrode that surrounds the trap cavity. As with quadrupole so with ion trap, for each ion type with a given value of m/z there is a corresponding value $\varphi 0$ when interactions between ion type and external quadrupole field are such as to enable the trapping of ion within the analyzer prior to release for detection.

Ion traps are relatively quite sensitive and robust, so are fairly widespread, despite being less accurate than TOF and quadrupole mass analyzers.

The final component of a mass spectrometer is “Detectors”. Its purpose is to detect and record the presence of ions coming out of the mass analyzer hitting the detector. An electron is emitted when an ion hits the recorder and creates a small current. The low level of signal from a small number of ions coming out of the mass analyzer is amplified from 1,000 to 1 million times to become delectable and then recorded. A detector may use an electron multiplier or a photomultiplier. Photomultipliers first convert and electron produced by the ion hitting the detector plate into photon, which is detected by a phosphorescent plate in a sealed tube. Photomultipliers are preferred in a detector because they are located in a sealed tube, which reduces the noise-to-signal ratio by not allowing any outside interference to come out from the mass analyzer. All mass spectrometer are equipped with photomultipliers. These signals are then recorded on a graph by plotting the amount of signal versus m/z ratio. Mass spectrometer graphs usually show the presence of proteins/peptides of different molecular size and their abundance.

LC-MS has become a powerful molecular biology tool and multiple strategies for peptide and protein quantitation by LC-MS have been developed and applied to address a wide range of biological questions. It is being used more and more to analyse differences between samples at the protein expression level, in post-translational modifications (PTMS), in the components of protein complexes and in intracellular protein localization, and this on a scale where thousands of proteins can now be compared in a single experiment. LC-MS has many applications in biomolecular research. In addition to quantification of gene expression at the protein level, as an alternative to microarray analysis, LC-MS can be used to comprehensively profile the protein composition of subcellular organelles and protein complexes and to provide a picture of the dynamic protein composition of macromolecular structures. Another important and gradually increasing application of LC-MS is for in-depth quantification of enzymatic activitites in cells. Enzymes are important mediators of biological functions, but since their levels may not reflect their activity status, standard proteomic approaches cannot be used to infer the activity status of biochemical pathways. Therefore, methods that can be used to quantify

enzymatic activities in a comprehensive manner will have many applications in biological research. In the future, related approaches that quantify other protein modifications, such as acetylation or methylation, may also be explored as these should provide equally important biological insights. Quantitative LC-MS is likely to continue to have important roles in these workflows (Cutillas and Timms, 2010)

4.3 Gel-based LC-MS approach

Gel separations are common experiments for evaluating the component of protein complexes. Early proteomics efforts were grafted onto existing gel separation technologies. The protocol for gel-based proteomics experiments begin with the denaturation of proteins. The proteins are separated by one-or 2D-GE, and bands or spots containing proteins of interest are cut from the gel. Disulfide bridges are reduced and alkylated, and proteolytic enzymes cleave proteins to peptides. These peptides are separated be reverse phase LC en route to a tandem mass spectrophotometer.

Gel electrophoresis is a standard technique for separating proteins. Typically, the proteins are first denatured by a detergent such as SDS. In one-dimentional electrophoresis, they are separated by size by electrophoretic mobilities through polyacrylamide. Because proteins contain different amino acid residues, the pH values at which they are neutral (call isoelectric point) differ. In 2D-GE, the proteins are separated by size in one dimension and by isoelectric focusing in another. Such gels have been shown effective for resolving up to 10,000 proteins in a single experiment. The positions of proteins are marked by applying a dye to the gel; Coomassie Blue dye is widely used but only highlights proteins for which at least 30-50 ng is present. Silver staining, on the other hand, can mark positions for proteins of which at least 1 ng is present. Fluorescent dyes have roughly the same sensitivity as silver staining but may be more easily to removed. Once proteins have been separated and positions are marked, gel bands (for 1D gels) or spots (for 2D gels) can be excised for extraction. Protein tertiary structures may be maintained by disulfide crosslinks between cysteine residues. Reduction of these bridges by dithiothreitol or tris(2-carboxyethyl)phosphine (TCEP) can break these links, allowing the protein to be fully denatures. Subsequence alkylation by iodoacetamide blocks off the cysteine side chains and adds 57 Da to their masses. Several enzymatic

digestions of proteins are available. The most commonly used in proteomics is the trypsin digest. This enzyme, which cleaves proteins after arginine and lysine residues, is available in a form bound to beads for removal from the sample after the digest. EndoK-C hs the same cleavage specificity as trypsin but show better efficiency in the present of urea and other denaturants; however, use of the enzyme increases experiment cost. Alternative enzymatic cleavages may increase the diversity of peptide produced; subtilisin, elastase, themolysin, and proteinase K can be employ to create peptides covering different portion of a protein sequence. These digestion yield peptides that can be separated en route to the mass spectrometer by reverse phase LC. Columns for these separations are commercially available, but they can also be produced from fused silica capillaries with inner diameters of 100 μm or less. A laser puller can be used to create tips with inner diameter of 5 μm C_{18}-coated beads and the sample's peptides are loaded into the column under pressure. A gradient of increasingly hydrophobic solvents elutes the peptides progressively from the column into the mass spectrophotometer (Veenstra and Yates, 2006)

Protein separation and comparison by 2D-GE, followed by mass spectrophotometry (MS) or tandem mass spectrometry (MS/MS) identification is the classical method for quantitative analysis of protein mixtures. In this method, the intensity of the protein stain is used to make a determination regarding the quantity of a particular protein. However, spots on a given 2D-GE often contain more than one protein, making quantification ambiguous since it is not immediately apparent which protein in the spot has changed. In addition, any 2D-GE approach is subject to the restrictions imposed by the gel method, which include limited dynamic range, difficulty handling hydrophobic proteins, and difficulty detecting proteins with extreme molecular weights and pI values (Zhu, Smith, and Huang, 2010)

An approach that constitutes an interesting compromise between the advantages and shortcomings of the method mentioned above is a combination of 1D PAGE protein separation and nanocapillary LC-MS/MS analysis (GeLC-MS/MS) of in-gel-generated peptides for protein identification. It is technically simple in nature and combines decent protein separation capability that also captures those proteins typically not accessible via

2D PAGE (notably large proteins and those with transmembrane domains) and the well established excellent sensitivity of gel-based protein identification using mass spectrometry for samples of low complexity (Schirle, Heurtier, and Kuster, 2003).

4.4 Proteomics study of salt stress response in plants

In recent years, the term proteomics has also been applied to all the protein expressed in a particular organelle or tissue or in response to a particular abiotic stress such as drought and salt stress with the emphasis being on a global analysis of how cell and organisms respond to these stress at the protein level. The recent development of proteomics approaches has emerged as a powerful tool for gaining insight into physiological changes at the cellular level, making possible a deeper exploration of the function and regulation of the plant response to environmental changes (Salekdeh, *et al.,* 2002, Wang, *et al.*, 2009., Liu, *et al.,* 2011). Comparative proteomics has been successfully applied for the systematic scrutiny of proteins in several plant species under a wide range of abiotic challenges, including salt stress, drought, high or low temperature and heavy metal (Pang, *et al.*, 2010., Liu *et al*., 2011)

Salt stress is one of the important factors limiting rice production as described previously in **section 3.2 and 3.4**. Under salt stress, numerous physiological, biochemical and metabolic functions are modified in plants that involving in the regulated expression of specific proteins, which leads to the re-establishment of proper cellular ion and osmotic homeostasis with other concomitant processes of repair and detoxification (Chinnusamy *et al.*, 2005). The research of plant responses to stress on the DNA or RNA level provided an important insight into defend processes (Gulick *et al.,* 2005), but it is known that the level of transcripts and proteins are not strictly correlated as shown in yeast (Ideker *et al.,* 2001). In addition, many proteins are modified by posttranslational modifications such as phosphorylation, glucosylation, ubiquitinylation, sumoylation, and many others (Canovas *et al.,* 2004) which significantly influence protein functions. Knowledge of the full complement of proteins expressed by the genome of a cell, tissue or organism at a specific time point is necessary to understand the biology of a cell or an organism. Therefore, proteomic analysis of stress-responsiveness provides a broad view of plant responses to salt stress at the protein level.

Roots are a site of perception and injury for several types of stress, including salinity, nutrient deficiency, and heavy metals. In many circumstances, it is the stress sensitivity of the root that limits the productivity of the entire plant (Steppuhn and Raney, 2005). An improved understanding of molecular responses of roots to NaCl treatment may therefore facilitate the development of crops with increased tolerance to NaCl. To build a useful description of the molecular mechanisms active in the response of roots to NaCl treatment, it is necessary to characterize the components of these mechanisms, including proteins. Proteomic profiles have been produced for various stresses and species, including NaCl-treated roots of pea (*Pisum sativum* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.), as well as drought-treated poplar (*Populus trichocarpa* Torr.), and cadmium- or arsenic-treated maize (*Zea mays* L.), rice, and *Arabidopsis thaliana* (Majoul *et al.*, 2000; Kav *et al.*, 2004; Requejo and Tena, 2005; Yan *et al.*, 2005; Aina *et al.*, 2006; Plomion *et al.*,2006; Roth *et al.*, 2006).

As described in the previous, **(section 3.4)** rice (*Oryza sativa*) is one of the most important food crops; almost half of the world's population is estimated to rely totally or partially on it. Moreover, rice considered a model organism because of its relatively small genome (12 chromosomes and approximately 430 Mbp) (Matsumuto, T. *et al.* 2008., Helmy, M. *et al.* 2011) The whole genome sequence and annotation have been published and updated several time (5 builds for the genome and 6 builds for the annotation to date) (Ouyang S, *et al*., 2007; Itoh T *et al*., 2007). However, there has been little attempt to include proteome information in the genome-wide annotation, except for the work of Itoh and colleagues, who used rice proteome data, available through the rice proteome database to confirm 834 ORFs. The virtual absence of proteome-based genome annotation for rice is possibly due to the absence of accurate and detailed rice proteome information. (Helmy, M. *et al*. 2011)

Rice proteomics were widely studied as a result from the completion of the draft sequence of the rice (*Oryza sativa*) genome and subsequent prediction of the protein complement (Goff *et al.*, 2002). Numerous published articles are dealing with the construction of proteomes from complex origins, such as the leaf, embryo, endosperm, root, stem, shoot, and callus proteome (van Wijk, 2001; Goff *et al.*, 2002; Tsugita *et al*.,

1994; Damerval *et al.*, 1986; Blum *et al*., 1987). However, various researches have also mainly focused on the alterations in genome expression that were triggered by environmental factors. For example, the global comparison of green and etiolated rice shoots (Tsugita *et al*., 1994) and an analysis of defense-associated responses in the rice leaf and leaf sheath following a jasmonic acid treatment (Gharahdaghi *et al.,* 1999). About salt stress, proteomic comparisons of salt-responsive proteins in roots of salt-tolerant cultivar Pokkali and the salt-sensitive IR29 were studied. Abundance of three proteins which are ASR1-like protein, ascorbate peroxidase, and Caffeoyl-cCoA *O*-methyltransferase (an enzyme in lignin biosynthesis) were found much higher in salt-tolerant Pokkali than in salt-sensitive IR29 in the absence of stress (Salekdeh *et al.*, 2002).

Among all of proteomic study, elucidation of the protein expression profile is the goal of proteome analysis that is used to determine the protein functions and to explore the functional network of proteins. Due to proteome analysis dealing with large numbers of proteins, the effective method for protein separation are necessary. In proteome analysis, the most popular method to achieve the profiling of a large number of proteins is two-dimensional gel electrophoresis (2-DE) before efficiently identify a numbers of proteins using mass spectrometry (MS) and comparing with the protein sequence database (Hirano et al., 2004). Although 2-DE allows simple, rapid and reproducible separation of a number of proteins, it is difficult to separate high molecular weight and basic proteins.

To overcome this problem, liquid chromatography (LC), which combines pH gradient and reversed-phase columns, is a new proteomics technique that promises to extend the range of protein separation (Komatsu et al., 2009). In this method, the proteins extracted from the cells are digested with protease such as trypsin and lysylendopeptidase, and the resultant peptides are analyzed by LC, followed by MS/MS to determine the sequences. Using this method, a comprehensive proteome analysis has been performed in many organisms including yeast and human.

Boonburapong, B. and Buaboocha, T., (2007) studied the Ca^{2+}-binding proteins of rice by phylogenetic analysis based on amino acid sequences similarity and classified proteins with a high degree of identity as “true” CaMs that probably function as typical CaMs. They were named *OsCam1-1*, *OsCam1-2*, *OsCam1-3*, *OsCam*2 and *OsCam3*.

OsCam1-1, *OsCam1-2* and *OsCam1-3* encode identical protein, whereas *OsCam2* and *OsCam3* encode a protein of only two amino acid differences and their sequences share 98.7% identity with those of *OsCam1* proteins. It is fascinating that the *OsCam1-1*, *OsCam1-2* and *OsCam1-3* genes encode identical proteins. How these protein sequences have been maintained with the natural selection pressure throughout evolution has no clear answer yet but it is likely that each of these genes has physiological significance. In addition, *OsCam1* amino acid sequences are identical to those of the typical CaMs from barley (*H. vulgare*) and wheat (*T. aestivum*) reflecting the close relationships among monocot cereal plants. On average, OsCaM amino acid sequences share about 99%, 90% and 60% identity with those from plants vertebrate and yeast, respectively.

Phean-o-pas *et al*. (2008) reported the structures and expression of *OsCam1-1* calmodulin gene in rice. In this work, the *OsCam1-1* gene along with its promoter was isolated from the *O. sativa L. cv. KhaoDok Ma li* 105 (KDML105). Fusion of the predicted promoter region to the GUS reporter gene showed GUS activity in all organs examined, particularly in the vascular bundles of leaves and the vascular cylinders of roots, indicating strong expression of *OsCam1-1* in these two specific tissues. Recombinant OsCaM1-1 was shown to bind to Ca^{2+} and bind to the peptide derived from CaM kinase II (CaMKII) with a 1:1 stoichiometry. The results thus suggest that OsCaM1-1 is a functional Ca^{2+} binding protein, which act as a calmodulin in signaling external stimuli via Ca^{2+} to generate the subsequent metabolic response cascade in rice.

Saeng-ngam *et al.* (2012) recently reported the role of *OsCam1-1* in abscisic acid (ABA) biosynthesis in response to salt stress in rice. Higher induction level of *OsCam1-1* transcription was found in FL530-IL, an isogenic salt resistant line derived from KDML105, compared to the salt susceptible KDML105. The *OsCam1-1* overexpressing salt tolerant line, which contained a significantly higher level of *OsCam1-1* transcript, was constructed and shown to have higher ABA accumulation in leaves, which was related to the up-regulation of ABA aldehyde oxidase and 9-cis-epoxycarotenoid dioxygenase 3 in ABA biosynthesis pathways. The results thus indicate that *OsCam1-1* signaling played an important role in ABA biosynthesis and the levels of *OsCam1-1* gene expression and the accumulated ABA levels would contribute to the salt tolerance mechanism in rice.

Therefore, in this study, comparative proteome analysis of transgenic rice *Oryza sativa* L. cv. KDML105, harboring *OsCam1-1* gene grown under salt stress was accomplished by using LC-MS/MS in order to identify potential proteins and/or pathways that may be regulated by OsCaM protein in the Ca^{2+}-signaling mediated responses to salt stress. Both differential root and shoot proteome among wild-type, vector-alone and *OsCam1-1* transgenic rice lines were investigated. In addition, activity assays of some selected enzymes involved in responses to oxidative stress, which results from salt stress were also investigated. The knowledge obtained from this study provided better understanding on the physiological function of the *OsCam1-1* gene under salt-stress and the rice salt tolerant mechanisms. In addition, the results in this study also contribute a valuable platform for further improvement of rice plants for abiotic stress tolerance in the future.

II. HYPOTHESIS

Salt stress results in oxidative stress, which can be combated against by the increased levels of antioxidative enzyme activities. If *OsCam1-1* confers the *OsCam1-1* transgenic rice salt stress tolerance ability by affecting the regulation pattern of the antioxidative-stress enzyme activities, *OsCam1-1* transgenic rice should exhibit up-regulated proteomic profile and high levels of antioxidative-stress enzymes.

III. OBJECTIVES

The aims of this study are to investigate protein profiles in wild-type *Oryza sativa* and *OsCam* transgenic lines by proteomic analysis and to examine activities of enzymes involved in oxidative-stress responses of the *OsCam1-1* overexpressing transgenic rice under salt-stress condition. The specific objectives of the project are as follows:

1. To analyse the global proteomic profiles of the wild-type *Oryza sativa* (WT), the control transgenic lines (VT), and the OsCam transgenic line (OS) under the non-stressed and salt stressed conditions in roots and shoots of each rice lines.

2. To analyse oxidative-stress enzyme activities in responses to NaCl among three rice lines.

IV. SCOPE OF THE STUDY

Analysis of the differentially expressed protein profiles of the wild-type *Oryza sativa* (WT), the control transgenic lines (VT), and the *OsCam* transgenic line (OS) in the presence and absence of 150 mM Sodium Chloride (NaCl) in roots and shoots were investigated by SDS-PAGE combined with nano-LC-MS/MS. (GeLC-MS). In quantitation of proteins, DeCyder MS Differential Analysis software was performed. MS/MS Results from DeCyder MS were submitted to MASCOT and searched against the *Oryza sativa* and NCBI databases for identification of proteins. List of identified proteins among the three rice lines of roots and shoots in both conditions were analysed by compatible proteomic software programs such as MeV and STRAP to classify biological functions and localizations. The protein profile datasets between each specific group in roots and shoots among the three rice lines were manually grouped using Venn diagram in order to elucidate the differences in responsive gene expression among the rice lines in our study. In addition, the complementary data on oxidative-stress enzyme activities analysis (Peroxidase, Ascorbate peroxidase, Glutathione-S-transferase and Adenosine Triphosphatase) in the wild-type and the transgenic rice lines were also investigated.

CHAPTER II

MATERIALS AND METHODS

I. MATERIALS

1. Plant materials

1.1 Rice seeds (*Oryza sativa* L. ssp. indica): Wild-type or original line

1.2 Rice seeds (*Oryza sativa* L. ssp. indica) harboring pCAMBIA1301: vector alone or control of transgenic line

1.3 Rice seeds (*Oryza sativa* L. ssp. indica) harboring pCAMBIA1301 with *OsCam1-1* gene or *OsCam1-1* transgenic line

2. Instruments

2.1 Equipment for plant germinating and growing

- Glass bottle with lid 8 oz.
- Aluminum foil
- Eppendrof tubes 1.5 ml
- Forceps
- Ruler
- Scissors
- Bunzen alcohol and matches
- Balance
- Shaker
- Sterile petri dishes

2.2 Equipment for proteomics and enzymatic analysis

- Motars and pestles
- Low-binding eppendrof tubes
- Spatula
- Micro plate reader
- Low-binding 96 well plates
- Centrifuge

- Spectrophotometer
- Shaker
- Sonicator
- Speed vac
- Ultimate 3000 LC system (Dionex, USA)
- Synapt HDMS system, Nano Acquity system (Waters Corp., Milford, MA)
- Screw Cap 12x32 mm Vial combination pack with bonded silicone/PTFE Septa

3. Chemicals and reagents

3.1 Chemicals for proteomic study

3.1.1 Protein extraction and precipitation

- Sodium dodecyl sulfate (SDS)
- Trichloroacetic acid (TCA)
- Acetone
- 2-mercaptoethanol

3.1.2 Salt stress treatment and protein determination

- Sodium Chloride (NaCl)
- Bovine serum albumin (BSA)
- Copper sulphate ($CuSO_4.7H_20$)
- Tartalic acid,
- Sodium dodecyl sulfate (SDS)
- Sodium hydroxide (NaOH)
- Sodium carbonate (Na_2CO_3)
- Sodium citrate dehydrate

3.1.3 SDS-PAGE preparation (Appendix B)

- 40% (w/v) acrylamide
- 1.5 M Tris-HCl, pH 8.8
- 0.5 M Tris-HCl, pH 6.8

- 10% SDS
- Deionized water
- 10 % Ammonium persulfate (APS)
- TEMED

3.1.4 Silver staining (Appendix B)

- Methanol (CH_3OH)
- 100% Acetic acid (CH_3COOH)
- 37% Formaldehyde
- Sodium thiosulfate ($Na_2S_2O_3$)
- Silver nitrate ($AgNO_3$)
- Sodium carbonate (Na_2CO_3)
- EDTA

3.1.5 In gel digestion for LC-MS/MS (Appendix B)

- 100% acetonitrile (ACN)
- 10 mM dithiothreitol (DTT)
- 10 mM ammonium bicarbonate $(NH_4)HCO_3$
- 100 mM Iodoacetamide (C_2H_4INO)
- 10 ng trypsin
- 0.1% Formic acid

3.2 Chemicals for enzymatic extraction and assay

3.2.1 Extraction buffer

- 0.1 M sodium phosphate buffer (pH 7.0)
- 2 mM ethylenediaminetetraacetic acid (EDTA)
- 4 mM dithiothreitol (DTT)
- 6 mg of polyvinylpyrrolidone (PVP)
- Bovine serum albumin (BSA); standard protein

3.2.2 Peroxidase (PX) activity assay

- 50 mM sodium phosphate buffer (pH 7.0)
- 117.9 mM guaiacol
- 11.6 mM H_2O_2

3.2.3 Ascorbate peroxidase (APX) activity assay

- 50 mM sodium phosphate buffer (pH 7.0)
- 100 mM ethylenediaminetetraacetic acid (EDTA)
- 11.6 mM H_2O_2
- 100 mM ascorbic acid (Vitamin C)

3.2.4 Glutathione-S-transferase (GST) activity assay

- 50 mM sodium phosphate buffer (pH 7.0)
- 20 mM reduced L-glutathione (GSH)
- 20 mM 1-chloro-2,4-dinitrobenzene (CDNB)
- 95% ethanol

3.2.5 ATPase activity assay

- 0.1 M Tris-HCl buffer (pH6.5)
- 50 mM magnesium sulphate ($MgSO_4$)
- 30 mM ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$)
- 15 mM adenosine-5'-triphosphate (ATP)
- 1.15 M perchloric acid ($HClO_4$)
- 1.62 mM Malachite green ($C_{23}H_{25}ClN_2.H_2O$)
- 1.5% Tween 20

3.2.6 Glucosidase activity assay

- 10 mM p-nitrophenyl-β-D-glucopyranoside (PNPG)
- 100 mM sodium acetate buffer (pH 5.0).
- 0.5 M sodium carbonate (Na_2CO_3)

II. METHODS

1. Plant material preparation

Seeds of three rice (*Oryza sativa* L. ssp. indica) lines, including wild type rice seeds, vector-alone rice seeds and *OsCam1-1* overexpressing rice seeds were hand-dehusked, rinsed with 70% ethanol, sterilized by soaking in 30% Clorox® (2% w/v sodium hypochlorite, Clorox Co, USA) with 2-3 drops of Tween 20 for 15 minutes. After that, seeds were washed extensively with sterile distilled water three times. Surface-sterilized seeds were germinated on sugar-free MS medium (Murashige and Skoog, 1962) containing 0.7% agar at pH 5.7. One week-old seedlings were then transferred to 50 ml liquid sugar-free MS media All seedlings were cultured under 25 ± 2^{o}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 provided by fluorescent lamps (TLD 36W/84, Cool White, Philips, Thailand).

2. Salt stress treatment

Two-week old rice seedlings were transferred to culture in 300 ml sugar-free liquid MS medium one day before treatment. Then, NaCl was added into the culture to the final concentration of 150 mM. Control treatments of transgenic plants grown in liquid MS medium were carried out in parallel with the salt stress treatment. After treatment, seedlings of each transgenic rice line were collected at various times (0, 1, 3, 6, 12 and 24 hours) and kept in liquid nitrogen before using as starting materials.

3. Proteomic analysis (GeLC-MS)

3.1 Protein extraction

Samples were ground in a mortar and pestle in liquid nitrogen to obtain a fine powder and then transfered into an eppendorf tube. One milliliter of TCA solution (10% TCA in acetone containing 0.07% 2-mercaptoethanol) was added to the tissue powder. Samples were vortexed, incubated at -20^{o}C for 1 h, and centrifuged at 12000

rpm for 30 min at 4^{o}C. Protein pellets were washed three times with acetone solution (acetone containing 0.07% 2-mercaptoethanol). The pellet from the final wash step was incubated in acetone solution at -20^{o}C overnight and centrifuged at 12000 rpm for 15 min at 4^{o}C. The pellet was dried in a Speedvac and stored at -20^{o}C prior to use.

3.2 Protein determination

Protein concentration was estimated by the method of Lowry using bovine serum albumin as standard (2-10 mg/ml BSA). The absorbance at 750 nm (OD_{750}) was measured and the protein concentration was calculated using the standard curve, plotted between OD_{750} on Y-axis and BSA concentration (μg/ml) on X-axis. Diluted samples were mixed with 0.2 ml freshly prepared alkaline copper solution made by mixing 0.4% $CuSO_4.7H_20$ in Tartalic acid, 5% SDS, 0.8 M NaOH and 20% sodium carbonate. The reaction was incubated for 30 min at room temperature before adding 0.05 ml 20% Folin-Ciocalteu phenol reagent. The mixture was vigorously mixed and allowed to stand at room temperature for 30 min. The absorbance at 750 nm was measured using a microplate reader (VERSAmax™, Cape Cod, Inc, UK).

3.3 Denaturing Gel Electrophoresis (SDS-PAGE)

Proteins were fractionated on SDS-PAGE mini slab gel (8 x 9 x 0.1 cm, HoeferminiVE, Amersham Biosciences, UK). SDS-PAGE was performed on 12.5 % polyacrylamide gels mixed according to standard method described by Laemmli (Laemmli, 1970). Prior to sample loading, the extracts were mixed with a fifth volume of a 5-fold concentrated sample buffer to yield a final concentration of 0.375 M Tris pH 6.8, 12% SDS, 60% glycerol, 0.6 M DTT, 0.06% bromophenol blue and heated at 95^{o}C for 10 min before applied to a gel lane. To estimate size of polypeptides, low molecular weight protein standard marker (Amersham Biosciences, UK) was used. The upper and lower reservoirs of the electrophoresis apparatus were filled with electrophoresis buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS). The individual proteins are separated electrophoretically at a constant voltage of 50V of constant current for a stacking gel until the bromophenol blue tracking dye enters the separating gel, and then the current was increased to 70 V until the tracking dye has reached the

bottom of the separating gel. After the electrophoresis finished, Protein bands were visualized by silver staining according to Blum *et al*. (1987).

3.4 Silver staining

At the end of each electrophoresis, the gel protein was fixed in the fixing solution (50% methanol, 12% acetic acid and 50 µl of 37% formaldehyde to 100 ml fixing solution) for 30 min. The gel was removed in the washing solution (35% ethanol) 2 times for 5min each and sensitizing in 0.02% sodium thiosulfate for 2 min. After washing in water twice for 5 min each, the gel was stained with silver nitrate (2%) for 20 min. The gel was shaken in the developing solution until regarded protein bands were visualized and stopped quickly in the stopping solution for 20 min. The gel was kept in 0.1% acetic acid at room temperature.

3.5 In-gel trypsin digestion

For testing the quality of digested proteins, Three BSA gel plugs were digested and analyzed with Ultraflex TOF/TOF with correct identification for all three. One BSA gel plug as control was digested along with the digestion of samples. Each stained lane in gel was excised into twelve sections. Each band was further chopped into approximately ~1x1x1 mm^3 and transferred into a well of a 96-well plate (6-8 pieces per well for SDS-PAGE) that was then subjected to in-gel digestion using an in-house method developed by the Proteomics Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand (Jaresitthikunchai *et al*., 2009). In order to stain, the gel pieces in each well of low-binding 96 well plates were briefly washed 2 times with 200 µl of 5% H_2O_2 with shaking at room temperature for 10 min. The gel pieces were then washed 2 times with 200 µl of sterile distilled water, with shaking at room temperature for 10 min. Water was removed and the gel was dehydrated with 200 µl of 100% acetonitrile (ACN), with shaking at room temperature for 5 min. ACN was removed and the gel plug allowed to dry at room temperature for 10 min. or until the gel plug in every well was dried. The gel pieces were reduced with 50 µl of 10 mM Dithiothreitol (DTT) in 10 mM ammonium bicarbonate (freshly prepared) per well at room temperature for 1 h. After DTT was removed, gel plugs in

each well were alkylated with 20 µl of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate at room temperature for 1 h in the dark. After alkylation, the gel pieces were dehydrated twice with 200 µl of 100% ACN, with shaking at room temperature for 5 min. ACN was removed and performed in-gel digestion

To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for three hrs. or overnight. Solution was transferred to another low binding 96 well plate. If the well was dried, 30 µl of 30% ACN was added, and mixed by shaking at room temperature for 5 min. before transferring) To extract the rest of peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gels, and the gels were then incubated at room temperature for 10 min in a shaker. Final solution in extraction process was evaporated or dried at 40°C for 3-4 hrs. or overnight. Samples were kept at -20°C for further analysis. The peptide samples from the extraction were resolved in 15 µl of 0.1% formic acid/LC-MS water grade only per well. Transferring of solution in each well was made to low-binding eppendrof tubes, before centrifuging at 12000 rpm for 10 min. Finalized clearly solution was transfer ed into the insert tube (vial) for further mass spectrometric analysis.

3.6 Nano-LC-MS Configuration for Synapt HDMS

The digested peptides were injected and separated in Nanoscale LC-MS for Synapt HDMS system, NanoAcquity system (Waters Corp., Milford, MA) equipped with a Symmetry C_{18} 5 µm, 180-µm x 20-mm Trap column and a BEH130 C_{18} 1.7 µm, 100-µm x 100-mm analytical reversed phase column (Waters Corp., Milford, MA). The samples were initially transferred with an aqueous 0.1% formic acid solution to the trap column with a flow rate of 15 µl/min for 1 min. Mobile phase A was water with 0.1% formic acid, and mobile phase B was 0.1% formic acid in acetonitrile. The peptides were separated with a gradient of 15–50% mobile phase B over 15 min at a flow rate of 600 nl/min followed by a 3-min rinse with 80% of mobile phase B. The column temperature was maintained at 35 °C. The lock mass was delivered from the

auxiliary pump of the NanoAcquity pump with a constant flow rate of 500 nl/min at a concentration of 200 fmol/µl of [Glu^1]fibrinopeptide B to the reference sprayer of the NanoLockSpray source of the mass spectrometer. All samples were analyzed in once. Analysis of tryptic peptides was performed using a SYNAPT^{TM} HDMS mass spectrometer (Waters Corp., Manchester, UK). For all measurements, the mass spectrometer was operated in the V-mode of analysis with a resolution of at 9,000 full-width half-maximum. All analyses were performed using positive nanoelectrospray ion mode. The time-of-flight analyzer of the mass spectrometer was externally calibrated with [Glu^1]fibrinopeptide B from m/z 50 to 1600 with acquisition lock mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu^1]fibrinopeptide B. The reference sprayer was sampled with a frequency of 20 sec. Accurate mass LC-MS data were acquired with data direct acquisition mode. The energy of trap was set at a collision energy of 6 V. In transfer collision energy control, low energy was set at 4 V. The quadrupole mass analyzer was adjusted such that ions from m/z 300 to 1800 were efficiently transmitted. The MS\MS survey is over range 50 to 1990 Da and scan time was 0.5 sec.

The peptide mixtures were separated and eluted with a 0-50% gradient solution (Buffer A, 0.1% formic acid in water; Buffer B, 0.1% formic acid in water and 50% acetonitrile and formic acid and 95% ACN) within 40 min and were then online detected in ESI-Ion Trap mass spectrometer.

3.7 Protein identification and Gene ontology categories

DeCyder MS Differential Analysis software (DeCyder MS, GE Healthcare (Johansson et al., 2006; Thorsell et al., 2007)) was used to quantify the peptide in all samples. Acquired LC-MS raw data were converted to mz XML file by CompassXport software and all peptides were detected with the PepDetect. The PepDetect module of the software was used for automated peptide detection, charge state assignments based on resolved isotopic peaks and consistent spacing between consecutive charge states, and quantitation based on MS signal intensities of individual LC-MS analyses. The final step was consisted of matching peptides across different signal intensity maps using the PepMatch module resulting in a quantitative comparison. Acquired MS/MS

data from the analysis of the DeCyderMS software were submitted to database search using the MS/MS Ions Search on Mascot software released in October, 2011, available on-line at www.matrixscience.com (Matrix Science, London, UK, (Perkins *et al*., 1999)).

The data were searched against the NCBI database for protein identification. Database interrogation was; taxonomy (*Oryza*); enzyme (trypsin); variable modifications carbamidomethyl and oxidation of 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+). Therefore the Mascot DAT files were merged and evaluated on the peptide level with the built-in DeCyder MS software and exported to Microsoft Excel.

3.8 Extraction of Gene Ontology information and classification

Gene ontology annotation was performed using Software Tool for Rapid Annotation of Proteins (STRAP) version 1.1.0.0 (Bhatia *et al.*, 2009). STRAP allows collection and annotation of information about the proteins in a data set. First, protein was imported from protein lists text file formats. It then downloads information on each protein from several online databases, focusing on information from the UniProt Knowledgebase database and then compiles all of the protein annotation information and displays it in a Gene ontology term that includes biological process, cellular component and molecular function, respectively. The final distribution pie charts were generated using Microsoft Excel.

3.9 Data partitioning and Mapping of protein networks

All proteins expressed at each time point were partitioned into groups similar to a Venn diagram, each group representing the reproducing presence/absence of a protein at a time point. These groups were further investigated to identified biological processes in the cell.

Moreover, the KEGG IDs of our identified proteins were simultaneously submitted to the iPath2 program (http://pathways.embl.de) to search for the visualization and analysis of cellular pathways (Takuji *et al*., 2011). The UniProt IDs

of our identified proteins were simultaneously submitted to The Search Tool for the Retrieval of Interacting Genes (STRING) (http://string-db.org) to search for understanding of cellular functions and annotate all functional interactions among proteins in the cell (Damian *et al.,* 2011).

3.10 Quantification of the changes in protein Analysis

Proteins expresses at all time points in non-stress and NaCl treatment among three lines (WT, VT and OS) were analyzed by a one-way ANOVA. An ANOVA was performed to identify proteins that showed different abundance patterns between the different time point and group. Proteins with *p*-values of less than 0.05 were selected as significantly differentially expressed.

Data normalization and quantification of the changes in protein abundance between the control and treated samples were performed and visualized using *Multi Experiment Viewer* (MeV) software version 4.6.1 (Howe *et al*., 2010). Briefly, peptide intensities from the LC-MS analyses were transformed and normalized using a mean central tendency procedure. They performed statistical tests of variance of differences (ANOVA) for these data sets that show statistically significant proteins ($p < 0.05$).

3.11 Annotation of identified proteins by using rice genome database

Interesting unnamed identified protein as performed in section 3.7 were additionally annotated by using rice genome databases which are available online in the following URL; http://riceplantbiology.msu.edu, http://rapd.dna.affrc.go.jp and http://oryzapg.iab.keio.ac.jp; 26 July 2012.

4. Assay of enzyme activities

Rice plants stressed with 150 mM NaCl at various times were used as starting materials to monitor the salt stress response via the activities of Peroxidase (PX), Ascorbate peroxidase (APX), Glutathione-S-transferase (GST), and Adenosine Triphosphatase (ATPase).

4.1 Enzyme extraction

Crude enzymes were extracted from the rice samples by the modified method of Corley and Wolosiuk (1985). Fifty milligrams of shoot and root tissues were homogenized with 600 µl of extraction buffer (0.1 M sodium phosphate buffer (pH 7.0), 2 mM EDTA, 4 mM DTT, and 6 mg of polyvinylpyrrolidone) by a micropestle. The whole extraction was performed on ice. The homogenates were sonicated for 15 minutes and centrifuged at 12,000 rpm for 15 minutes. The resulting supernatants were used as crude enzymes that used for the assays of enzyme activity. Protein concentration in each samples was estimated by the method of Lowry using bovine serum albumin as standard as described in **section 3.3.2**

4.2 Enzyme assay

All enzyme assays were performed in a microtiter plate with 4 replications and activities of the enzymes were determined with a microtiterplate reader.

4.2.1 Peroxidase (PX)

Peroxidase (PX) activity was measured according to the modified method of Nakano & Asada (1981). The substrate solution containing 0.17 mM H_2O_2, and 2.36 mM guaiacol in 50 mM sodium phosphate buffer (pH 7.0) was prepared and pH adjusted into 3 different values (5, 7, and 10) in order to determine acidic (pH 5), neutral (pH7) and basidic (pH 10) PX. The reaction was started by adding 200 µl of substrate solution into enzyme extract in microtiter plate. The kinetic of the formation of tetraguaiacol was measured by monitoring changes in absorbance at 436 nm for 3 minutes. One unit of peroxidase was defined as that which formed 1 nmol of tetraguaiacol per minute.

4.2.2 Ascorbate peroxidase (APX)

Ascorbate peroxidase activity was determined according to the modified method of Nakano & Asada (1981). The substrate solution containing 0.8 mM EDTA, 0.17 mM H_2O_2 and 0.5 mM ascorbic acid in 50 mM sodium phosphate buffer (pH 7.0) was prepared. The oxidation of ascorbate was started by adding 200 µl of substrate solution into enzyme extract in microtiter plate. The decrease in absorbance at 290 nm

due to the oxidation of ascorbate was monitored. One unit of enzyme activity was defined as that which oxidized 1 μmol of ascorbate per minute.

4.2.3 Glutathione-*S*-transferase (GST)

Glutathione-*S*-transferase activity was measured according to the modified method of Mannervik & Guthenberg, 1981. The substrate solution containing 1 mM glutathione (GSH) and 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) in 95% ethanol was prepared in 50 mM sodium phosphate buffer (pH 7.0). The reaction was initiated by adding 200 μl of substrate solution into enzyme extract in microtiterplate. The kinetic of the formation of 2, 4-dinitrophenylglutathione was measured by monitoring changes in absorbance at 340 nm for 3 minutes. One unit of enzyme activity was defined as that which formed 1 nmol of 2, 4-dinitrophenylglutathione per minute.

4.2.4 Adenosine Triphosphatase (ATPase)

<u>*Color reagent*</u>: The color reagent was prepared by mixing 1 volume of 8.5 mM ammonium molybdate in 5 M hydrochloric acid, 3 volume of 1.62 mM Malachite green, 1.15 M perchloric acid, and 1.5% Tween 20, stirred 20-30 minutes, and filtered.

<u>*Standard curve*</u>: A solution of KH_2PO_4 (1 mg/ml) containing 0, 1, 2, 4, and 8 μl was added into separated well of microtiterplate. 50 μl of substrate solution and 200 μl color reagent were added. The solution was mixed and read at 660 nm.

V-type ATPase activity was measured according to the modified method of Wortsman *et al*., 2001. The substrate solution containing 5 mM $MgSO_4$, 0.2 mM Ammonium molybdate, 3 mM ATP, and 100 mM KNO_3 was prepared in 100 mM Tris-HCl buffer (pH 6.5). The reaction was initiated by adding 50 μl of substrate solution into 5 μl of enzyme extract in microtiterplate and incubated 37 ^{o}C for 30 minutes. The reaction was stopped by adding 200 μl of the color reagent. After mixing, the absorbance at 660 nm of the mixture was measured and compared to standard curve. One unit of ATPase activity is defined as the amount of enzyme releasing 1 μmol of Pi released per min. *P-type* ATPase activity was measures as previously described in *V-type* ATPase activity except no adding 100 mM KNO_3 in the substrate solution.

CHAPTER III

RESULTS AND DISCUSSION

Salt stress has been reported to result in alteration in gene expression, i.e. induction and down-regulation of a set of genes, which leads to changes at the protein expression level. In this study, comparative proteome analysis of transgenic rice *Oryza sativa* L. cv. KDML105, harboring *OsCam1-1*gene grown under salt stress was conducted by using LC-MS/MS. in order to identify potential proteins and/or pathways that may be regulated by OsCaM1 protein in the Ca^{2+}-signaling mediated responses to salt stress.

In order to explore the protein expression patterns in response to salt stress, which are affected by *OsCam1-1* over-expression, the protein profiles in *Oryza sativa* wild-type (WT), *OsCam1-1*-overexpressing transgenic (OS) line, and its transgenic control line containing only vector with no insert (VT) were examined **(Figure 3.1).** Physiological morphology of the three rice lines (1 week old seedings) before transferring to MS-liquid medium and treating with salt treatments (150 mM NaCl) was similar (data not shown).

Each group comprised the control samples and the salt-stressed samples (150 mM NaCl) of the three rice lines at different time intervals (0-24 hrs). For each sample, proteins from shoot or root tissues of individuals in each treatment were extracted and pooled into a single sample for LC/MS/MS analysis. The proteins were annotated using MASCOT and the protein candidates with statistically significant alteration in expression level between different treatments were identified using ANOVA. Proteomic analysis in response to salt stress in the wild-type and the transgenic rice lines in this study was described in section 1. In addition, activities of the enzymes previously reported to be related to stress response in plant were analysed. The complementary data on enzyme activity analysis in the wild-type and the transgenic rice lines are described in section 2.

