Proteomic Analysis of Germinated Rice (Oryza sativa L.)
Under Salt Stress

Mr. Li Rui

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Biotechnology
Faculty of Biotechnology
Assumption University
Academic year 2016
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<td>Thesis Advisor</td>
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Accepted by the Faculty of Biotechnology, Assumption University in
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PROTEOMIC ANALYSIS OF GERMINATED RICE (*Oryza sativa* L.) UNDER SALT STRESS

KEYWORDS: SALT STRESS / RICE/ GERMINATION/ PROTEOMICS

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ABSTRACT

Salt stress caused a dramatic decline of rice production. Proteome study of salt tolerance mechanism supplied a span-new viewpoint and valuable clue to rice tolerant improvement projects. Aim of this study was to identify the salt tolerant capacity and stress response proteins of seven Thai rice (*Oryza sativa* L.) cultivars at germination stage. To achieve these goals, seven Thai rice varieties: Pathumthani, Phitsanulok2, RD29, RD31, RD41, RD47, and Riceberry were germinated under 200mM NaCl for 4 days. Based on germination rate, Pathumthani, Phitsanulok2 and RD31 cultivars were categorized as tolerant, while RD29, RD41 and Riceberry were moderately tolerant and RD47 as susceptible. GeLC-MS/MS analysis of total proteins prepared from 7 rice seeds grown under salt stress identified 1339 proteins, 51 of which were expressed only in salt tolerant cultivars including Pathumthani, Phitsanulok02 and RD31. These proteins were distributed on cell membrane, cytoplasm, peroxisome, plastid, mitochondrion and nucleus. They played role in development, protein modification, signal transduction, stress response, transport and transcription. Proteome mechanism during the process of seed germination under salt stress was proposed.
ACKNOWLEDGEMENT

It is obviously that I would not complete such a challenging project without the spiritual encouragement from my beloved parents and the academic aspect support from my advisor Dr. Viyada Kunathigan and co-advisor Dr. Sittiruk Roytrakul. Take this wonderful opportunity, I express my deep sense of gratitude and appreciation to them via this simple words.

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Rui Li
2016
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<td>°C</td>
<td>degree Celsius</td>
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<tr>
<td>et al.</td>
<td>et. alli (Latin) and others</td>
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<td>g</td>
<td>gram</td>
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<td>h</td>
<td>hour</td>
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<td>L</td>
<td>litre</td>
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<td>M</td>
<td>Molar (concentration)</td>
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<td>minute(s)</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>molecular weight</td>
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<td>µg</td>
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<td>microliter</td>
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<td>micromolar</td>
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<td>µmol</td>
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<td>rpm</td>
<td>round per minute</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>sec</td>
<td>second(s)</td>
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<tr>
<td>v</td>
<td>volume</td>
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<tr>
<td>v/v</td>
<td>percent “volume in volume” express the number of milliliters of an active constituent in 100 milliliters of solution</td>
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<tr>
<td>w</td>
<td>weight</td>
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<tr>
<td>w/v</td>
<td>percent “weight in volume” express the number of gram of an active constituent in 100 milliliters of solution, and is used regardless of whether water or another liquid is the solvent</td>
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CHAPTER I

INTRODUCTION

Salt stress is considered as a major environmental limiting factor of farming land productivity. It was proved that excess salt exist in cultivate soil not only sharply reduced the yield of crops but it also significant impacted the quality of final product. Unfortunately, according to the estimated data from Food and Agriculture Organization of the United Nations (FAO), salt contaminated land area has been occupied 6.5% of total world's cultivable farming land and this number is continuing to grow by 10 million hectare each year. Former intrinsic mechanism investigations of salt stress showed that excessive salt containing in growth matrix will initiate both osmotic and ionic imbalance in cells to start, via its increased soluble solutes. Subsequence studies found that these impaired intercellular balances will further influence all regular living activities in plant. However, many evidence demonstrated that plants have developed series of effective adaption strategies to face such a brutal stimulation. Among them, the strategies that manipulated water transport, osmotic potential balance, and plant hormone synthesis were intensively studied.