Section I: Proteomic Analysis

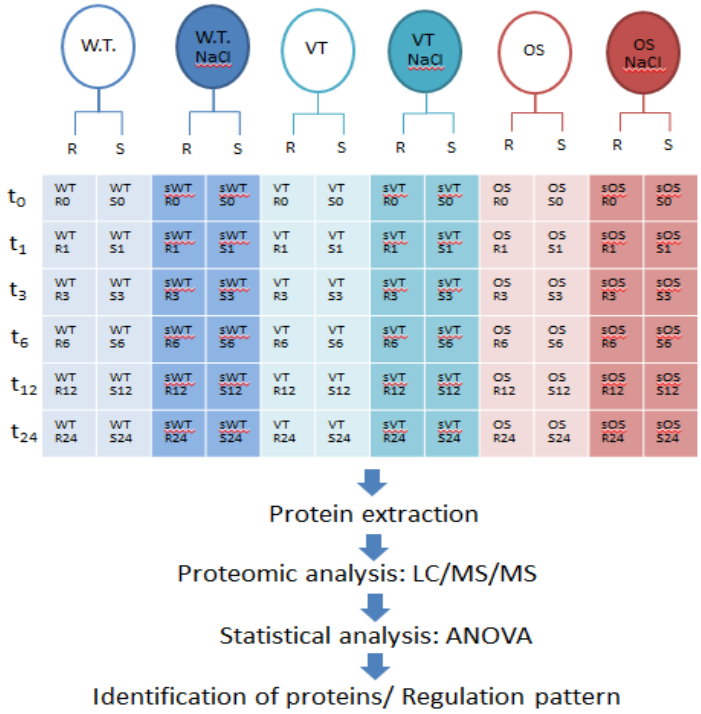


Figure 3.1: Overall research plan on proteomic analysis of the *O. sativa* response to salt stress. WT: wild-type; VT: transgenic control line (harboring T-DNA with no insert); OS: *OsCam1-1*-overexpressing transgenic line. Letter 's' indicates samples that experienced salt stress conditions. R = Root, S = Shoot

1. Proteomic analysis of salt-responsive proteins among wild type (WT), transgenic control (VT) and *OsCam1-1*-overexpressing transgenic (OS) rice lines

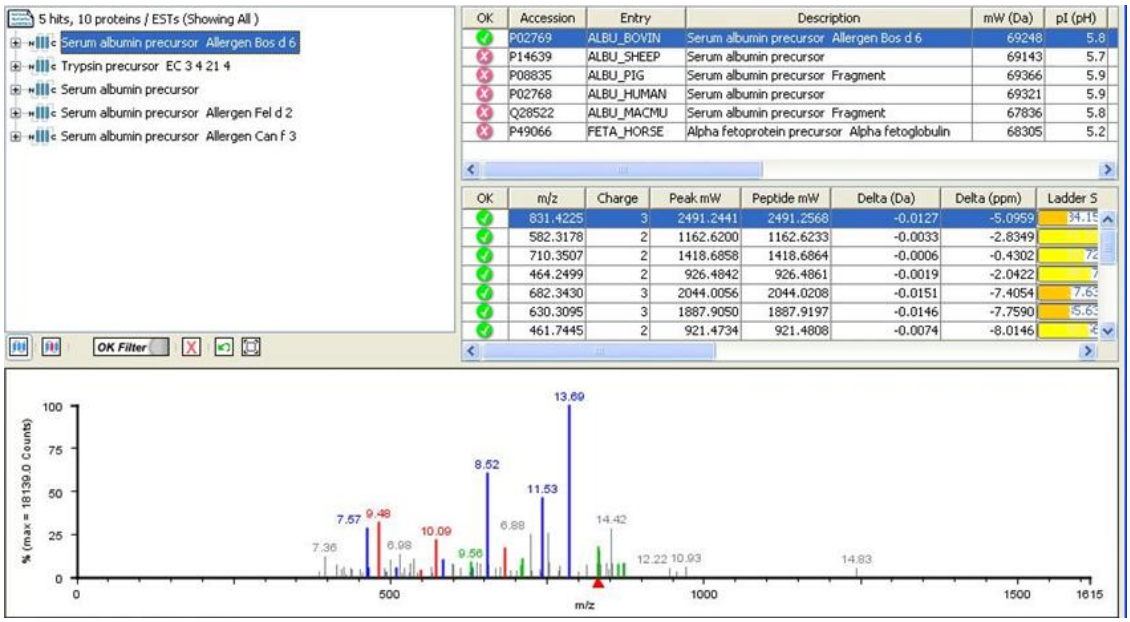
In this study, three lines of *O. sativa* were cultivated, including the wild-type (WT), the transgenic control (VT), and the *OsCam1-1*-overexpressing transgenic (OS) rice lines. The samples were then separated into two groups, the non-stressed control samples and the salt-stressed samples. The rice was cultivated under identical conditions and the samples were collected from each line at different time intervals in order to determine differences in gene expression profile at different induction times.

After three weeks of cultivation, the rice seedlings in each line were treated with 150 mM NaCl and the root and shoot samples were collected at 0, 1, 3, 6, 12 and 24 hrs after salt stress. Overall, there was no significant difference in phenotypes and physical characteristics of these three rice lines and between the non-stressed control samples and the salt-stressed samples in the first 6 hrs. After that, rice seedlings of all lines then started to senesce, which can be clearly observed at 24 hrs time point by naked eye especially in the shoot samples compared to the starting samples at 0 hr.

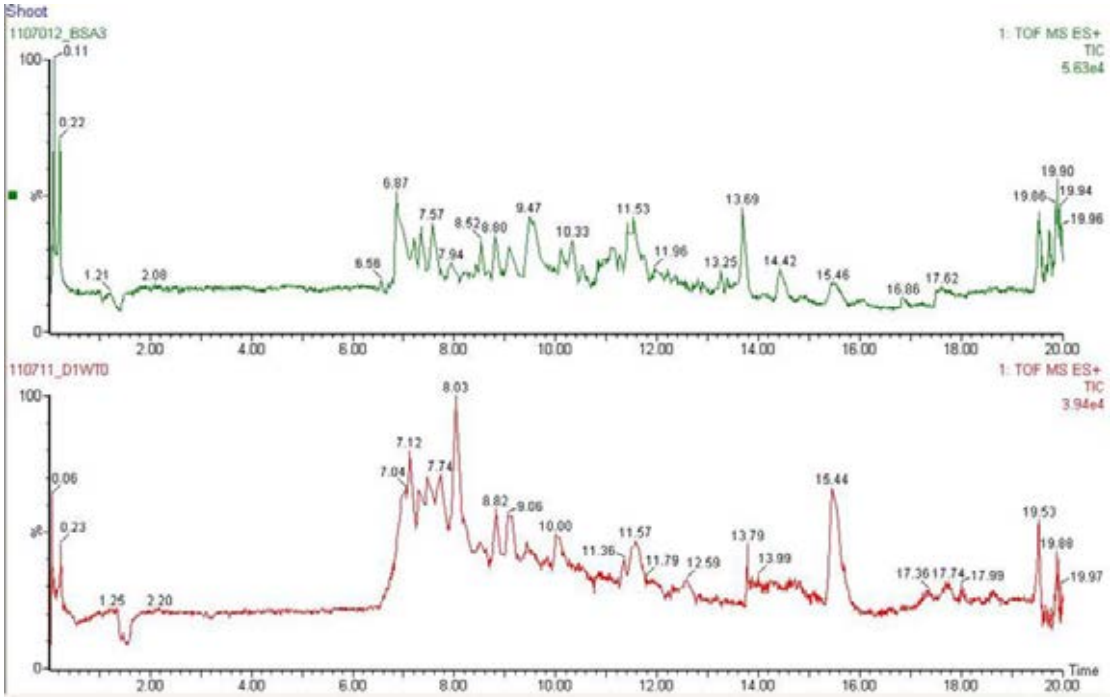
Proteins were then extracted separately from the root and the stem parts of individual samples. In total, there were 72 protein samples, representing proteins from the root and the shoot of the non-stressed and the salt-stressed WT/ VT/ OS collected at 0, 1, 3, 6, 12, and 24 h. Protein profiles in each sample were then separated by one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE). Alteration in protein expression profiles in these samples was then subsequently analysed by LC/MS/MS using BSA as an internal or technical control.

Examples of chromatograms for tryptic peptides of a shoot sample of *Oryza sativa* L. were demonstrated in **Figure 3.2** as a representative for chromatograms for other root and shoot samples in this study

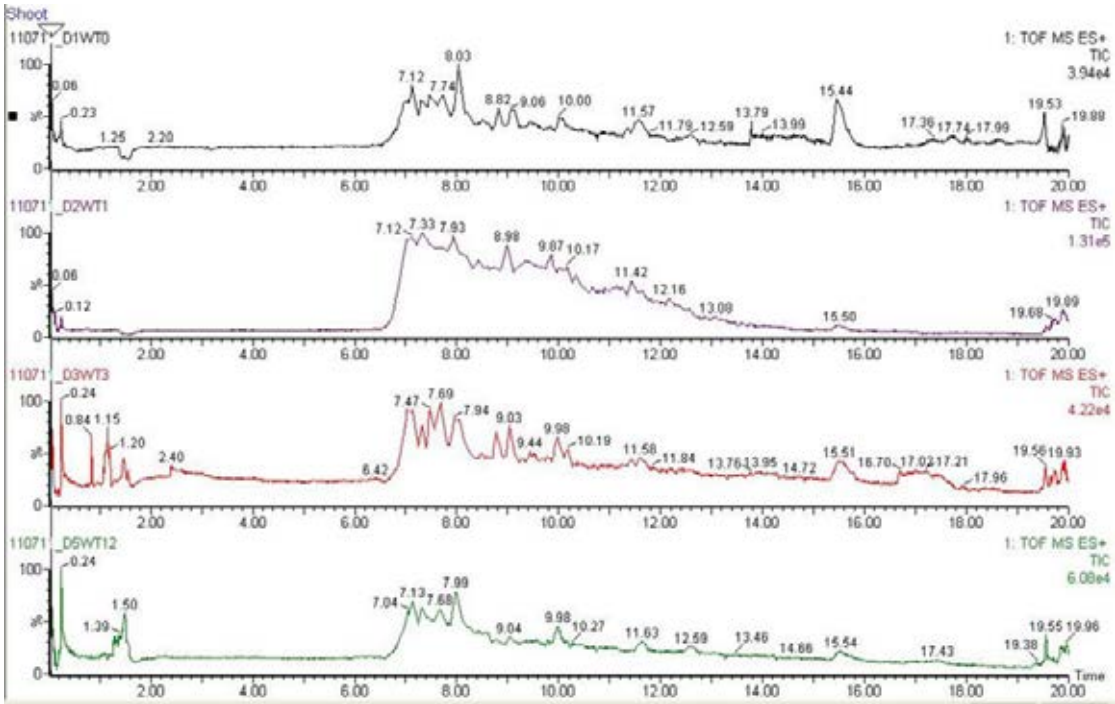
a)



b)


Shoot
1107012_BSA3
1: TOF MS ES+
TIC
5.63e4
110711_D1WT0
1: TOF MS ES+
TIC
3.94e4
Time

c)


Shoot
11071_D1WT0
1: TOF MS ES+
TIC
3.94e4
11071_D2WT1
1: TOF MS ES+
TIC
1.31e5
11071_D3WT3
1: TOF MS ES+
TIC
4.22e4
11071_D5WT12
1: TOF MS ES+
TIC
6.08e4
Time

d)

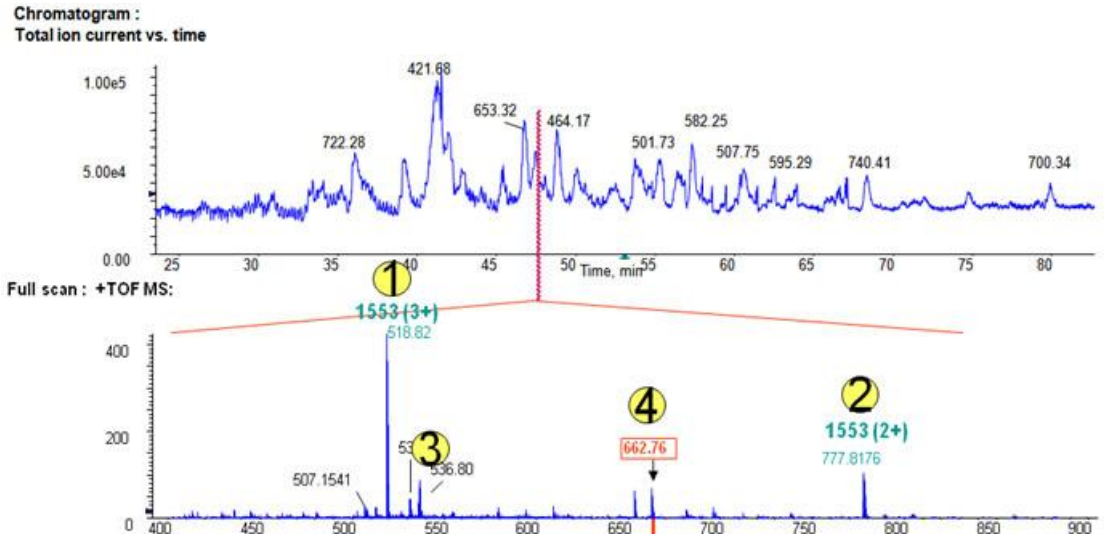


Figure 3.2 Examples of Chromatograms from tryptic peptides of a gel plug for shoot of *Oryza sativa* L. as analysed by GeLC-MS. a) BSA sample: Internal control and normalization b) Upper panel: BSA sample, Lower panel: Root sample, normal condition, wild type line c) Shoot samples at 0, 1, 3 and 12 hr. after treated with 150 mM NaCl, wild type line and d) Root samples at 12 hr. after treated with 150 mM NaCl, *OsCam1-1* overexpresssing line

After the process of LC-MS/MS analysis (see section 2.3.6) for shoot and root samples of the three rice lines, the acquired LC-MS raw data were converted to mz XML file by CompassXport (Bridge) to .TXT files and other proteomic software programs including PepDetect and PepMatch to get the files which can be used for DeCyder MS Differential Analysis Software Program. All protein patterns among three rice lines of the wild-type (WT), vector alone (VT) and *OsCam* transgenic lines (OS) of *O. sativa* (non-stressed/ stress-stressed with NaCl treatment) were analysed by using compatible proteomic software program as described in **Chapter II, Methods, section 3.7**

For protein quantitation analsyis, DeCyder MS Differential Analysis software (DeCyder MS), 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 MS mode. The analyzed MS/MS data from DeCyder MS was submitted to database search using the Mascot software (Matrix Science, London, UK, (Perkins et al., 1999)). For protein identification, data were searched against the NCBI database. Database interrogation included taxonomy; enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1 Da); fragment mass tolerance (±0.4 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages. Proteins considered as identified proteins have at least two peptides with an individual mascot score corresponding to $p<0.05$.

By using this proteomic analysis procedure, 207 different proteins which showed significant change in response to salt stress were positively identified by ANOVA. The list of these proteins in close-up views is shown in **Table 7 (Appendix B).** Overall, the majority of the proteins are matched to *Oryza sativa* database, for which the identity score was ranging from 0.19- 42.21. The identified proteins also showed the theoretical MW (MH+ (Da) in Table 7) in the range of 402.68-3,435.28 Da. Nevertheless, only 25.6% (53 of 207) could be identified with known function or showed significant homology with proteins available in NCBI database.

Each identified protein was functionally classified according to their known and putative functions by using STRAP software program. These proteins were categorized in to 7 groups based on their biological functions **(Figure 3.3)** including cellular process (42%), metabolic process (14%), regulation (12%), developmental process (2%), localization (2%), response to stimulus (2%) while the rest are proteins in other processes or of unknown function. Furthermore, these proteins were then further analyzed by compatible proteomic software program as described in **Chapter II, section 3.8** to predict their subcellular localization. **(Figure 3.4)** and molecular

function**(Figure3.5)**

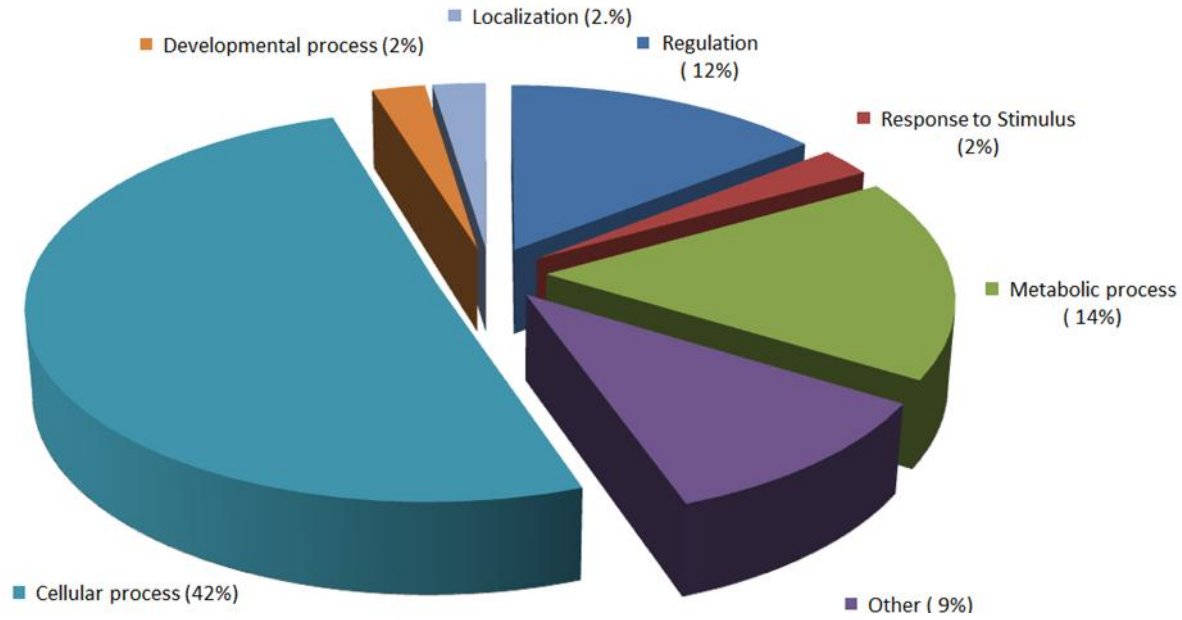


Figure 3.3: Classification of all identified proteins in shoot and root significantly responded to salt stress based on biological functions

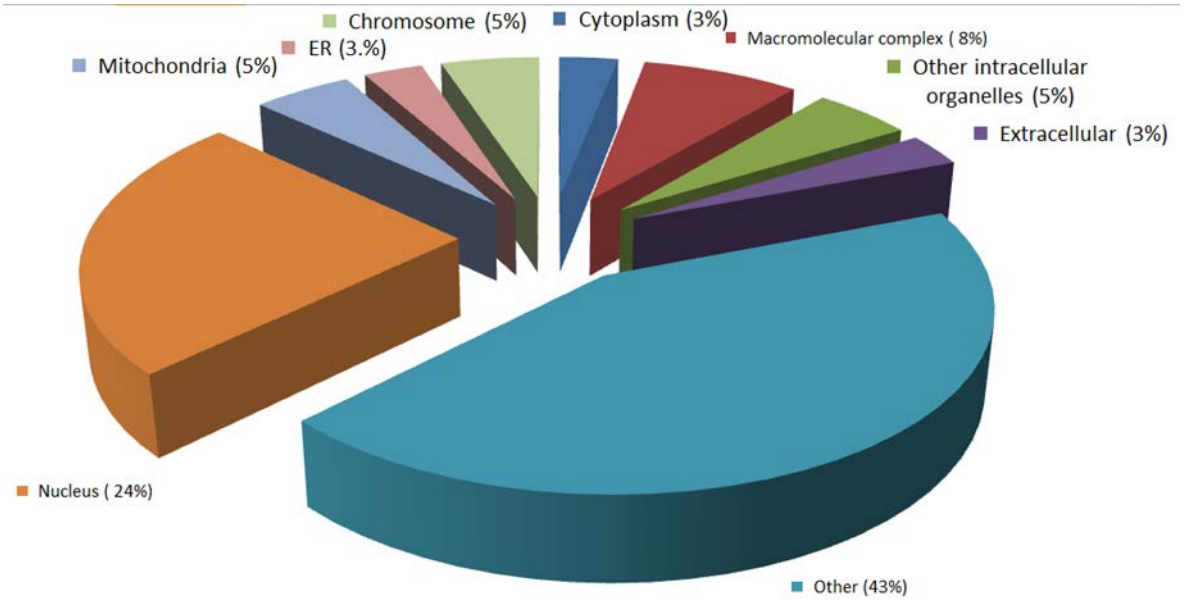


Figure 3.4: Classification of all identified proteins in shoot and root significantly responded to salt stress based on subcellular localization.

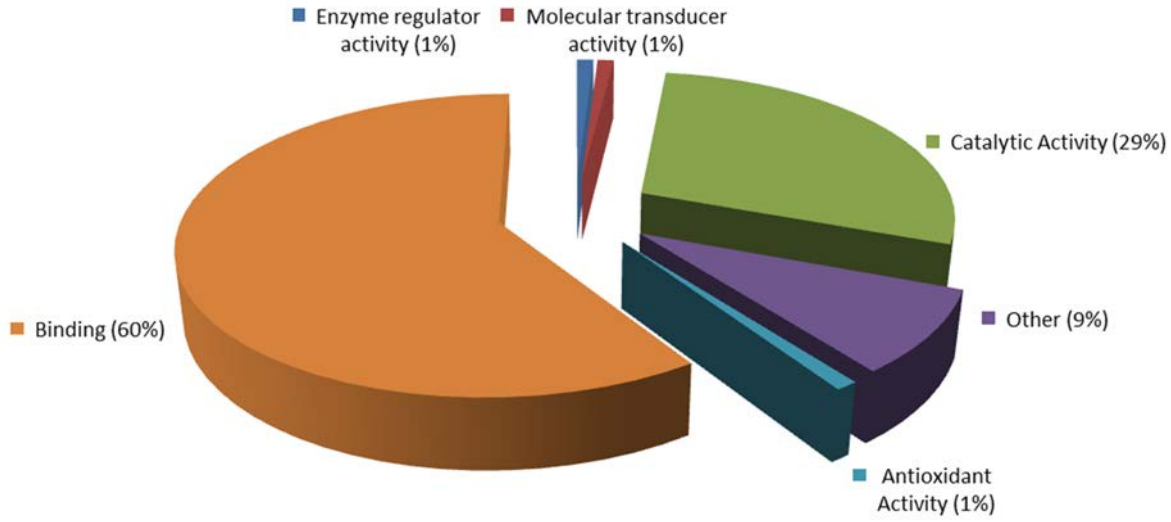


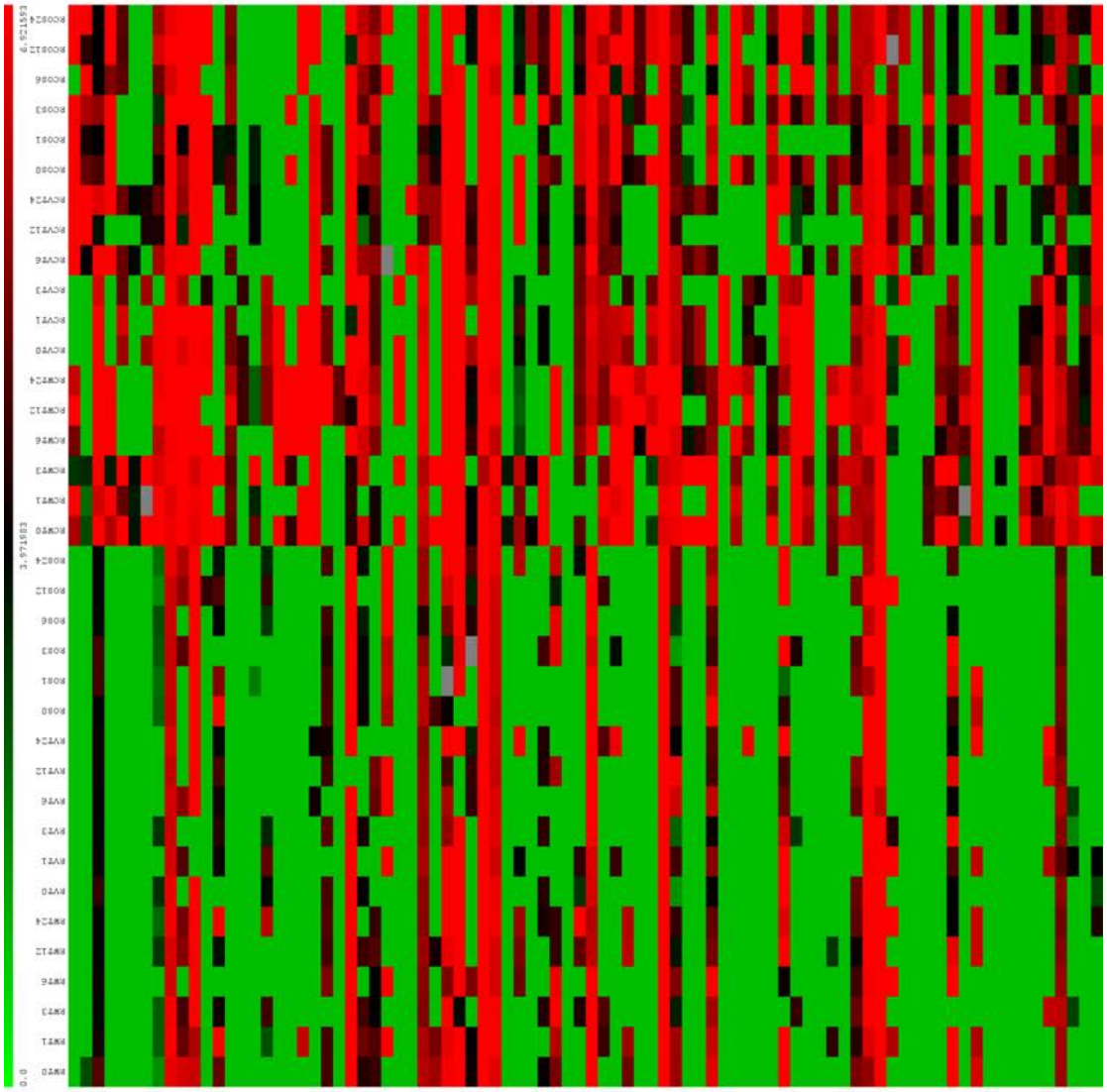
Figure 3.5: Classification of all identified proteins in shoot and root significantly respond to salt based on molecular function.

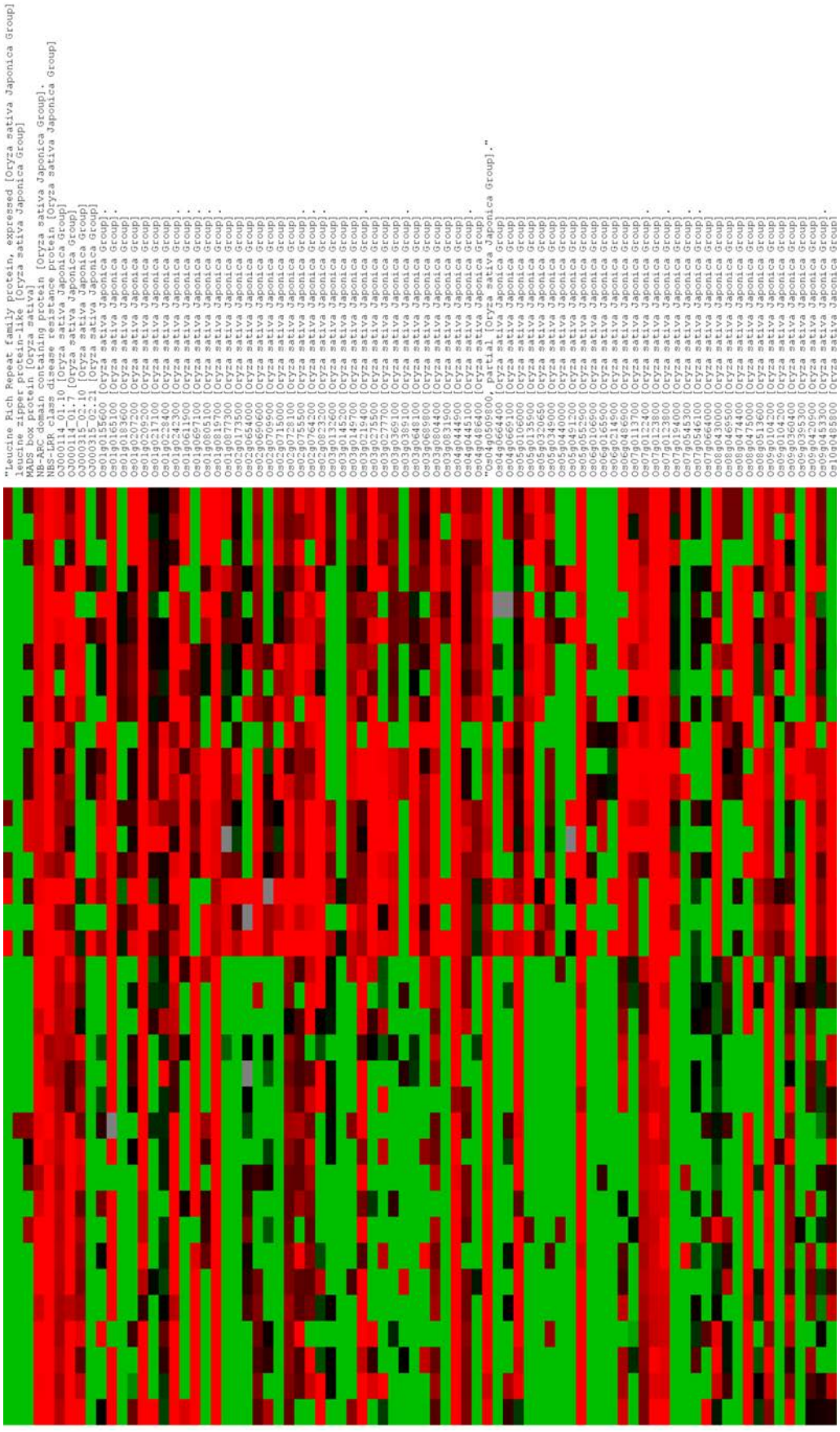
Although a large number of identified responded proteins cannot be localized into any subcellular location, many of the proteins in shoot and root responded to the induced salt stress were localized in nucleus (24%) and also in chromosome (5%), which could suggest their function in regulation of gene expression in response to stress. Significant fractions were also located in mitochondria (5%), ER (3%), and other intracellular organelles (5%) while only a small fraction was located in cytoplasm. Some are part of macromolecular complex (8%). According to classification based on molecular functions, the majority of the identified proteins (60%) are related to binding function. A substantial number of them (29%) are related to enzymes with various catalytic activities. Only few proteins were those working as enzyme regulators and molecular transducers. The results thus suggested systematic response of gene expression to salt stress for proteins in various organelles and subcellular locations, which are related to overall cellular responses by different mechanisms to the induced salt stress.

2. Dynamics of proteins under salt treatment in root of *O. sativa* L. among the three rice lines

Root is the first organ of plants to sense salt stress. Some salt stress-responsive genes were found to be mainly or more strongly induced in roots than in other organs.

Comparative proteomic analysis was used to investigate the protein profiles in roots of all rice lines under salt stress at different time after stress induction. In order to distinguish stress responses from developmental changes in protein accumulation, both control and treated roots were harvested at the same time point of treatment. Total proteins extracted from the control and the treated samples were separated and analysed by GeLC-MS.


6.921593
0.0
4-alpha-glucanotransferase [Oryza sativa Japonica Group]
Blight resistance protein [Oryza sativa Japonica Group]
bZIP protein [Oryza sativa Japonica Group]
cellulase [Oryza sativa Japonica Group]
chitinase [Oryza sativa Japonica Group]
copia-type pol polyprotein [Oryza sativa Japonica Group]
cytochrome P450 [Oryza sativa Japonica Group]
disease resistance protein [Oryza sativa Japonica Group]
disease resistance protein RPM1 [Oryza sativa Japonica Group]
gamma-adaptin 1 [Oryza sativa Japonica Group]
kinase [Oryza sativa Japonica Group].
MtN21 [Oryza sativa Japonica Group]
O-deacetylbaccatin III-10-O-acetyltransferase [Oryza sativa Japonica Group]
peroxidase [Oryza sativa Japonica Group]
polyprotein [Oryza sativa Japonica Group]
pre-mRNA-splicing factor cwc-22 [Oryza sativa Indica Group]
receptor kinase [Oryza sativa Japonica Group]
red chlorophyll catabolite reductase [Oryza sativa Japonica Group]
RNA and export factor binding protein [Oryza sativa Japonica Group]
RPS2 [Oryza sativa Japonica Group].
SecA [Oryza sativa Japonica Group]
T-complex protein 1 beta subunit (TCP-1-beta) (CCT-beta) [Oryza sativa Japonica Group]
wall-associated protein kinase [Oryza sativa Japonica Group]
WD domain containing protein [Oryza sativa Japonica Group]
Adaptin NBP35-protein [Oryza sativa Japonica Group]
ANKYRIN REPEAT-
BPI-XD interacting protein 103 [Oryza sativa Japonica Group]
"Catalase-1, , expressed [Oryza sativa Japonica Group]"
"Cytochrome P450 family protein, expressed [Oryza sativa Japonica Group]"
DNA polymerase lambda [Oryza sativa Japonica Group]
E3 ubiquitin-protein ligase BRE1-like 1
Epstein-Barr virus EBNA-1-like protein [Oryza sativa Japonica Group]
Fructose-bisphosphate aldolase
Heat shock protein 81-1
"homeodomain protein, [Oryza sativa Japonica Group]"
hypothetical protein LOC_Os03g15800 [Oryza sativa Japonica Group]
hypothetical protein LOC_Os03g37850 [Oryza sativa Japonica Group]
hypothetical protein LOC_Os03g44340 [Oryza sativa Japonica Group].
hypothetical protein LOC_Os11g04630 [Oryza sativa Japonica Group]
hypothetical protein LOC_Os11g38730 [Oryza sativa Japonica Group]
hypothetical protein LOC_Os11g38330 [Oryza sativa Japonica Group]
hypothetical protein LOC_Os11g46060 [Oryza sativa Japonica Group].
hypothetical protein LOC_Os11g40120 [Oryza sativa Japonica Group]
hypothetical protein LOC_Os11g01470 [Oryza sativa Japonica Group]
hypothetical protein LOC_Os11g16530 [Oryza sativa Japonica Group]
hypothetical protein LOC_Os11g35790 [Oryza sativa Japonica Group]
hypothetical protein OsI_00907 [Oryza sativa Indica Group].
hypothetical protein OsI_06779 [Oryza sativa Indica Group].
hypothetical protein OsI_07204 [Oryza sativa Indica Group]
hypothetical protein OsI_10150 [Oryza sativa Indica Group]
hypothetical protein OsI_13807 [Oryza sativa Indica Group]
hypothetical protein OsI_14894 [Oryza sativa Indica Group]
hypothetical protein OsI_15451 [Oryza sativa Indica Group]
hypothetical protein OsI_19531 [Oryza sativa Indica Group]
hypothetical protein OsI_23143 [Oryza sativa Indica Group]
hypothetical protein OsI_23247 [Oryza sativa Indica Group].
hypothetical protein OsI_25164 [Oryza sativa Indica Group]
hypothetical protein OsI_25502 [Oryza sativa Indica Group]
hypothetical protein OsI_26696 [Oryza sativa Indica Group]
hypothetical protein OsI_26982 [Oryza sativa Indica Group].
hypothetical protein OsI_29778 [Oryza sativa Indica Group]
hypothetical protein OsI_32427 [Oryza sativa Indica Group]
hypothetical protein OsI_33557 [Oryza sativa Indica Group]
hypothetical protein OsI_34842 [Oryza sativa Indica Group]
hypothetical protein OsI_36657 [Oryza sativa Indica Group]
hypothetical protein Os3_00067 [Oryza sativa Japonica Group]
hypothetical protein Os3_04402 [Oryza sativa Japonica Group]
hypothetical protein Os3_06804 [Oryza sativa Japonica Group]
hypothetical protein Os3_07524 [Oryza sativa Japonica Group]
hypothetical protein Os3_07825 [Oryza sativa Japonica Group]
hypothetical protein Os3_10072 [Oryza sativa Japonica Group]
hypothetical protein Os3_14601 [Oryza sativa Japonica Group]
hypothetical protein Os3_17642 [Oryza sativa Japonica Group]
hypothetical protein Os3_21464 [Oryza sativa Japonica Group]
hypothetical protein Os3_23643 [Oryza sativa Japonica Group]
hypothetical protein Os3_26947 [Oryza sativa Japonica Group]
hypothetical protein Os3_30069 [Oryza sativa Japonica Group]
hypothetical protein Os3_30125 [Oryza sativa Japonica Group]
hypothetical protein Os3_31157 [Oryza sativa Japonica Group]
hypothetical protein OSJNBa0079E14.12 [Oryza sativa Japonica Group]


"Leucine Rich Repeat family protein, expressed [Oryza sativa Japonica Group]
leucine zipper protein-like [Oryza sativa Japonica Group]
MADS box protein [Oryza sativa]
NB-ARC domain containing protein [Oryza sativa Japonica Group].
NBS-LRR class disease resistance protein [Oryza sativa Japonica Group]
Os01g0165100 [Oryza sativa Japonica Group]
Os01g0183600 [Oryza sativa Japonica Group]
Os01g0207200 [Oryza sativa Japonica Group]
Os01g0209200 [Oryza sativa Japonica Group]
Os01g0217100 [Oryza sativa Japonica Group]
Os01g0228400 [Oryza sativa Japonica Group]
Os01g0242300 [Oryza sativa Japonica Group]
Os01g0611900 [Oryza sativa Japonica Group]
Os01g0669700 [Oryza sativa Japonica Group]
Os01g0885100 [Oryza sativa Japonica Group]
Os01g0917300 [Oryza sativa Japonica Group]
Os02g0173500 [Oryza sativa Japonica Group]
Os02g0654000 [Oryza sativa Japonica Group]
Os02g0660600 [Oryza sativa Japonica Group]
Os02g0709900 [Oryza sativa Japonica Group]
Os02g0715000 [Oryza sativa Japonica Group]
Os02g0728100 [Oryza sativa Japonica Group]
Os02g0755500 [Oryza sativa Japonica Group]
Os02g0764200 [Oryza sativa Japonica Group]
Os02g0833200 [Oryza sativa Japonica Group]
Os03g0126600 [Oryza sativa Japonica Group]
Os03g0134700 [Oryza sativa Japonica Group]
Os03g0149700 [Oryza sativa Japonica Group]
Os03g0219400 [Oryza sativa Japonica Group]
Os03g0275500 [Oryza sativa Japonica Group]
Os03g0277700 [Oryza sativa Japonica Group]
Os03g0369100 [Oryza sativa Japonica Group]
Os03g0648100 [Oryza sativa Japonica Group]
Os03g0689800 [Oryza sativa Japonica Group]
Os03g0804400 [Oryza sativa Japonica Group]
Os03g0831600 [Oryza sativa Japonica Group]
Os04g0445100 [Oryza sativa Japonica Group]
Os04g0486400 [Oryza sativa Japonica Group].
"Os04g0509800, partial [Oryza sativa Japonica Group]."
Os04g0664400 [Oryza sativa Japonica Group]
Os04g0669100 [Oryza sativa Japonica Group]
Os05g0106000 [Oryza sativa Japonica Group]
Os05g0135500 [Oryza sativa Japonica Group]
Os05g0320650 [Oryza sativa Japonica Group]
Os05g0349000 [Oryza sativa Japonica Group]
Os05g0440000 [Oryza sativa Japonica Group]
Os05g0541200 [Oryza sativa Japonica Group]
Os06g0106600 [Oryza sativa Japonica Group]
Os06g0165500 [Oryza sativa Japonica Group]
Os06g0214900 [Oryza sativa Japonica Group]
Os06g0486900 [Oryza sativa Japonica Group]
Os07g0113700 [Oryza sativa Japonica Group]
Os07g0122400 [Oryza sativa Japonica Group]
Os07g0123800 [Oryza sativa Japonica Group]
Os07g0154000 [Oryza sativa Japonica Group]
Os07g0545300 [Oryza sativa Japonica Group]
Os07g0654100 [Oryza sativa Japonica Group]
Os08g0430000 [Oryza sativa Japonica Group]
Os08g0469500 [Oryza sativa Japonica Group]
Os08g0474400 [Oryza sativa Japonica Group]
Os08g0475000 [Oryza sativa Japonica Group]
Os08g0513600 [Oryza sativa Japonica Group]
Os09g0104200 [Oryza sativa Japonica Group]
Os09g0360400 [Oryza sativa Japonica Group]
Os09g0355300 [Oryza sativa Japonica Group]
Os09g0443900 [Oryza sativa Japonica Group]
Os09g0459300 [Oryza sativa Japonica Group]
Os10g0459800 [Oryza sativa Japonica Group]

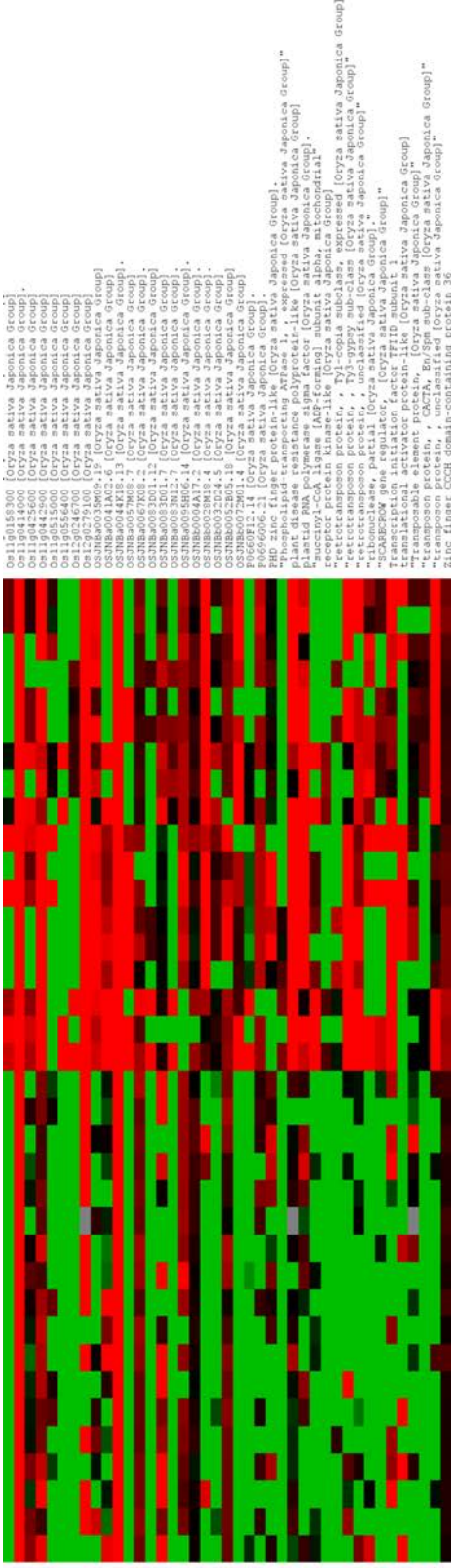


Figure 3.6: Heat map of salt stress-responsive proteins in roots of *O. sativa* L. among three rice lines (WT, VT, and OS)

Significantly expressed proteins in roots with no NaCl treatment and NaCl treatment, respectively at 0, 1, 3, 6, 12, and 24 hr were identified. The heat map was constructed by using Multi experiment Viewer (MeV) software. The upper bar from pale green to red colors indicated low to high protein expression. Red is up-regulated; green is down-regulated; black is no change in protein expression.

Hierarchical analyses of the expression profiles of salt-responsive proteins are demonstrated in **figure 3.6.** The first group of columns represents protein expression changes in the control group with no salt treatment of the three rice lines including wild-type, vector alone and *OsCam1-1*-overexpressing lines, respectively. The second group of columns represents protein expression changes in the salt (150 mM NaCl) stress treatment group of the three rice lines The heat map profiles in each group contained the data for protein profiles at 0, 1, 3, 6, 12, and 24 hr after salt stress induction. The rows represent the identified individual proteins. The up- or down-regulated proteins are indicated in red or green, respectively. The intensity of the colors increases with the increases in differences of expression levels compared to the control treatments.