As a conventional carbohydrate resource, rice (Oryza sativa. L) has been widely planted in 115 tropical and subtropical countries, served energy to more than half world's population. But massive research evidences showed that rice is a salt sensitive species, excess salt directly declines rice yield. Since rice production is important to human society, the salt tolerance improvement in rice is thus received intensive attention all over the world. Numerous methods have been developed to increase the salt resistant capacity of rice. Briefly, these attempts can be divide into two branches: the chemical embranchment and the genetic breeding embranchment. Till now, several chemical components such as abscisic acid (ABA), hydrogen peroxide, melatonin, polyamines, and 24-epibrassinolide (EBL) were reported to enhance salt tolerant capability of rice. However, the limitation concerning stability and high-efficiency traits in real production process still remains, the cultivar breeding is therefore the popular approach for improvement of salt tolerance in rice. Based on the advance of molecular biology in the past decades, many new concepts and techniques (like molecular marker, marker-associated selection, genetic engineering etc.) were introduced to rice breeding that greatly improved this time-consuming process and opened a new era (molecular breeding) for this conventional method. While, abundant launched biological database and it continuing augmented inclusive information boosted the exertion of potential competence of these newly developed advance techniques in rice breeding research. However, the basic knowledge concerning salt stress resistant mechanism is needed.
In plant, salt stress defense pathway started from the initial sensing of extracellular stimulation signal, then converse and amplifies these physical stress signal elements into perceivable chemical signal component. Finally, stress-resistant responses to changed environmental condition were controlled downstream by mediating intercellular signal transduction, gene expression, protein synthesis and metabolism. Hence, the exploration of defense pathway could provide a systemic viewpoint that makes our understanding of rice salt tolerance more complete. So far, numerous remarkable studies have been done in this field, and many stresses resistance related signal transduction pathways (e.g. calcium ions mediated pathway, ABA regulated pathway, and phospholipid controlled signaling pathway), resistant genes and proteins were identified. But, how these signaling molecular elements, resistant genes and proteins work together to maintain intercellular ionic homeostasis and osmotic equilibrium re-establishment under salt stress is still underexplored. As a bridge, protein precisely connected up-stream function gene to down-stream physiological response, the investigation of salt response protein may provide an understanding the rice salt stress tolerance mechanism systemically.

The proteomic approach has been widely used to investigate protein alteration in various organisms because it provides the entire data network of protein regulation. In addition this technique can be used to detect the proteins regulated by post-translational modification. In this study, this powerful technique was applied to investigate the protein profiles of seven widely cultivated rice varieties including Pathumthani, Phitsanulok02, RD29, RD31, RD41, RD47, and Riceberry. The alteration of protein expressed in rice seeds germinated under 200 mM NaCl was monitored. The protein characteristics derived from proteomics and bioinformatics provided insight into the potential salt defense pathway in Thai indica rice. The obtained stress responsive proteins might be used as molecular marker in future breeding project for yield improvement and salt tolerance.
CHAPTER II

LITERATURE REVIEW

1. Rice

As a major carbohydrate carrier, rice has been grown in Southeast Asia region more than 7000 years and successfully cultivated in 115 tropical and subtropical countries around the world (FAO, 2015). Nowadays, rice production achieved a remarkable level. According to Food and Agriculture Organization of the United Nations (FAO) statistic data, 500 million tones rice were produced annually and supplied energy to more than half world’s population. However, to equilibrate ever-increasing population, additional 176 million tones rice is needed by 2025 (FAO, 2000). Therefore, rice production still face change. In the other hand, rice is also an important crop for economy, it was reported that rice trade occupied 2.7 percentage of south Asia GDP in 2007 (Bishwajit et al., 2013). The value of rice is not merely related to food security and socio-economic development, since its smallest genome size (400-430Mb) within gramineous plants and closely gene amount with the most well studied dicotyledonous model plant—Arabidopsis thaliana, rice has also been became a useful model plant for monocotyledons at both genetic and molecular biology research field (Arumuganathan and Earle, 1991).