3. Functional classification and localization of differentially expressed proteins in roots of among the three rice lines (wild-type, vector alone and transgenic *OsCam1-1* overexpressing *O. sativa* L. lines)

Proteins up-regulated or down-regulated in roots were functionally classified according to their known and putative functions by using STRAP software. These proteins were classified in to 6 groups based on their biological functions **(Figure 3.7)** including cellular process (51%), response to stimulus (18%), metabolic process (11%), regulation (11%), or developmental process (2%) while the rest are unknown or proteins in other processes (7%). Furthermore, these proteins were analyzed by compatible proteomic software program as described in **Chapter 2, section 3.8** to predict their subcellular location **(Figure 3.8)** and molecular function **(Figure 3.9)**.

Biological functions

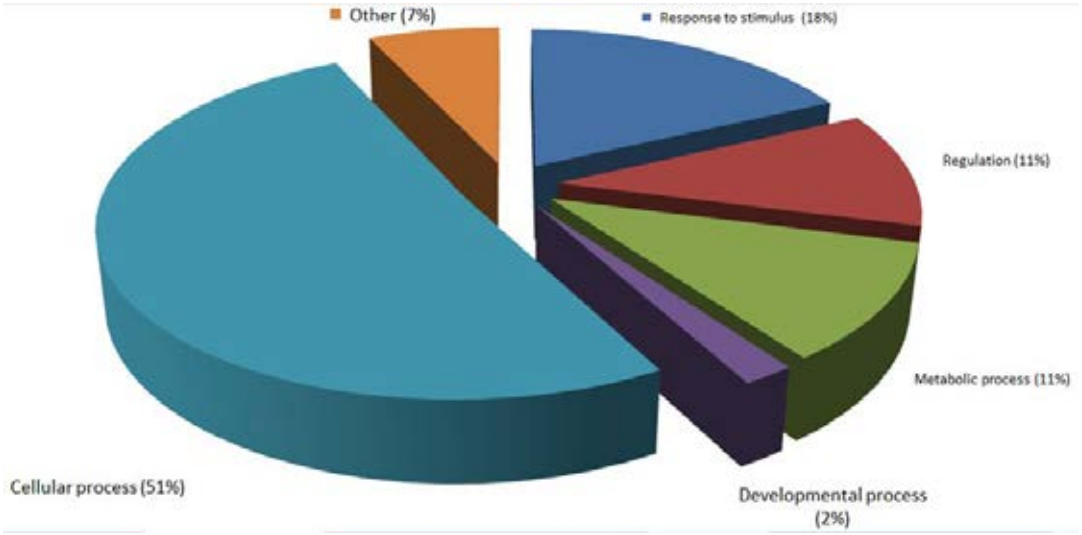


Figure 3.7: Classification of all identified proteins in roots significantly responded to salt stress based on functional characteristics

Localization

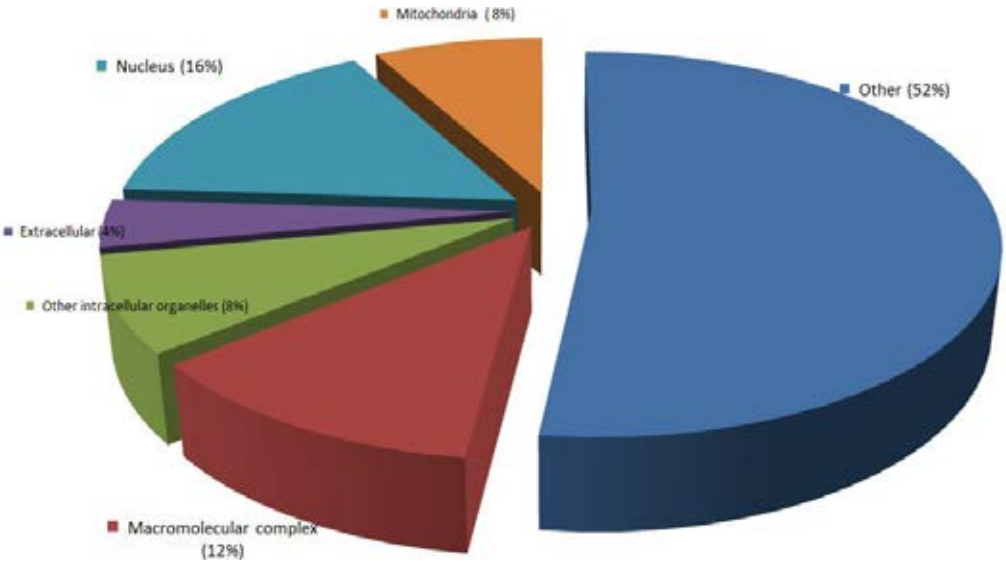


Figure 3.8: Classification of all identified proteins in roots significantly responded to salt stress based on subcellular localization.

Molecular functions

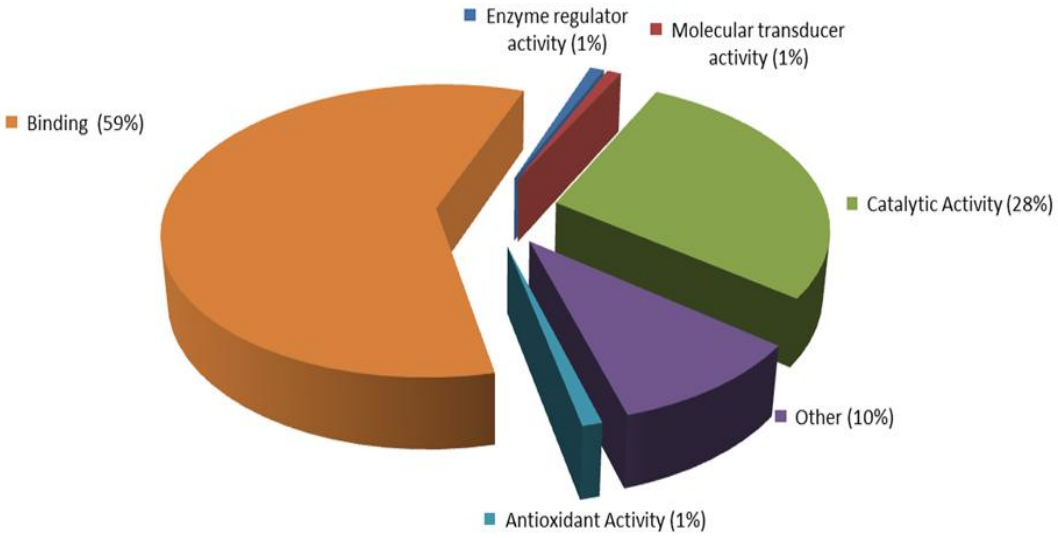


Figure 3.9: Classification of all identified proteins in roots significantly responded to salt stress based on molecular function

According to classification by STRAP, the majority of the identified responsive proteins could not be assigned to any subcellular locations. However, similar to those found in the classification of all proteins in the whole plants described in the previous section, many of the proteins in roots responded to salt stress were localized in nucleus (16%), which could suggest their function in regulation of gene expression in response to salt treatment. Significant fractions were also located in mitochondria (8%) and other intracellular organelles (8%) while only a small fraction was related to extracellular proteins. A substantial fraction of identified proteins were associated with macromolecular complex (12%). Relatively similar profiles were obtained from classification of the salt responsive proteins based on their molecular function in roots compared to the whole plant. The majority of the identified proteins (59%) are related to binding function. A substantial number of them (28%) are related to enzymes with various catalytic activities. Only few proteins were those working as enzyme regulators, molecular transducers, and antioxidants. The results thus suggested systematic response of gene expression in roots to induce salt stress resulting in up- or

down regulations of proteins in various organelles and subcellular locations which are related to overall cellular responses by different mechanisms to the induced salt stress.

4. Dynamics of proteins under salt treatment in shoots of *O. sativa* L. among the three rice lines

In this study, protein profiles in shoots of rice seedling were also investigated to study the sensing of salt stress to other parts of the plant. Comparative proteomic analysis was used to investigate the protein profiles in shoots under salt stress. In order to distinguish stress responses from developmental changes in protein accumulation, both control and treated shoots were harvested at the same time points of treatment using the same procedure as for roots. Total proteins extracted from the control and the treated samples were separated on SDS-PAGE and analysed by GeLC-MS. The result of the heat map of salt stress-responsive proteins in the shoot of rice *O. sativa* L. was shown in **figure 3.10**

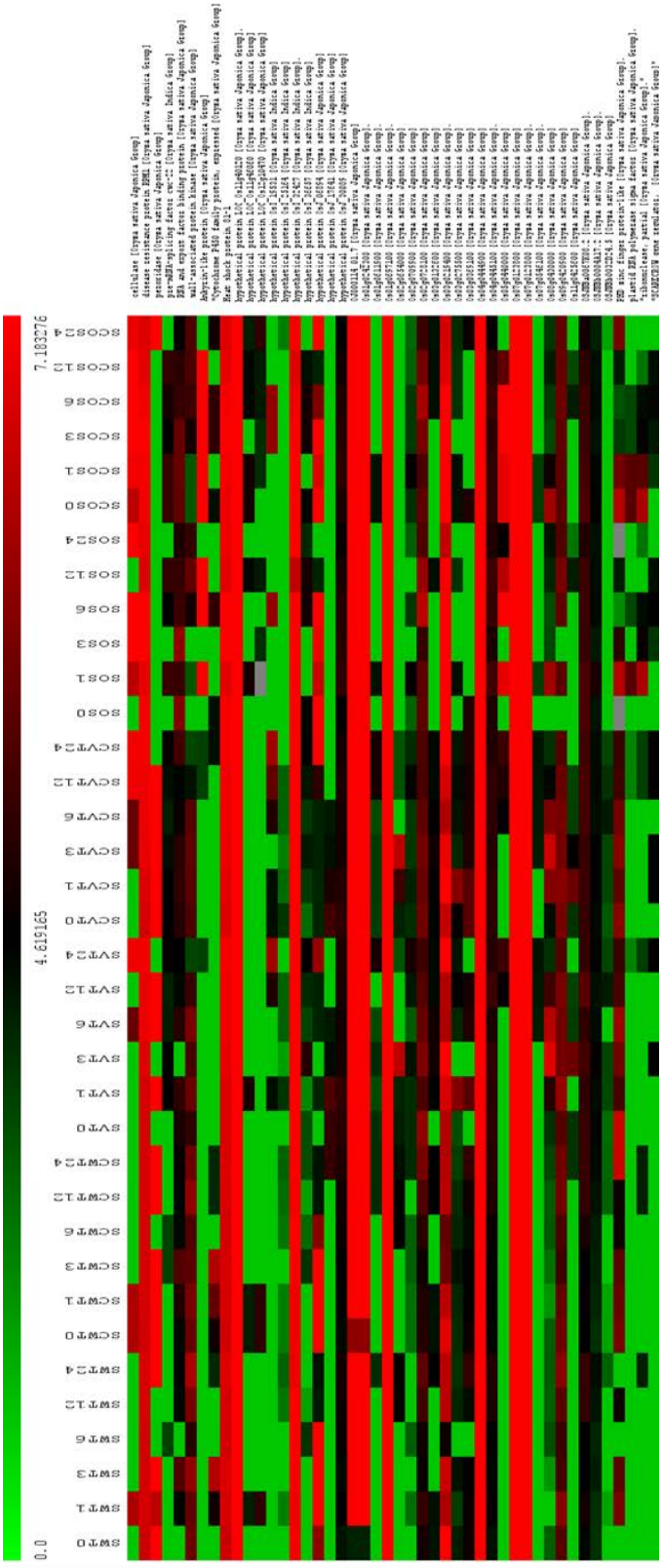


Figure 3.10: Heat map of salt stress-responsive proteins in shoots of *O. sativa*L. among three rice lines (WT, VT, and OS)

Significant expressed proteins in roots with no NaCl treatment and NaCl treatment, respectively at 0, 1, 3, 6, 12, and 24 hr were identified. The heat map was constructed by using Multi experiment Viewer (MeV) software. The upper bar from pale green to red colors indicated low to high protein expression. Red is up-regulated; green is down-regulated; black is no change in protein expression.

Hierarchical analyses of the expression profiles of salt-responsive proteins are demonstrated in **figure 3.10.** The first group of columns represents protein expression changes in the control group of the three rice lines with no salt treatment including wild-type, vector alone, and *OsCam1-1*-overexpressing lines. The second group of columns represents protein expression changes in the salt (150 mM NaCl) stress treatment group of the three rice lines at 0, 1, 3, 6, 12, and 24 hr after salt stress induction, respectively. The rows represent the identified individual proteins. The up- or down-regulated proteins are indicated in red or green, respectively. The intensity of the colors increases with the increases of expression differences between the control treatments.

When compared the expression profiles in shoot proteins in the control and the salt stress treatment groups, most proteins were not significantly changed in their expression levels.

5. Functional classification and localization of differentially expressed proteins in shoots of three *O. sativa* L. rice lines

Each identified protein was functionally classified according to their known and putative functions by using STRAP software. Among proteins found in shoots, 23 proteins have assigned functions. These proteins were classified in to 5 groups based on their biological functions **(Figure 3.11)** including cellular process (39%), response to stimulus (22%), regulation (17%), metabolic process (13%) while the rest are unknown or related to other biological functions (9%). Furthermore, these proteins were analyzed by compatible proteomic software as described in **Chapter 2, section 3.8** to predict their subcellular location **(Figure 3.12)** and molecular function **(Figure 3.13).**

Biological functions

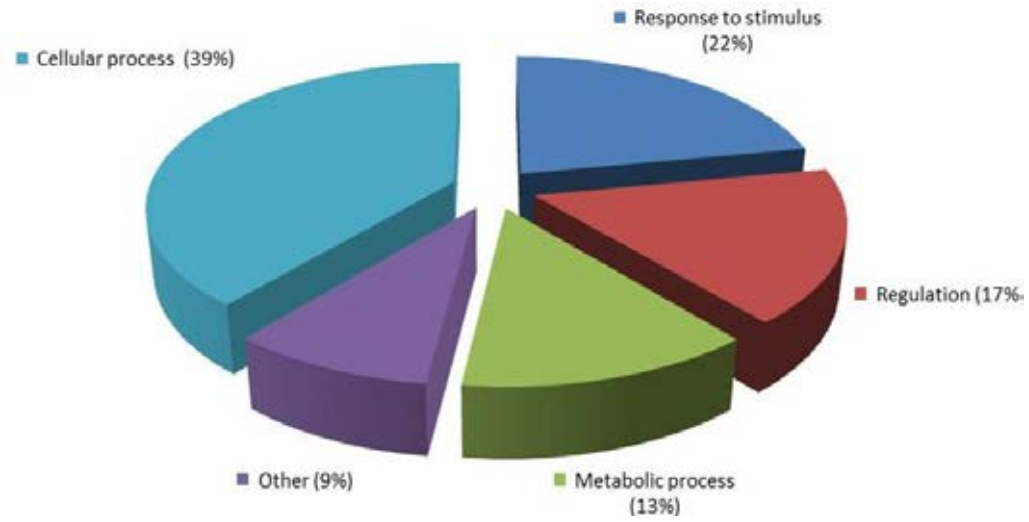


Figure 3.11: Classification of all identified proteins in shoots significantly responded to salt stress based on functional characteristics

Localization

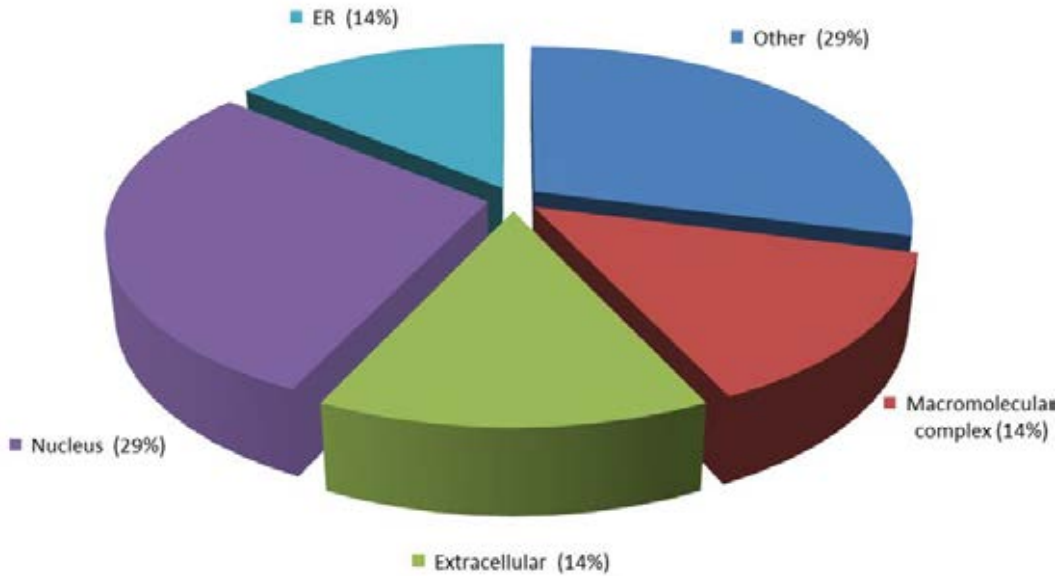


Figure 3.12: Classification of all identified proteins in shoots significantly responded to salt stress based on subcellular localization.

Molecular functions

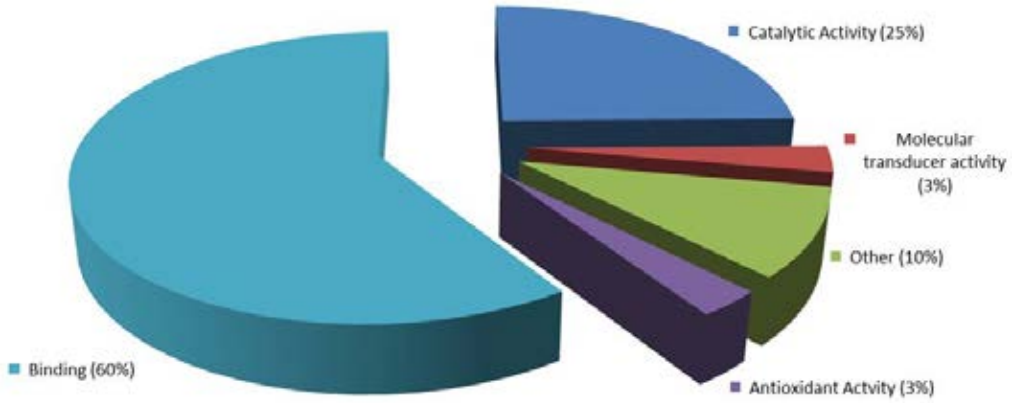


Figure 3.13: Classification of all identified proteins in shoots significantly responded to salt stress based on molecular function.

According to classification by STRAP, similar to those found in the classification of overall protein in the whole plants and roots described in the previous section, many of the proteins in roots responded to the induced salt stress were localized in nucleus (29%), which could suggest their function in regulation of gene expression in response to salt treatment. However, a large number of salt responsive proteins were localized in ER (14%) and are predicted as extracellular proteins (14%) while no proteins were assigned to mitochondrial origin, which are different to those found in roots. A substantial fraction of proteins were associated with macromolecular complex. Relative similar profiles were obtained from classification of the salt responsive proteins in shoots based on their molecular function compared to the whole plants and the roots. The majority of the identified proteins (60%) are related to binding function, which could involve a large number of regulatory proteins functioning by binding. A substantial number of them (25%) is related to enzymes with various catalytic activities. Only a small fraction of proteins were those functioning as molecular transducers and antioxidants. The results thus suggested sensing of signal for salt stress from roots to shoots which leads to systematic response of gene expression in shoots. This results in up- or down regulation of proteins in

various organelles and subcellular locations which are related to the overall cellular responses by different mechanisms to the induced salt stress.

6. Comparison of annotated proteins based on gene ontology (GO) terms

Overall protein expression responses in roots and shoots of rice seedlings from the three rice lines (WT, VT, and OS) are compared based on gene ontology terms (GO) in order to compare the gene function based on its biological roles **(Figure 3.14).** In total, there were 48 annotated proteins in roots, which responded to salt stress compared to that in shoots (24 genes). Most responsive proteins were classified as proteins involved in biological regulation, accounting for 47.9 and 37.5 % of salt responsive proteins in roots and in shoots, respectively. A substantial fraction of annotated proteins in roots were related to those functioning in plant defensive mechanisms (16.6 %), growth (10.4 %), developmental process (10.4 %), interaction with cells and organisms (6.25 %), and cellular process (2.08 %). In addition, in shoots, proteins involved in growth (20.8 %) and developmental process (16.6 %) were detected, and proteins related to cellular process were more pronounced (12.5 %). Overall, these would thus suggest differences in systematic gene expression responses in roots and in shoots of rice seedling during salt stress treatment.

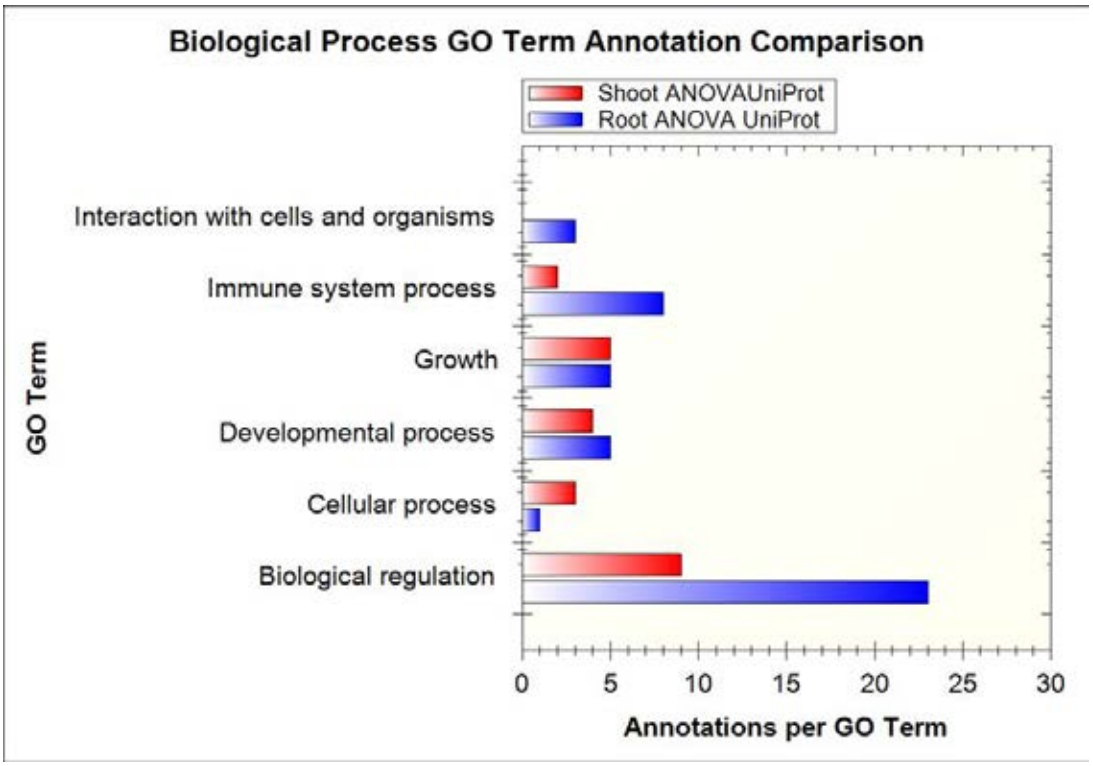


Figure 3.14: Comparison of biological process GO Term in shoots and roots of *Oryza sativa* L.

In summary, there were significant alterations in protein expression patterns in rice in response to salt stress when analysed based on the proteomic profiles of all three rice lines (WT, VT, and OS). Changes in protein expression profiles and levels in response to salt stress treatment were more pronounced in roots compared to shoots of the rice seedings. The majority of the proteins induced in the root (23 genes) and the shoot (9 genes) are related to those with biological regulation. Proteins in defensive system process (8 genes in roots and 3 genes in shoots) also showed significant up-regulation during salt stress. A substantial number of responded genes are classified to those related to growth (5 genes in both roots and shoots) and developmental process (5 genes in roots and 4 genes in shoots). Several responsive genes (1 gene in roots and 3 genes in shoots) are also related to those in cellular process. The rest are related in those function in cellular interaction.

7. Comparison of protein expression profiles in wild type (WT), transgenic control (VT) and *OsCam1-1*-overexpressing transgenic (OS) rice lines

This section focuses on the comparison of differences in protein expression profiles in the three rice lines in this study including the wild-type *O. sativa* (WT), the vector-control transgenic line (VT), and the *OsCam1-1*-overexpressing transgenic line (OS), previously reported for increasing salt tolerance ability. Differences in protein expression profiles under non-stressed and in responses to salt stress in these rice lines were determined based on Venn diagrams.

Venn diagrams or set diagrams are diagrams that show all possible logical relations between a finite collection of sets (aggregation of things). Venn diagrams normally comprise overlapping circles. Venn diagram uses intersecting circles to illustrate the similarities, differences, and relationships between groups. Similarities between groups are represented in the intersecting portions of the circles, while differences are represented in the non-intersecting portions of the circles (http://creately.com/Draw-Venn-Diagrams-Online). The protein profile datasets between each specific group were grouped into Venn diagram manually in order to elucidate the differences in responsive gene expression among the rice lines in our study.

7.1 Comparison of protein expression profiles in roots and shoots

7.1.1 Comparison of protein profiles in roots and shoots under non-stressed conditions

The protein expression profiles in roots and shoots of the wild-type and transgenic lines under non-stressed conditions were compared. Focusing on protein expression profile in the wild-type, there were 128 ANOVA annotated proteins that were commonly expressed in WT under non-induced conditions **(Figure 3.15)**. There were 17 proteins which were uniquely expressed in shoots and 19 proteins which were uniquely identified in roots under non-stressed conditions. The lists of these proteins with known function are shown in **Table 2**. The complete list of proteins is summarized in the appendix section. **(Appendix B)**

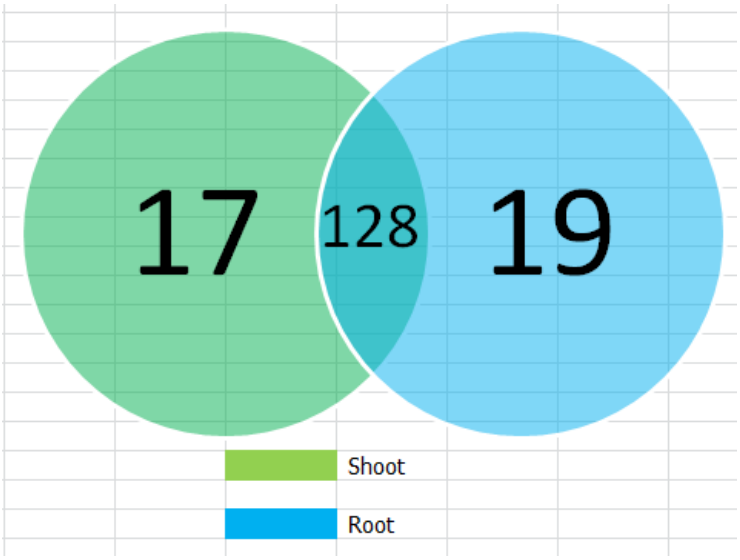


Figure 3.15: Venn diagram of annotated proteins which are differently expressed in root and shoots of WT under non-stressed conditions

Focusing on protein expression profile in the vector control transgenic line, there were 117 ANOVA annotated proteins that were commonly expressed in VT under non-induced conditions **(Figure 3.16)**. There were 36 proteins that were uniquely expressed in shoots and 13 proteins that were uniquely identified in roots under non-stressed conditions. The lists of these proteins with known function are shown in **Table 2**. The complete list of proteins is summarized in the appendix section. **(Appendix B)**

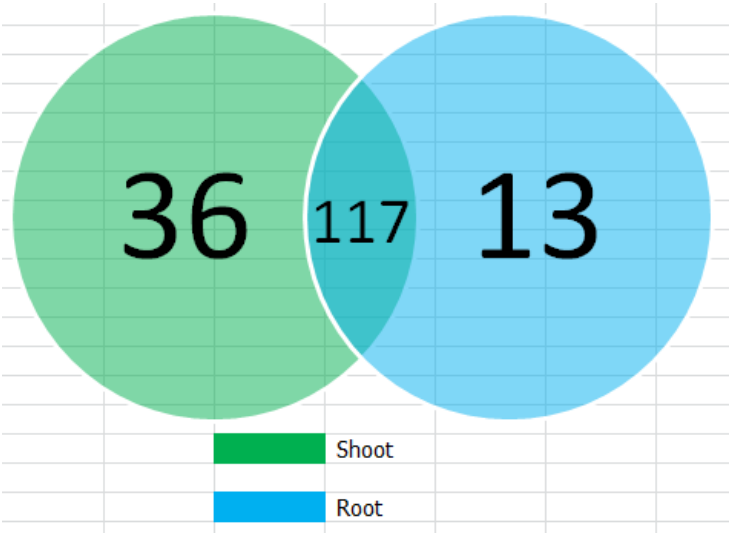


Figure 3.16: Venn diagram of annotated proteins which are differently expressed in root and shoots of VT under non-stressed conditions

Focusing on protein expression profile in the *OsCam1-1*-overexpressing transgenic line, there were 113 ANOVA annotated proteins which were commonly expressed in OS under non-induced conditions **(Figure 3.17)**. There were 41 proteins which were uniquely expressed in shoots and 14 proteins which were uniquely identified in roots under non-stressed conditions. The lists of these proteins with known function are shown in **Table 2**. The complete list of proteins is summarized in the appendix section. **(Appendix B)**

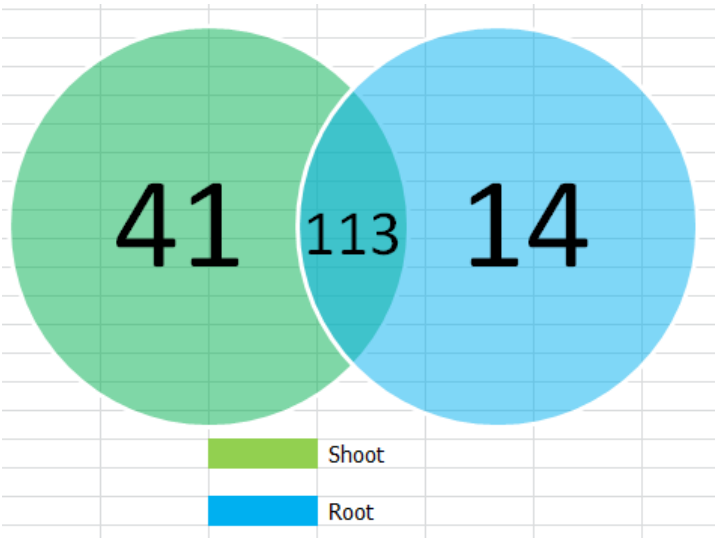


Figure 3.17: Venn diagram of annotated proteins which are differently expressed in root and shoots of OS under non-stressed conditions

7.1.2 Comparison of protein profiles in roots and shoots under salt-stressed conditions

The protein expression profiles in roots and shoots of the wild-type and transgenic lines under salt-stressed conditions were compared in order to identify differences in expression of specific proteins in these parts in response to increasing salt concentration. Focusing on protein expression profile in the wild-type, there were 143 ANOVA annotated proteins that were commonly expressed in WT under non-induced conditions **(Figure 3.18)**. There were 7 proteins which were uniquely expressed in shoots and 48 proteins which were uniquely identified in roots under salt-stressed conditions. The lists of these proteins with known function are shown in **Table 3.** The complete list of proteins is summarized in the appendix section. **(Appendix B)**

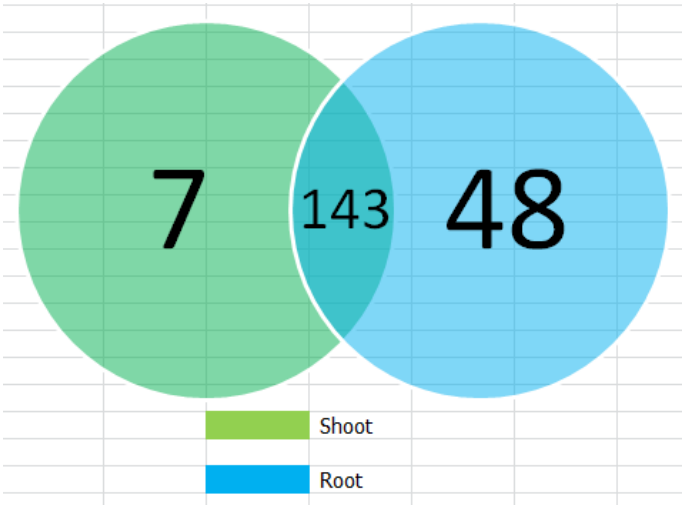


Figure 3.18: Venn diagram of annotated proteins which are differently expressed in root and shoots of WT under salt-stressed conditions

Focusing on protein expression profile in the vector control transgenic line, there were 141 ANOVA annotated proteins that were commonly expressed in VT under non-induced conditions **(Figure 3.19)**. There were 13 proteins which were uniquely expressed in shoots and 41 proteins which were uniquely identified in roots under non-stressed conditions. The lists of these proteins with known function are

shown in **Table 3.** The complete list of proteins is summarized in the appendix section. **(Appendix B)**

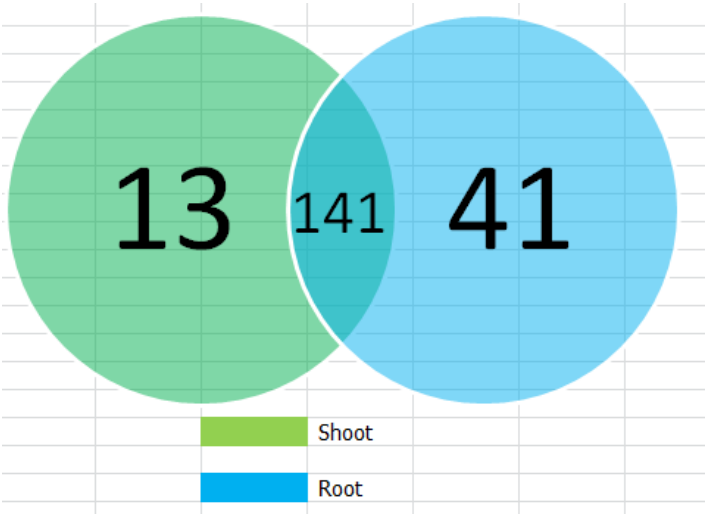


Figure 3.19: Venn diagram of annotated proteins which are differently expressed in root and shoots of VT under salt-stressed conditions

Focusing on protein expression profile in the *OsCam1-1*-overexpressing transgenic line, there were 140 ANOVA annotated proteins which were commonly expressed in OS under non-induced conditions **(Figure 3.20)**. There were 34 proteins which were uniquely expressed in shoots and 20 proteins which were uniquely identified in roots under non-stressed conditions. The lists of these proteins with known function are shown in **Table 3.** The complete list of proteins is summarized in the appendix section. **(Appendix B)**

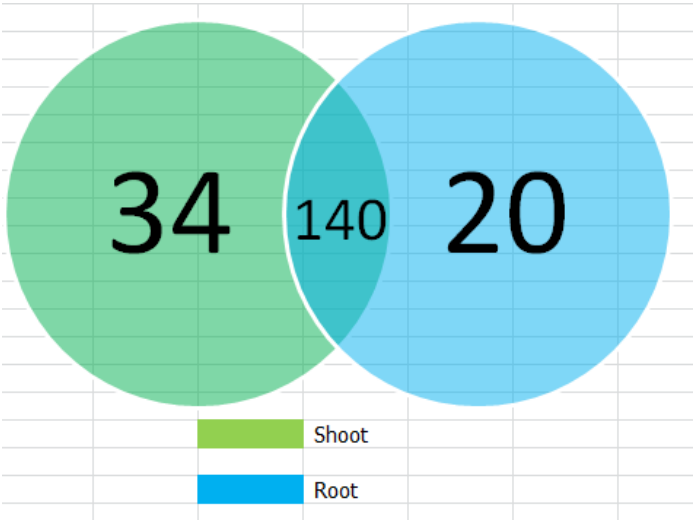


Figure 3.20: Venn diagram of annotated proteins which are differently expressed in root and shoots of OS under salt-stressed conditions

7.2 Analysis of biomarkers in responses to salt stress

This section focuses on the comparison of differences in protein expression profiles between each rice line (WT, VT, and OS) under the non-stressed and salt stress conditions in order to identify potential biomarkers in response to increasing salt stress in roots and shoots of these wild-type and transgenic lines. Differences in protein expression profiles under non-stressed and in responses to salt stress in these rice lines were determined based on Venn diagrams

7.2.1 Identification of biomarkers in roots in responses to salt stress

Protein expression profiles in roots of each rice line under the non-stressed and salt-stressed conditions were compared in order to determine the expression of specific proteins in the different lines in response to salt stress. Focusing on protein expression profile in the wild-type, there were 125 ANOVA annotated proteins that were commonly expressed in WT under both conditions **(Figure 3.21)**. There were 4 proteins which were uniquely expressed under non-stressed conditions, which were Ankyrin-like protein gi|47848255, Os11g0515000 gi|115485653, Os08g0469500 gi|115476832 and Os03g0389100 gi|115453337. In addition, there were 66 proteins, which were identified only under the salt-stressed conditions. The list of these

uniquely expressed proteins with known function is shown in **Table 5**. The complete list of proteins is summarized in the appendix section. **(Appendix B)**

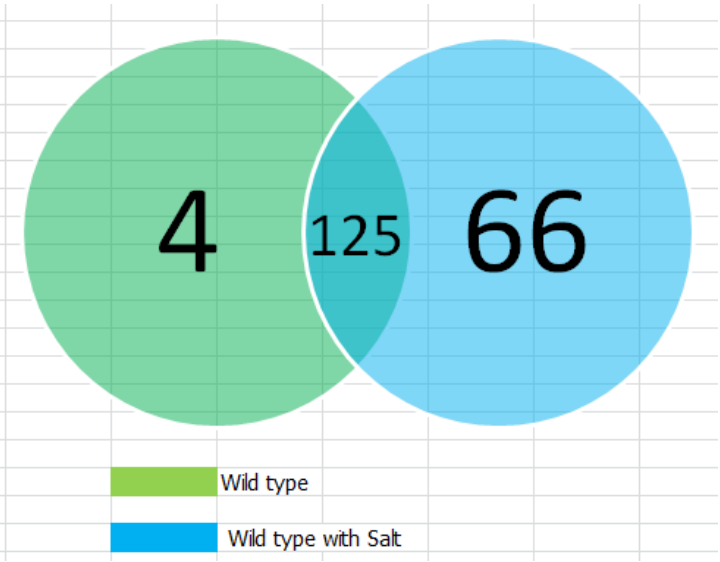


Figure 3.21: Venn diagram of annotated proteins which are differently expressed in roots of WT under salt stressed conditions

Focusing on the vector-control transgenic line, there were 124 ANOVA annotated proteins that were commonly expressed in VT under both conditions **(Figure 3.22)**. There were 6 proteins uniquely expressed under non-stressed conditions, which were identified as Os05g0440000 gi|115464133, hypothetical protein LOC_Os11g04630 gi|77548646, MtN21 gi|50508502, Os03g0132600 gi|297721777, Os07g0664000 gi|297607764 and leucine zipper protein-like gi|49387676.

In addition, there were 58 proteins, which were identified only under the salt-stressed conditions. The list of these uniquely expressed proteins is shown in **Table 5**. The complete list of proteins is summarized in the appendix section. **(Appendix B)**

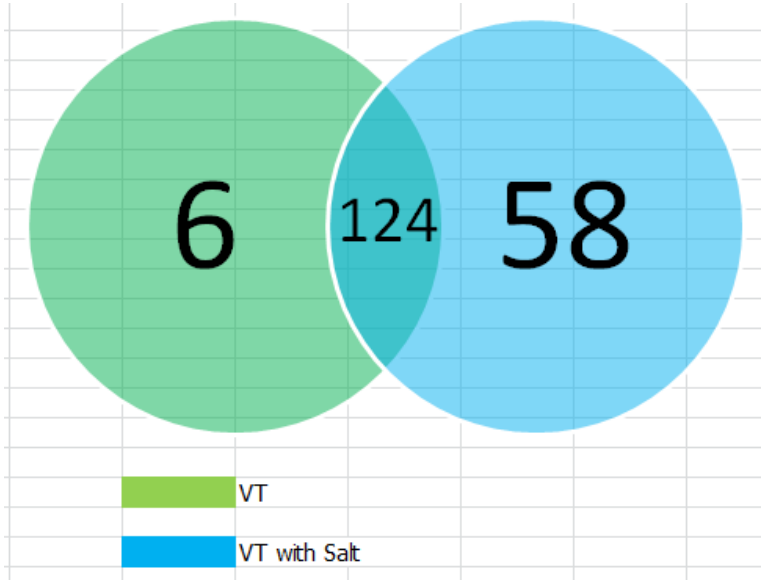


Figure 3.22: Venn diagram of annotated proteins which are differently expressed in roots of VT under salt stressed conditions

Focusing on the *OsCam1-1*-overexpressing transgenic line, there were 145 ANOVA annotated proteins that were commonly expressed in OS under both conditions **(Figure 3.23)**. There were 7 proteins uniquely expressed under non-stressed conditions, which were annotated assuccinyl-CoA ligase [ADP-forming] subunit alpha, mitochondrial gi|75294330, Os02g0709900 gi|115448227, Os09g0395300 gi|115479007, pre-mRNA-splicing factor cwc-22 gi|156622338, OSJNBa0041A02.6 gi|38344139, Os10g0485800 gi|297727701 and Os01g0697100 gi|115439381.

In addition, there were 55 proteins, which were identified only under the salt-stressed conditions. The list of these uniquely expressed proteins is shown in **Table 5.** The complete list of proteins is summarized in the appendix section. **(Appendix B)**

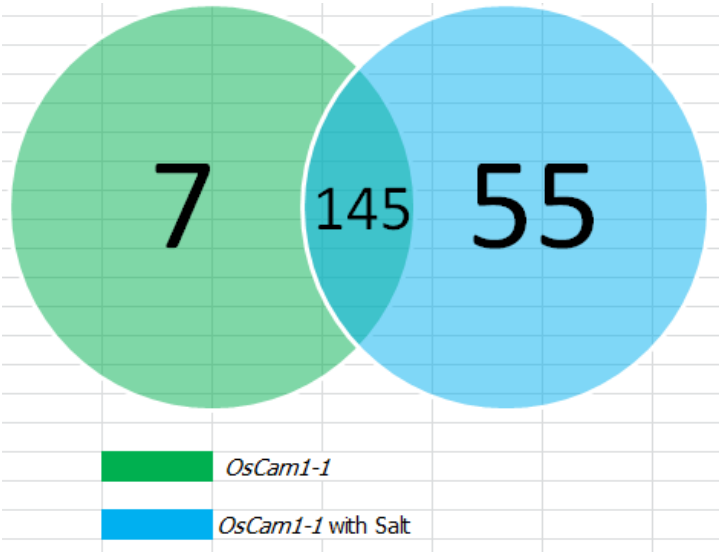


Figure 3.23: Venn diagram of annotated proteins which are differently expressed in roots of OS under salt stressed conditions

7.2.2 Identification of biomarkers in shoots in responses to salt stress

Protein expression profiles in shoots of each rice line under the non-stressed and salt-stressed conditions were compared in order to determine the expression of specific proteins in the different lines in response to salt stress. Focusing on protein expression profile in the wild-type, there were 143 ANOVA annotated proteins that were commonly expressed in WT under both conditions **(Figure 3.24)**. There were 2 proteins which were uniquely expressed under non-stressed conditions, which were OSJNBa0083N12.7 gi|38345769 and BRI1-KD interacting protein 103 gi|50252361.

In addition, there were 7 proteins, which were identified only under the salt-stressed conditions. These involved Os09g0453300 gi|115479499, translational activator protein-like gi|50251987, Os11g0425600 gi|115485265, Os07g0113700 gi|115470301, Os05g0135900 gi|115461955, hypothetical protein OsJ_17641 gi|222630706, and O-deacetylbaccatin III-10-0-acetyltransferase gi|19920081. The list of these uniquely expressed proteins is shown in **Table 6.**

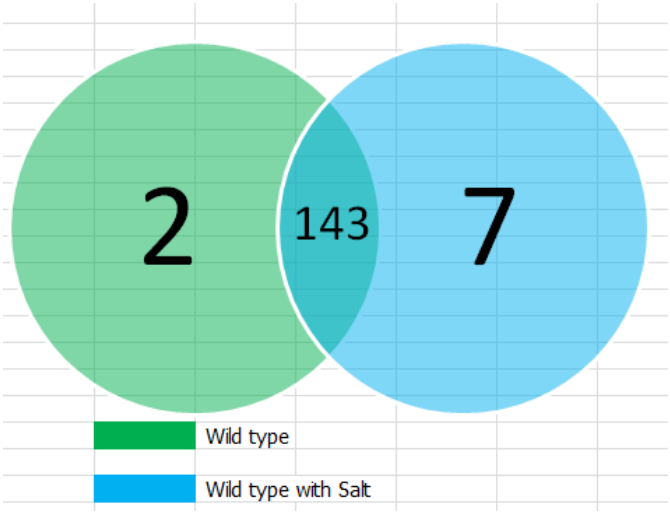


Figure 3.24: Venn diagram of annotated proteins which are differently expressed in shoots of WT under non-stressed and salt stressed conditions

Focusing on the vector-control transgenic line, there were 152 ANOVA annotated proteins that were commonly expressed in VT under both conditions **(Figure 3.25)**. There was only one protein uniquely expressed under non-stressed conditions, which was identified as hypothetical protein LOC_Os11g46060 gi|77552574.

In addition, there were 2 proteins, which were identified only under the salt-stressed conditions. These proteins were Cytochrome P450 family protein, expressed gi|108706099 and Phospholipid-transporting ATPase 1, expressed gi|108707922. The list of these uniquely expressed proteins is shown in **Table 6.**

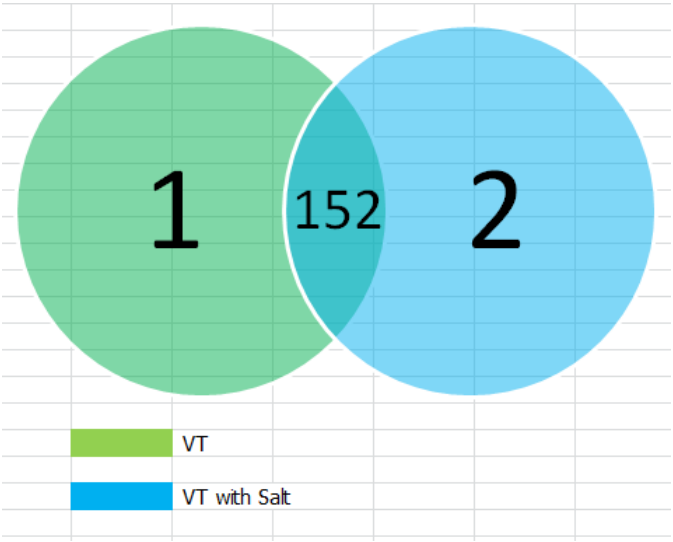


Figure 3.25: Venn diagram of annotated proteins which are differently expressed in shoots of VT under salt stressed conditions

Focusing on the *OsCam-1-1*-overexpressing transgenic line, there were 153 ANOVA annotated proteins that were commonly expressed in WT under both conditions **(Figure 3.26)**. There was only one protein uniquely expressed under non-stressed conditions, which was annotated as hypothetical protein LOC_ Os11g46060 gi|77552574.

In addition, there were 7 proteins that were identified only under the salt-stressed conditions. These were OSJNBb0032D24.5 gi|32487629, hypothetical protein OsI_25164gi|218199206, Transcription initiation factor TFIID subunit 1 gi|75116324, hypothetical protein OsI_00373 gi|125524397, OSJNBa0083D01.12 gi|39546279, hypothetical protein OsI_14854 gi|125547231 and peroxidase gi|12039346. The list of these uniquely expressed proteins is shown in **Table 6.**

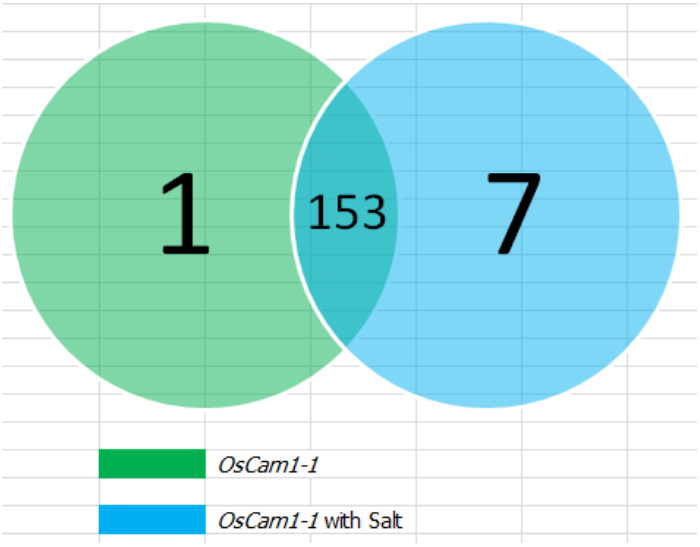


Figure 3.26: Venn diagram of annotated proteins which are differently expressed in shoots of OS under salt stressed conditions

7.3 Comparative protein expression responses among the three rice lines

According to **Figure 3.27**, there are 111 proteins which were normally expressed in roots of all rice lines under the non-stressed conditions. There are 5 proteins, which were only significantly expressed in WT, which involved blight resistance protein gi|57899196, retroelement gi|14091849, ribonuclease gi|259130093, retrotransposon protein, Ty1-copia subclass, expressed gi|108707437 and hypothetical protein LOC_Os12g16530 gi|77554222. There are 8 proteins which were only expressed in VT, which were Os05g0135900 gi|115461955, Os06g0165900 gi|297724507, Os07g0545300 gi|115472631, Os09g0104200 gi|297609027, RNA and export factor binding protein gi|33146678, leucine zipper protein-like gi|49387676, MADS box protein gi|2055376, and hypothetical protein OsI_19435 gi|218196538. In addition, 4 proteins are significantly identified only in OS, which included polyprotein gi|20177631, Os01g0877300 gi|115441387 mytotic checkpoint, Os03g0648100 gi|115454351, and mitogen activated protein kinase gi|55297327.

Figure 3.27: Venn diagram of annotated proteins which are differently expressed in roots under non-stressed conditions

According to **Figure 3.28**, there are 153 proteins which were normally expressed in roots of all rice lines under the salt stressed conditions. There are 4 proteins, which were only significantly expressed in WT, which included homeodomain protein, gi|50872469, Os03g0145200 gi|115450693, Phospholipid-transporting ATPase 1 gi|108707922, and SecA gi|52075758. There are 4 proteins which were only expressed in VT, which were hypothetical protein OsI_19531 gi|218196583, Os09g0395300 gi|115479007, hypothetical protein OsJ_02067 gi|222618593 and BRI1-KD interacting protein 103 gi|50252361. In addition, 3 proteins are significantly identified only in OS, which included Os05g0440000 gi|115464133, hypothetical protein OsJ_17641 gi|222630706, and Ankyrin-like protein gi|47848255.

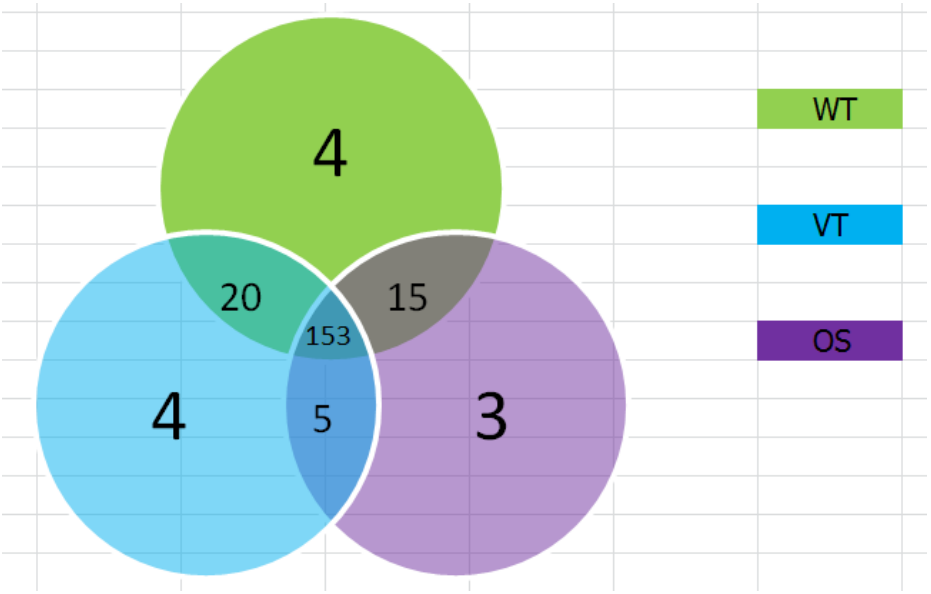


Figure 3.28: Venn diagram of annotated proteins which are differently expressed in roots under salt stressed conditions

According to **Figure 3.29**, there are 123 proteins which were normally expressed in shoots all rice lines under the non-stressed conditions. There are 4 proteins, which were only significantly expressed in WT, which included OSJNBa0083D01.12 gi|39546279, hypothetical protein OsI_14854 gi|125547231, OSJNBa0083N12.7 gi|38345769, and Os03g0277700 gi|115452243. There are 6 proteins which were only expressed in VT, which were O-deacetylbaccatin III-10-0-acetyltransferase gi|19920081, hypothetical protein OsJ_17641 gi|222630706, Os04g0664400 gi|297603514, hypothetical protein LOC_Os11g04630 gi|77548646, hypothetical protein OsI_26986 gi|218200079, and hypothetical protein OsJ_02067 gi|222618593.

In addition, 4 proteins are significantly identified only in OS, which included hypothetical protein OsI_28982 gi|218201046, Os08g0474400 gi|297726519, hypothetical protein LOC_Os11g14700 gi|62733035 and Phospholipid-transporting ATPase 1, expressed gi|108707922.

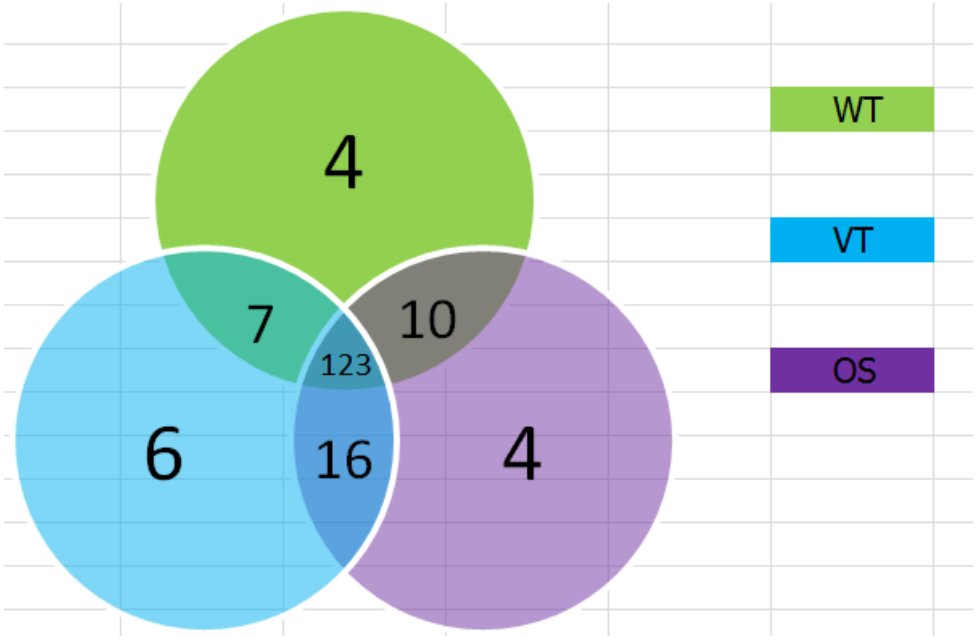


Figure 3.29: Venn diagram of annotated proteins which are differently expressed in shoots under non-stressed conditions

According to **Figure 3.30**, there are 135 proteins which were normally expressed in shoots of all rice lines under the salt stressed conditions. There are only 1 protein, which was only significantly expressed in WT, which was identified as Os03g0277700 gi|115452243. There are 4 proteins which were only expressed in VT, which were Os04g0664400 gi|297603514, hypothetical protein LOC_Os11g04630 gi|77548646, hypothetical protein OsI_26986 gi|218200079 and hypothetical protein OsJ_02067 gi|222618593. In addition, 4 proteins are significantly identified only in OS, which included hypothetical protein OsI_28982 gi|218201046, BRI1-KD interacting protein 103 gi|50252361, Os08g0474400gi|297726519, and hypothetical protein LOC_Os11g14700 gi|62733035.

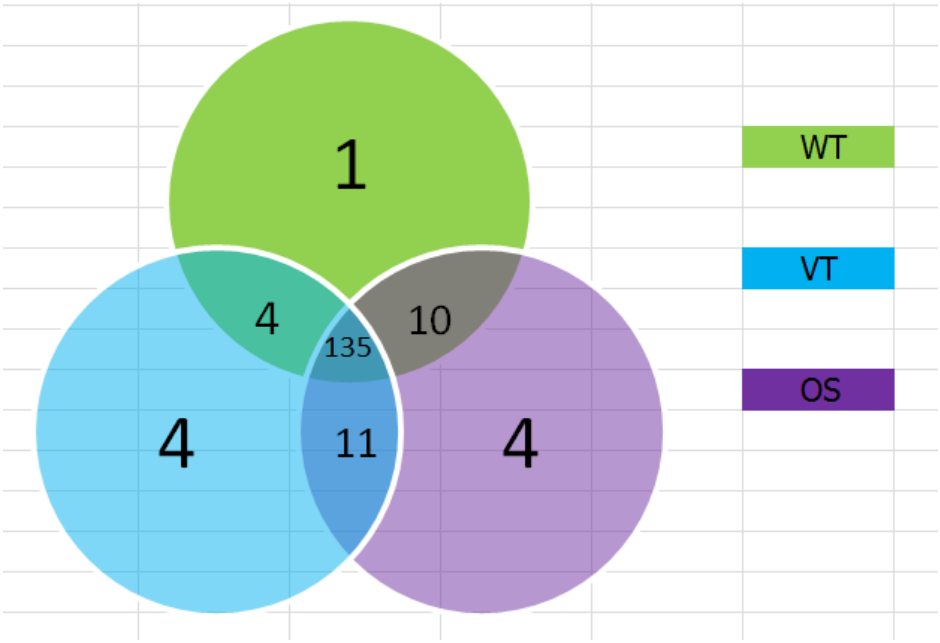


Figure 3.30: Venn diagram of annotated proteins which are differently expressed in shoots under salt stressed conditions

7.4 Analysis of protein expression in root and shoots among WT, VT and OS under salt stress and non-stressed conditions

Protein expression profiles in WT and OS roots under salt stress and non-stressed conditions were compared in order to identify any specific proteins expressed under each condition. Overall, there were 88 ANOVA identified proteins commonly shared in all samples. **(Figure 3.31)** Seventeen proteins were specially identified for WT under salt stressed conditions. Only one protein, namely Os09g0395300 gi|115479007, which was annotated as myb-like DNA-binding domain were uniquely identified in non-stressed OS while 3 proteins were only specifically annotated in salt-stressed OS namely hypothetical protein OsJ_17641 gi|222630706, hypothetical protein OsI_36657 gi|218186020 and Os07g0545300 gi|115472631 which was annotated as Histone H2A

In addition, two commonly shared proteins in the *OsCam 1-1* overexpressing line under normal and stressed conditions were Os09g0395300 gi|115479007 and

Os05g0440000 gi|115464133 which were annotated as myb-like DNA-binding domain and Zinc finger, Dof-type family protein.

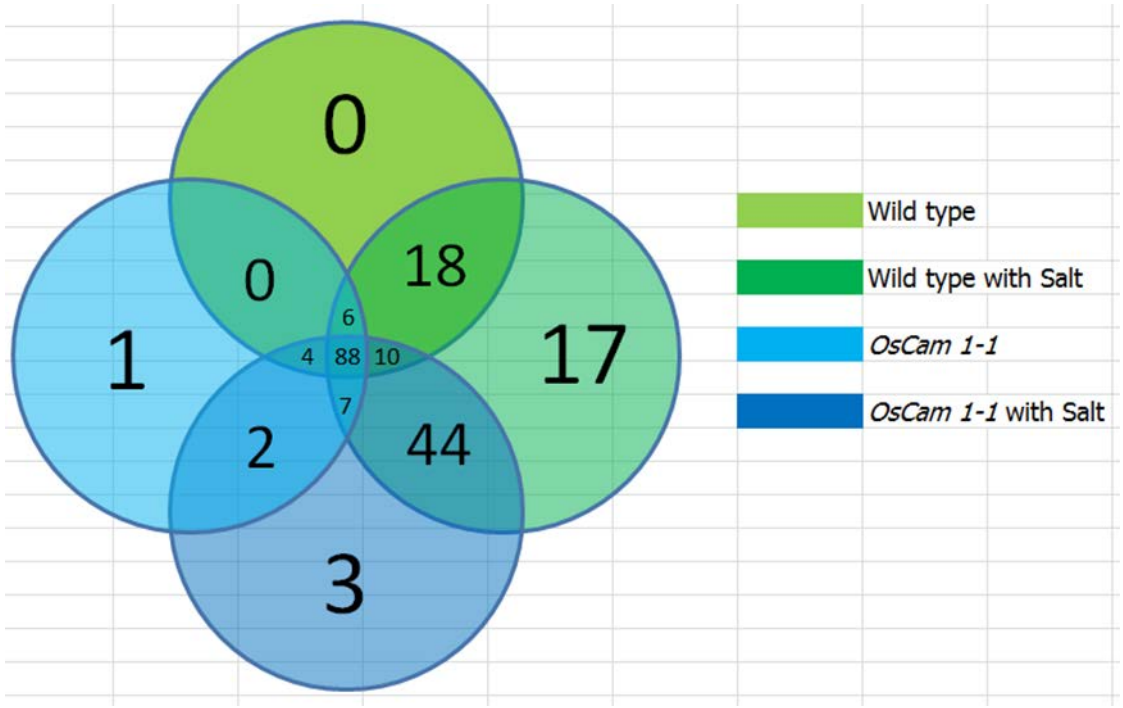


Figure 3.31: Venn diagram of annotated proteins which are differently expressed in roots of WT and OS under non-stressed and stressed conditions

Comparing protein expression profile in the transgenic OS line with the vector control line VT. **(Figure 3.32)** the results showed that 83 ANOVA annotated proteins were commonly expressed in both lines under the non-stressed and stressed conditions. Only one protein, namely leucine zipper protein-like gi|49387676 was specifically expressed in non-stressed VT while 15 proteins were uniquely identified in VT under salt stress. Interestingly, there were 12 proteins specifically identified in OS under salt stress condition which were annotated as OSJNBa0083D01.12 gi|39546279, retrotransposon protein, Ty1-copia subclass gi|108707437, hypothetical protein OsJ_07225 gi|222623105, hypothetical protein OsI_28982 gi|49387758, red chlorophyll catabolite reductase gi|14018063, Os05g0491200 gi|115464617 SNF1 kinase complex anchoring protein, hypothetical protein OsJ_17641 gi|222630706, hypothetical protein LOC_Os12g35790 gi|77556676, Os03g0831900 gi|115456395

Zinc finger U1-type domain containing protein, Leucine Rich Repeat family protein gi|77552081 hypothetical protein LOC_Os03g37850 gi|108709446 and hypothetical protein OsI_14854 gi|125547231.

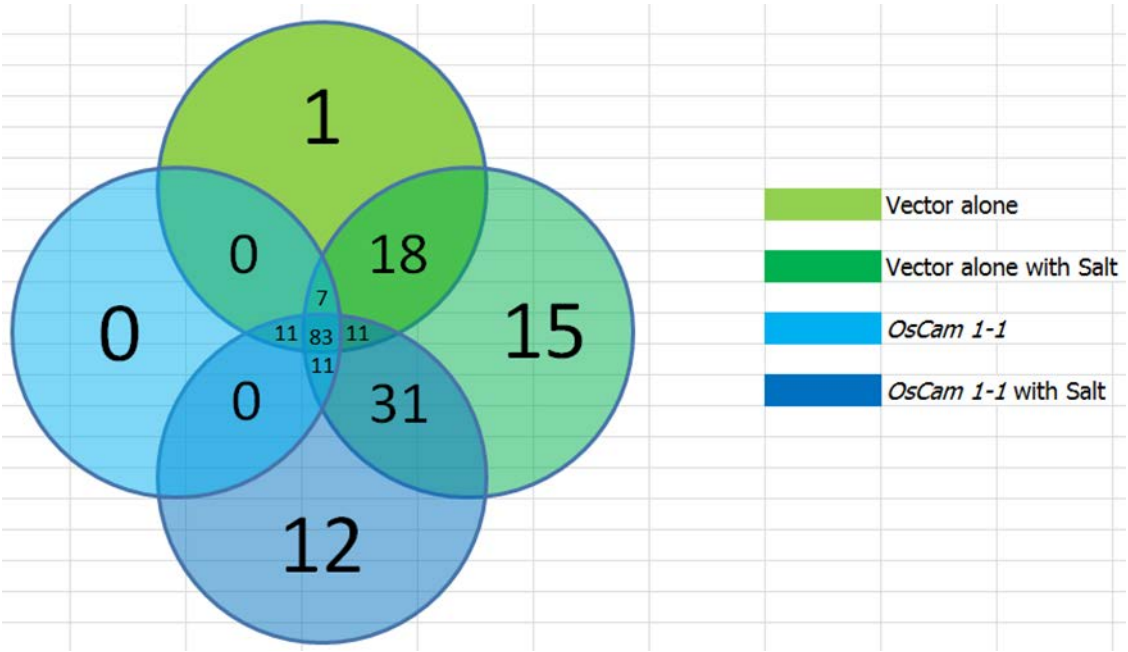


Figure 3.32: Venn diagram of annotated proteins which are differently expressed in roots of VT and OS under non-stressed and stressed conditions

In contrast to protein expression in roots, most ANOVA annotated proteins from WT and OS shoots were commonly expressed in both lines under the non-stressed and stressed conditions. **(Figure 3.33 and 3.34)** Only few proteins were specifically identified in salt stressed OS. Similarly, almost all ANOVA annotated proteins were also shared by VT and OS under the non-stressed and stressed conditions. Only few proteins were shown for specific expression in salt stressed OS.

Focusing on **Figure 3.33**, comparison of the annotated proteins which are differently expressed in shoots of WT and OS under non-stressed and stressed conditions was shown using Venn Diagram. By using rice genome database as mentioned above, 14 commonly shared identified proteins in the *OsCam 1-1* overexpressing line under normal and stressed conditions were annotated as Os07g0546100 gi|115472643 (unknown), hypothetical protein OsI_28982

gi|218201046, Os08g0474400 gi|297726519, F-box domain, cyclin-like domain containing protein, hypothetical protein LOC_Os11g14700 gi|62733035, Phospholipid-transporting ATPase 1 gi|108707922, plastid RNA polymerase sigma factor gi|2780746, P0660F12.14 gi|15290159, Os11g0515000 gi|115485653 Armadillo-like helical domain containing protein, hypothetical protein OsI_19531 gi|218196583, retrotransposon protein, Ty3-gypsy sub-class gi|62733004 Transcription in Nucleus, SCARECROW gene regulator gi|62733159, Os05g0349000 gi|297724023 (unknown), Os05g0440000 gi|115464133 Zinc finger, Dof-type family protein and Ankyrin-like protein gi|47848255.

Figure 3.33: Venn diagram of annotated proteins which are differently expressed in shoots of WT and OS under non-stressed and stressed conditions

Focusing on **Figure 3.34**, comparison of the annotated proteins which are differently expressed in shoots of VT and OS under non-stressed and stressed conditions was shown using Venn Diagram. By using rice genome database as mentioned above, 11 commonly shared identified proteins in the *OsCam 1-1* overexpressing line under normal and stressed conditions were annotated as

hypothetical protein OsI_28982 gi|218201046, BRI1-KD interacting protein 103 gi|50252361, Os08g0474400 gi|297726519 F-box domain, cyclin-like domain containing protein, hypothetical protein LOC_Os11g14700 gi|62733035, Os05g0491200 gi|115464617 SNF1 kinase complex anchoring protein, Os01g0155600 gi|115434616 Splicing factor RSZ33, hypothetical protein LOC_Os12g10470 gi|77553333, retrotransposon protein, Ty1-copia subclass gi|108707437, Os03g0145200 gi|115450693 Zinc finger, NHR/GATA-type domain containing protein, hypothetical protein OsI_23247 gi|125555615 and Os07g0545300 gi|115472631 Histone H2A. In addition, 2 proteins only found in the *OsCam1-1* overexpressing line under salt stressed conditions were OSJNBa0083D01.12 gi|39546279 and hypothetical protein OsI_14854 gi|125547231.

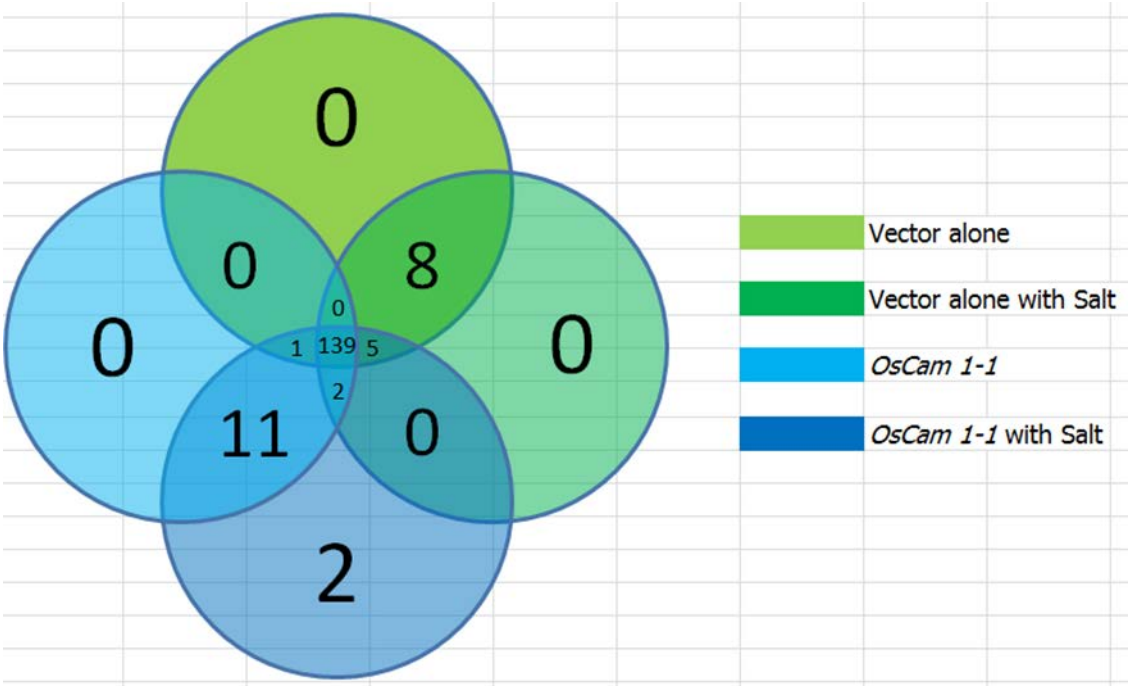


Figure 3.34: Venn diagram of annotated proteins which are differently expressed in shoots of VT and OS under non-stressed and stressed conditions

Overall, there were significant differences in protein expression profiles in WT, VT, and OS under non-stressed and salt stressed conditions. Based on Venn diagram analysis in section 2.1, most of the ANOVA annotated proteins were commonly expressed in all rice lines under both conditions. Overall, there are no remarkable differences between protein expression profiles in these wild-type and transgenic rice lines under both non-stressed and salt-stressed conditions. However, some differences in protein expression were observed between WT, VT, and OS as shown by identification of several proteins uniquely expressed in each line. Salt stress resulted in remarkable changes in protein expression profiles in all rice lines as shown by a large number of induced proteins in responses in salt stress as shown in **section 2.2.** The responses in expression of these proteins were correlated with previous works in rice and other plants, in which salt-stress led to changes in protein expression profiles. The results thus indicate the systematic changes in gene expression in our rice lines which are related to several responsive mechanisms in responses to the increasing salt stress.

Salt stress resulted in different effects on protein expression in roots and shoots of the rice seedlings of the wild-type and the transgenic lines. Under the non-stressed conditions, significant differences in protein profiles in roots and shoots of WT, VT, and OS were observed. Changes in protein profiles were further identified under the salt stress conditions. Many of the ANOVA annotated proteins were specific to roots or shoots. Roots are seen as the plant tissue direct contact to stress and would result in pronounced response to the induced salt stress. Shoots are not in direct contact with the salt stress and the effects of salt stress in shoots are considered as the result of systematic sensing of stress signal in plants. A range of proteins were also specific to shoots in response in salt stress. Induction of expression of several groups of proteins in specific plant tissues in response to salt stress has been previously reported in shoots of rice and other plants. Overall, effects of salt stress specific to roots and shoots were different among the three rice lines. The results thus indicate differences in protein expression specific to roots and shoots of all rice lines in our study in response to salt stress.

In this study, the effects of salt stress on protein profiles were studied in the first 24 h of salt stress induction because our previous study showed that *OsCam1-1* was rapidly and highly increased when exposed to salt stress and slowly decreasing afterward (Phean-opas., *et al.*, 2008 and Saeng-ngam, *et al.*, 2012). However, OsCaM1-1 protein was not identified in proteomics of the OS line. This could be due to the marginal increase in this protein level in the transgenic line, of which its difference in expression level was not observed compared to the WT and VT lines using the proteomic approach. Crosstalk between *OsCam1-1* and other OsCaM protein homologs may also lead to modification of their expression levels to balance calcium equilibrium in plant cells, and thus no marked changes in the overall OsCaM level.

The use of several transgenic lines in the experimental study may exclude misinterpretation of the effects from random gene insertion in the genome of the transgenic rice which might lead to difference in metabolism. This would thus increase the accuracy from data interpretation of the results.

To recapitulate a number of proteins identified as specifically expressed in roots and shoots of OS compared to those of WT or VT in our study, most of proteins were transcription regulatory proteins, involving in regulation of gene expression in nucleus and also in other organelles. Several families of transcription factors in nucleus were identified involving those functions as zinc finger or leucine zipper proteins, which bind specifically to the target regions or so called, regulatory sites on DNA, resulting in up- or down-regulation of a specific set of proteins under a specific physiological or environmental condition. Sun J. *et al*., 2007 reported that AtSZF1 and AtSZF2, two closely related CCCH-type zinc finger proteins, involved in salt stress responses in Arabidopsis. The expression of AtSZF1 and AtSZF2 is quickly and transiently induced by NaCl treatment. Functions of these proteins resulting from our study complement with those in the Arabidopsis transgenic plants overexpressing Zinc Finger Protein (AtSZF1) and localized in the nucleus (Sun J. *et al.*, 2007 and Wang, M. *et al.*, 2011) which suggesting that genes in this group probably play an important roles in modulating the tolerance to salt stress of rice used in our study.

Furthermore, we also found group of proteins namely “Sigma factors” in our studies which play roles in regulation of gene expression in plant organelles e.g. plastid and mitochondria by binding to RNA polymerases and turning on expression of target genes. Roles of transcription regulatory proteins in controls of genes expression in response to abiotic stress were reported in different plants. Transcription in higher plant plastids is performed by two types of RNA polymerases called NEP and PEP, and expression of photosynthesis genes in chloroplasts is largely dependent on PEP, a eubacteria-type multi-subunit enzyme. For examples, Nagashima A., *et al*., 2004 reported one of the six sigma factors, SIG5, is induced under various stress conditions, such as high salt and high osmotic conditions and postulated that SIG5 protects plants from stresses by enhancing repair of the PSII reaction center**.** In addition, Nikkinen H.L. *et al.*, 2012 and his colleagues reported the 2 sigma factor and concluded that SigB was found to be important for the growth of the cyanobacterium Synechocystis sp. PCC 6803 in high-salt (0.7 m NaCl) stress and play to regulate many salt acclimation processes.

Most of them were identified in OS under both non-stressed and salt stressed conditions, our findings thus suggest the specific expression of several potential transcription regulatory proteins which may involve in control of genes related to salt tolerance mechanisms in rice, particularly for the transgenic OS line.

However, the functions of these proteins need further experimental study in order to specifically annotate their roles in adaptive physiological response to salt stress in the transgenic OS line. In addition to transcription regulatory proteins, some identified proteins specifically expressed in OS were annotated as proteins with specific functions e.g. kinase, and ATPase. Protein bound kinases may involve in the signal cascade in transduction of external salt stress signals to cellular mechanisms as shown in other plants e.g. Baisakh, N. *et al.*, 2012 discovered that salt stress tolerance of rice plants expressing a vacuolar H^+ -ATPase subunit c1 (SaVHAc1) gene from the halophyte grass *Spartina alterniflora* Löisel was enhanced. The increased K(+) /Na(+) ratio and other cations established an ion homoeostasis in SaVHAc1-expressing plants to protect the cytosol from toxic Na(+) and thereby maintained higher chlorophyll retention than the WT plants under salt stress. Besides, the role of SaVHAc1 in cell

wall expansion and maintenance of net photosynthesis was implicated by comparatively higher root and leaf growth and yield of rice expressing SaVHAc1 over WT under salt stress. (Baisakh, N. *et.al*. 2012). ATPases may involve in transport of ions or metabolites and control of osmotic pressure in plant cells in response to salt stress (Golldack D., *et al*., 2001, Lunda C., *et. al*., 2007 and Baisakh N., *et. al*. 2012).

A large fraction of the identified proteins was hypothetical proteins with unknown function, which can be considered as interesting new salt responsive protein candidates for further study. Functions of these proteins can be investigated through several approaches such as gene silencing like RNAi. Advances in bioinformatics strategies can also assist annotation of genes with unknown functions. Altogether this work provides a number of potent protein candidates involving in salt stress response in rice and particularly for the transgenic OS line which are of interest for further study.

Table 2: List of uniquely expressed proteins between shoots and roots of three rice lines under non-stressed conditions

Protein	Accession number	Peptide	MH+ (Da)	No Salt Treatment (- NaCl)					
				Wild-type Shoot	Wild-type Root	Vector alone Shoot	Vector alone Root	*OsCam 1-1* Shoot	*OsCam 1-1* Root
4-alpha-glucanotransferase [Oryza sativa Japonica Group]	gi|22093785	MPSSVSFDSLSPEAAK	1668.000985						
blight resistance protein [Oryza sativa Japonica Group]	gi|57899196	LVLENLIPPR	1163.633924						
cellulase [Oryza sativa Japonica Group]	gi|24476042	SDYRYQPFVSR	1415.835456						
cinnamoyl CoA reductase [Oryza sativa Japonica Group]	gi|51535204	AEGGKMVCVTGAGGFIGSWVVK	2224.855119						
copia-type pol polyprotein [Oryza sativa Japonica Group]	gi|29126367	ASCSKIAPSNEPCAR	1647.785181						
mitogen activated protein kinase [Oryza sativa Japonica Group]	gi|55297327	KHSSLPR	824.6650145						
O-deacetylbaccatin III-10-0-acetyltransferase [Oryza sativa Japonica Group]	gi|19920081	GLPSAPSVTPVR	1180.721465						
peroxidase [Oryza sativa Japonica Group]	gi|12039346	MAAVDVKNGYQGEIR	1668.095297						
retroelement [Oryza sativa Japonica Group]	gi|14091849	GNTGGANPGR	899.9042545						
RNA and export factor binding protein [Oryza sativa Japonica Group]	gi|33146678	AAPPAAR	653.337129						
SecA [Oryza sativa Japonica Group]	gi|52075758	SSSYEDLQEYLRTR	1747.653242						
XS domain containing protein [Oryza sativa Japonica Group]	gi|28875992	ATHLALAR	852.40007						
Ankyrin-like protein [Oryza sativa Japonica Group]	gi|47848255	RGRSLPR	840.7296119						
BRI1-KD interacting protein 103 [Oryza sativa Japonica Group]	gi|50252361	RILSSLNSR	1046.552422						
homeodomain protein, [Oryza sativa Japonica Group]	gi|50872469	AVPGRAARGSAREGWR	1697.390034						
hypothetical protein LOC_Os03g37850 [Oryza sativa Japonica Group]	gi|108709446	GLGGVIGVR	829.0534433						
hypothetical protein LOC_Os11g04630 [Oryza sativa Japonica Group]	gi|77548646	QGGMA	477.9854401						
hypothetical protein LOC_Os11g14700 [Oryza sativa Japonica Group]	gi|62733035	LGSAYRPALR	1045.575078						
hypothetical protein LOC_Os12g10470 [Oryza sativa Japonica Group]	gi|77553333	MDFVFAGKSATAVVIVDLIKK	2267.859685						
hypothetical protein LOC_Os12g16530 [Oryza sativa Japonica Group]	gi|77554222	SGLTPSIPR	926.993523						
hypothetical protein OsI_00373 [Oryza sativa Indica Group]	gi|125524397	AAIVKISLAVALNVR	1537.524495						
hypothetical protein OsI_14854 [Oryza sativa Indica Group]	gi|125547231	MVDSSGDR	881.8990083						
hypothetical protein OsI_19435 [Oryza sativa Indica Group]	gi|218196538	VEAKDSLTIVAR	1302.55001						
hypothetical protein OsI_19531 [Oryza sativa Indica Group]	gi|218196583	GRREAPR	1468.066106						
hypothetical protein OsI_23143 [Oryza sativa Indica Group]	gi|125555508	LPLALAVLGGYLSK	1415.389814						
hypothetical protein OsI_23247 [Oryza sativa Indica Group].	gi|125555615	VSSSA	462.6931378						
hypothetical protein OsI_25164 [Oryza sativa Indica Group]	gi|218199206	KSPSVTPR	870.6611144						
hypothetical protein OsI_26986 [Oryza sativa Indica Group]	gi|218200079	REVAGDRGSR	1065.562661						
hypothetical protein OsI_28982 [Oryza sativa Indica Group].	gi|218201046	IMEAR	635.1744616						
hypothetical protein OsI_36657 [Oryza sativa Indica Group]	gi|218186020	MSTIRGQ	809.8883308						
hypothetical protein OsJ_02067 [Oryza sativa Japonica Group]	gi|222618593	AGEPEMVAKR	1254.604451						
hypothetical protein OsJ_05171 [Oryza sativa Japonica Group]	gi|125580613	LSMLLKDEPELPR	1558.391355						
hypothetical protein OsJ_07225 [Oryza sativa Japonica Group]	gi|222623105	DQVSQR	730.5290261						
hypothetical protein OsJ_10072 [Oryza sativa Japonica Group]	gi|222624530	SPADLSPR	807.2833461						
hypothetical protein OsJ_14601 [Oryza sativa Japonica Group]	gi|125590203	GMPMAPGPR	929.8296938						
hypothetical protein OsJ_17641 [Oryza sativa Japonica Group]	gi|222630706	ERGSGARGRR	830.6455516						
hypothetical protein OsJ_21286 [Oryza sativa Japonica Group]	gi|222635541	VASNISQIFERGR	1477.172629						
hypothetical protein OsJ_28947 [Oryza sativa Japonica Group]	gi|222641370	LICTDFVAISTDVICNWGK	2210.497578						
hypothetical protein OsJ_30129 [Oryza sativa Japonica Group]	gi|222641983	MPPDQLMVMSK	1277.778365						

Table 2: List of uniquely expressed proteins between shoots and roots of three rice lines under non-stressed conditions (cont.)