2. Rice seeds and germination

Rice is an annual grass plant. Thus, mature seeds are extremely important for rice production of next year. According to cultivar, the morphological characteristics and weight of rice seeds are differ greatly, but the basic morphological structure is the same. Generally, inside the lemma of rice seeds contain bran, endosperm and embryo. If stored rice seeds under a proper environment, which the surrounding humidity lower than 14%, temperature between 2 to 20°C and seeds moisture content at 6%, its seeds viability can remain more than one year (Ricepedia, 2015).

Germination is a vital process for all spermatophytes. A successful germination could end seeds dormancy, while, start another new life cycle. Briefly, the germinating process of rice seeds can be fall into three main phases. In the first phase, rice seeds undergo a water potential drive physical imbibition period. During this phase, the dry seeds rehydrate rapidly, and increases its weight dramatically whatever they are living or death. At the second phase, seeds look like stay in a motionless status, because the radicle still not breaks through testa at this stage. But inside lemma, series of important bio-reactions are undergoing that prepare
energy and material for subsequence germination. These indiscernible inner activities involved in seeds storage energy substance (starch, lipid, protein) degradation, crucial enzyme activation, reserve redistribution. And they are indispensable for germinating. The other rapid water uptake period appears again at the third phase of germination that will boost elongation growth of seeds and finally leads to root break the testa. Once the length of radicle equal to the seeds itself or the length of coleoptile achieve half of seeds, the germinating process is finished. Generally, the whole germination process of rice seeds takes at least 72h at 30°C (IRRI.2013; Yang et al., 2007).

However, numerous environmental factors (e.g. water, light, temperature, and oxygen) and endogenous hormone (GA, ABA) and even seeds itself (seed coat) is demonstrated to inhibit germinating process of rice seeds. Therefore, a few physiological and chemical treatment such as priming, seeds soaking, and GA3 are developed to improve germination capability of rice seeds through ensure degradation of endosperm stored energy and expression of embryo carried information (kim et al., 2009; Lee et al., 2000; Han et al., 2014).

3. Salt stress

Plants cannot escape from unfavorable conditions like animals did. Therefore, drastic environmental change let plants expose to various extreme conditions (such as cold, drought, salt, UV radiation, insert) frequently, and even worse, different types of environmental stress can be occurred overlapping. Under stress condition, the internal metabolism, substance transport and external morphogenesis are interfered and finally affecting plant growth, development, and reproduction. Some extreme statuses even lead to plant death. Due to its deeply impact on agriculture, massive research related to plant stress have been carried out through physiological, biochemical and molecular biological manner. Many attempts have been created to reduce (or eliminate) the negative influence of environment stresses on plant. Previously study broadly divided environmental stresses into two types: 1) biotic stress and 2) abiotic stress. However, the abiotic stress is reported to be the main barrier of world agriculture productivity (He et al., 2014).

Salt stress, which is one of the most adverse stress factor that causes 10 million hectare cultivatable land degradation each year, and inhabited crop growth and yield improvement. In some worst region even lead to crop failure and death (Owens, 2001). Recent investigation indicated that both natural activity and anthropogenic perturbation cause soil salinization. At natural activity aspect, plenty evidences show that the high salt content mineral rock, seawater evaporation, and climate change induced sea level rise are the major natural contributor of soil salinization (Rengasamy, 2006; Adam et al., 2009; Dimmock et al.,
Meanwhile, the most effective yield improvement technique—irrigation that double total yield of world rice production, has been proved as a key anthropic impact factor of farming land salinization (Biggs et al., 2010). Numerous salt stress researches in plant reveal that high concentration of salt existed in soil will increase the soluble solute of soil, that will initiates both osmotic potential and ionic imbalance immediately. Then, regular plant water uptake and mineral transport will be disturbed, and finally inhabited plants growth.

Ca\(^{2+}\), Mg\(^{2+}\), Na\(^+\), Cl\(^-\) and SO\(_4^{2-}\) are major salt ions that caused soil salinization, and naturally exist in pedologic component. Before achieved sufficient amount, they do not influence growth, even some of them (Na\(^+\), Cl\(^-\)) are the essential micronutrients of plants. But high concentration makes them become noxious factors (Aroca et al., 2012; Ehsan et al., 2010; White et al., 2001). Further investigation shows that the toxicity of Na\(^+\) and Cl\(^-\) are higher than other salinization related salt ions. Once the ionic concentration accumulated to virulence-required amount, they will bring more impairment to plant (Slabu et al., 2009). Since Na\(^+\) and Cl\(^-\) both are essential micronutrients, so they could inflows and accumulated in plant easier. When Na\(^+\) accumulated to high concentration, it will compete the functional binding site with the critical detoxification ion potassium, disturb Na\(^+\)/K\(^+\) ratio and then impeded K\(^+\) mediated stomata movement. Subsequently, water transport inhibition will induce the water lack of plant (Ma et al., 2011; Edgar et al., 1990). The main impairment of excessive Cl\(^-\) is proved on plant photosynthesis system via decreased chlorophyll biosynthesis. However, since chloride ion participates diverse intracellular activities (such as regulation of cytoplasmic pH value, cell turgor management, and enzyme activation). Thus, high concentration of Cl\(^-\) will trigger damage worse than Na\(^+\) did in plant (Slabu et al., 2009; Jennings, 1976).

For long-term naturally adaptation and evolution, plants have been developed series of physiology strategies to face high salt concentration induced stress condition. It was known that plant could change its roots growth direction to avoid salinity area in growth matrix or accumulate organic component (like sugar) in its root to avert excessive salt influx into plant. However, if additional salt influx in plant, they will be transported to upper part by xylem vessel using symplasm and apoplastic pathways, then these excessive salt could be accumulated in some specific tissues (such as old leaf) of plant or secreted out directly (Li et al., 2008; Siringam et al., 2001; Betty and Merrill., 1966; Rush and Epstein., 1980; Wang et al., 2012). But, the complicated molecular mechanisms that drive these resistant responses are still unclear (Flowers and Colmer, 2015).
4. Proteomic analysis of salt tolerant rice

Depend on the rapid advance on biotechnology and biology digital database establishment, the investigation of plant salt stress entered into a new era. Diverse newly developed analytical techniques make plant stress mechanism study could reach molecular level through metabolomics, proteomics, genomics, and transcriptomics manner. Among these ‘Omics’, the proteomics provide a high-throughput and systematic analysis approach to identify and understand the structure, function and interaction of all expressed proteins in an organism under a specific condition. While, since cells response to external or internal stimulate signals by mediating its protein synthesis level and activity. Hence, proteomics study could provide a snapshot into internal cellular actions (Yang et al., 2015).

Rice is a valuable glycophyte. Excessive salt appeared in soil caused germinating inhibition, development and growth delay, senescence acceleration or even to die (Zhu, 2001). Thus, massive remarkable research has been carried out to explore its molecular tolerant mechanisms. Through these researches, huge number of salt stress responsive proteins were isolated and identified. Meanwhile, many probable signal transduction pathways, salt tolerant mechanisms and models have been proposed. According to previously investigation, the salt stress adaptation response of rice is started from initial sensing of extracellular stimulation signal. Then, these physical signals are conversed and amplified into perceivable chemical signal components by cell membrane localized protein sensor. Protein sensor generated secondary signal (like ABA, Ca⁺) will continue transmit at intercellular environment to regulate gene expression, protein synthesis and metabolism. Finally, mediated cells make corresponding stress response actions to adapt adverse condition. Within rice salt resistant responses, the signal transduction plays crucial rule. Abscisic acid (ABA) and calcium ion (Ca⁺) are two major controlling elements of rice salt tolerant signal transduction pathways. At the early stage of salt stress, calcium ion (Ca⁺) and its mediated Salt Overly Sensitive (SOS) pathway will be activated promptly to resist high concentration of salt induced ionic imbalance via enhanced expression of ionic homeostasis related proteins such as Na⁺/K⁺ antiporter, vacuolar H⁺/ATPase and k⁺ transporter (Juliana et al., 2007; Kumar et al., 2013; Tomoaki et al., 2007; Chen et al., 2015). As exposure time goes on, the biosynthesis of phytohormone ABA and it regulated signal transduction pathways are activated to re-establish the osmotic potential of rice cells. More recent proteomics research shows that increased ABA level will trigger the expression of Mitogen-Activated Protein Kinase (MAPK), calcium-dependent protein kinases (CDPK), receptor-like kinases (RLK), SNF1-related protein kinases (SnRK) and some transcription factors (like OSNAC6) to enhance intercellular organic osmolytes (proline, trehalose, amino acid), accumulation for a better maintenance of rice cell osmotic potential (Fu et al., 2002; Yusuke et al., 2000; Ouyang et al.,
2010; Calliste et al., 2008; Kazuo et al., 2007; Vaidyanathan et al., 2003). However, due to the high degree of genetic variability for salt tolerance and the spatio-temporal difference of gene expression, the exploration of salt tolerance mechanism in rice still has long way to go.