Protein	Accession number	Peptide	MH+ (Da)	No Salt Treatment (- NaCl)					
				Wild-type Shoot	Wild-type Root	Vector alone Shoot	Vector alone Root	*OsCam 1-1* Shoot	*OsCam 1-1* Root
leucine zipper protein-like [Oryza sativa Japonica Group]	gi|49387676	RLTLSPR	872.6587021						
MADS box protein [Oryza sativa]	gi|2055376	EQMLCEANKCLR	1566.544826						
Os01g0155600 [Oryza sativa Japonica Group].	gi|115434616	GSASP	418.0580906						
Os01g0183600 [Oryza sativa Japonica Group]	gi|115434940	AAVAASTSRK	962.0815983						
Os01g0207200 [Oryza sativa Japonica Group]	gi|115435180	SCTAEPR	819.4359918						
Os01g0805100 [Oryza sativa Japonica Group].	gi|297597805	VAPTK	514.199155						
Os01g0877300 [Oryza sativa Japonica Group]	gi|115441387	SSHTNVELLK	1128.774552						
Os02g0654000 [Oryza sativa Japonica Group]	gi|297599699	AGGTQR	587.6783342						
Os03g0145200 [Oryza sativa Japonica Group]	gi|115450693	VMEMRLQSEEDASAASR	1941.644578						
Os03g0277700 [Oryza sativa Japonica Group]	gi|115452243	AIISSK	619.1910151						
Os03g0389100 [Oryza sativa Japonica Group].	gi|115453337	GDTSR	521.1275759						
Os03g0648100 [Oryza sativa Japonica Group]	gi|115454351	LPCLIK	742.3009706						
Os04g0486400 [Oryza sativa Japonica Group]	gi|115459074	AAASGGGGGPKK	956.4526994						
Os04g0664400 [Oryza sativa Japonica Group]	gi|297603514	GEPRGK	642.6777901						
Os05g0135900 [Oryza sativa Japonica Group]	gi|115461955	ILSDVSPR	887.7828578						
Os05g0320650 [Oryza sativa Japonica Group]	gi|297723967	QTWPELLGK	1071.977367						
Os05g0349000 [Oryza sativa Japonica Group]	gi|297724023	YSISTK	697.5231268						
Os05g0491200 [Oryza sativa Japonica Group]	gi|115464617	MGNASGK	679.8321254						
Os06g0165900 [Oryza sativa Japonica Group]	gi|297724507	EREGDRIQR	1158.603726						
Os07g0113700 [Oryza sativa Japonica Group]	gi|115470301	GIAQVNEGR	943.6046984						
Os07g0545300 [Oryza sativa Japonica Group].	gi|115472631	AGSSK	450.62655						
Os07g0546100 [Oryza sativa Japonica Group].	gi|115472643	SEFAK	581.3644419						
Os07g0664000 [Oryza sativa Japonica Group]	gi|297607764	ATVIAIVR	821.3371472						
Os08g0474400 [Oryza sativa Japonica Group]	gi|297726519	TVLAGLDNDK	725.6195699						
Os08g0513600 [Oryza sativa Japonica Group]	gi|115477260	QAAELAIR	870.5101805						
Os09g0104200 [Oryza sativa Japonica Group]	gi|297609027	FAVPS	519.3124499						
Os09g0395300 [Oryza sativa Japonica Group]	gi|115479007	CEPASLDQK	823.9240272						
Os09g0453300 [Oryza sativa Japonica Group].	gi|115479499	GSRAS	477.9842623						
Os11g0425600 [Oryza sativa Japonica Group]	gi|115485265	AGGAKK	531.5179615						
Os11g0515000 [Oryza sativa Japonica Group]	gi|115485653	DVGDAIPR	1340.135921						
OSJNBa0083D01.12 [Oryza sativa Japonica Group]	gi|39546279	KVPKPTSSKKTSQPK	1640.688396						
OSJNBa0083N12.7 [Oryza sativa Japonica Group].	gi|38345769	NPSAK	515.4798734						
OSJNBb0028M18.4 [Oryza sativa Japonica Group].	gi|38345641	EMGAK	552.0264168						
OSJNBb0032D24.5 [Oryza sativa Japonica Group]	gi|32487629	AKVPIGK	711.9054181						
OSJNBb0072M01.4 [Oryza sativa Japonica Group]	gi|32482939	IAISLGIRRLIVR	1479.914369						
P0660F12.14 [Oryza sativa Japonica Group].	gi|15290159	GPVGK	457.4027818						
Phospholipid-transporting ATPase 1, , expressed [Oryza sativa Japonica Group]	gi|108707922	SLRSVGDMPSVTFAGDMR	1941.079769						
plastid RNA polymerase sigma factor [Oryza sativa Japonica Group].	gi|2780746	QGVSR	545.7428184						
receptor protein kinase-like [Oryza sativa Japonica Group]	gi|56202196	HVLDSGDVRSLVDAR	1640.658256						

Table 2: List of uniquely expressed proteins between shoots and roots of three rice lines under non-stressed conditions (cont.)

Protein	Accession number	Peptide	MH+ (Da)	No Salt Treatment (- NaCl)					
				Wild-type Shoot	Wild-type Root	Vector alone Shoot	Vector alone Root	*OsCam 1-1* Shoot	*OsCam 1-1* Root
retrotransposon protein, , Ty1-copia subclass, expressed [Oryza sativa Japonica Group]	gi|108707437	EASSSAAMADRGR	1310.149114						
retrotransposon protein, , Ty3-gypsy sub-class [Oryza sativa Japonica Group]	gi|62733004	NRWTKWR	1046.296405						
retrotransposon protein, , unclassified [Oryza sativa Japonica Group]	gi|77555660	ATAGT	421.3796022						
ribonuclease, partial [Oryza sativa Japonica Group].	gi|259130093	TYTLG	553.7022193						
succinyl-CoA ligase [ADP-forming] subunit alpha, mitochondrial	gi|75294330	GTAQDK	619.5091243						
Transcription initiation factor TFIID subunit 1	gi|75116324	SHAKK	571.3825952						
translational activator protein-like [Oryza sativa Japonica Group]	gi|50251987	YKAGLK	870.1734249						
transposon protein, , CACTA, En/Spm sub-class [Oryza sativa Japonica Group]	gi|77552302	MYANKK	772.0774301						

Table 3: List of uniquely expressed proteins between shoots and roots of three rice lines under stressed conditions

Protein	Accession number	Peptide	MH+ (Da)	Salt Treatment (+ NaCl)					
				Wild-type Shoot	Wild-type Root	Vector alone Shoot	Vector alone Root	*OsCam 1-1* Shoot	*OsCam 1-1* Root
cinnamoyl CoA reductase [Oryza sativa Japonica Group]	gi|51535204	AEGGKMVCVTGAGGFIGSWVVK	2224.855119		■		■	■	
copia-type pol polyprotein [Oryza sativa Japonica Group]	gi|29126367	ASCSKIAPSNEPCAR	1647.785181					■	■
cytochrome P450 [Oryza sativa Japonica Group]	gi|47777427	GASFRAAMSELIGDGLFAADGR	2210.827484		■		■		
mitogen activated protein kinase [Oryza sativa Japonica Group]	gi|55297327	KHSSLPR	824.6650145		■		■	■	
MtN21 [Oryza sativa Japonica Group]	gi|50508502	MSSGGAK	653.2204316			■			
O-deacetylbaccatin III-10-0-acetyltransferase [Oryza sativa Japonica Group]	gi|19920081	GLPSAPSVTPVR	1180.721465					■	
peroxidase [Oryza sativa Japonica Group]	gi|12039346	MAAVDVKNGYQGEIR	1668.095297						■
polyprotein [Oryza sativa Japonica Group]	gi|20177631	GLDSDGEK	819.4328523		■		■		
pre-mRNA-splicing factor cwc-22 [Oryza sativa Indica Group]	gi|156622338	HDDSPPR	1289.876321						■
receptor kinase [Oryza sativa Japonica Group]	gi|52077114	GHAFHPAPLR	1101.928612		■		■		
red chlorophyll catabolite reductase [Oryza sativa Japonica Group]	gi|14018063	VPAPPR	635.248504		■			■	
retroelement [Oryza sativa Japonica Group]	gi|14091849	GNTGGANPGR	899.9042545		■		■	■	
SecA [Oryza sativa Japonica Group]	gi|52075758	SSSYEDLQEYLRTR	1747.653242			■			■
Aquaporin NIP3-2	gi|75295453	MEGGKMSSMGMDAASASVTVPPMQMQAGDQSNE	3435.28145		■		■		
BRI1-KD interacting protein 103 [Oryza sativa Japonica Group]	gi|50252361	RILSSLNSR	1046.552422				■		■
homeodomain protein, [Oryza sativa Japonica Group]	gi|50872469	AVPGRAARGSAREGWR	1697.390034			■			■
hypothetical protein LOC_Os03g37850 [Oryza sativa Japonica Group]	gi|108709446	GLGGVIGVR	829.0534433			■			
hypothetical protein LOC_Os11g04630 [Oryza sativa Japonica Group]	gi|77548646	QGGMA	477.9854401		■	■		■	
hypothetical protein LOC_Os11g14700 [Oryza sativa Japonica Group]	gi|62733035	LGSAYRPALR	1045.575078						■
hypothetical protein LOC_Os11g46060 [Oryza sativa Japonica Group]	gi|77552574	HGGIGDGGR	717.4504641				■		
hypothetical protein LOC_Os12g10470 [Oryza sativa Japonica Group]	gi|77553333	MDFVFAGKSATAVVIVDLIKK	2267.859685				■		
hypothetical protein LOC_Os12g35790 [Oryza sativa Japonica Group]	gi|77556676	LMGASPMDK	981.1343585		■			■	
hypothetical protein OsI_07204 [Oryza sativa Indica Group]	gi|218190738	HGFGSAGPCAS	1049.031069		■		■	■	
hypothetical protein OsI_08502 [Oryza sativa Indica Group]	gi|125540708	AQLTSAVIADVIR	1356.684739		■		■		
hypothetical protein OsI_13807 [Oryza sativa Indica Group]	gi|218193866	IDGDSPPR	856.0064791		■		■		
hypothetical protein OsI_19435 [Oryza sativa Indica Group]	gi|218196538	VEAKDSLTIVAR	1302.55001		■		■	■	
hypothetical protein OsI_19531 [Oryza sativa Indica Group]	gi|218196583	GRREAPR	1468.066106						■
hypothetical protein OsI_23247 [Oryza sativa Indica Group].	gi|125555615	VSSSA	462.6931378				■		
hypothetical protein OsI_25902 [Oryza sativa Indica Group]	gi|125558238	SMALGCTAKLGVADAIHRAGGR	2210.451172		■		■	■	
hypothetical protein OsI_26986 [Oryza sativa Indica Group]	gi|218200079	REVAGDRGSR	1065.562661			■			
hypothetical protein OsI_28982 [Oryza sativa Indica Group].	gi|218201046	IMEAR	635.1744616		■		■	■	
hypothetical protein OsI_28982 [Oryza sativa Indica Group].	gi|49387758	TPKGN	470.802164		■				
hypothetical protein OsI_36657 [Oryza sativa Indica Group]	gi|218186020	MSTIRGQ	809.8883308	■					
hypothetical protein OsJ_06402 [Oryza sativa Japonica Group]	gi|222622678	ATDALVQK	844.7946465		■		■		
hypothetical protein OsJ_07225 [Oryza sativa Japonica Group]	gi|222623105	DQVSQR	730.5290261			■			
hypothetical protein OsJ_10072 [Oryza sativa Japonica Group]	gi|222624530	SPADLSPR	807.2833461	■		■			■
hypothetical protein OsJ_17641 [Oryza sativa Japonica Group]	gi|222630706	ERGSGARGRR	830.6455516	■		■		■	
hypothetical protein OsJ_23643 [Oryza sativa Japonica Group]	gi|222636720	KPCHPLPEKK	1231.817228		■		■	■	
hypothetical protein OsJ_28947 [Oryza sativa Japonica Group]	gi|222641370	LICTDFVAISTDVICNWGK	2210.497578		■		■	■	

Table 3: List of uniquely expressed proteins between shoots and roots of three rice lines under stressed conditions (cont.)

Protein	Accession number	Peptide	MH+ (Da)	Salt Treatment (+ NaCl)					
				Wild-type Shoot	Wild-type Root	Vector alone Shoot	Vector alone Root	*OsCam 1-1* Shoot	*OsCam 1-1* Root
hypothetical protein OsJ_31157 [Oryza sativa Japonica Group]	gi|222612660	RRGLWWPTPPR	1421.184935						
Leucine Rich Repeat family protein, expressed [Oryza sativa Japonica Group]	gi|77552081	IIINDSIR	944.5886482						
leucine zipper protein-like [Oryza sativa Japonica Group]	gi|49387676	RLTLSPR	872.6587021						
MADS box protein [Oryza sativa]	gi|2055376	EQMLCEANKCLR	1566.544826						
OJ000315_02.21 [Oryza sativa Japonica Group]	gi|32487917	LHATVESDKLLVR	1479.544231						
Os01g0155600 [Oryza sativa Japonica Group]	gi|115434616	GSASP	418.0580906						
Os01g0207200 [Oryza sativa Japonica Group]	gi|115435180	SCTAEPR	819.4359918						
Os01g0697100 [Oryza sativa Japonica Group]	gi|115439381	EAASA	449.3706143						
Os02g0173500 [Oryza sativa Japonica Group]	gi|115444521	KLGKFKETQR	1235.37737						
Os02g0654000 [Oryza sativa Japonica Group]	gi|297599699	AGGTQR	587.6783342						
Os02g0709900 [Oryza sativa Japonica Group]	gi|115448227	LEIEGK	688.8466036						
Os02g0715000 [Oryza sativa Japonica Group]	gi|115448287	MAGNNSLLAMDSK	1383.843437						
Os03g0132600 [Oryza sativa Japonica Group]	gi|297721777	AAMDR	578.6281201						
Os03g0145200 [Oryza sativa Japonica Group]	gi|115450693	VMEMRLQSEEDASAASR	1941.644578						
Os03g0277700 [Oryza sativa Japonica Group]	gi|115452243	AIISSK	619.1910151						
Os03g0369100 [Oryza sativa Japonica Group]	gi|115453181	VVYVAK	679.5140827						
Os03g0389100 [Oryza sativa Japonica Group]	gi|115453337	GDTSR	521.1275759						
Os03g0831900 [Oryza sativa Japonica Group]	gi|115456395	GPPVQR	653.8407817						
Os04g0664400 [Oryza sativa Japonica Group]	gi|297603514	GEPRGK	642.6777901						
Os05g0349000 [Oryza sativa Japonica Group]	gi|297724023	YSISTK	697.5231268						
Os05g0440000 [Oryza sativa Japonica Group]	gi|115464133	GGGGGGAAAFSHR	1262.627846						
Os06g0106900 [Oryza sativa Japonica Group]	gi|115465932	MDKAGGNQGGKVLK	1418.48031						
Os06g0165900 [Oryza sativa Japonica Group]	gi|297724507	EREGDRIQR	1158.603726						
Os06g0214900 [Oryza sativa Japonica Group]	gi|115467068	LGGTPMKFGLPSRLFR	1794.332029						
Os07g0194000 [Oryza sativa Japonica Group]	gi|115471021	SQAQDFRQQGTK	1393.909319						
Os07g0545300 [Oryza sativa Japonica Group]	gi|115472631	AGSSK	450.62655						
Os07g0546100 [Oryza sativa Japonica Group]	gi|115472643	SEFAK	581.3644419						
Os07g0664000 [Oryza sativa Japonica Group]	gi|297607764	ATVIAIVR	821.3371472						
Os08g0469500 [Oryza sativa Japonica Group]	gi|115476832	NSLKNLDSR	1033.441161						
Os08g0474400 [Oryza sativa Japonica Group]	gi|297726519	TVLAGLDNDK	725.6195699						
Os08g0513600 [Oryza sativa Japonica Group]	gi|115477260	QAAELAIR	870.5101805						
Os09g0395300 [Oryza sativa Japonica Group]	gi|115479007	CEPASLDQK	823.9240272						
Os10g0485800 [Oryza sativa Japonica Group]	gi|297727701	RWAASPPTAAR	1183.023685						
Os11g0158300 [Oryza sativa Japonica Group]	gi|115484287	VHGRVDVAGRVHGLMR	1772.378037						
Os11g0556400 [Oryza sativa Japonica Group]	gi|115485891	MNKSGK	679.509904						
Os12g0246700 [Oryza sativa Japonica Group]	gi|115488014	KETPSVELLK	1142.517916						
OSJNBa0041A02.6 [Oryza sativa Japonica Group]	gi|38344139	MLDDITR	878.9971547						
OSJNBa0057M08.7 [Oryza sativa Japonica Group]	gi|58532075	AAPLPAER	824.7667881						
OSJNBa0083N12.7 [Oryza sativa Japonica Group]	gi|38345769	NPSAK	515.4798734						

Table 3: List of uniquely expressed proteins between shoots and roots of three rice lines under stressed conditions (cont.)

Protein	Accession number	Peptide	MH+ (Da)	Salt Treatment (+ NaCl)					
				Wild-type Shoot	Wild-type Root	Vector alone Shoot	Vector alone Root	*OsCam 1-1* Shoot	*OsCam 1-1* Root
OSJNBb0028M18.4 [Oryza sativa Japonica Group].	gi|38345641	EMGAK	552.0264168						
P0660F12.14 [Oryza sativa Japonica Group].	gi|15290159	GPVGK	457.4027818						
Phospholipid-transporting ATPase 1, , expressed [Oryza sativa Japonica Group]	gi|108707922	SLRSVGDMPSVTFAGDMR	1941.079769						
plastid RNA polymerase sigma factor [Oryza sativa Japonica Group].	gi|2780746	QGVSR	545.7428184						
receptor protein kinase-like [Oryza sativa Japonica Group]	gi|56202196	HVLDSGDVRSLVDAR	1640.658256						
retrotransposon protein, , Ty3-gypsy sub-class [Oryza sativa Japonica Group]	gi|62733004	NRWTKWR	1046.296405						
SCARECROW gene regulator, [Oryza sativa Japonica Group]	gi|62733159	SATELLKQIKQNSSAR	1201.493043						
succinyl-CoA ligase [ADP-forming] subunit alpha, mitochondrial	gi|75294330	GTAQDK	619.5091243						

Table 4: List of uniquely expressed proteins in identification of biomarkers in roots of three rice lines under non-stressed and salt stressed conditions

Protein	Accession number	Peptide	MH+ (Da)	WT	WT (+Salt)	VT	VT (+Salt)	OS	OS (+Salt)
4-alpha-glucanotransferase [Oryza sativa Japonica Group]	gi|22093785	MPSSVSFDSLSPEAAK	1668.000985						
blight resistance protein [Oryza sativa Japonica Group]	gi|57899196	LVLENLIPPR	1163.633924						
cellulase [Oryza sativa Japonica Group]	gi|24476042	SDYRYQPFVSR	1415.835456						
cinnamoyl CoA reductase [Oryza sativa Japonica Group]	gi|51535204	AEGGKMVCVTGAGGFIGSWVVK	2224.855119						
copia-type pol polyprotein [Oryza sativa Japonica Group]	gi|29126367	ASCSKIAPSNEPCAR	1647.785181						
cytochrome P450 [Oryza sativa Japonica Group]	gi|47777427	GASFRAAMSELIGDGLFAADGR	2210.827484						
mitogen activated protein kinase [Oryza sativa Japonica Group]	gi|55297327	KHSSLPR	824.6650145						
MtN21 [Oryza sativa Japonica Group]	gi|50508502	MSSGGAK	653.2204316						
O-deacetylbaccatin III-10-0-acetyltransferase [Oryza sativa Japonica Group]	gi|19920081	GLPSAPSVTPVR	1180.721465						
peroxidase [Oryza sativa Japonica Group]	gi|12039346	MAAVDVKNGYQGEIR	1668.095297						
polyprotein [Oryza sativa Japonica Group]	gi|20177631	GLDSDGEK	819.4328523						
pre-mRNA-splicing factor cwc-22 [Oryza sativa Indica Group]	gi|156622338	HDDSPPR	1289.876321						
receptor kinase [Oryza sativa Japonica Group]	gi|52077114	GHAFHPAPLR	1101.928612						
red chlorophyll catabolite reductase [Oryza sativa Japonica Group]	gi|14018063	VPAPPR	635.248504						
retroelement [Oryza sativa Japonica Group]	gi|14091849	GNTGGANPGR	899.9042545						
RNA and export factor binding protein [Oryza sativa Japonica Group]	gi|33146678	AAPPAAR	653.337129						
SecA [Oryza sativa Japonica Group]	gi|52075758	SSSYEDLQEYLRTR	1747.653242						
XS domain containing protein [Oryza sativa Japonica Group]	gi|28875992	ATHLALAR	852.40007						
Ankyrin-like protein [Oryza sativa Japonica Group]	gi|47848255	RGRSLPR	840.7296119						
Aquaporin NIP3-2	gi|75295453	MEGGKMSSMGMDAASASVTVPPMQMQAGDQSNR	3435.28145						
BRI1-KD interacting protein 103 [Oryza sativa Japonica Group]	gi|50252361	RILSSLNSR	1046.552422						
Cytochrome P450 family protein, expressed [Oryza sativa Japonica Group]	gi|108706099	ATMASC	654.8040849						
homeodomain protein, [Oryza sativa Japonica Group]	gi|50872469	AVPGRAARGSAREGWR	1697.390034						
hypothetical protein LOC_Os03g37850 [Oryza sativa Japonica Group]	gi|108709446	GLGGVIGVR	829.0534433						
hypothetical protein LOC_Os11g04630 [Oryza sativa Japonica Group]	gi|77548646	QGGMA	477.9854401						
hypothetical protein LOC_Os12g10470 [Oryza sativa Japonica Group]	gi|77553333	MDFVFAGKSATAVVIVDLIKK	2267.859685						
hypothetical protein LOC_Os12g16530 [Oryza sativa Japonica Group]	gi|77554222	SGLTPSIPR	926.993523						
hypothetical protein LOC_Os12g35790 [Oryza sativa Japonica Group]	gi|77556676	LMGASPMDK	981.1343585						
hypothetical protein OsI_00373 [Oryza sativa Indica Group]	gi|125524397	AAIVKISLAVALNVR	1537.524495						
hypothetical protein OsI_07204 [Oryza sativa Indica Group]	gi|218190738	HGFGSAGPCAS	1049.031069						
hypothetical protein OsI_08502 [Oryza sativa Indica Group]	gi|125540708	AQLTSAVIADVIR	1356.684739						
hypothetical protein OsI_13807 [Oryza sativa Indica Group]	gi|218193866	IDGDSPPR	856.0064791						
hypothetical protein OsI_14854 [Oryza sativa Indica Group]	gi|125547231	MVDSSGDR	881.8990083						
hypothetical protein OsI_19435 [Oryza sativa Indica Group]	gi|218196538	VEAKDSLTIVAR	1302.55001						
hypothetical protein OsI_19531 [Oryza sativa Indica Group]	gi|218196583	GRREAPR	1468.066106						
hypothetical protein OsI_23143 [Oryza sativa Indica Group]	gi|125555508	LPLALAVLGGYLSK	1415.389814						
hypothetical protein OsI_25902 [Oryza sativa Indica Group]	gi|125558238	SMALGCTAKLGVADAIHRAGGR	2210.451172						
hypothetical protein OsI_28982 [Oryza sativa Indica Group].	gi|218201046	IMEAR	635.1744616						

Table 4: List of uniquely expressed proteins in identification of biomarkers in roots of three rice lines under non-stressed and salt stressed conditions (cont.)

Protein	Accession number	Peptide	MH+ (Da)	WT	WT (+Salt)	VT	VT (+Salt)	OS	OS (+Salt)
hypothetical protein OsI_36657 [Oryza sativa Indica Group]	gi|218186020	MSTIRGQ	809.8883308						
hypothetical protein OsJ_02067 [Oryza sativa Japonica Group]	gi|222618593	AGEPEMVAKR	1254.604451						
hypothetical protein OsJ_05171 [Oryza sativa Japonica Group]	gi|125580613	LSMLLKDEPELPR	1558.391355						
hypothetical protein OsJ_06402 [Oryza sativa Japonica Group]	gi|222622678	ATDALVQK	844.7946465						
hypothetical protein OsJ_07225 [Oryza sativa Japonica Group]	gi|222623105	DQVSQR	730.5290261						
hypothetical protein OsJ_14601 [Oryza sativa Japonica Group]	gi|125590203	GMPMAPGPR	929.8296938						
hypothetical protein OsJ_17641 [Oryza sativa Japonica Group]	gi|222630706	ERGSGARGRR	830.6455516						
hypothetical protein OsJ_21286 [Oryza sativa Japonica Group]	gi|222635541	VASNISQIFERGR	1477.172629						
hypothetical protein OsJ_23643 [Oryza sativa Japonica Group]	gi|222636720	KPCHPLPEKK	1231.817228						
hypothetical protein OsJ_28947 [Oryza sativa Japonica Group]	gi|222641370	LICTDFVAISTDVICNWGK	2210.497578						
hypothetical protein OsJ_30129 [Oryza sativa Japonica Group]	gi|222641983	MPPDQLMVMSK	1277.778365						
hypothetical protein OsJ_31157 [Oryza sativa Japonica Group]	gi|222612660	RRGLWWPTPPR	1421.184935						
Leucine Rich Repeat family protein, expressed [Oryza sativa Japonica Group]	gi|77552081	IIINDSIR	944.5886482						
leucine zipper protein-like [Oryza sativa Japonica Group]	gi|49387676	RLTLSPR	872.6587021						
MADS box protein [Oryza sativa]	gi|2055376	EQMLCEANKCLR	1566.544826						
OJ000315_02.21 [Oryza sativa Japonica Group]	gi|32487917	LHATVESDKLLVR	1479.544231						
Os01g0183600 [Oryza sativa Japonica Group]	gi|115434940	AAVAASTSRK	962.0815983						
Os01g0697100 [Oryza sativa Japonica Group].	gi|115439381	EAASA	449.3706143						
Os01g0805100 [Oryza sativa Japonica Group].	gi|297597805	VAPTK	514.199155						
Os01g0877300 [Oryza sativa Japonica Group]	gi|115441387	SSHTNVELLK	1128.774552						
Os02g0173500 [Oryza sativa Japonica Group]	gi|115444521	KLGKFKETQR	1235.37737						
Os02g0709900 [Oryza sativa Japonica Group]	gi|115448227	LEIEGK	688.8466036						
Os02g0715000 [Oryza sativa Japonica Group]	gi|115448287	MAGNNSLLAMDSK	1383.843437						
Os03g0132600 [Oryza sativa Japonica Group]	gi|297721777	AAMDR	578.6281201						
Os03g0145200 [Oryza sativa Japonica Group]	gi|115450693	VMEMRLQSEEDASAASR	1941.644578						
Os03g0389100 [Oryza sativa Japonica Group].	gi|115453337	GDTSR	521.1275759						
Os03g0648100 [Oryza sativa Japonica Group]	gi|115454351	LPCLIK	742.3009706						
Os03g0831900 [Oryza sativa Japonica Group]	gi|115456395	GPPVQR	653.8407817						
Os04g0486400 [Oryza sativa Japonica Group]	gi|115459074	AAASGGGGGPKK	956.4526994						
Os04g0664400 [Oryza sativa Japonica Group]	gi|297603514	GEPRGK	642.6777901						
Os05g0135900 [Oryza sativa Japonica Group]	gi|115461955	ILSDVSPR	887.7828578						
Os05g0320650 [Oryza sativa Japonica Group]	gi|297723967	QTWPELLGK	1071.977367						
Os05g0349000 [Oryza sativa Japonica Group]	gi|297724023	YSISTK	697.5231268						
Os05g0440000 [Oryza sativa Japonica Group]	gi|115464133	GGGGGGAAAFSHR	1262.627846						
Os05g0491200 [Oryza sativa Japonica Group]	gi|115464617	MGNASGK	679.8321254						
Os06g0106900 [Oryza sativa Japonica Group]	gi|115465932	MDKAGGNQGGKVLK	1418.48031						
Os06g0165900 [Oryza sativa Japonica Group]	gi|297724507	EREGDRIQR	1158.603726						
Os06g0214900 [Oryza sativa Japonica Group]	gi|115467068	LGGTPMKFGLPSRLFR	1794.332029						

Table 4: List of uniquely expressed proteins in identification of biomarkers in roots of three rice lines under non-stressed and salt stressed conditions (cont.)

Protein	Accession number	Peptide	MH+ (Da)	WT	WT (+Salt)	VT	VT (+Salt)	OS	OS (+Salt)
Os07g0194000 [Oryza sativa Japonica Group]	gi|115471021	SQAQDFRQQGTK	1393.909319						
Os07g0545300 [Oryza sativa Japonica Group].	gi|115472631	AGSSK	450.62655						
Os07g0664000 [Oryza sativa Japonica Group]	gi|297607764	ATVIAIVR	821.3371472						
Os08g0469500 [Oryza sativa Japonica Group]	gi|115476832	NSLKNLDSR	1033.441161						
Os08g0474400 [Oryza sativa Japonica Group]	gi|297726519	TVLAGLDNDK	725.6195699						
Os09g0104200 [Oryza sativa Japonica Group]	gi|297609027	FAVPS	519.3124499						
Os09g0395300 [Oryza sativa Japonica Group]	gi|115479007	CEPASLDQK	823.9240272						
Os10g0485800 [Oryza sativa Japonica Group]	gi|297727701	RWAASPPTAAR	1183.023685						
Os11g0158300 [Oryza sativa Japonica Group]	gi|115484287	VHGRVDVAGRVHGLMR	1772.378037						
Os11g0515000 [Oryza sativa Japonica Group]	gi|115485653	DVGDAIPR	1340.135921						
Os11g0556400 [Oryza sativa Japonica Group]	gi|115485891	MNKSGK	679.509904						
Os12g0246700 [Oryza sativa Japonica Group]	gi|115488014	KETPSVELLK	1142.517916						
OSJNBa0041A02.6 [Oryza sativa Japonica Group]	gi|38344139	MLDDITR	878.9971547						
OSJNBa0057M08.7 [Oryza sativa Japonica Group]	gi|58532075	AAPLPAER	824.7667881						
OSJNBa0083D01.12 [Oryza sativa Japonica Group]	gi|39546279	KVPKPTSSKKTSQPK	1640.688396						
OSJNBa0083N12.7 [Oryza sativa Japonica Group].	gi|38345769	NPSAK	515.4798734						
OSJNBb0028M18.4 [Oryza sativa Japonica Group].	gi|38345641	EMGAK	552.0264168						
OSJNBb0032D24.5 [Oryza sativa Japonica Group]	gi|32487629	AKVPIGK	711.9054181						
OSJNBb0072M01.4 [Oryza sativa Japonica Group]	gi|32482939	IAISLGIRRLIVR	1479.914369						
Phospholipid-transporting ATPase 1, , expressed [Oryza sativa Japonica Group]	gi|108707922	SLRSVGDMPSVTFAGDMR	1941.079769						
receptor protein kinase-like [Oryza sativa Japonica Group]	gi|56202196	HVLDSGDVRSLVDAR	1640.658256						
retrotransposon protein, , Ty1-copia subclass, expressed [Oryza sativa Japonica Group]	gi|108707437	EASSSAAMADRGR	1310.149114						
retrotransposon protein, , unclassified [Oryza sativa Japonica Group]	gi|77555660	ATAGT	421.3796022						
ribonucleasse, partial [Oryza sativa Japonica Group].	gi|259130093	TYTLG	553.7022193						
SCARECROW gene regulator, [Oryza sativa Japonica Group]	gi|62733159	SATELLKQIKQNSSAR	1201.493043						
succinyl-CoA ligase [ADP-forming] subunit alpha, mitochondrial	gi|75294330	GTAQDK	619.5091243						
translational activator protein-like [Oryza sativa Japonica Group]	gi|50251987	YKAGLK	870.1734249						
transposon protein, , CACTA, En/Spm sub-class [Oryza sativa Japonica Group]	gi|77552302	MYANKK	772.0774301						

Table 5: List of uniquely expressed proteins in identification of biomarkers in shoots of three rice lines under non-stressed and salt stressed conditions

Protein	Accession number	Peptide	MH+ (Da)	WT	WT (+Salt)	VT	VT (+Salt)	OS	OS (+Salt)
O-deacetylbaccatin III-10-0-acetyltransferase [Oryza sativa Japonica Group]	gi|19920081	GLPSAPSVTPVR	1180.721465						
Peroxidase [Oryza sativa Japonica Group]	gi|12039346	MAAVDVKNGYQGEIR	1668.095297						
BRI1-KD interacting protein 103 [Oryza sativa Japonica Group]	gi|50252361	RILSSLNSR	1046.552422						
Cytochrome P450 family protein, expressed [Oryza sativa Japonica Group]	gi|108706099	ATMASC	654.8040849						
hypothetical protein LOC_Os11g46060 [Oryza sativa Japonica Group]	gi|77552574	HGGIGDGGR	717.4504641						
hypothetical protein OsI_00373 [Oryza sativa Indica Group]	gi|125524397	AAIVKISLAVALNVR	1537.524495						
hypothetical protein OsI_14854 [Oryza sativa Indica Group]	gi|125547231	MVDSSGDR	881.8990083						
hypothetical protein OsI_25164 [Oryza sativa Indica Group]	gi|218199206	KSPSVTPR	870.6611144						
hypothetical protein OsJ_17641 [Oryza sativa Japonica Group]	gi|222630706	ERGSGARGRR	830.6455516						
Os05g0135900 [Oryza sativa Japonica Group]	gi|115461955	ILSDVSPR	887.7828578						
Os07g0113700 [Oryza sativa Japonica Group]	gi|115470301	GIAQVNEGR	943.6046984						
Os09g0453300 [Oryza sativa Japonica Group].	gi|115479499	GSRAS	477.9842623						
Os11g0425600 [Oryza sativa Japonica Group]	gi|115485265	AGGAKK	531.5179615						
OSJNBa0083D01.12 [Oryza sativa Japonica Group]	gi|39546279	KVPKPTSSKKTSQPK	1640.688396						
OSJNBa0083N12.7 [Oryza sativa Japonica Group].	gi|38345769	NPSAK	515.4798734						
OSJNBb0032D24.5 [Oryza sativa Japonica Group]	gi|32487629	AKVPIGK	711.9054181						
Phospholipid-transporting ATPase 1, , expressed [Oryza sativa Japonica Group]	gi|108707922	SLRSVGDMPSVTFAGDMR	1941.079769						
Transcription initiation factor TFIID subunit 1	gi|75116324	SHAKK	571.3825952						
Translational activator protein-like [Oryza sativa Japonica Group]	gi|50251987	YKAGLK	870.1734249						

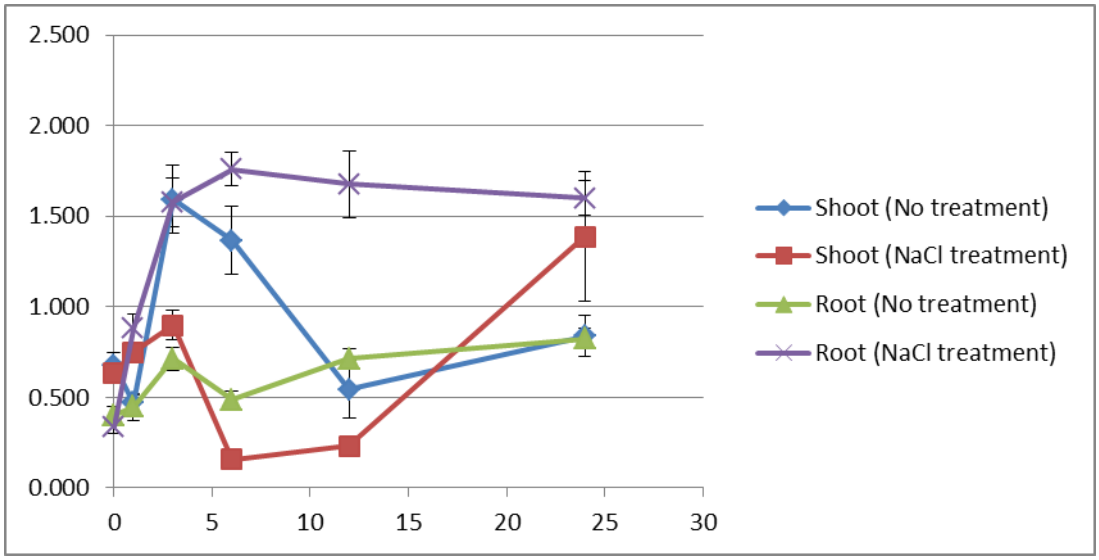
Section 2. Enzyme activity analysis

1. Acidic Peroxidase

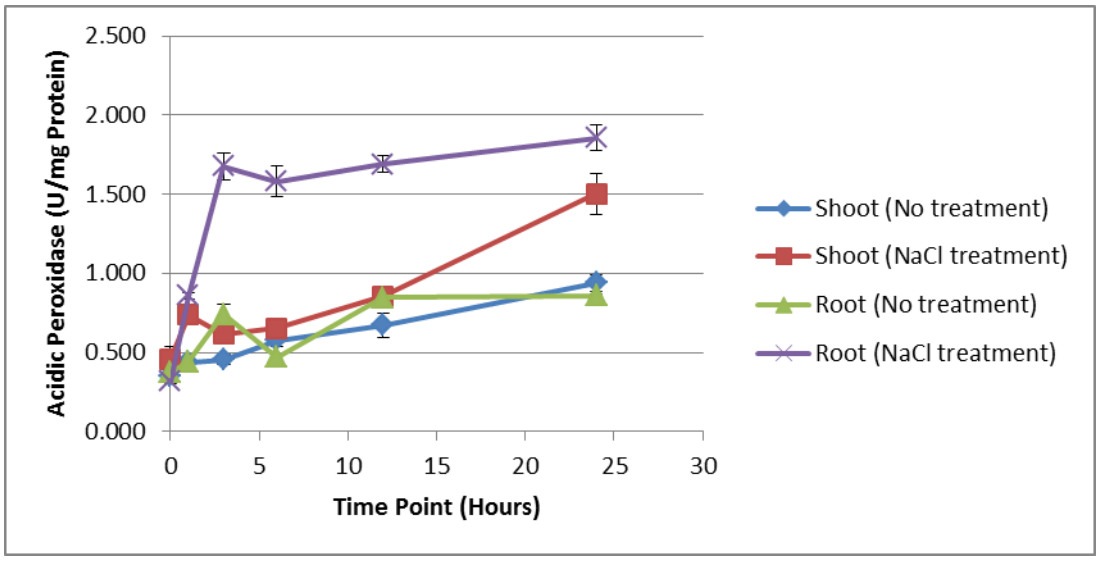
Overall, salt stress (150 mM) exposure led to a rapid increase in acidic peroxidase activity in roots of all rice lines as shown in **Figure 3.35**. Significant increase in activity was observed at 1-3 hours after salt stress, which led to high acid peroxidase levels of 0.86- 1.73 U/mg protein for root samples in WT, VT, and OS while less increase in activity was found in the range of 0.44 - 0.9 U/mg protein for the non-stressed rice lines. After 3 hours, similar activity levels in roots of all rice lines under salt stress were maintained until 24 hours after treatment. When comparing the trend of neutral peroxidase activity in roots among the three rice lines, no difference was observed.

In shoots, increases in acid peroxidase activity in the salt stressed OS was observed as early as 6 hours until 24 h (0.77 U/mg protein) after treatment compared to the non-stressed controls (0.37 U/mg protein) while significantly higher activity was observed during the late hour (24 h) for salt stressed WT and VT (1.39-1.50 U/mg protein) compared to the non-stressed controls (0.84 - 0.90 U/mg protein) Noticeably, the enzyme activity of the non-stressed wild type was higher than when under salt stress during the early period (3-6 hrs). Nonetheless, these results suggest that the *OsCam1-1* transgenic lines affect the acid peroxidase activity in shoots compared with the wild-type and the vector-alone rice lines.

(A)


2.500
2.000
1.500
1.000
0.500
0.000
0
5
10
15
20
25
30
Shoot (No treatment)
Shoot (NaCl treatment)
Root (No treatment)
Root (NaCl treatment)

(B)


Acidic Peroxidase (U/mg Protein)
2.500
2.000
1.500
1.000
0.500
0.000
0
5
10
15
20
25
30
Time Point (Hours)
Shoot (No treatment)
Shoot (NaCl treatment)
Root (No treatment)
Root (NaCl treatment)

(C)

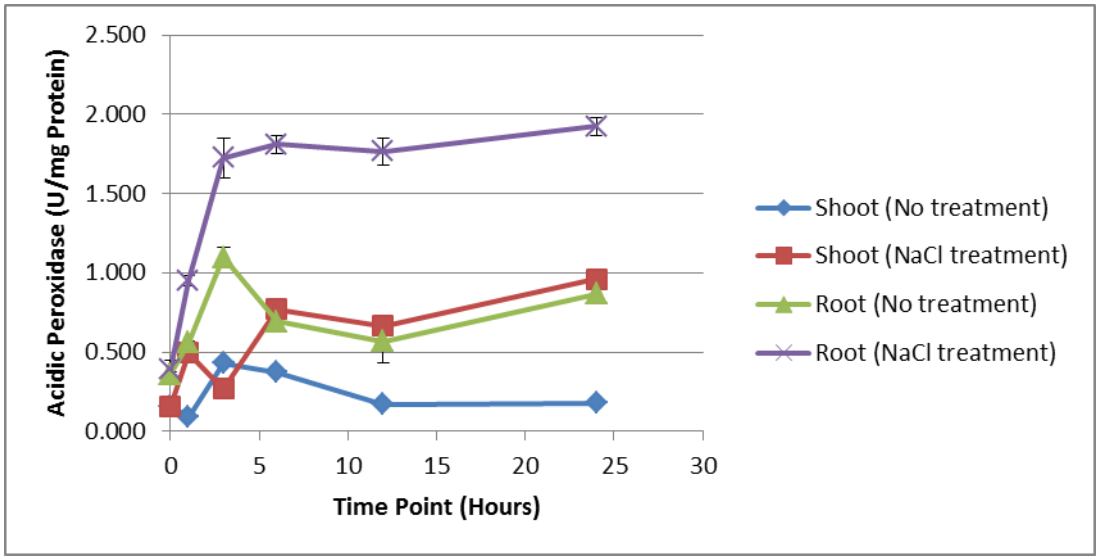
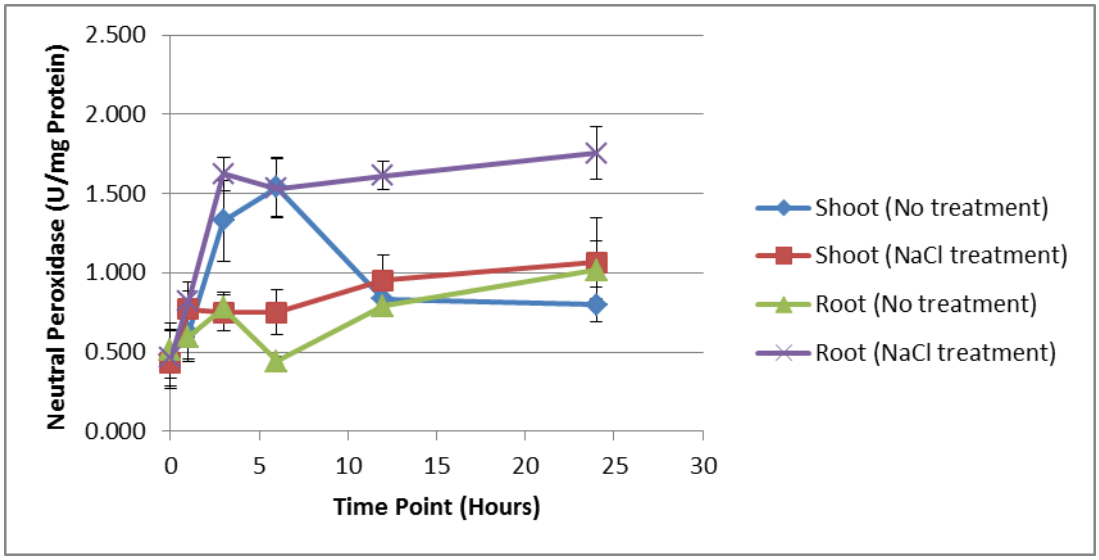


Figure 3.35: Acidic Peroxidase activity subjected to salt stress for 0, 1, 3, 6, 12 and 24 hr. in shoots and roots among three rice lines; (A) Wild-type, (B) Vector-alone, (C) *OsCam1-1*-overexpressing transgenic line

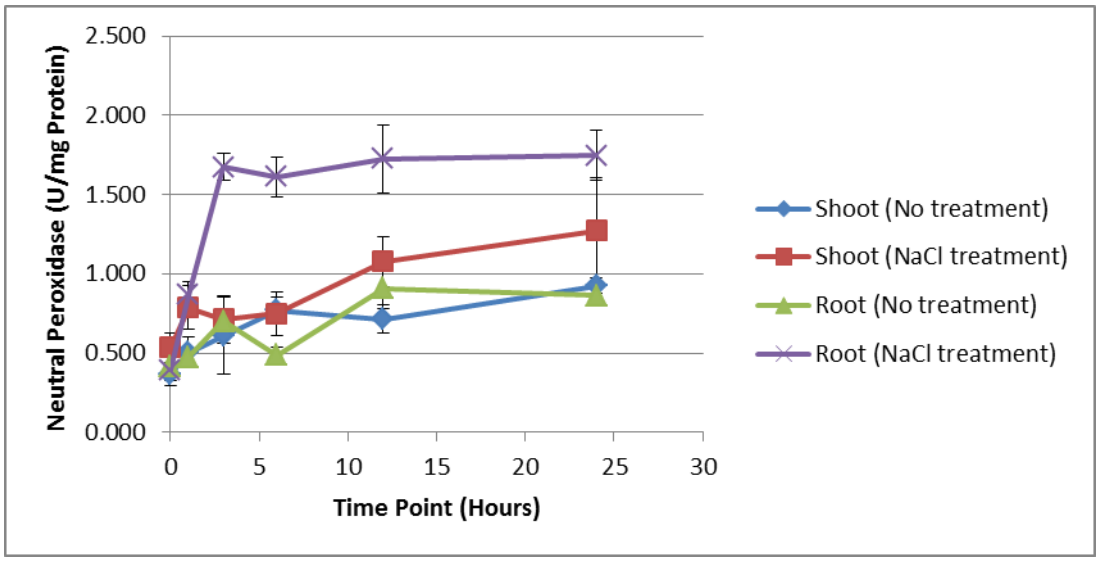
2. Neutral Peroxidase

Overall, salt stress (150 mM NaCl) exposure showed a marked effect on neutral peroxidase activity in roots of all rice lines as shown in **Figure 3.36**. A rapid increase in the enzyme activity was shown at the early hour after salt stress in root samples after salt treatment and maintained until the late hour after treatment. This led to higher neutral peroxidase levels in salt stressed WT, VT, and OS (1.52-1.85 U/mg protein) at 24 hr compared to the non-stressed control samples (0.86 - 0.88 U/mg protein) at the same time points. When comparing the trend of this enzyme activity in roots among the three rice lines, no difference was observed. In contrast, no significant difference was observed between shoot samples under salt stress conditions compared to the non-stressed controls in all rice lines examined except that the enzyme activity of the non-stressed wild type was higher than when under salt stress during the early period (3-6 hrs) of treatment.

(A)


Neutral Peroxidase (U/mg Protein)
2.500
2.000
1.500
1.000
0.500
0.000
0
5
10
15
20
25
30
Time Point (Hours)
Shoot (No treatment)
Shoot (NaCl treatment)
Root (No treatment)
Root (NaCl treatment)

(B)


Neutral Peroxidase (U/mg Protein)
2.500
2.000
1.500
1.000
0.500
0.000
0
5
10
15
20
25
30
Time Point (Hours)
Shoot (No treatment)
Shoot (NaCl treatment)
Root (No treatment)
Root (NaCl treatment)

(C)

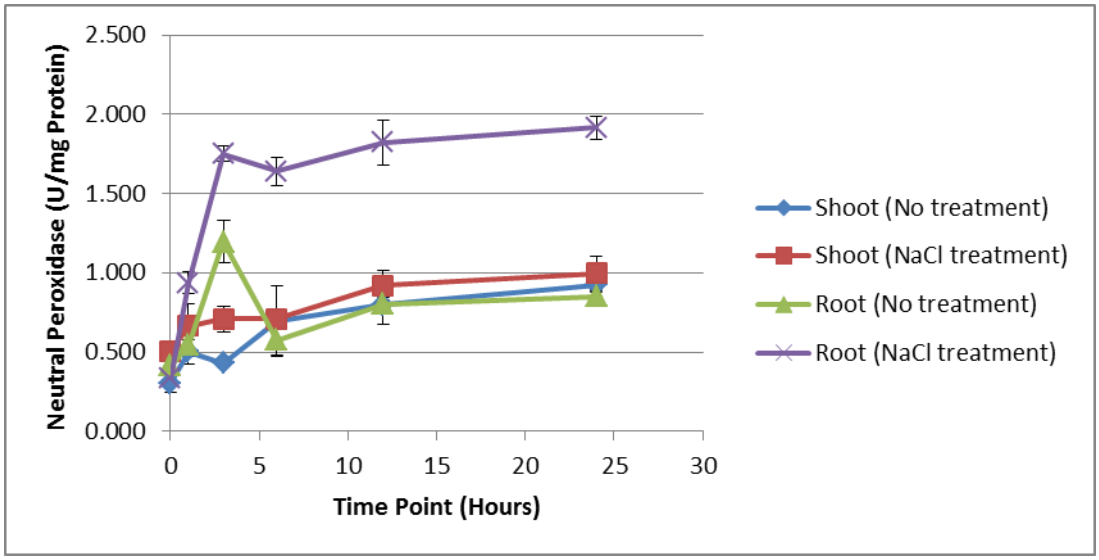


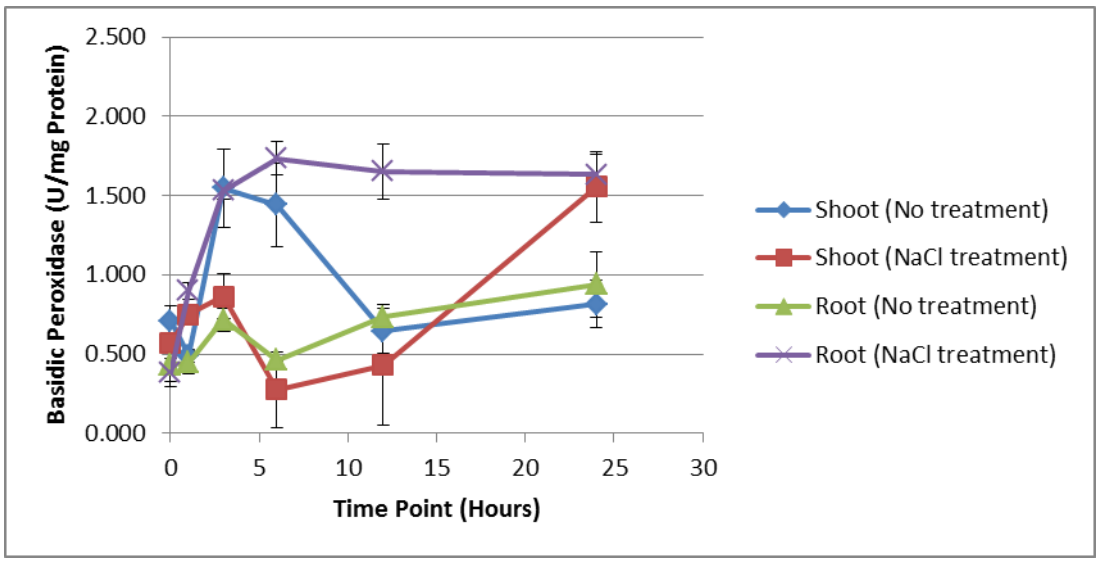
Figure 3.36: Neutral Peroxidase activity subjected to salt stress for 0, 1, 3, 6, 12 and 24 hr. in shoots and roots among three rice lines; (A) Wild-type, (B) Vector-alone, (C) *OsCam1-1*-overexpressing transgenic line.

3. Basidic Peroxidase

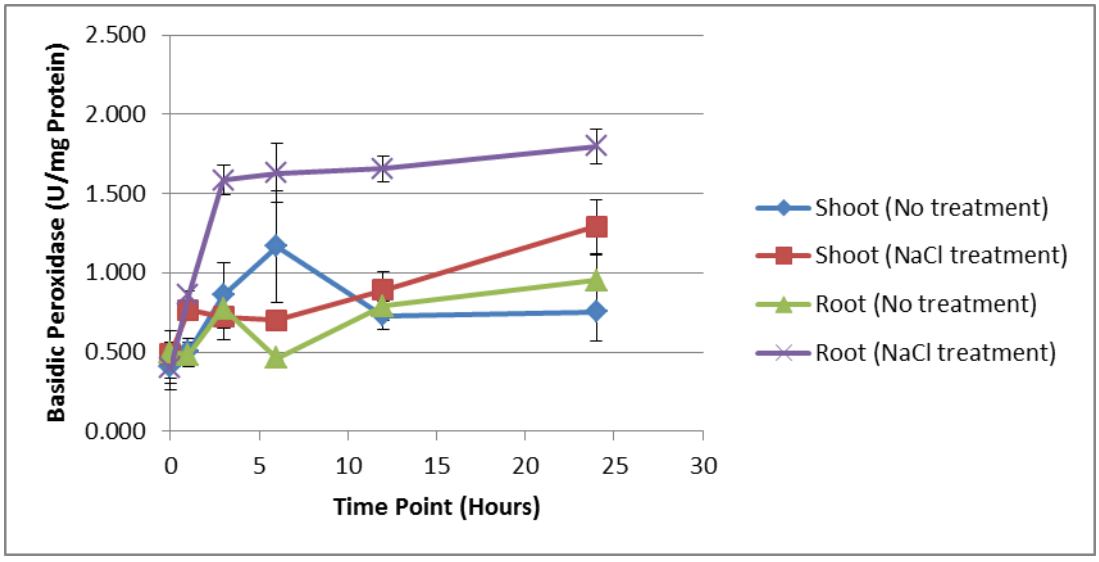
Overall, salt stress (150 mMNaCl) exposure led to a rapid increase in basidic peroxidase activity in roots of all rice linesas shown in **Figure 3.37**. Significant increase in activity was observed at 1-3 hours after salt stress and maintained until the late hours after treatment. This led to high basidic peroxidase levels of 1.63-1.83 U/mg protein for root samples in WT, VT, and OS at 24 h while less increase in activity was found in the range of 0.83-0.95 U/mg for the non-stressed rice lines at the same time points. When comparing the trend of this enzyme activity in roots among the three rice lines, no difference was observed. In contrast, no significant difference in the basidic peroxidase activity level was observed among the shoot samples from WT, VT, and OS.

In shoots, significantly higher basidic peroxidase activity was observed during the late hour (24 h) in shoots of the salt stressed WT and VT (1.29-1.55 U/mg protein) compared to the non-stressed controls (0.75-0.81 U/mg) while, in contrast to the acidic peroxidase, no significant difference of basidic peroxidase activity was observed for OS under the salt stress and non-stressed conditions 0.883-0.958 U/mg protein) at 24 h. The results thus showed some differences in basidic peroxidase response in shoots to salt stress of the *OsCam* line compared to the wild-type and vector control lines.

(A)



(B)



(C)

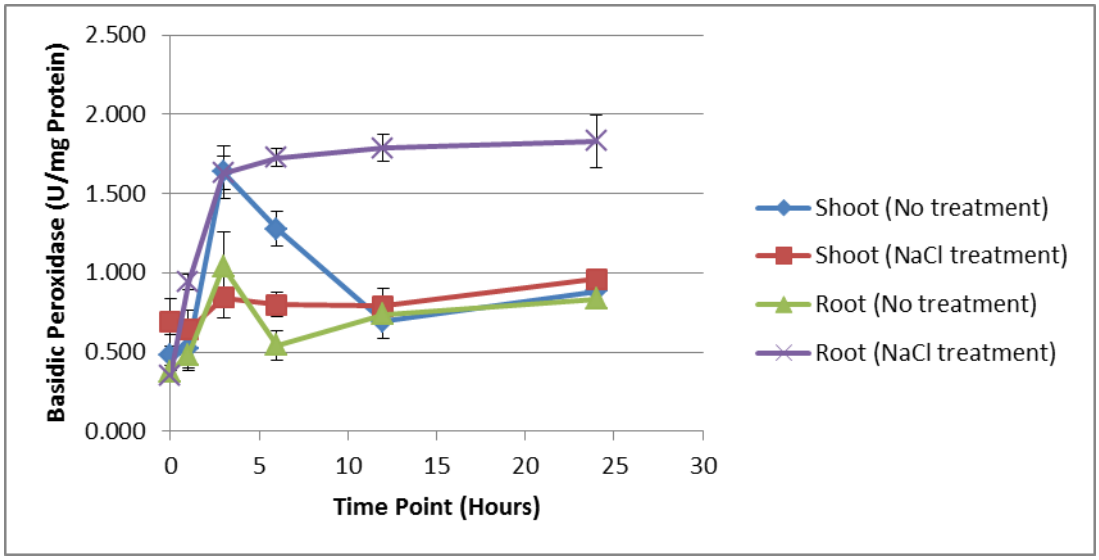


Figure 3.37: Basidic Peroxidase activity subjected to salt stress for 0, 1, 3, 6, 12 and 24 hr. in shoots and roots among three rice lines; (A) Wild-type, (B) Vector-alone, (C) *OsCam1-1*-overexpressing transgenic line.

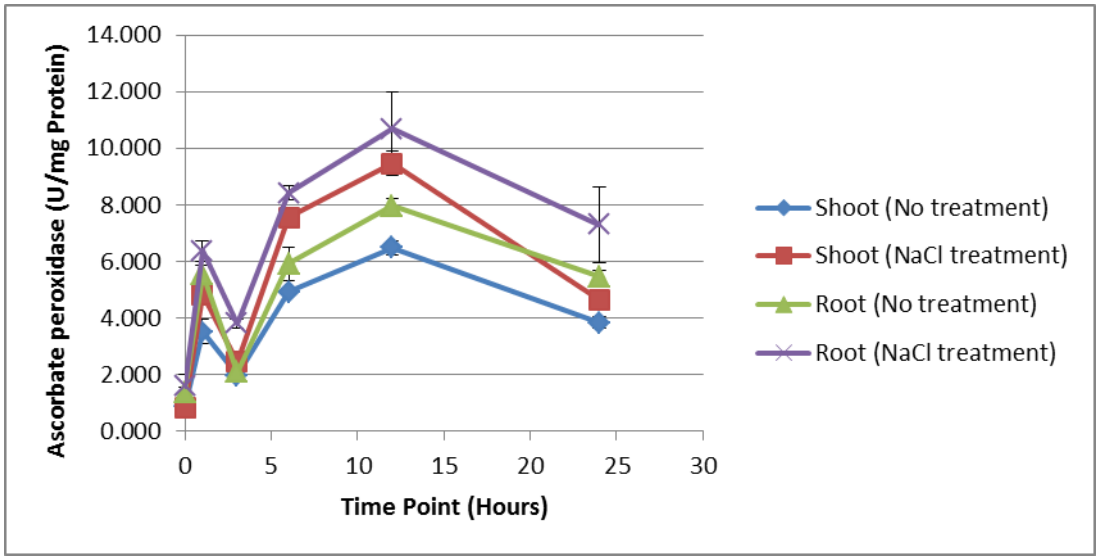
4. Ascorbate peroxidase (APx)

Overall, both salt stressed and non-stressed rice exhibited similar patterns of increases in ascorbate peroxidase activity with the highest levels detected at 12 hours after treatment in all rice lines as shown in **Figure 3.38.** However, salt stress exposure led to a higher increase in ascorbate peroxidase activity in both roots and shoots of all rice lines. At 12 hours after salt stress, significant increase in activity was observed, which led to high ascorbate peroxidase levels of 8.38-10.48 U/mg protein for root samples in WT, VT, and OS while less increase in activity was found in the range of 7.27-7.96 U/mg protein for the non-stressed rice lines.

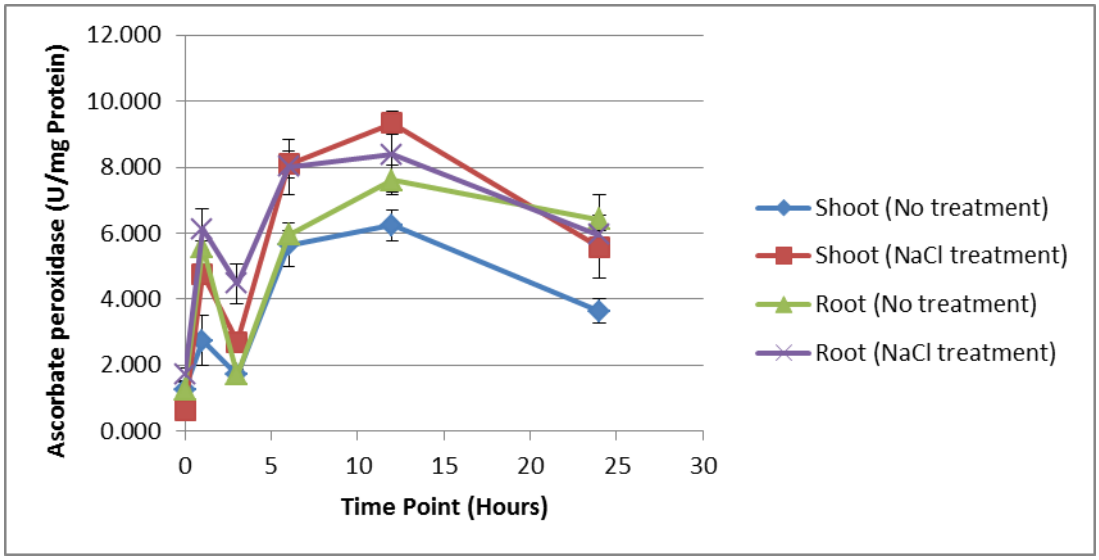
In shoots at 12 hours after salt stress, high ascorbate peroxidase activity levels of 8.75- 9.46 U/mg protein were observed in WT, VT, and OS while less increase in activity was found in the range of 6.14-6.47 U/mg protein for the non-stressed rice lines.No significant difference in the peroxidase activity level was observed among the samples from WT, VT, and OS.

When comparing the trend of this enzyme activity in shoots or roots among three rice lines; wild-type, vector alone and *OsCam1-1* lines, no significant difference was observed.

(A)



(B)



(C)

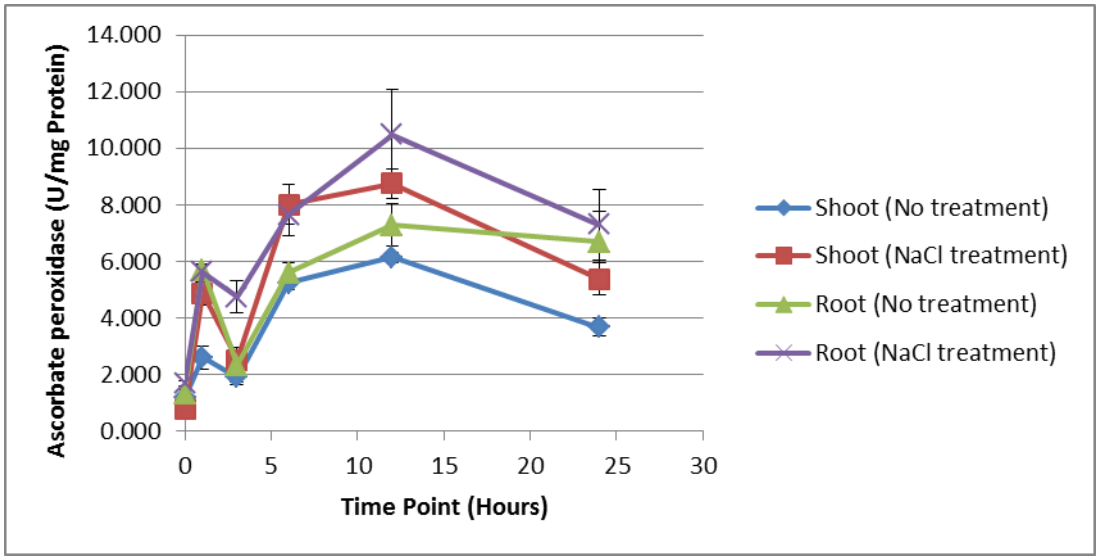


Figure 3.38: Ascorbate Peroxidase activity subjected to salt stress for 0, 1, 3, 6, 12 and 24 hr. in shoots and roots among three rice lines; (A) Wild-type, (B) Vector-alone, (C) *OsCam1-1*-overexpressing

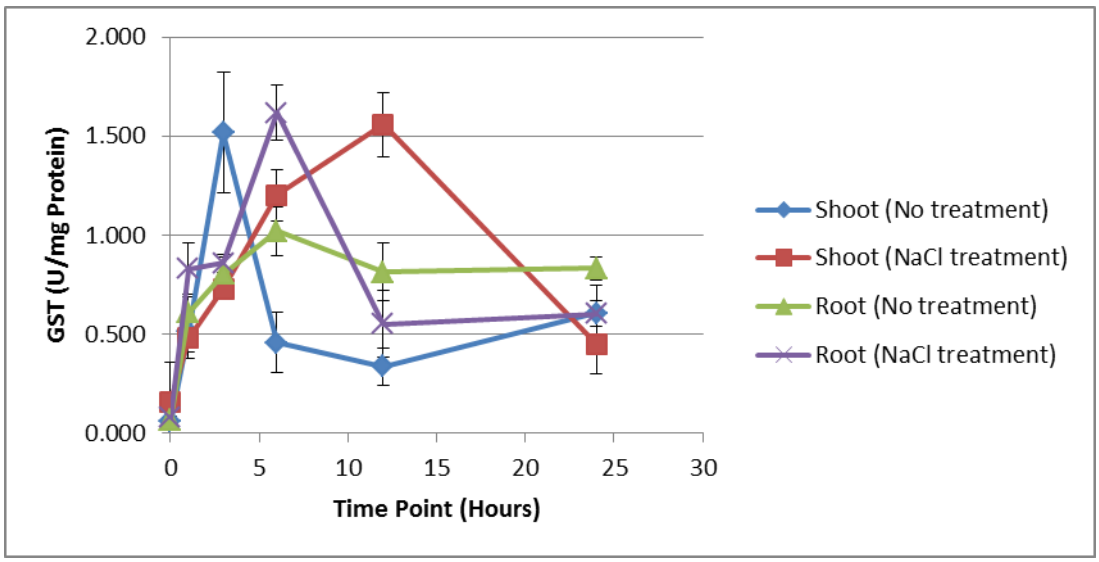
5. Gluthatione-*S*-transferase (GST)

Overall, both salt stressed and non-stressed rice exhibited an increase in glutathione-S-transferase activity in all rice lines as shown in **Figure 3.39**. However, the increases in the salt-stressed plants were either of higher levels or lasted longer than those in the non-stressed plants. In roots, glutathione-S-transferase activity reached the highest levels at 6 hours after treatment as salt stress led to a higher increase in all rice lines reaching the levels of 1.60- 1.65 U/mg protein for root samples in WT, VT, and OS while less increase in activity was found in the range of 0.85- 1.157 U/mg for the non-stressed rice lines. No significant difference in the activity level was observed among the root samples from WT, VT, and OS except that glutathione-S-transferase activity in the OS line after exposure to salt stress was found to reach to a higher level earlier at 3 hours compared to those from WT and VT.

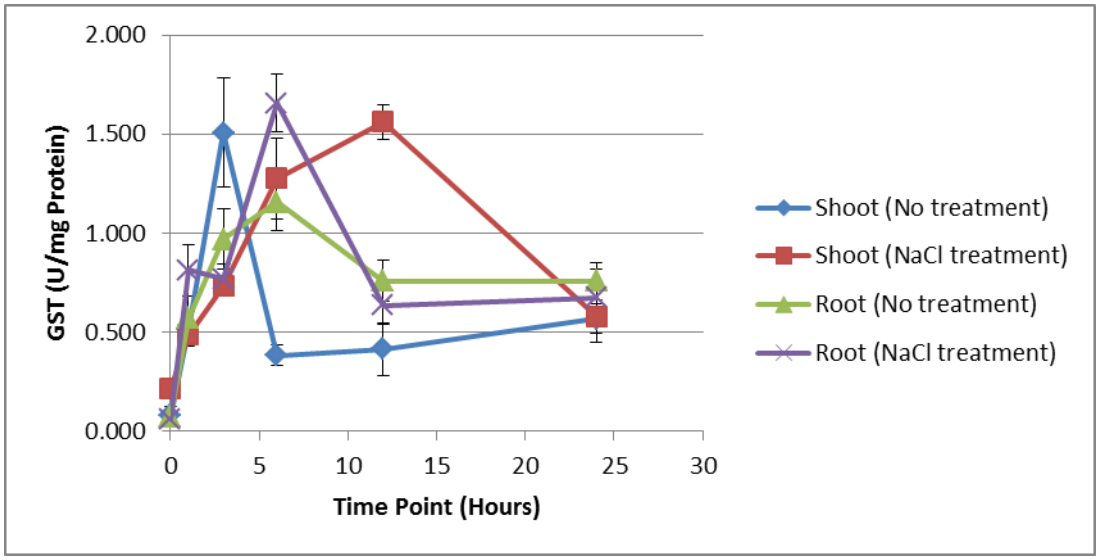
In shoots, increases in glutathione-S-transferase activity in WT and VT appeared to last longer, reaching the highest levels at 12 hours after treatment for the

salt stressed WT and VT (1.53-1.56 U/mg protein) compared to the non-stressed controls (0.33-0.41 U/mg protein). On the other hand, OS exhibited similar increases to those in roots, reaching the highest level earlier at 6 hours after salt stress (1.31 U/mg protein). In contrast to WT and VT, glutathione-S-transferase activity in OS under salt stress was decreased to similar levels to the non-stressed plants at 12 hours after treatment.

(A)



(B)



(C)

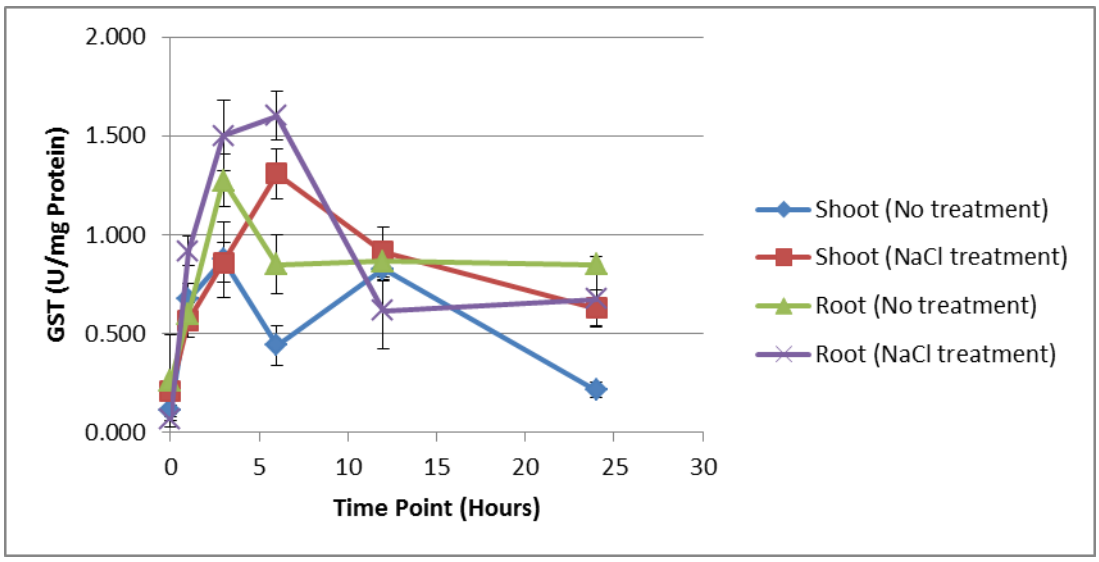


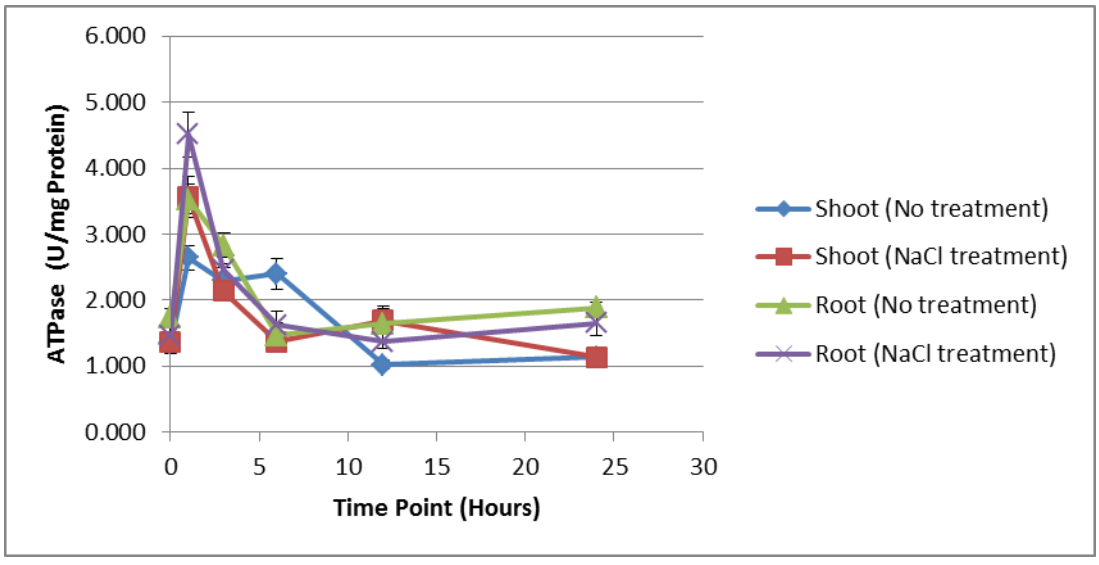
Figure 3.39: Glutatione-S-transferase activity subjected to salt stress for 0, 1, 3, 6, 12 and 24 hr. in shoots and roots among three rice lines; (A) Wild-type, (B) Vector-alone, (C) *OsCam1-1*-overexpressing transgenic line.

6. ATPase

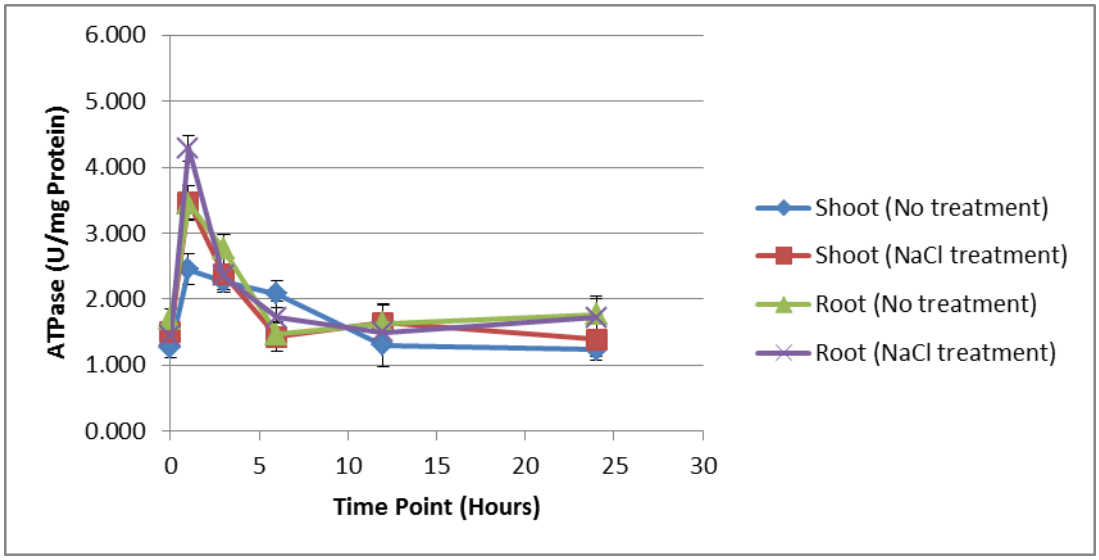
6.1 *V-type* ATPase (+KNO_3) and *P-type* ATPase (No KNO_3)

Overall, both salt stressed and non-stressed rice exhibited similar patterns of rapid increases in*V-type* and *P-type* ATPase activity, reaching the highest levels at 1 hour after treatment and then declined to their original levels at 6-12 hours after treatment onwards in all rice lines as shown in **Figure 3.40 and Figure 3.41**, respectively. However, salt stress exposure led to a slightly higher increase in ATPase activity in both roots and shoots of all rice lines. At 1 hour after salt stress, significant increase in activity was observed, which led to high ATPase levels of 4.28-4.53 U/mg protein for roots= samples in WT, VT, and OS under salt stress while less increase in activity was found in the range of 3.32-3.49 U/mg protein for the non-stressed rice lines. In shoots at 1 hour after salt stress, high ATPase activity levels of 3.41-3.58 U/mg protein were observed in WT, VT, and OS while less increase in activity was found in the range of 2.35-2.74 U/mg protein for the non-stressed rice lines. No significant difference in the ATPase (P or V-type) activity level in both roots and shoots was observed among the samples from WT, VT, and OS.

(A)



(B)



(C)

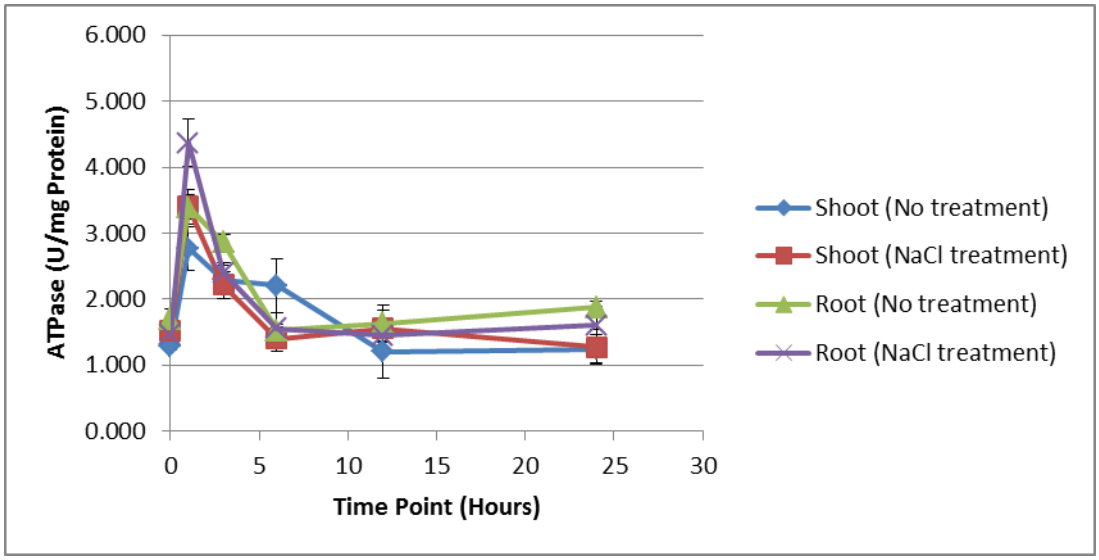
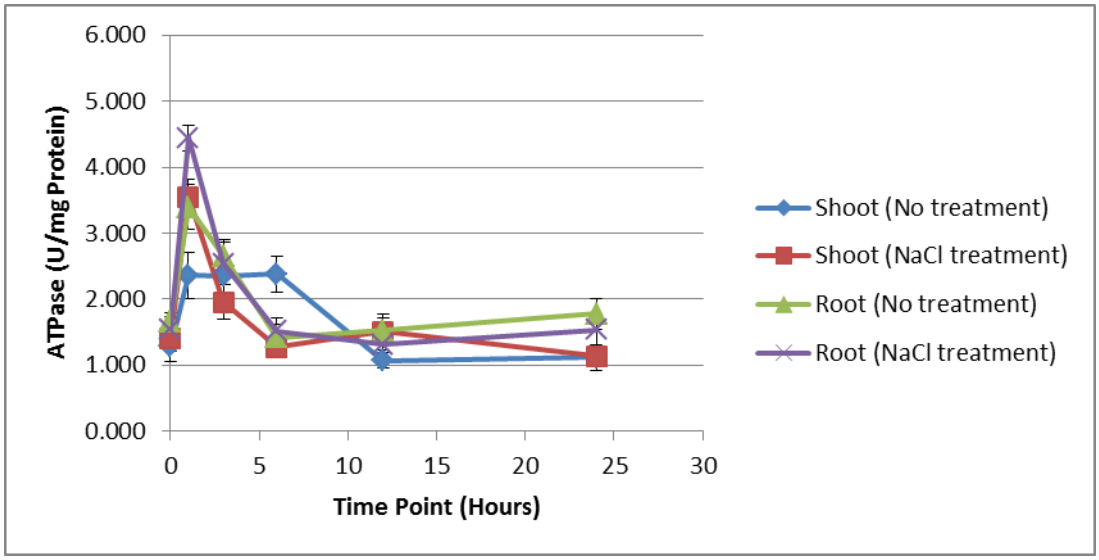
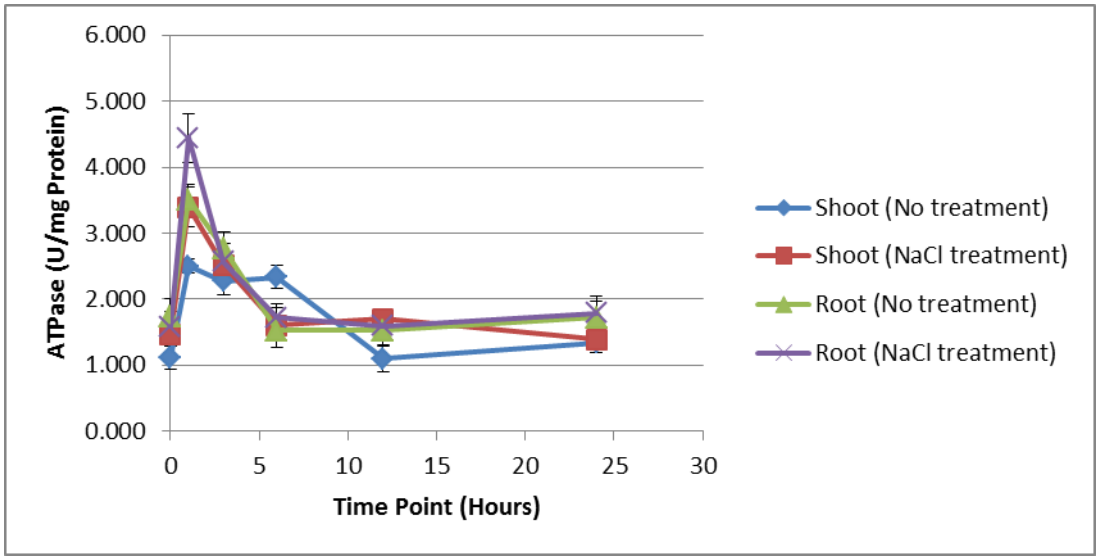


Figure 3.40: *V-type* ATPase activity subjected to salt stress for 0, 1, 3, 6, 12 and 24 hr. in shoots and roots among three rice lines; (A) Wild-type, (B) Vector-alone, (C) *OsCam1-1*-overexpressing transgenic line.

(A)


6.000
5.000
4.000
3.000
2.000
1.000
0.000
ATPase (U/mg Protein)
0
5
10
15
20
25
30
Time Point (Hours)
Shoot (No treatment)
Shoot (NaCl treatment)
Root (No treatment)
Root (NaCl treatment)

(B)


6.000
5.000
4.000
3.000
2.000
1.000
0.000
ATPase (U/mg Protein)
0
5
10
15
20
25
30
Time Point (Hours)
Shoot (No treatment)
Shoot (NaCl treatment)
Root (No treatment)
Root (NaCl treatment)

(C)

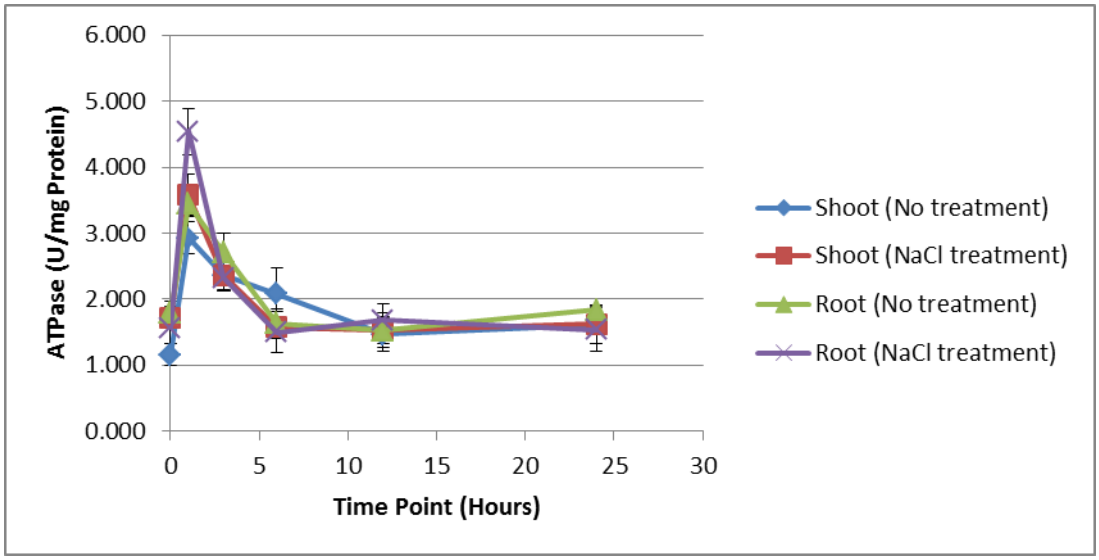


Figure 3.41: *P-type* ATPase activity subjected to salt stress for 0, 1, 3, 6, 12 and 24 hr. in shoots and roots among three rice lines; (A) Wild-type, (B) Vector-alone, (C) *OsCam1-1*-overexpressing transgenic line.

In this study, changes in activities of key stress-responded enzymes involving in salt stress of plants were studied in roots and shoots of rice seedlings. Overall, salt stress by sodium chloride led to increases in most stress-responded enzyme activities examined with varying levels compared to the non-stressed controls in wild-type and transgenic rice and its control transgenic line, suggesting overall similar mechanism of the plants in response to increasing salt concentrations. Increasing enzyme activities in roots were more pronounced than those observed in shoots. This was due to direct effects of salt to physiological change in roots which were in direct contact to the stress factor. Less or no significant changes in enzyme activity in shoots were observed as response in shoots are indirect and considered to be due to signal relay process from the primary contact site .