Thailand is one of the most important contributors of world’s rice production and commerce. The yield improvement research of Thai rice lines is a very valuable issue. But the literature reviews show that most of salt tolerance investigation in Thai rice are from mature tissue (such as root and leaf) or young seedling. Only few researches focus on germinating stage seeds, and there is no proteomics study (Ahsan et al., 2007; Liu et al., 2015). Therefore, this project aimed to study the salt tolerant ability and their proteome profiles of seven widely cultivated Thai rice varieties including Pathumthani, Phitsanulok02, RD29, RD31, RD41, RD47 and Riceberry.
CHAPTER III

MATERIALS AND METHODS

1. Plant material preparation

Seven widely cultivated Thai rice (*Oryza sativa* Indica) cultivar including Pathumthani, Phitsanulok02, RD29, RD31, RD41, RD47, and Riceberry were used to investigate the salt tolerant response protein during germination. Hundred rice seeds were selected, soaked in 10% H$_2$O$_2$ for 10 minutes, and immersed in 1% calcium hypochlorite for 1h. Then, the seeds were rinsed five times with sterile water (5 min each) (Miché L and Balandreau J., 2001) and germinated in petri dish on filter paper in the presence of 0, 100, 150, 200 and 250 mM NaCl solution (10ml) at 30°C for 4 days under dark condition.

2. Protein extraction and separation

The 120mg germinated seeds under 200 mM NaCl was weighed and milled in liquid nitrogen with a porcelain mortar. Fine powder was transferred into a 0.5ml Eppendorf tube, mixed with 1 ml prechilled acetone and kept at -20°C for 1h to precipitate protein. After centrifugation at 10,000 rpm for 15 min the supernatant was discarded and the protein pellet was air dried for 1.5h before dissolving in 0.5% anionic detergent sodium dodecyl sulfate (SDS). The clear supernatant was then transferred to a new tube and stored at -20°C until use. The protein concentration was measured according to Lowry method (1951) using Bovine serum albumin (BSA) as standard protein. The one-dimensional polyacrylamide gel electrophoresis was employed to prefractionate the protein by their molecular size (Laemmli, 1970). The protein visualization will be achieved by coomassie blue R250 staining dye method (Syrový, 1991).

Protein concentration reduction formula:

$$\text{Concentration (µg/µl)} = \text{dilution factor/testing volume} \times \text{OD750 average of sample/m}$$

* m is the slope of standard curve

3. In-gel trypsin digestion

An in-house in-gel digestion procedure developed by Proteomics Research Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand (Jaresittikhunchai et al., 2009) was followed after gel excision. The gel plugs were dehydrated with 100% acetonitrile (ACN) and rehydrated by 10mM DTT in 10mM ammonium...
bicarbonate at 56°C for 1h, and then the alkylation step was performed at room temperature under dark condition for another 1h by 100mM iodoacetamide (IAA) in 10mM ammonium bicarbonate solution. The alkylated gel pieces were dehydrated with 100% ACN for 5 min with shaking (repeated two times). Once the ACN is evaporated, 20µl trypsin solution (10ng/µl trypsin dissolved in 50% ACN/10mM ammonium bicarbonate) was added to the dehydrated gel plugs and incubated at 4°C for 20 min, and then 20µl of 10mM ammonium bicarbonate solution was added into tube to immerse gel plugs. The protein digestion process was performed at 37°C overnight. To extract all protein digestion product, 90µl of 50% ACN in 0.1% formic acid (FA) was used and incubated for 10 min before transferred to a new tube. After extraction for three times, the tryptic peptides will be pooled to a new tube and dried at 40°C for overnight before kept at -20°C prior to mass spectrometric analysis.