Increasing concentration of NaCl in environments led to increases in H_2O_2 level in plants, particularly in roots, which is related to systematic plant response in gene expression profiles. Salt was reported to cause up-regulation and down-

regulation of various genes in rice seedling at the transcription level using microarray approach (Kawasaki S., 2001). It was shown that approximately 10% of the transcripts in Pokkali were significantly up-regulated or down-regulated during the first hour of salt stress. The initial differences between control and stressed plants was shown to continue for several hours but became less pronounced as the plants adapted over time. This is related to the changes in expression levels of a range of stress-responded enzymes as observed in our study. Most enzymes examined in this study involving several peroxidases, and glutathione-S-transfeaseare oxidative enzymes functioning in antioxidation mechanism (e.g. degradation of free radical from H_2O_2) These enzymes are previously shown to be key enzymes involved in salt stress responses in rice and other plants e.g. eastern cottonwood, *Populus deltoids*, Rio Fuego plant, *Solanum lycopersicum* L. (Li G. *et al*., 2003, Yan. S., *et. al*., 2005, Hong C.Y. *et. al*., 2008, Nasrabadi H.T. *et. al*., 2011 and Csiszár J. *et al.,* 2011)

Increasing concentrations of NaCl from 50 to 150 mM was reported to reduce root growth and increase ionically bound cell-wall peroxidase activity in rice seedling while it showed no effect on covalently bound cell-wall peroxidase activities. The inhibition of root growth and the increase in ionically bound peroxidase activity in roots by NaCl was shown to be reversible and associated with ionic rather than osmotic component. Treatment with NaCl was reported to cause an increase in the ascorbate peroxidase activity and glutathione reductase and the their gene expression in rice roots (Tsaia Y.C., *et al.,* 2004). The activities of several antioxidant enzymes including peroxidase, ascorbate peroxidase, superoxide dismutase, and glutathione reductase were also observed to be higher in NaCl-stressed rice leaves than in control leaves, suggesting signal relay of salt stress from roots, which led to systematic enzyme up-regulation in leaves (Chuan Chi Lin. *et al.,* 2000). Increases in the activity of antioxidant enzymes such as superoxide dismutase, peroxidase, catalase, glutathione reductase, glutathione-*S*-transferasewere also reported in salt tolerant mulberry compared to the salt sensitive cultivar under high salinity conditions (Sudhakar C. *et al.*, 2001). Previous work in mungbean reported the stimulation of the plasma lemma ATPase activity in roots in parallel with increases in the intracellular concentration of Na^+ (Nakamura Y. *et al*., 1992). In overall, the results in our study

showed the changes in activity of key enzymes involved in salt stress response mechanism in which was the effects of primary stress contact as shown in roots and as the signal relay response in shoots in all rice lines under high salinity conditions.

However, significant differences in some enzyme activity profiles were also observed in the wild-type and the transgenic control lines compared to the *OsCam* transgenic line. These would be related to differences in physiology and salt stress response mechanism in *OsCam1-1*. Further study is needed for investigation of the salt stress response mechanisms by *OsCam1-1*, which would possibly be related to activities of these enzymes.

CHAPTER IV

CONCLUSIONS

1. By using a comparative proteomic strategy, we provided an overview of the systematic rice response to salt stress. The identified 207 rice proteins with significant change among the three rice lines: wild-type, *OsCam1-1*-overexpressing transgenic line, and its transgenic control line containing only vector with no insert, possibly play important roles in response to salt stress. These proteins were categorized into 7 groups based on their biological functions including cellular process (42%), metabolic process (14%), regulation (12%), developmental process (2%), localization (2%), response to stimulus (2%) while the rest are proteins in other processes or of unknown function.

2. Many of the proteins in the shoot and the root responded to the induced salt stress were localized in nucleus (24%) and also in chromosome (5%), which could suggest their function in regulation of gene expression in response to salt stress. Significant fractions were also located in mitochondria (5%), ER (3%), and other intracellular organelles (5%) while only a small fraction was located in cytoplasm. Some are part of macromolecular complex (8%).

3. According to classification based on molecular functions, the majority of the identified proteins (60%) are related to binding function. A substantial number of them (29%) are related to enzymes with various catalytic activities. Only few proteins were those working as enzyme regulators and molecular transducers. The results thus suggested systematic response of gene expression to salt stress for proteins in various organelles and subcellular locations, which are related to overall cellular responses by different mechanisms to the induced salt stress.

4. Analysis of oxidative-stress enzyme activity revealed that activity of all peroxidases examined significantly increased in roots more than in shoots when exposed to salt stress. When comparing activity of these enzymes in roots among the three rice lines, no significant difference was observed except activity of acidic and basidic peroxidases between WT/VT and OS rice lines at the latter stage of salt stress treatment.

5. Increases in glutathione-S-transferase activity of the salt-stressed plants were either of higher levels or lasted longer than those in the non-stressed plants. No significant difference in the activity level was observed among the WT, VT, and OS except that glutathione-S-transferase activity in the OS line after exposure to salt stress was found to reach to a higher level earlier compared to those from WT and VT. ATPase activity was only slightly increased when exposed to salt.No significant difference was observed among the three rice lines.

6. When comparing protein profiles between wild-type and *OsCam 1-1* overexpressing line, both of them use common salt responsive-proteins. Only a few different proteins were determined between both rice lines. These proteins give an insight into the physiological function of the *OsCam1-1* gene and mechanism of rice in response to salt stress, providing a platform for further development of salt tolerant rice varieties.

7. To recapitulate a number of proteins identified as specifically expressed in roots and shoots of OS compared to those of WT or VT in our study, most of proteins were transcription regulatory proteins, involving in regulation of gene expression in nucleus and also in other organelles. Several families of transcription factors in nucleus were identified involving those functions as zinc finger or leucine zipper proteins, which bind specifically to the target regions or so called, regulatory sites on DNA, resulting in up- or down-regulation of a specific set of proteins under a specific physiological or environmental condition.

8. Furthermore, we also found group of proteins namely “Sigma factors” in our studies which play roles in regulation of gene expression in plant organelles e.g. plastid and mitochondria by binding to RNA polymerases and turning on expression of target genes. Roles of transcription regulatory proteins in controls of genes expression in

response to abiotic stress were reported in different plants which related in our studies. Most of them were identified in *OsCam 1-1* overexpressing line under both non-stressed and salt stressed conditions, our findings thus suggest the specific expression of several potential transcription regulatory proteins which may involve in control of genes related to salt tolerance mechanisms in rice, particularly for the transgenic OS line.

9. A large fraction of the identified proteins was hypothetical proteins with unknown function, which can be considered as interesting new salt responsive protein candidates for further study. Functions of these proteins can be investigated through several approaches such as gene silencing like RNAi. Advances in bioinformatics strategies can also assist annotation of genes with unknown functions. Altogether this work provides a number of potent protein candidates involving in salt stress response in rice and particularly for the transgenic OS line which are of interest for further study.

REFERENCES

Abdrakhamanova, A., Wang, Q. Y., Khokhlova, L., & Nick, P. (2003). Is microtubule disassembly a trigger for cold acclimation? *Plant and Cell Physiology*, 44, 676–686

Ahloowalia, B. S., Maluszynski M., & Nichterlein K. (2004). Global impact of mutation-derived varieties. *Euphytica*, 135, 187-204.

Agrawal, G.K., and Rakwal, R. (2006). Rice proteomics: A cornerstonefor cereal food crop proteomes. *Mass Spectrom. Rev*. 25: 1-53.

Aiken, R. M., & Smucker, A. J. M. (1996). Root system regulation of whole plant growth. *Annual Review of Phytophatology*, 34, 325-246

Aina, R., Labra, M., Fumagalli, P., Vannini, C., Marsoni, M., Cucchi, U., Bracale, M., Sgorbati, S., & Citterio, S. (2006). Thiolpeptide level and proteomic changes in response to cadmium toxicity in *Oryza sativa* L. roots. *Environmental and Experimental Botany*, 59, 381–392.

Alamgir, A. N. M., & Ali, M. Y., (1999). Effect of salinity on leaf pigments, sugar and protein concentrations and chloroplast ATPase activity of rice (*Oryza sativa* L.). *Bangladesh Journal of Botany*, 28, 145–149.

Alscher, R.G., Erturk, N., & Heath, L. S. (2002). Role of superoxide and develop mental specificity of salt-stress tolerance dismutases in controlling oxidative stress in plants. *Journal of Experimental Botany,* 53, 1331–1341.

Amirjani M.R. (2010). Effect of NaCl on Some Physiological Parameters of Rice *EJBS*, 3(1), 6-16.

Ardrey, R.E. (2003). *Liquid Chromatography-Mass Spectrophotometry: An Introduction.* England: John Wiley & Sons.

Ashraf, M. (1997). Changes in soluble carbohydrates and soluble proteins in three arid-zone grass species under salt stress. *Journal of Tropical Agriculture*. 74, 234–237.

Ashraf, M., & Harris, P. J. C. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Science*, 166, 3-16.

Ayala, F., O'Leary, J. W., & Schumaker, K.S. (1996). Increased vacuolar and plasma membrane H+-ATPase activities in *Salicornia bigelovii* Torr. in response to NaCl. *Journal of Experimental Botany*, 47, 25–32.

Badawi, G.H. *et al*., (2004). Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. Physiol Plant. Jun;121(2):231-238.

Baisakh, N. *et al*., (2012). Enhanced salt stress tolerance of rice plants expressing a vacuolar H^+-ATPase subunit c1 (SaVHAc1) gene from the halophyte grass *Spartina alterniflora* Löisel. Plant Biotechnol J. May;10(4):453-64.

Batistič, O. and Kudla, J. (2012) Analysis of calcium signaling pathways in plants Biochim Biophys Acta. Aug;1820(8):1283-93. Epub 2011 Oct 26.

Bhatia VN, Perlman DH, Costello CE, McComb ME. (2009) Software tool for researching annotations of proteins: open-source protein annotation software with data visualization. *Analytical Chemistry*, 81 (23), 9819-23.

Bhushan, D., Jaiswal, D.K. , Ray, D. Basu, D., Datta A., Chakraborty, S. and Chakraborty N. (2011). Dehydration-Responsive Reversible and Irreversible Changes in the Extracellular Matrix: Comparative Proteomics of Chickpea Genotypes with Contrasting Tolerance. *J. Proteome Res.*, 10, 2027–2046

Blum, H., Beier, H., & Gross, H. J. (1987). Silver stain protocol. *Electrophoresis* 8, 93–99

Bohnert, H.J., & Jensen, R.G. (1996). Strategies for engineering water stress tolerance in plants. *Trends in Biotechnology*, 14, 89–97.

Boonburapong, B., and Buaboocha, T. (2007). Genome-wide identification and analyses of the rice calmodulin and related potential calcium sensor proteins. *BMC Plant Biol*. 7:4.

Bor, M., Ozdemir, F., & Turkan, I. (2003). The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet Beta *Vulgaris* L. and wild beet *Beta maritima* L. *Plant Science*, 164, 77-84.

Bray, E.A. (1997). Plant responses to water deficit. *Trends in Plant Science*, 2**,** 48–54.

Brun, T., Roche, E., Kim, K. H., & Prentik, M. (1993). Glucose regulates acetyl CoA carboxylase gene expression in a pancreatic *β*-cell line (INS-1). *Journal of Biological Chemistry*, 268**,** 18905–18911.

Buaboocha, T., and Zielinski, R.E. (2002). Calmodulin; in Protein-Protein Interactions in Plant Biology, McManus, M.T., Liang, W.A., and Allen, A.C. (eds.), pp. 285-313, Sheffield Academic Press, Sheffield, UK.

Cao, F.Q., Werner, A. K., Dahncke, K., Romeis, T., Liu, L.H. and Witte, C.P. (2010) Identification and Characterization of Proteins Involved in Rice Urea and Arginine Catabolism *Plant Physiology*, 154, 98–108.

Canovas, F.M., Dumas-Gaudot, E., Recorbet, G., Jorrin, J., Mock, H. P., & Rossignol, M. (2004). Plant proteome analysis. *Proteomics*, 4, 285–298.

Castrillo, M. (1992). Sucrose metabolism in bean plants under water deficit. *Journal of Experimental Botany*, 43**,** 1557–1561.

Cervilla, L. M., Blasco, B. A., Rios, J. J., Romero, L., & Ruiz J. M. (2007). Oxidative stress and antioxidants in tomato (*Solanum lycopersicum*) plants subjected to Boron toxicity. *Annals of Botany*, 1–10.

Cha-um, S., Kirdmanee, C., & Supaibulwatana K. (2004). Biochemical and physiological responses of Thai jasmine rice (*Oryza sativa* L. spp. Indica cv. KDML105) to salt stress. *Science Asia*, 30, 247-253.

Cha-um, S., Singh, H. P., Samphumphuang, T. & Kirdmanee C. (2012). Calcium-alleviated salt tolerance in indica rice (*Oryza sativa* L. spp. indica): Physiological and morphological changes *Australian Journal of Crop Science* 6(1):176-182

Chen, Y. F., Etheridge, N., & Schaller, G. E. (2005). Ethylene signal transduction. *Annals of Botany*, 95, 901–915.

Chinnusamy, V., Jagendorf, A., Zhu, J-K. (2005). Understanding and improving salt tolerance in plants. *Crop Science*, 45, 437–448.

Corley, E. & Wolosiuk, R. A. (1985). The effect of organic solvents on the activation and the activity of spinach chloroplast Fructose-1,6-bisphosphatase. *Journal of Biologigal Chemistry*, 260, 3978-3983.

Coruzzi, G., & Bush, D. R. (2001). Nitrogen and carbon nutrient and metabolite signaling in plants. *Plant Physiology*, 125, 61–64.

Couée, I., Sulmon, C., Gouesbet, G., & Amrani, A. E. (2006). Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *Journal of Experimental Botany*, 57, 2006.

Cram, W.J. (1976). Negative feedback regulation of transport in cells. The maintenance of turgor, volume and nutrient supply. *Encyclopaedia of Plant Physiology, New Series, Springer-Verlag, Berlin*, 2, 284–316.

Cutillas, P.R., & Timms, J.F. (2010). *LC-MS/MS in proteomics: Methods and Applications*. United state of America: Springer.

Damerval, C., de Vienne, D., Zivy, M., & Thiellement, H. (1986). Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. *Electrophoresis* 7, 52–54.

Damian et al., (2011). The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res.* Jan;39 (Database issue):D561-8. Epub 2010 Nov 2.

Das, A. B., & Parida, A. K. (2005). Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety*, 60, 324-349

Dass,C. (2007). *Fundamentals of Contemporary Mass Spectrophotometry*. United state of America: John Wiley & Sons.

Deepak S.A., Shibato J., Ishii H., Ogawa, Y., Yoshida Y., Iwahashi, H. Masuo, Y. Agrawal, G.K. and Rakwal, R. (2008) Proteomics Approach for Investigating the Disease Resistance Using Cucumber as Model Plant. *American Journal of Biochemistry and Biotechnology* 4 (3): 231-238

Dhonukshe, P., Laxalt, A. M., Goedhart, J., Gadella, T. W. J., & Munnik, T. (2003). Phospholipase D activation correlates with microtubule reorganization in living plant cells. *The Plant Cell*, 15, 2666–2679.

Downton, W. J. S. (1977). Photosynthesis in salt-stressed grapevines, *Australian Journal of Plant Physiology*,4, 183–192.

Drose, S. and Altendrof, K. (1997) Bafilomycins and concanamycins as inhibitors of V-ATPase and P-ATPase. Journal of Experimental Biology 200, 1–8.

Dubey, R. S., & Singh, A. K., (1999). Salinity induces accumulation of soluble sugars and alters the activity of sugar metabolizing enzymes in rice plants. *Plant Biology*, 42, 233-239.

Eastmond, P.J., & Graham, I. A. (2001). Re-examining the role of the glyoxylate cycle in oilseeds. *Trends in Plant Science*, 6, 72–78.

Eimer, M. (2004). Transgenic drought- and salt-tolerant plants. *Genetic Engineering Newsletter*, 1-13.

El-Shourbagy, M. N., & Koshk, H.T. (1975) Sodium chloride effects on the sugar metabolism of several plants. *Phytochemistry*, 17, 101–108.

Espartero, J., Pintor-Toro, J. A., & Pardo, J. M. (1994). Differential accumulation of *S*-adenosylmethionine synthetase transcripts in response to salt stress. *Plant Molecular Biology*, 25, 217–227.

FAO. (2005) *Global Network on Integrated Soil Management for SustainableUse of Salt-affected Soils.* Rome, Italy: FAO Land and Plant Nutrition Management Service. http://www.fao.org/ag/agl/agll/spush

Filho, E. G., Costa, P. H., Neto, A. D., Bezerra, M. A., & Prisco, J.T. (2005). Antioxidant-enzymatic system of two sorghum genotypes differing in salt tolerance. *Journal of Plant Physiology*, 17, 353-361.

Flowers, T. J., Troke, P. F., & Yeo, A. R. (1977). The mechanism of salt tolerance in halophytes. *Annual Review of Plant Physiology,* 28, 89-121.

Fridovich, I. (1986). Biological effects of the superoxide radical. *Archieves of Biochemistry and Biophysics*, 247, 1-11.

Gao, Z.F., Sagi, M., Lips, S.H., 1998. Carbohydrate metabolism in leaves and assimilate partitioning in fruits of tomato (*Lycopersicon esculentum* L.) as affected by salinity. *Plant Science*, 135, 149–159.

Galiba, G. & Kerepesi, L. (2000). Osmotic and salt-induced alteration in soluble carbohydrate content in wheat seedlings. *Crop Science*, 40, 482-487.

Garham, J., Hughes, L.Y., & Wynjanes, R. G. (1981). Low molecular weight carbohydrates in some salt stressed plants. *Physiology of Plant*, 53, 27–33.

Gauch, H. G., & Eaton, F. M. (1942) Effect of saline substrate on levels of carbohydrates and inorganic constituents of barley plants. *Plant Physiology*, 17, 347–365.

Gazzarrini, S., & McCourt, P. (2001). Genetic interactions between ABA, ethylene and sugar signaling pathways. *Current Opinion in Plant Biology*, 4, 387–391.

Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., Imai, B. S., & Mische, S. M. (1999). Mass spectrometric identification of proteins from silver-stained

polyacrylamide gel: A method for the removal of silver ions to enhance sensitivity. *Electrophoresis*, 20, 601–605.

Giri, C. C., & Laxmi, G. V. (2000). Production of transgenic rice with agronomically useful genes: an assessment. *Biotechnology Advances*, 18, 653-683.

Gill, P. K., Sharma, A. D., Singh, P., & Bhullar, S. S. (2002). Osomtic stress-induced changes in germination, growth and soluble sugars content of *Sorghum Bicolor* L. moench seeds. *BULG. J. PLANT PHYSIOL.*, 2002, 28(3–4), 12–25.

Goff, S. A., Ricke, D., Lan, T. H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., & Varma, H. (2002). A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science*, 296, 92–100.

Gossett, D. R., Millhollon, E.P., & Cran, L. M. (1994). Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. *Crop Science*, 34, 706-714.

Gulick, P. J., Drouin, S., Yu, Z. H., Danyluk, J., Poisson, G., Monroy, A. F., & Sarhan, F. (2005). Transcriptome comparison of winter and spring wheat responding to low temperature. *Genome*, 48, 913–923.

Gupta, A. K., & Kuar, N. (2005). Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *Journal of Bioscience*, 30, 101-113

Guy, M., Mittova, V., Tal, M., & Volokita, M. (2004). Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. *Journal of Experimental Botany*, 55, 1105-1113.

Helmy M., Tomita M. & Ishihama Y. (2011). OryzaPG-DB: Rice Proteome Database based on Shotgun Proteogenomics. *BMC Plant Biology*, 11:63

Hirano, H., Islam, N., & Kawasaki, H. (2004). Technical aspects of functional proteomics in plants. *Phytochemistry*, 65, 1487–1498.

Hong, Z., Lakkineni, K., Zhang, Z., & Verma, D. P. S. (2000). Removal of feedback inhibition of D1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiology*, 122, 1129-1136.

Hussain, T. M., Chandrasekhar, T., Hazara, M. , Sultan, Z., Brhan Khiar Saleh, B. K., & Gopal, G. R. (2008). Recent advances in salt stress biology – a review. *Biotechnology and Molecular Biology Review*, 3, 8-13.

Ideker, T., Thorsson, V., Ranish, J. A., Christmas, R., Buhler, J., Eng, J. K., Bumgarner, R., Goodlett, D. R., Aebersold, R., & Hood, L. (2001): Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science*, 292, 929–934.

Imlay, J.A., & Linn, S. (1988). DNA damage and oxygen radical toxicity. *Science*, 240, 1302-1309.

Itoh T, Tanaka T, Barrero RA, Yamasaki C, Fujii Y, Hilton PB, Antonio BA, Aono H, Apweiler R, Bruskiewich R, *et al* (2007): Curated genome annotation of *Oryza sativa* ssp. japonica and comparative genome analysis with *Arabidopsis thaliana*. *Genome Research*, 17:175-183.

Jang, J. C., & Sheen, J. (1994). Sugar sensing in higher plants. *Plant Cell*, 6**,** 1665–1679

Jaresitthikunchai, J., Phaonakrop, N., Kittisenachai, S., and Roytrakul, S. Rapid in-gel digestion protocol for protein identification by peptide mass fingerprint. Proceeding of The 2nd Biochemistry and Molecular Biology Conference: Biochemistry and Molecular Biology for Regional Sustainable Development. May 7-8, 2009 KhonKaen, Thailand, p.29.

Jbir, N., Chaïbi, W., Saïda, A., Ahmed, J., & Abdelkader, A. (2001). Root growth and lignification of two wheat species differing in their sensitivity to NaCl, in response to salt stress. *Comptes Rendus de l'Académie des Sciences - Series III – Sciences de la Vie.*, 342, 1065-1021.

Jiang, Y., & Deyholos, M. K. (2006). Comprehensive transcriptional profiling of NaCl-stressed Arabidopsis roots reveals novel classes of responsive genes. *BMC Plant Biology*, 6, 25.

Jiang, Y., Yang, B., Harris, N. S., & Deyholos M. K. (2007). Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots. *Journal of Experimental Botany*, 1-17.

Jinglan, W., & Denise, M. S. (1998). Salinity adaptation of plasma membrane H^+ - ATPase in the salt marsh plant *Spartina patens*: ATP hydrolysis and enzyme kinetics. *Journal of Experimental Botany,* 49, 1005-1013.

Johansson, C., Samskog, J., Sundstrom, L., Wadensten, H., Bjorkesten, L., Flensburg, J. (2006). Differential expression analysis of Escherichia coli proteins using a novel software for relative quantitation of LC-MS/MS data. *Proteomics.* 6: 4475-4485.

Karkacier, M., Erbas, M., Usiu, M. K., & Aksu M. (2003). Comparison of different extraction and detection methods for sugar using amino-bonded phase HPLC. *Journal of Chromatagraphic Science*, 41, 331-333.

Kaur, S., Gupta, A. K., & Kaur, N., (2000). Effect of GA_3, kinetin and indole acetic acid on carbohydrate metabolism in chickpea seedlings germinating under water stress; *Plant Growth and Regulation,* 30, 61–70

Kav, N. V., Srivastava, S., Goonewardene, L., Blade, S.F., (2004). Proteome-level changes in the roots of Pisum sativum in response to salinity. *Annals of Applied Biology*. 145, 217–230.

Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D., & Bohnert, H. L. (2001). Gene expression profiles during the initial phase of salt stress in Rice. *Plant Cell,* 13, 889–905.

Kerepesi, I., & Galiba, G., (2000). Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. *Crop Science*, 40, 482–487.

Khatkar, D., & Kuhad, M. S., (2000). Short-term salinity induced changes in two wheat cultivars at different growth stages. *Biologia Plantarum*, 43, 629–632.

Koch, K. E. (1996). Carbohydrate-modulated gene expression in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 47, 509–540.

Koller, A., Washburn, M. P., Lange, B. M., Andon, N. L., Deciu, C., Haynes, P. A., Hays, L., Schieltz, D., Ulaszek, R., Wei, J., Wolters, D., & Yates, J. R. (2002). Proteomic survey of metabolic pathways in rice. *Proceedings of the National Academy of Sciences*, 99, 11969–11974.

Komatsu, S., Zang, X., & Tanaka N. (2009). Comparison of two proteomics techniques used to identify proteins regulated by gibberellin in rice. *Journal of Proteome Research*, 5, 270-276.

Khatun, S., Rizzo, C.A., & Flowers, T. J. (1995). Genotypic variation in the effect of salinity on fertility in rice. *Plant Soil*, 173, 239–250.

Khavarinejad, R. A., & Mostofi, Y., (1998). Effects of NaCl on photosynthetic pigments, saccharides, and chloroplast ultrastructure in leaves of tomato cultivars. *Photosynthetica*, 35, 151–154.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680–685.

Laura, L., *et.al*., (2009). A novel plant leucine-rich repeat receptor kinase regulates the response of *Medicago truncatula* roots to salt stress. The Plant Cell, Vol. 21: 668–680.

Lazof, D.B., & Bernstein, N. (1999). The NaCl induced inhibition of shoot growth: the case for distributed nutrition with special consideration of calcium. *Advances in Botanical Research*, 29, 113-189.

Lee, S.H.,, Kim, J.C., Lee, M.S., Heo, W.D., Seo, H.Y., Yoon, H. W., Hong, J.C., Lee, S. Y., Bahk, J. D., Hwang, I., & Cho, M.J. Identification of a Novel Divergent Calmodulin Isoform from Soybean Which Has Differential Ability to Activate Calmodulin-dependent Enzymes. (1995) *J. Biol. Chem.*, 270, 37, 21806–21812.

Lee, S.H., Seo, H.Y., Kim, J.C., Heo, W.D., Chung,W.S., Lee, K. J., Kim, M.C. Cheong, Y. H., Choi, J. Y., Lim, C.O. & Cho, M.J. (1997). Differential Activation of NAD Kinase by Plant Calmodulin Isoforms. The critical role of domain I. *J. Biol. Chem*., 272, 14, 9252–9259.

Lopez, F., Vansuyt, G., Casse-Delbart, F., & Fourcroy, P. (1996). Ascorbate peroxidase activity, not the mRNA level, is enhanced in salt-stressed *Raphanus sativus* plants. *Physiologia Plantarum*, 97, 13-20.

Lowry, O.H., Rosbrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265.

Luan, S., Kudla, J., Rodriguez-Concepcion, M., Yalovsky, S., and Gruissem, W. (2002). Calmodulins and Calcineurin B–like Proteins: Calcium Sensors for Specific Signal Response Coupling in Plants. *Plant Cell*. 14: S389-S400.

Majoul, T., Chahed, K., Zamiti, E., Ouelhazi, L., & Ghrir, R. (2000). Analysis by two-dimensional electrophoresis of the effect of salt stress on the polypeptide

patterns in roots of a salt-tolerant and a salt-sensitive cultivar of wheat. *Electrophoresis*, 21, 2562–2565.

Malamy, J. E. (2005). Intrinsic and environmental response pathways that regulate root system architecture. *Plant Cell and Environment*, 28, 67-77.

Mannervik, B. & Guthenberg, C. (1981). Glutathione Transferase (Human Plancenta). *Methods in Enzymology*, 77, 231-235.

Muller, M.L, Jensen, M. and Taiz L., (1999) The vacuolar H^+-ATPase of lemon fruits is regulated by variable H^+/ATP coupling and slip *J. Biol. Chem* Vol. 274, No. 16, Issue of April 16, pp. 10706–10716.

McCormack, E., and Braam, J. (2003). Calmodulins and related potential calcium sensors of Arabidopsis. *New Phytol.* 159: 585-598.

Meeir, H., & Reid, J. S. G., (1982). Reserve polysaccharides other than starch in higher plants. *Encyclopedia of Plant Physiology, new series, Springer-Verlag, Berlin*, 13a, 418–471.

Miflin, B. J., & Habash, D. Z., (2002). The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *Journal of Experimental Botany*, 53, 979–987.

Mittova, V., Guy, M., Tal, M., & Volokita, M. (2002). Response of the cultivated tomato and its wild salt-tolerant relative Lycopersicon pennellii to salt-dependent oxidative stress: increased activities of antioxidant enzymes in root plastids. *Free Radical Research*, 36, 195-202.

Moller, I. M. (2001). Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annual Review of Plant Physiology and Plant Molecular Biology*, 52, 561–591.

Moore, B., Rolland, F., & Sheen, J. (2002). Sugar sensing and signaling in plants. *The Plant Cell*, 185-205.

Munns, R. (2005). Genes and salt tolerance: bringing them together. *New Phytologist,* 167, 645-663.

Murakeozy, E. P., Nagy, Z., Duhaze, C., Bouchereau, A., & Tuba, Z. (2003). Seasonal changes in the levels of compatible osmolytes in three halophytic species of inland saline vegetation in Hungary. *Journal of Plant Physiology*, 160, 395–401.

Nakano, Y., & Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in Spinach chloroplasts. *Plant and Cell Physiology*, 22, 867-880**.**

Nagashima, A. *et. al.* (2004) The multiple-stress responsive plastid sigma factor, SIG5, directs activation of the psbD blue light-responsive promoter (BLRP) in *Arabidopsis thaliana* Plant Cell Physiol. 45(4): 357–368

Nematol, J. (1982). Chloroplast differentiation in tomato root galls induced by the root knot nematode *Meloidogyne incognita*. *The journal of nematology*, 14, 77-83

Nguyen, H. T., Shim, I. S., Kobayashi, K., & Usui, K. (2005). Effects of salt stress on ion accumulation and antioxidative enzyme activities of *Oryza sativa* L. and *Echinochloa oryzicola* Vasing. *Weed Biology Management*, 5, 1-7

Plant Physiol. 2012 Jan;158(1):514-23. Epub 2011 Nov 17.

Nikkinen H.L., *et.al*. (2012) The SigB σ factor regulates multiple salt acclimation responses of the cyanobacterium Synechocystis sp. PCC 6803. Plant Physiol. Jan;158(1):514-23. Epub 2011 Nov 17.

Noctor, G., & Foyer, C., (1998). Ascorbate and glutathione: keeping active oxygen species under control. *Annual Review of Plant Physiology and Plant Molecular Biology*, 49, 249–279.

Nuccio, M. L., Rhodes, D., McNeil, S. D., & Hanson, A. D. (1999). Metabolic engineering of plants for osmotic stress resistance. *Current Opinion in Plant Biology*, 2, 128-134.

Oliveira, L. (2006). The development of chloroplasts in root meristematic tissue of *Secale cereale* L. seedlings. *New Phytologist*, 2, 263-275.

Oliver Batistič and Jörg Kudla, (2011) Analysis of calcium signaling pathways in plants. Biochimica et Biophysica Acta (BBA), 1820 (8), Pages 1283–1293

Ouyang S, Zhu W, Hamilton J, Lin H, Campbell M, Childs K, Thibaud-Nissen F, Malek RL, Lee Y, Zheng L, *et al* (2007): The TIGR Rice Genome Annotation Resource: improvements and new features. *Nucleic Acids Research*, 35: D883-887.

Park, O. K. (2003). Proteomic studied in plants. *Journal of Biochemistry and Molecular Biology*, 37, 133-138.

Parvaiz, A., & Satyawati, S. (2008). Salt stress and phyto-biochemical responses of plants – a review. *Plant Soil & Environment*, 54, 88-99.

Pawlak, S., & Deckert, J. (2007). Histone modifications under environmental stress. *Biological Letters*, 44, 65-73

Pelleschi, S., Guy, S., Kim, J. Y., Pointe, C., Mahe, A., Barthens, L., Leonardi, A., & Prioul, J. L. (1999). *IVR2*, a candidate gene for a QTL of vacuolar invertase activity in maize leaves. Gene specific repression under water stress. *Plant Molecular Biology*, 39, 373–380.

Pelleschi, S., Rocher, J-P., & Prioul, J-L. (1997). Effect of water restriction on carbohydrate metabolism and photosynthesis in mature maize leaves. *Plant Cell and Environment*, 20, 493–503.

Perkins, D.N., Pappin, D.J.C., Creasy, D.M., and Cottrell, J.S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*. 20: 3551-3567.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29: 2002–2007.

Phean-o-pas, S., Limpaseni, T., and Buaboocha, T. (2008). Structure and expression analysis of the OsCam1-1 calmodulin gene from *Oryza sativa* L. *BMB Rep.* 41(11): 771-777.

Pieters, A. J., & Souki, S. E. (2005). Effects of drought during grain filling on PS II activity in rice. *Journal of Plant Physiology*, 162, 903-911

Plomion, C., Lalanne, C., & Claverol, S. (2006). Mapping the proteome of poplar and application to the discovery of drought stress responsive proteins. *Proteomics*, 6, 6509–6527.

Price, A. H., Tomos, A. D., & Virk, D. S. (1997). Genetic dissection of root growth in rice (*Oryza sativa* L.) I. A hydrophonic screen. *Theoretical and Applied Genetics*, 95, 132-142.

Price, J., Laxmi, A., Martin, S. K., & Jang J-C. (2004). Global transcription profiling reveals multiple sugar signal transduction mechanisms in *Arabidopsis*. *Plant Cell*, 16, 2128–2150

Quintero, J. M., Monila, R., Fournier, J. M., Benlloach, M., & Romos, J. (2001). Glucose induced activation of rubidium transport and water flux in sunflower root systems. *Journal of Experimental Botany*, 52, 99-104

Rabello, A.R., Guimarães, C.M., Rangel, P.H., Silva, F.R., Seixas, D., Souza, E. Brasileiro1, A.C., Spehar, C.R., Ferreira, M.E., & Mehta, A. (2008) Identification of drought-responsive genes in roots of upland rice (*Oryza sativa* L) *BMC Genomics*, 9:485

Raharjo, T. J., Widjaja, I., Roytrakul, S. and Verpoorteb, R. (2004). Comparative Proteomics of Cannabis sativa Plant Tissues. *Journal of Biomolecular Techniques*. 15, 97–106

Rathert, G., (1985). The influence of high salt stress on starch, sucrose, and degradative enzymes of two *Glycine max* varieties that differ in salt tolerance. *Journal of Plant Nutrition*, 8, 199–209.

Razavizadeh, R., Ehsanpour, A.A., Ahsan, N., and Komatsu S. (2009) Proteome analysis of tobacco leaves under salt stress. Peptides. Sep;30(9):1651-9.

Requejo, R., & Tena, M. (2005). Proteome analysis of maize roots reveals that oxidative stress is a main contributing factor to plant arsenic toxicity. *Phytochemistry* , 66, 1519–1528.

Roitsch, T., Balibrea, H. E., Hofmann, M., Proels, R., & Sinha, A. K. (2003). The upregulation of extracellular invertase was suggested to be a common response to various biotic and abiotic stress related stimuli like pathogen infection and salt stress. *Journal of Experimental Botany*, 54 513–524

Roth, U., von Roepenack-Lahaye, E., Clemens, S. (2006). Proteome changes in *Arabidopsis thaliana* roots upon exposure to Cd^{2+}. *Journal of Experimental Botany*, 57, 4003–4013.

Roxas, V. P., Lodhi, S. A., Garrett, D. K., Mahan, J. R., & Allen, R. D. (2000). Stress tolerance in transgenic tobacco seedlings that overexpress glutathione *S*-transferase/ glutathione peroxidase. *Plant Cell and Physiology*, 41, 1229–1234.

Saeng-ngam S., Takpirom W., Buaboocha, T. and Chadchawan, S. (2012) The role of the *OsCam1-1* salt stress sensor in ABA accumulation and salt Tolerance in rice. J. Plant Biol. 55:198-208

Salekdeh, Gh. H., Siopongco, J., Wade, L. J., Ghareyazie B., & Bennett, J. (2002). A proteomic approach to analyzing drought- and salt-responsiveness in rice. *Field Crops Research*, 76, 199-219.

Salerno, G. L., & Curatti, L. (2003). Origin of sucrose metabolism in higher plants: when, how and why? *Trends in Plant Science*, 8, 63–69.

Schirle, M., Heurtier, M.A., & Kuster, B. (2003). Profiling core proteomes of human cell lines by one-dimensional PAGE and liquid chromatography-tandem mass spectrometry. *Molecular & Cellular Proteomics*, 2.12, 1297-1305.

Seki, M., Narusaki, M., Ishida, J., Nanjo, T., Fujita, M., Oone, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Shinozaki, K. Y., Carninci, P., Kawai, J., Hayashizaki. K. Y., & Shinozaki, K. (2002). Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high salinity stresses using a full length cDNA micro array. *Plant Journal*, 31, 279–292.

Shalata, A., & Tal, M. (1998). The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt tolerant relative *Lycopersicon pennellii*. *Plant Physiology*, 104, 169–174.

Shi, H., Ishitani, M., Kim, C., & Zhu, J. K. (2000). The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na^+/H^+ antiporter. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 6896-6901.

Shuji, Y., Ray, A. B., & Paul M. H. (2002). Salt stress tolerance of plants. *JIRCAS Working Report*, 25-33.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Experimental Physiology*, 82, 291-295.

Singh, S. K., Sharma, H. C., Goswami, A. M., Datta, S. P., Singh, S. P. (2000). *In vitro* growth and leaf composition of grapevine cultivars as affected by sodium chloride. *Biologia Plantarum*, 43, 283–286.

Sivaguru, M., Pike, S., Gassmann, W., & Baskin, T. I. (2003). Aluminum rapidly depolymerizes cortical microtubules and depolarizes the plasma membrane: evidence that these responses are mediated by a glutamate receptor. *Plant and Cell Physiology*, 44, 667–675.

Skopelitis, D. S., Paranychianakis, N. V., Paschalidis, K. A., Pliakonis, E. D., Delis, I. D., Yakoumakis, D. I., Kouvarakis, A., Papadakis, A. K., Stephanou, E. G., & Roubelakis-Angelakis, K. A. (2006). Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for praline synthesis in tobacco and grapevine. *The Plant Cell*, 18, 2767–2781.

Smeekens, S. (2000). Sugar-induced signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 51, 49-81.

Smirnoff, N., Conklin, P. L., & Loewus, F. A. (2001). Biosynthesis of ascorbic acid in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 52, 437–467.

Steppuhn, H., & Raney, J. P. (2005). Emergence, height, and yield of canola and barley grown in saline root zones. *Canadian Journal of Plant Science*, 85, 815–827.

Sun, J., *et.al.* (2007). The CCCH-type zinc finger proteins AtSZF1 and AtSZF2 regulate salt stress responses in Arabidopsis. Plant Cell Physiol. 48(8): 1148–1158.

Tada, Y., & Kashimura, T. (2009). Proteomic analysis of salt-responsive proteins in the Mangrove Plant, *Bruguiera gymnorhiza*. *Plant Cell and Physiology*, 50, 439-446.

Tanji, K. K. (2002). Salinity in the soil environment. In *Salinity:Environment-Plant-Molecules* (eds A. Lauchli & U. Luttge). Dordrecht, Netherlands, 21-51.

Tarczynski, M. C., Jensen, R. G., & Bohnert, H. J. (1993). Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science*, 259, 508-510.

Teichmann, T., Guan, C., Kristoffersen, P., Muster, G., Tietz, O., & Palme K. (2004). Cloning and Biochemical Characterization of an Anionic Peroxidase from *Zea Mays*. *European Journal of Biochemistry*, 247, 826-832.

Termaat, A., Munns, R. (1986). Use of concentrated macronutrient solutions to separate osmotic from NaCl-specific effects on plant growth. *Australian Journal of Plant Physiology*, 13, 509–522

Thorsell, A., Portelius, E., Blennow, K., and Westman, B.A. (2007). Evaluation of sample fractionation using microscale liquid-phase isoelectric focusing on mass

spectrometric identification and quantitation of proteins in a SILAC experiment. *Rapid Commun Mass Spectrom.* 21: 771-778.

Tianbao Yang, B.W Poovaiah (2003) Calcium/calmodulin-mediated signal network in plants *Trends in plant science* Volume 8, Issue 10, October 2003, Pages 505–512

Tsai, Y. C., Hong, C. Y., Liu, L. F., & Kao, C. H. (2005). Expression of ascorbate peroxidase and glutathione reductase in response to NaCl and H_2O_2 in roots of rice seedlings. *Journal of Plant Physiology*, 162, 291-299

Tsugita, A., Kawakami, T., Uchiyama, Y., Kamo, M., Miyatake, N. & Nozu, Y. (1994). Separation and characterization of rice proteins. *Electrophoresis*, 15, 708–720.

Turner, W.L., Waller, J.C., Vanderbeld, B. & Snedden, W.A. (2004). Cloning and Characterization of Two NAD Kinases from Arabidopsis. Identification of a Calmodulin Binding Isoform. *Plant Physiology*, 135, 1243–1255.

Ueda, A., Yamamoto-Yamane Y., and Takabe, T. Salt stress enhances proline utilization in the apical region of barley roots. Biochem Biophys Res Commun. 2007 Mar 30;355(1):61-6. Epub 2007 Jan 26.

Vaidyanathan, H., Sivakumar, P., Chakrabarty, R. & Thomas, G. (2003). Scavenging of reactive oxygen species in NaCl-stressed rice (*Oryza sativa* L.) - differential response in salt-tolerant and sensitive varieties. *Plant Science*, 165, 1411-1418.

van Wijk, K. J. (2001). Challenges and prospects of plant Proteomics, *Plant Physiology* 126, 501–508.

Veenstra, T.D., & Yates, J. R. (2006). *Proteomics for Biological Discovery*. United States of America: Wiley-Liss.

Vetter, S.W. and Leclerc, E. (2003): Novel aspects of calmodulin target recognition and activation. *Eur. J. Biochem*. 270, pp. 404-414.

Viswanathan, C. & Zhu, J. K. (2003). Plant salt tolerance. *Topics in Current Genetics*, 4, 241 270.

Wang, J., Zhang, H., & Allen, R. D. (1999). Overexpression of an Arabidopsis peroxisomal ascorbate peroxidase gene in tobacco increases protection against oxidative stress. *Plant Cell and Physiology*, 40, 725–732.

Wang, M., Xu, Q., and Yuan, M. (2011). Zinc homeostasis is involved in unfolded protein response under salt stress. Plant Signaling & Behavior 6:1, 77-79.

Wang, W., Vinocur, B., Shoseyov, O., & Altman, A. (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science*, 9, 244–252.

White, P.J., and Broadley, M.R. (2003). Calcium in Plants. Ann. Bot. 92: 478-511.

Wilkins, M. R., Sanchez, J. C., Gooley, A. A., Appel, R. D., Humphery-Smith, I., Hochstrasser, D. F., & Williams, K. L. (1996). Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnology Genetic Engineering Review*, 13, 19-50.

Wortsman, J., Slominski, A., Coburn, S. P., & Mahuren, J. D. (2001). Microassay of phosphate provides a general method for measuring the activity of phosphatases using physiological, nonchromogenic substrates such as lysophosphatidic acid. *Analytical Biochemistry*, 298, 241-245.