4. LC-MS/MS

LC-MS/MS analysis of digested peptide mixtures was performed using a Waters SYNAPT™ HDMS™ system. The 1D-nanoLC was carried out with a Waters nanoACQUITY UPLC system. Four microlitres of tryptic digests were injected onto the RP analytical column (20 cm x 75 µm) packed with a 1.7 µm Bridged Ethyl Hybrid (BEH) C18 material. Peptides were eluted with a linear gradient from 2% to 40% acetonitrile developed over 60 minutes at a flow rate of 350 nl/min. This was followed by a 15 min period of 80% acetonitrile to clean the column before returning to 2% acetonitrile for the next sample. The effluent samples were electrosprayed into a mass spectrometer (Synapt HDMS) for MS/MS analysis of peptides and then generated the spectral data for further protein quantitation and identification against database search.

5. Protein quantitation and identification

The quantitation of proteins was achieved by DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare) (Johansson et al., 2006; Thorsell 2007). The raw data obtained from LC-MS were converted and imported to the PepDetect module for automated peptide detection. The charge state assignments, and quantitation based on the peptide ions signal intensities in MS model were performed. The DeCyderMS analyzed MS/MS data was then send to database searching through the Mascot software (Matrix Science, London, UK) (Perkins et al., 1999). Protein identification was performed by searching against the *Oryza sativa* non-redundant subset database of National Center for Biotechnology Information (NCBI). The following parameters were selected for searching: peptide tolerance ±2 Da; fragment mass tolerance ±2 Da; peptide charge 1+, 2+ and 3+; maximum allowed missed cleavage 1; instrument type, ESI-Q-TOF. Protein scores were derived from ion scores as a
non-probabilistic ranking protein hits and obtained as the sum of peptide scores. The score threshold was set at $p<0.05$ by Mascot algorithm. The subcellular localization and molecular function were assigned to protein identification according to the Gene Ontology (Go) cat (http://eagl.unige.ch/GOCat/) and Uniprot (http://uniprot.org). Protein-protein interaction was analyzed according to STITCH 4.0 database (http://stitch.embl.de/).

6. Statistics analysis

The germination testing of each rice cultivar was designed in Completely Randomized Design (CRD) with 2 replications. The obtained data was further analyzed statistically by using Statistical Analysis System (SAS) program, and the mean difference was determined by one-way ANOVA tukey’s test ($p<0.05$).
CHAPTER IV

RESULTS

1. Germination testing

At the final day of germination test, germination rate of all rice varieties under 100mM, 150mM and 200mM NaCl were recorded. The percentage of successful germinated seeds was continually dropped accompany with gradually increased salt concentration (Figure 1). However, 250mM salt stress condition caused no growth of RD29 and Riceberry. Since, 200mM NaCl let seven rice cultivars survive till the final day of testing. While, this salt concentration could significantly reduce seeds germination. Under 200mM salt stress condition, the germination rates of all seven rice varieties were lower than 30%. Among them the most salt tolerant cultivar is Pathumthani, 26% seeds of this cultivar could successfully germinated. On the contrary, RD47 was classified as the most salinity sensitive (6% germination rate). Further statistical analysis divided rice into 3 group based on their germination rate under salt stress (Figure 2). Pathumthani, Phitsanulok02 and RD31 were salt tolerant cultivar while RD29, RD41 and Riceberry were moderately tolerant cultivars. RD47 were classified as salt sensitive cultivars.

![Figure 1. Salt tolerant ability of all 7 Thai rice seeds germinated under various concentrations of NaCl.](image-url)
Figure 2. Germination percentage of seven rice cultivars under 200mM NaCl. All experiments were done in replicates.