Yamada *et al.* (2011). iPath2.0: interactive pathway explorer. *Nucleic Acids Res.* 39(suppl 2): W412-W415.

Yan, S., Tang, Z., Su, W., & Sun, W. (2005). Proteomic analysis of salt stress-responsive proteins in rice root. *Proteomics*, 5, 235-244

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlis, R. D., & Somero, G. N. (1982). Living with water stress: evolution of osmolyte system. *Science*, 217, 1214–1222.

Yang, C., Yang, L., Yang, Y. & Ouyangc, Z. (2004). Rice root growth and nutrient uptake as influenced by organic manure in continuously and alternately flooded paddy soils. *Agricultural Water Management*, 70, 67–81.

Yang, J. C., Peng, S. B., Visperas, R. M., Sanico, A. L., Zhu, Q. S., & Gu, S. L. (2000). Grain filling pattern and cytokinin content in the grains and roots of rice plants. *Plant Growth Regulation*, 30, 261-270

Yuvaniyama, A., Dissataporn C., Pongwichian P., Yamclee P. (2007). Salinity problems and sustainable management in Nakhon Ratchasima Province, Northeast Thailand. *The 2nd International Salinity Forum*, Salinity, water and society global issues., 1-3.

Zhanga , C., Yina, Y., Zhanga, A., Lua, Q., Wena, X., Zhub, Z., Zhanga, L. & Lua,C. (2012). Comparative proteomic study reveals dynamic proteome changes between superhybrid rice LYP9 and its parents at different developmental stages *Journal of Plant Physiology*, 169, 387– 398

Zhu, J. K. (2003). Regulation of ion homeostasis under salt stress. *Current Opinion in Plant Biology*, 6, 441–445.

Zhu, J. K., Chinnusamy, V., & Jagendorf, A. (2005). Understanding and improving salt tolerance in plants. *Crop Science*, 45, 437-448.

Zhu, J. K., Xiong, L. (2002). Salt tolerance. *American Society of Plant Biologists*, 1-24

Zhu, W., Smith, J.W., & Huang, C.M. (2010). Mass spectrometry-based label-free quantitative proteomics. *Journal of Biomedicine and Biotechnology*, 1-6.

APPENDICES

APPENDIX A. Reagent for Germinating rice seeds, Protein extraction and precipitation

1. Preparation of 0.1 M potassium phosphate buffer at 25°C

pH	VOLUME OF 1 M K_2HPO_4 (ml)	VOLUME OF 1 M KH_2PO_4 (ml)
5.8	8.5	91.5
6.0	13.2	86.8
6.2	19.2	80.8
6.4	27.8	72.2
6.6	38.1	61.9
6.8	49.7	50.3
7.0	61.5	38.5
7.2	71.7	28.3
7.4	80.2	19.8
7.6	86.6	13.4
7.8	90.8	9.2
8.0	94.0	6.0

Dilute the combined 1 M stock solutions to 1 liter with distilled H_2O. pH is calculated according to the Henderson-Hasselbalch equation:

$$pH = pK' + \log \left\{ \frac{(\text{proton acceptor})}{\text{proton donor}} \right\}$$

where $pK' = 6.86$ at 25°C.

2. MS medium for germinating rice seeds

2.1 Conventional approach

MS medium 1 L		**Final concentrations (1X)**
100X MS Nitrate stock 1 L		
$(NH_4)NO_3$	165 g	(1650 mg/l)
KNO_3	190 g	(1900 mg/l)

100X MS Sulfate stock 1 L

$MgSO_4.7H_2O$	37 g	(370 mg/l)
$MnSO_4.H_2O$	1.69 g	(16.9 mg/l)
$ZnSO_4.7H_2O$	0.86 g	(8.6 mg/l)
$CuSO_4.5H_2O$	0.0025 g	(0.025 mg/l)

100X MS Halide stock 1 L

$CaCl_2.2H_2O$	44 g	(440 mg/l)
KI	0.083 g	(0.83 mg/l)
$CoCl_2.2H_2O$	0.0025 g	(0.025 mg/l)

100X MS PBMO stock 1 L

$KH_2.PO_4$	17 g	(170 mg/l)
H_3BO_3	0.62 g	(6.2 mg/l)
$Na_2MoO_4.2H_2O$	0.025 g	(0.25 mg/l)

100X MS NaFeEDTA stock 1 L – store in a refrigerator

$FeSO_4.7H_2O$	2.78 g	(27.8 mg/l)
Na_2EDTA	3.78 g	(37.8 mg/l)

Mix the followings:

	Final concentrations
100X MS Nitrate stock	10 ml
100X MS Sulfate stock	10 ml
100X MS Halide stock	10 ml
100X MS PBMO stock	10 ml
100X MS NaFeEDTA stock	10 ml

Add plant growth regulator to the desired concentrations.

Adjust pH to 5.7-5.8 with 1 N HCl or 1 N KOH/NaOH.

Add H_2O to 1 L.

Add 8 g of agar. (final concentrations 8 g/l)

2.2 Instant approach

Weigh instant MS. medium (Pacific Science) 25 g. Add plant growth regulator to the desired concentrations. Adjust pH to 5.7-5.8 with 1 N HCl or 1 N KOH/NaOH. Add H_2O to 1 L. Add 20 g of Gelco Gel or Phyta Gel (Sigma)

For all media without antibiotics prepared in 2.1 or 2.2, boil until melt and pour the media into the desired container. Autoclave.

3. Reagent for Protein determination by Lowry assay

3.1 Alkaline copper solution

3.1.1 Solution A (10ml)

$CuSO_4.7H_20$	40 mg
Sodium citrate dehydrate	100 mg
Deionized water	add to 10 ml

3.1.2 Solution B (100 ml)

Sodium carbonate	2 g
Sodium hydroxide	0.4 g
Deionized water	add to 100 ml

3.1.3 Solution C (51 ml)

Solution A	1ml
Solution B	50ml

3.2 Folin-Ciocalteu phenol reagent (20%) 20 ml

Folin-Ciocalteu phenol reagent	10 ml
Deionized water	10 ml

4. Reagent preparation for SDS-PAGE

4.1 SDS loading buffer (5X stock) 25 ml

1 M Tris-HCl, pH 6.8	3.25 ml
SDS	2.5 g
87% glycerol	12.5 ml
DTT (M.W. = 154.25)	1.925 g
Deionized water	8.75 ml
Bromophenol blue	0.006 g

4.2 electrophoresis buffer (10X stock) 100 ml

Tris-base	3 g
Glycine	14 g
10% SDS	10 ml
Deionized water	add to 100 ml

4.3 separating gel (12% polyacrylamide gel) 10 ml

1.5 M Tris-HCl, pH 8.8	2.5 ml
40% Acrylamide	3.0 ml
10% SDS	125 µl
Deionized water	4.35 ml
10 % Ammonium persulfate (APS)	50 µl
TEMED	5 µl

4.4 stacking gel (4% polyacrylamide gel) 3 ml

0.5 M Tris-HCl, pH 6.8	0.75 ml
40% Acrylamide	0.3 ml
10% SDS	30 µl
Deionized water	1.82 ml
10 % Ammonium persulfate (APS)	50 µl
TEMED	5 µl

5. Silver staining processes

5.1 Stock solution for silver staining

5.1.1 Fixing solution 200 ml

99.8% Methanol	100 ml
100% Acetic acid	24 ml
37% Formaldehyde	100 µl
Deionized water	add to 200 ml

5.1.2 Washing solution 200 ml

96% Ethanol	73 ml
Deionized water	add to 200 ml

5.1.3 Sensitizing solution 200 ml

Sodium thiosulfate	0.04 g
Deionized water	add to 200 ml

5.1.4 Staining solution 200 ml

Silver nitrate	0.4 g
Deionized water	add to 200 ml

5.1.5 Developing solution 200 ml

Sodium carbonate	12 g
37% Formaldehyde	100 µl
0.02% sodium thiosulfate	4 ml
Deionized water	add to 200 ml

5.1.6 Stopping solution 100 ml

Disodium, EDTA	1.4 g
Deionized water	add to 100 ml

Table 6 Gel staining protocol

Step	Processes	Reagent	time
1	Fix	fixing solution	30 min.
2	Wash	washing solution	2 X 5 min.
3	Sensitize	sensitizing solution	2 min.
4	wash	deionized water	2 X 5 min.
5	Stain	staining solution	20 min.
6	wash	deionized water	2 X 1 min.
7	Develop	developing solution	15 min. (max.) or completed visual band marker protein on gel
8	Stop	stopping solution	20 min.
9	wash	deionized water	3 X 5 min.

Gel can be stored in 5% acetic acid solution at 4°C for several weeks prior to in-gel digestion.

6. Reagent for In-gel trypsin digestion

6.1 Ammonium bicarbonate (10mM) 50 ml

Ammonium bicarbonate (m.w. 79.056)	39.5 mg
Deionized water (18 MΩ)	add to 50 ml

6.2 Reducing solution 10 ml (freshly prepare)

DTT	15.42 mg
10 mM Ammonium bicarbonate	add to 10 ml

6.3 Alkylating solution 10 ml

Iodoacetamide (m.w. 184.96)	185 mg
10 mM Ammonium bicarbonate	add to 10 ml

6.4 50% acetonitrile /10mM ammonium bicarbonate 10 ml

100% acetonitrile	5 ml
10mM ammonium bicarbonate	5 ml

6.5 Trypsin solution (10 ng/µl) 1 ml

Trypsin	10 mg
50% acetonitrile /10mM ammonium bicarbonate	1 ml

6.6 Acetonitrile (30%) 10 ml

100% Acetonitrile	3 ml
Deionized water	7 ml

6.7 Acetonitrile (50%) 10 ml

100% Acetonitrile	5 ml
Deionized water	5 ml

6.8 Formic acid (0.1%) 5 ml

Formic acid	5 µl
Deionized water	add to 5 ml

6.9 Peptide extracts solution 10 ml

0.1 % Formic acid	5 ml
100% Acetonitrile	5 ml

Appendix B: Table 7: List of significant differentially expressed protein investigated by GeL-LC-MS/MS and ANOVA

Protein name	Accession number	ID Score	t-test	ANOVA	Peptide sequence	MH+ (Da)
4-alpha-glucanotransferase [Oryza sativa Japonica Group]	gi|22093785	16.65	0.000066	0.000066	MPSSVSFDSLSPEAAK	1668.001
blight resistance protein [Oryza sativa Japonica Group]	gi|57899196	26.55	0.037525	0.037525	LVLENLIPPR	1163.634
bZIP protein [Oryza sativa Japonica Group]	gi|51038218	9.42	1.28E-05	1.28E-05	MAFDLK	741.2314
cellulase [Oryza sativa Japonica Group]	gi|24476042	15.24	7.92E-05	7.92E-05	SDYRYQPFVSR	1415.835
cinnamoyl CoA reductase [Oryza sativa Japonica Group]	gi|51535204	22.88	0.000111	0.000111	AEGGKMVCVTGAGGFIGSWVVK	2224.855
copia-type pol polyprotein [Oryza sativa Japonica Group]	gi|29126367	18.68	0.037216	0.037216	ASCSKIAPSNEPCAR	1647.785
cytochrome P450 [Oryza sativa Japonica Group]	gi|47777427	18.59	0.023602	0.023602	GASFRAAMSELIGDGLFAADGR	2210.827
disease resistance protein [Oryza sativa Japonica Group]	gi|22953955	3.61	0.004216	0.004216	APVAGGK	598.7048
disease resistance protein RPM1 [Oryza sativa Japonica Group]	gi|15528838	1.29	0.006732	0.006732	GAMPK	519.1466
gamma-adaptin 1 [Oryza sativa Japonica Group]	gi|15451585	18.03	0.008788	0.008788	LMLGNGRR	933.4581
kinase [Oryza sativa Japonica Group].	gi|18071364	0.71	0.001313	0.001313	LVAGA	429.1641
mitogen activated protein kinase [Oryza sativa Japonica Group]	gi|55297327	22.46	0.016408	0.016408	KHSSLPR	824.665
MtN21 [Oryza sativa Japonica Group]	gi|50508502	15.49	0.002543	0.002543	MSSGGAK	653.2204
O-deacetylbaccatin III-10-0-acetyltransferase [Oryza sativa Japonica Group]	gi|19920081	9.88	0.035367	0.035367	GLPSAPSVTPVR	1180.721
peroxidase [Oryza sativa Japonica Group]	gi|12039346	18.37	0.020822	0.020822	MAAVDVKNGYQGEIR	1668.095
polyprotein [Oryza sativa Japonica Group]	gi|20177631	22.28	0.001359	0.001359	GLDSDGEK	819.4329
pre-mRNA-splicing factor cwc-22 [Oryza sativa Indica Group]	gi|156622338	12.83			HDDSPPR	1289.876
receptor kinase [Oryza sativa Japonica Group]	gi|52077114	12.71	0.039287	0.039287	GHAFHPAPLR	1101.929
red chlorophyll catabolite reductase [Oryza sativa Japonica Group]	gi|14018063	8.38	3.01E-05	3.01E-05	VPAPPR	635.2485
retroelement [Oryza sativa Japonica Group]	gi|14091849	24.03	0.020433	0.020433	GNTGGANPGR	899.9043
RNA and export factor binding protein [Oryza sativa Japonica Group]	gi|33146678	10.95	0.029699	0.029699	AAPPAAR	653.3371
RPS2 [Oryza sativa Japonica Group].	gi|51091438	2.17	2.74E-13	2.74E-13	SDDIK	577.2648

Appendix B: Table 7: List of significant differentially expressed protein investigated by GeL-LC-MS/MS and ANOVA (cont.)

Protein name	Accession number	ID Score	t-test	ANOVA	Peptide sequence	MH+ (Da)
SecA [Oryza sativa Japonica Group]	gi|52075758	12.66	0.002311	0.002311	SSSYEDLQEYLRTR	1747.653
T-complex protein 1 beta subunit (TCP-1-beta) (CCT-beta) [Oryza sativa Japonica Group]	gi|49328009	14.45	0.001347	0.001347	VRVDSMAK	905.6863
wall-associated protein kinase [Oryza sativa Japonica Group]	gi|14029045	3.49	0.024419	0.024419	VSCPP	559.1759
XS domain containing protein [Oryza sativa Japonica Group]	gi|28875992	15.19	0.017047	0.017047	ATHLALAR	852.4001
Ankyrin-like protein [Oryza sativa Japonica Group]	gi|47848255	24.79	0.170081	0.170081	RGRSLPR	840.7296
Aquaporin NIP3-2	gi|75295453	9.54	0.028939	0.028939	MEGGKMSSMGMDAASASVTVPPMQMQA GDQSNR	3435.281
BRI1-KD interacting protein 103 [Oryza sativa Japonica Group]	gi|50252361	20.28	0.000485	0.000485	RILSSLNSR	1046.552
Catalase-1, , expressed [Oryza sativa Japonica Group]	gi|3929924	10.34	0.002034	0.002034	GFAIK	536.1969
Cytochrome P450 family protein, expressed [Oryza sativa Japonica Group]	gi|108706099	14.22	0.000424	0.000424	ATMASC	654.8041
DNA polymerase lambda [Oryza sativa Japonica Group]	gi|47232548	21.16	0.002718	0.002718	ISSDGPENRK	1102.498
E3 ubiquitin-protein ligase BRE1-like 1	gi|152013369	33.77	0.00321	0.00321	ALVSSIPR	842.5629
Epstein-Barr virus EBNA-1-like protein [Oryza sativa Japonica Group]	gi|34394514	26.13	0.007665	0.007665	EEGNGGEATRGR	1233.101
Fructose-1,6-bisphosphatase	gi|152032435	14.52	5.47E-05	5.47E-05	SPNGK	503.1164
Heat shock protein 81-1	gi|158513648	4.7	0.00593	0.00593	ADADK	519.1418
homeodomain protein, [Oryza sativa Japonica Group]	gi|50872469	18.93	0.005723	0.005723	AVPGRAARGSAREGWR	1697.39
hypothetical protein LOC_Os03g19800 [Oryza sativa Japonica Group]	gi|108707790	6.18	0.046989	0.046989	SPGRK	543.5066
hypothetical protein LOC_Os03g37850 [Oryza sativa Japonica Group]	gi|108709446	23.79	2.03E-05	2.03E-05	GLGGVIGVR	829.0534
hypothetical protein LOC_Os03g44340 [Oryza sativa Japonica Group].	gi|108710075	4.47	0.000222	0.000222	EVKME	651.1669
hypothetical protein LOC_Os11g04630 [Oryza sativa Japonica Group]	gi|77548646	0.53	0.046233	0.046233	QGGMA	477.9854
hypothetical protein LOC_Os11g14700 [Oryza sativa Japonica Group]	gi|62733035	16.24	0.00045	0.00045	LGSAYRPALR	1045.575

Appendix B: Table 7: List of significant differentially expressed protein investigated by GeL-LC-MS/MS and ANOVA (cont.)

Protein name	Accession number	ID Score	t-test	ANOVA	Peptide sequence	MH+ (Da)
hypothetical protein LOC_Os11g38380 [Oryza sativa Japonica Group]	gi|77551845	17.56	0.00124	0.00124	MDPPATDAAGR	1159.78
hypothetical protein LOC_Os11g40120 [Oryza sativa Japonica Group].	gi|77551897	11.17	0.007856	0.007856	VAVSK	503.394
hypothetical protein LOC_Os11g46060 [Oryza sativa Japonica Group]	gi|77552574	10.15			HGGIGDGGR	717.4505
hypothetical protein LOC_Os12g10470 [Oryza sativa Japonica Group]	gi|77553333	9.58	0.043407	0.043407	MDFVFAGKSATAVVIVDLIKK	2267.86
hypothetical protein LOC_Os12g16530 [Oryza sativa Japonica Group]	gi|77554222	22.13	0.018986	0.018986	SGLTPSIPR	926.9935
hypothetical protein LOC_Os12g35790 [Oryza sativa Japonica Group]	gi|77556676	10	0.000196	0.000196	LMGASPMDK	981.1344
hypothetical protein OsI_00373 [Oryza sativa Indica Group]	gi|125524397	8.16	0.004907	0.004907	AAIVKISLAVALNVR	1537.524
hypothetical protein OsI_03605 [Oryza sativa Indica Group].	gi|125527585	10.92	1.78E-06	1.78E-06	SGDPK	503.1332
hypothetical protein OsI_06779 [Oryza sativa Indica Group].	gi|218190503	0.19	0.009828	0.009828	LALAA	459.7067
hypothetical protein OsI_07204 [Oryza sativa Indica Group]	gi|218190738	9.97	0.004297	0.004297	HGFGSAGPCAS	1049.031
hypothetical protein OsI_08502 [Oryza sativa Indica Group]	gi|125540708	7.55	0.036912	0.036912	AQLTSAVIADVIR	1356.685
hypothetical protein OsI_10198 [Oryza sativa Indica Group]	gi|218192176	12.6	0.006571	0.006571	SIVAPPPSR	922.6624
hypothetical protein OsI_13807 [Oryza sativa Indica Group]	gi|218193866	40.43	0.019747	0.019747	IDGDSPPR	856.0065
hypothetical protein OsI_14854 [Oryza sativa Indica Group]	gi|125547231	14.09	0.0012	0.0012	MVDSSGDR	881.899
hypothetical protein OsI_19435 [Oryza sativa Indica Group]	gi|218196538	19.55	0.03793	0.03793	VEAKDSLTIVAR	1302.55
hypothetical protein OsI_19531 [Oryza sativa Indica Group]	gi|218196583	15.22			GRREAPR	1468.066
hypothetical protein OsI_23143 [Oryza sativa Indica Group]	gi|125555508	17.38	0.00321	0.00321	LPLALAVLGGYLSK	1415.39
hypothetical protein OsI_23247 [Oryza sativa Indica Group].	gi|125555615	4.37	0.065512	0.065512	VSSSA	462.6931
hypothetical protein OsI_25164 [Oryza sativa Indica Group]	gi|218199206	29.33	0.0329	0.0329	KSPSVTPR	870.6611
hypothetical protein OsI_25902 [Oryza sativa Indica Group]	gi|125558238	17.78	0.017839	0.017839	SMALGCTAKLGVADAIHRAGGR	2210.451
hypothetical protein OsI_26986 [Oryza sativa Indica Group]	gi|218200079	15.69			REVAGDRGSR	1065.563
hypothetical protein OsI_28982 [Oryza sativa Indica Group].	gi|218201046	13.91	0.001727	0.001727	IMEAR	635.1745

Appendix B: Table 7: List of significant differentially expressed protein investigated by GeL-LC-MS/MS and ANOVA (cont.)

Protein name	Accession number	ID Score	t-test	ANOVA	Peptide sequence	MH+ (Da)
hypothetical protein OsI_28982 [Oryza sativa Indica Group].	gi|49387758	0.87	0.00391	0.00391	TPKGN	470.8022
hypothetical protein OsI_29778 [Oryza sativa Indica Group].	gi|218201406	7.03	0.002387	0.002387	YGENK	610.3294
hypothetical protein OsI_32427 [Oryza sativa Indica Group].	gi|125564736	3.8	0.000854	0.000854	TGNAR	519.2892
hypothetical protein OsI_33597 [Oryza sativa Indica Group]	gi|125531939	14.75	0.001284	0.001284	SPGAP	429.1139
hypothetical protein OsI_34842 [Oryza sativa Indica Group]	gi|125533146	23.37	0.01084	0.01084	KATVAVSPR	927.4224
hypothetical protein OsI_36657 [Oryza sativa Indica Group]	gi|218186020	14.65	0.005214	0.005214	MSTIRGQ	809.8883
hypothetical protein OsJ_02067 [Oryza sativa Japonica Group]	gi|222618593	8.41			AGEPEMVAKR	1254.604
hypothetical protein OsJ_05171 [Oryza sativa Japonica Group]	gi|125580613	17.37	0.024887	0.024887	LSMLLKDEPELPR	1558.391
hypothetical protein OsJ_06402 [Oryza sativa Japonica Group]	gi|222622678	22.97	0.005802	0.005802	ATDALVQK	844.7946
hypothetical protein OsJ_06894 [Oryza sativa Japonica Group]	gi|222622939	11.82	0.001349	0.001349	AHLDAK	653.0802
hypothetical protein OsJ_07225 [Oryza sativa Japonica Group]	gi|222623105	11.32	0.000409	0.000409	DQVSQR	730.529
hypothetical protein OsJ_07524 [Oryza sativa Japonica Group]	gi|125582880	17.85	0.049772	0.049772	LVDQTLPPY	927.4046
hypothetical protein OsJ_10072 [Oryza sativa Japonica Group]	gi|222624530	24.13			SPADLSPR	807.2833
hypothetical protein OsJ_14601 [Oryza sativa Japonica Group]	gi|125590203	23.05	0.028132	0.028132	GMPMAPGPR	929.8297
hypothetical protein OsJ_17641 [Oryza sativa Japonica Group]	gi|222630706	11.88			ERGSGARGRR	830.6456
hypothetical protein OsJ_21286 [Oryza sativa Japonica Group]	gi|222635541	14.58	0.017085	0.017085	VASNISQIFERGR	1477.173
hypothetical protein OsJ_23643 [Oryza sativa Japonica Group]	gi|222636720	11.88	0.003048	0.003048	KPCHPLPEKK	1231.817
hypothetical protein OsJ_28947 [Oryza sativa Japonica Group]	gi|222641370	18.98	0.007397	0.007397	LICTDFVAISTDVICNWGK	2210.498
hypothetical protein OsJ_30089 [Oryza sativa Japonica Group]	gi|125606403	16.57	0.039162	0.039162	AAVPPR	610.2462

Appendix B: Table 7: List of significant differentially expressed protein investigated by GeL-LC-MS/MS and ANOVA (cont.)

Protein name	Accession number	ID Score	t-test	ANOVA	Peptide sequence	MH+ (Da)
hypothetical protein OsJ_30129 [Oryza sativa Japonica Group]	gi|222641983	5.35	0.044327	0.044327	MPPDQLMVMSK	1277.778
hypothetical protein OsJ_31157 [Oryza sativa Japonica Group]	gi|222612660	5.27	0.003206	0.003206	RRGLWWPTPPR	1421.185
hypothetical protein OSJNBa0079E14.12 [Oryza sativa Japonica Group]	gi|34015317	9.85			LGRDTAQEGR	845.1848
Leucine Rich Repeat family protein, expressed [Oryza sativa Japonica Group]	gi|77552081	12.75	0.0053	0.0053	IIINDSIR	944.5886
leucine zipper protein-like [Oryza sativa Japonica Group]	gi|49387676	24.73			RLTLSPR	872.6587
MADS box protein [Oryza sativa]	gi|2055376	11.83	0.005219	0.005219	EQMLCEANKCLR	1566.545
NB-ARC domain containing protein [Oryza sativa Japonica Group].	gi|77553104	15.58	0.035135	0.035135	GTDVK	519.0697
NBS-LRR class disease resistance protein [Oryza sativa Japonica Group]	gi|241989376	28.52	0.114995	0.114995	VDSRLPR	899.3778
OJ000114_01.10 [Oryza sativa Japonica Group]	gi|21952849	13.86	0.001508	0.001508	MGEGK	536.1485
OJ000114_01.7 [Oryza sativa Japonica Group]	gi|39545652	5.46	0.013789	0.013789	MMGGD	543.376
OJ000315_02.10 [Oryza sativa Japonica Group]	gi|38569175	11.81	0.003584	0.003584	FGEDTR	725.2828
OJ000315_02.21 [Oryza sativa Japonica Group]	gi|32487917	20.41	3.13E-05	3.13E-05	LHATVESDKLLVR	1479.544
Os01g0155600 [Oryza sativa Japonica Group].	gi|115434616	3.72	0.04513	0.04513	GSASP	418.0581
Os01g0165100 [Oryza sativa Japonica Group].	gi|115434700	9.96	7.76E-12	7.76E-12	SGPNK	503.1691
Os01g0183600 [Oryza sativa Japonica Group]	gi|115434940	15.43	0.000458	0.000458	AAVAASTSRK	962.0816
Os01g0207200 [Oryza sativa Japonica Group]	gi|115435180	19.29	0.005224	0.005224	SCTAEPR	819.436
Os01g0209200 [Oryza sativa Japonica Group]	gi|115435206	9.36	0.031579	0.031579	GDPAA	429.1715
Os01g0217100 [Oryza sativa Japonica Group]	gi|115435290	17.55			LSPSAAGSVEK	943.6501
Os01g0228400 [Oryza sativa Japonica Group]	gi|115435438	7.09	0.006515	0.006515	ASMSLK	653.1595
Os01g0242300 [Oryza sativa Japonica Group].	gi|39545652	5.46	0.013789	0.013789	MMGGD	543.376
Os01g0611900 [Oryza sativa Japonica Group].	gi|115438490	1.4	0.008606	0.008606	AAFDK	551.7047
Os01g0697100 [Oryza sativa Japonica Group].	gi|115439381	10.67	0.018951	0.018951	EAASA	449.3706

Appendix B: Table 7: List of significant differentially expressed protein investigated by GeL-LC-MS/MS and ANOVA (cont.)

Protein name	Accession number	ID Score	t-test	ANOVA	Peptide sequence	MH+ (Da)
Os01g0805100 [Oryza sativa Japonica Group].	gi|297597805	7.13	0.005784	0.005784	VAPTK	514.1992
Os01g0819700 [Oryza sativa Japonica Group].	gi|115440725	2.44	0.000622	0.000622	AAGRK	503.1013
Os01g0877300 [Oryza sativa Japonica Group]	gi|115441387	23.92	0.047743	0.047743	SSHTNVELLK	1128.775
Os02g0173500 [Oryza sativa Japonica Group]	gi|115444521	13.15	0.022716	0.022716	KLGKFKETQR	1235.377
Os02g0654000 [Oryza sativa Japonica Group]	gi|297599699	10.44	0.001099	0.001099	AGGTQR	587.6783
Os02g0690600 [Oryza sativa Japonica Group]	gi|115448025	18.66	0.00396	0.00396	VLLLLK	697.5277
Os02g0709900 [Oryza sativa Japonica Group]	gi|115448227	28.61	0.02931	0.02931	LEIEGK	688.8466
Os02g0715000 [Oryza sativa Japonica Group]	gi|115448287	15.77	0.036201	0.036201	MAGNNSLLAMDSK	1383.843
Os02g0728100 [Oryza sativa Japonica Group]	gi|115448437	9.53	1.43E-06	1.43E-06	AAMSR	536.1186
Os02g0755500 [Oryza sativa Japonica Group].	gi|115448767	10.04	5.37E-07	5.37E-07	GSNSR	520.9484
Os02g0764200 [Oryza sativa Japonica Group].	gi|115448869	3.94	0.000189	0.000189	GSDDK	520.7717
Os02g0823200 [Oryza sativa Japonica Group].	gi|297600147	3.12	0.003906	0.003906	THGTT	514.2212
Os03g0132600 [Oryza sativa Japonica Group]	gi|297721777	5.07	0.000246	0.000246	AAMDR	578.6281
Os03g0145200 [Oryza sativa Japonica Group]	gi|115450693	9.85	0.032003	0.032003	VMEMRLQSEEDASAASR	1941.645
Os03g0149700 [Oryza sativa Japonica Group]	gi|297721811	17.04			GHGGGGARR	923.6209
Os03g0219400 [Oryza sativa Japonica Group].	gi|115451601	0.76	0.01242	0.01242	GSNGK	463.4472
Os03g0275500 [Oryza sativa Japonica Group]	gi|115452215	23.27	0.029465	0.029465	EPEPEPVK	923.5398
Os03g0277700 [Oryza sativa Japonica Group]	gi|115452243	8.5	0.012862	0.012862	AIISSK	619.191
Os03g0369100 [Oryza sativa Japonica Group]	gi|115453181	14.55	0.002442	0.002442	VVYVAK	679.5141
Os03g0389100 [Oryza sativa Japonica Group].	gi|115453337	3.26			GDTSR	521.1276
Os03g0648100 [Oryza sativa Japonica Group]	gi|115454351	3.95	0.019105	0.019105	LPCLIK	742.301
Os03g0689800 [Oryza sativa Japonica Group]	gi|297722461	18.63	0.029072	0.029072	LANVLVGMAK	1031.255
Os03g0804400 [Oryza sativa Japonica Group]	gi|115456037	18.71	0.012649	0.012649	VVADEK	661.5295
Os03g0831900 [Oryza sativa Japonica Group]	gi|115456395	19.89	0.009942	0.009942	GPPVQR	653.8408
Os04g0444900 [Oryza sativa Japonica Group]	gi|115458594	7.27	7.11E-11	7.11E-11	SGIVK	503.0822
Os04g0445100 [Oryza sativa Japonica Group].	gi|115458598	4.62	5.27E-05	5.27E-05	MASMR	610.1972

Appendix B: Table 7: List of significant differentially expressed protein investigated by GeL-LC-MS/MS and ANOVA (cont.)

Protein name	Accession number	ID Score	t-test	ANOVA	Peptide sequence	MH+ (Da)
Os04g0486400 [Oryza sativa Japonica Group]	gi|115459074	16.65	0.021531	0.021531	AAASGGGGGPKK	956.4527
Os04g0509800, partial [Oryza sativa Japonica Group].	gi|115459364	8.43	8.88E-07	8.88E-07	GRGTK	519.1492
Os04g0664400 [Oryza sativa Japonica Group]	gi|297603514	6.38	0.00161	0.00161	GEPRGK	642.6778
Os04g0669100 [Oryza sativa Japonica Group]	gi|115461200	24.32	0.032565	0.032565	TVEAPR	672.4112
Os05g0106000 [Oryza sativa Japonica Group]	gi|115461631	14.91	0.008338	0.008338	CALYK	653.2416
Os05g0135900 [Oryza sativa Japonica Group]	gi|115461955	16.62	0.03194	0.03194	ILSDVSPR	887.7829
Os05g0320650 [Oryza sativa Japonica Group]	gi|297723967	32.47	0.000301	0.000301	QTWPELLGK	1071.977
Os05g0349000 [Oryza sativa Japonica Group]	gi|297724023	18.12	0.000285	0.000285	YSISTK	697.5231
Os05g0440000 [Oryza sativa Japonica Group]	gi|115464133	7.48			GGGGGGAAAFSHR	1262.628
Os05g0491200 [Oryza sativa Japonica Group]	gi|115464617	12.74	0.011466	0.011466	MGNASGK	679.8321
Os05g0552900 [Oryza sativa Japonica Group]	gi|297604880	7.39	0.008326	0.008326	SGNPK	503.2214
Os06g0106900 [Oryza sativa Japonica Group]	gi|115465932	5.91	0.006344	0.006344	MDKAGGNQGGKVLK	1418.48
Os06g0165900 [Oryza sativa Japonica Group]	gi|297724507	17.52	0.020482	0.020482	EREGDRIQR	1158.604
Os06g0214900 [Oryza sativa Japonica Group]	gi|115467068	4.09	0.021523	0.021523	LGGTPMKFGLPSRLFR	1794.332
Os06g0486900 [Oryza sativa Japonica Group]	gi|115468136	17.03	0.010697	0.010697	GVTIVNNAR	635.1632
Os07g0113700 [Oryza sativa Japonica Group]	gi|115470301	18.54	0.015832	0.015832	GIAQVNEGR	943.6047
Os07g0122400 [Oryza sativa Japonica Group].	gi|115470389	17.22	0.000239	0.000239	SSSNK	520.4521
Os07g0123800 [Oryza sativa Japonica Group]	gi|115470403	11.6	0.018878	0.018878	VAAVA	429.3818
Os07g0123800 [Oryza sativa Japonica Group].	gi|297596423	13.35	0.01194	0.01194	AATPA	429.3821
Os07g0194000 [Oryza sativa Japonica Group]	gi|115471021	10.17	0.018515	0.018515	SQAQDFRQQGTK	1393.909
Os07g0545300 [Oryza sativa Japonica Group].	gi|115472631	5.38			AGSSK	450.6266
Os07g0546100 [Oryza sativa Japonica Group].	gi|115472643	2.71	0.027952	0.027952	SEFAK	581.3644
Os07g0664000 [Oryza sativa Japonica Group]	gi|297607764	22.02			ATVIAIVR	821.3371
Os08g0430000 [Oryza sativa Japonica Group]	gi|115476512	4.95	0.012124	0.012124	AGGAK	402.6788
Os08g0469500 [Oryza sativa Japonica Group]	gi|115476832	23.55			NSLKNLDSR	1033.441
Os08g0474400 [Oryza sativa Japonica Group]	gi|297726519	20.17			TVLAGLDNDK	725.6196

Appendix B: Table 7: List of significant differentially expressed protein investigated by GeL-LC-MS/MS and ANOVA (cont.)

Protein name	Accession number	ID Score	t-test	ANOVA	Peptide sequence	MH+ (Da)
Os08g0475000 [Oryza sativa Japonica Group]	gi|297608735	25.4			VDPELPR	856.4632
Os08g0513600 [Oryza sativa Japonica Group]	gi|115477260	16.91	0.017665	0.017665	QAAELAIR	870.5102
Os09g0104200 [Oryza sativa Japonica Group]	gi|297609027	6.99	0.001439	0.001439	FAVPS	519.3124
Os09g0104200 [Oryza sativa Japonica Group]	gi|297609027	11.87	0.020069	0.020069	MISVAMILKK	1166.048
Os09g0360400 [Oryza sativa Japonica Group]	gi|115478767	12.21	0.03169	0.03169	AGGKK	459.2789
Os09g0395300 [Oryza sativa Japonica Group]	gi|115479007	7.22			CEPASLDQK	823.924
Os09g0420900 [Oryza sativa Japonica Group]	gi|115479213	18.5	0.01003	0.01003	VMVAVK	661.529
Os09g0453300 [Oryza sativa Japonica Group].	gi|115479499	4.44	0.038571	0.038571	GSRAS	477.9843
Os10g0485800 [Oryza sativa Japonica Group]	gi|297727701	15.86	0.010833	0.010833	RWAASPPTAAR	1183.024
Os11g0158300 [Oryza sativa Japonica Group]	gi|115484287	15.05	0.019797	0.019797	VHGRVDVAGRVHGLMR	1772.378
Os11g0414000 [Oryza sativa Japonica Group].	gi|115485233	8.79	0.004965	0.004965	GSNPK	503.1021
Os11g0425600 [Oryza sativa Japonica Group]	gi|115485265	13.4	0.01268	0.01268	AGGAKK	531.518
Os11g0442900 [Oryza sativa Japonica Group]	gi|115485349	40.81	0.046444	0.046444	VTVVAEPR	870.6878
Os11g0515000 [Oryza sativa Japonica Group]	gi|115485653	21.9			DVGDAIPR	1340.136
Os11g0556400 [Oryza sativa Japonica Group]	gi|115485891	11.45			MNKSGK	679.5099
Os12g0246700 [Oryza sativa Japonica Group]	gi|115488014	35.59	0.001641	0.001641	KETPSVELLK	1142.518
Os12g0279100 [Oryza sativa Japonica Group]	gi|115488170	18.1	0.027825	0.027825	VQDEASTR	905.7221
OSJNBa0035M09.19 [Oryza sativa Japonica Group]	gi|38346558	2.64	0.000117	0.000117	SVPAAK	572.8049
OSJNBa0041A02.6 [Oryza sativa Japonica Group]	gi|38344139	17.4	0.040032	0.040032	MLDDITR	878.9972
OSJNBa0044K18.13 [Oryza sativa Japonica Group].	gi|38605770	6.75	0.00406	0.00406	AGGPK	429.0792
OSJNBa0057M08.7 [Oryza sativa Japonica Group]	gi|58532075	20.9	0.006061	0.006061	AAPLPAER	824.7668
OSJNBa0067K08.2 [Oryza sativa Japonica Group].	gi|21740783	8.22	0.015225	0.015225	AARHR	610.1913
OSJNBa0083D01.12 [Oryza sativa Japonica Group]	gi|39546279	23.1	0.009902	0.009902	KVPKPTSSKKTSQPK	1640.688
OSJNBa0083D01.7 [Oryza sativa Japonica Group]	gi|39546274	9.85	0.005197	0.005197	IINPAK	654.2326
OSJNBa0083N12.7 [Oryza sativa Japonica Group].	gi|38345769	2.42	0.002934	0.002934	NPSAK	515.4799
OSJNBa0095H06.14 [Oryza sativa Japonica Group].	gi|38346789	7.02	0.024231	0.024231	TSGIK	505.1368

Appendix B: Table 7: List of significant differentially expressed protein investigated by GeL-LC-MS/MS and ANOVA (cont.)

Protein name	Accession number	ID Score	t-test	ANOVA	Peptide sequence	MH+ (Da)
OSJNBb0004A17.2 [Oryza sativa Japonica Group].	gi|70663938	3.63	0.006726	0.006726	HIEIR	667.2355
OSJNBb0028M18.4 [Oryza sativa Japonica Group].	gi|38345641	0.52	0.025803	0.025803	EMGAK	552.0264
OSJNBb0032D24.5 [Oryza sativa Japonica Group]	gi|32487629	13.4	0.045669	0.045669	AKVPIGK	711.9054
OSJNBb0052B05.18 [Oryza sativa Japonica Group]	gi|21742486	2.97	0.013912	0.013912	TATSR	536.2125
OSJNBb0072M01.4 [Oryza sativa Japonica Group]	gi|32482939	26.76	0.025119	0.025119	IAISLGIRRLIVR	1479.914
P0660F12.14 [Oryza sativa Japonica Group].	gi|15290159	3.59	0.000125	0.000125	GPVGK	457.4028
P0696G06.21 [Oryza sativa Japonica Group].	gi|21952849	2.58	0.028551	0.028551	MGEGK	537.1658
PHD zinc finger protein-like [Oryza sativa Japonica Group].	gi|55296653	5.01	0.000973	0.000973	AIPSS	474.1575
Phospholipid-transporting ATPase 1, , expressed [Oryza sativa Japonica Group]	gi|108707922	4.8	0.017086	0.017086	SLRSVGDMPSVTFAGDMR	1941.08
plant disease resistance polyprotein-like [Oryza sativa Japonica Group]	gi|54291382	18.34	0.011202	0.011202	GWVSGGEPR	943.6311
plastid RNA polymerase sigma factor [Oryza sativa Japonica Group].	gi|2780746	7.32	0.032702	0.032702	QGVSR	545.7428
succinyl-CoA ligase [ADP-forming] subunit alpha, mitochondrial	gi|75294330	0.53	0.025102	0.025102	GTAQDK	619.5091
receptor protein kinase-like [Oryza sativa Japonica Group]	gi|56202196	23.91	0.027333	0.027333	HVLDSGDVRSLVDAR	1640.658
retrotransposon protein, , Ty1-copia subclass, expressed [Oryza sativa Japonica Group]	gi|108707437	10.28	0.03002	0.03002	EASSSAAMADRGR	1310.149
retrotransposon protein, , Ty3-gypsy sub-class [Oryza sativa Japonica Group]	gi|62733004	31.48	0.038443	0.038443	NRWTKWR	1046.296
retrotransposon protein, , unclassified [Oryza sativa Japonica Group]	gi|77555660	7.63	0.005339	0.005339	ATAGT	421.3796
ribonuclease, partial [Oryza sativa Japonica Group].	gi|259130093	15.79	0.021667	0.021667	TYTLG	553.7022
SCARECROW gene regulator, [Oryza sativa Japonica Group]	gi|62733159	22.07	0.00017	0.00017	SATELLKQIKQNSSAR	1201.493
Transcription initiation factor TFIID subunit 1	gi|75116324	3.28	0.030388	0.030388	SHAKK	571.3826
translational activator protein-like [Oryza sativa Japonica Group]	gi|50251987	18.43			YKAGLK	870.1734

Appendix B: Table 7: List of significant differentially expressed protein investigated by GeL-LC-MS/MS and ANOVA (cont.)

Protein name	Accession number	ID Score	t-test	ANOVA	Peptide sequence	MH+ (Da)
Transposable element protein, [Oryza sativa Japonica Group]	gi|62732688	6.1	0.013526	0.013526	AIVPK	525.7953
transposon protein, , CACTA, En/Spm sub-class [Oryza sativa Japonica Group]	gi|77552302	12.84	0.013356	0.013356	MYANKK	772.0774
transposon protein, , unclassified [Oryza sativa Japonica Group]	gi|77549280	42.21	0.033638	0.033638	NVTDLTK	789.5329
Zinc finger CCCH domain-containing protein 36	gi|75226408	12.25	0.006494	0.006494	GADFDK	651.2653

BIOGRAPHY

Mr. Trilert Chaicherdsakul was born in September 7th, at Bangkok, Thailand. He graduated in Bachelor degree in Biotechnology from Mahidol university. After that he graduated in Master degree in Biochemistry (International Program) from Mahidol university.

For more detail, please visit http://www.biotech.au.edu