2. Protein separation

1D-SDS-PAGE analysis in Figure 3 showed the distribution of separated protein bands, which were very different between treatment and control of each cultivar. Compared with protein standard marker, the proteins band in range of 23 to 50 kDa were changed significantly after 200mM NaCl treatment. The protein profile of Pathumthani, Phitsanulok02, RD31 and Riceberry were changed after salinity treatment higher than more other cultivars.
3. LC-MS/MS

By shotgun proteomics analysis, 1339 different expressed proteins in seeds of 7 Thai rice cultivars germinated under 200 mM NaCl were identified (Figure 4). The most salt tolerant cultivar Pathumthani contained 1294 differently expressed proteins and 44% of them were up-regulated expressed. However, 1288 differently expressed proteins were observed in the salt sensitive cultivar RD47 and just 27% of these proteins were up-regulated (Table 1).
Figure 4. Heatmap depicting the level of expression (absent in green, lowest in dark green, and highest in red) of 7 Thai rice cultivars germinated under 200 mM NaCl.
Table 1. Identified differently expressed proteins in seeds of 7 Thai rice cultivars after 200mM NaCl treatment

<table>
<thead>
<tr>
<th>Rice cultivar</th>
<th>Total detected</th>
<th>Upregulated proteins</th>
<th>Down regulated proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathumthani</td>
<td>1294</td>
<td>569</td>
<td>725</td>
</tr>
<tr>
<td>Phitsanulok2</td>
<td>1278</td>
<td>514</td>
<td>764</td>
</tr>
<tr>
<td>RD31</td>
<td>1272</td>
<td>470</td>
<td>802</td>
</tr>
<tr>
<td>RD29</td>
<td>1252</td>
<td>422</td>
<td>830</td>
</tr>
<tr>
<td>Riceberry</td>
<td>1257</td>
<td>595</td>
<td>662</td>
</tr>
<tr>
<td>RD41</td>
<td>1309</td>
<td>717</td>
<td>592</td>
</tr>
<tr>
<td>RD47</td>
<td>1288</td>
<td>344</td>
<td>944</td>
</tr>
</tbody>
</table>

4. Ontology and localization of identified proteins

Functional prediction result showed that these salt stress response proteins involved in many biological processing (e.g. protein modification, transport, development, signal transduction and transcription). These identified salt stress response proteins were predicted to function in stress response (18%), metabolic (11%), protein modification (9%), transport (9%), signal transduction (7%), cell growth (4%) and transcription (4%). However, 34% of these rice proteins’ functions were not known.
The subcellular localization (Figure 4) and protein function (Figure 5) of rice seed proteins were obtained. They appeared in the cell membrane (19%), nucleus (12%) and plastid (10%). However, the localization of 37% proteins were still unknown.
5. Proteins detected only in germinated seeds of salt tolerant cultivars

The specific seed proteins present in each rice cultivar were analyzed via an online interactive Venn diagram viewer program or jvenn (Philippe et al., 2014). The result showed that three salt tolerant cultivars expressed 51 specific proteins (Table 2). Among them, 8 proteins were uniquely observed in Pathumthani and 2 proteins in RD31. The salt sensitive RD47 contained 2 specific proteins. Remaining 1171 different expressed proteins were found in both salt sensitive and salt tolerant cultivars (Figure 7).
Figure 7. Venn diagram showing the number of expressed proteins detected in germinated seeds of 4 Thai rice cultivars analyzed by shotgun proteomics. Pathumthani, Phitsanulok2 and RD31 were salt tolerant cultivar while RD47 was a salt sensitive cultivar.
Table 2. Identified proteins observed only in salt tolerant cultivars including Pathumthani, Phitsanulok02 and RD31.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>NCBI Accession number</th>
<th>Uniprot Accession number (STITCH number)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pathumthani specific proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP2 domain containing protein</td>
<td>gil108706208</td>
<td>Q8H075 (4331633)</td>
<td>Response to ethylene</td>
</tr>
<tr>
<td>Os01g0634600 or Pectinesterase</td>
<td>gil255673491</td>
<td>Q0JL04 (OsJ_02732)</td>
<td>Cell wall metabolism</td>
</tr>
<tr>
<td>Os01g0227500 or Cytochrome P450 family protein</td>
<td>gil113532003</td>
<td>Q5N7Z8 (4327182)</td>
<td>Secondary metabolism</td>
</tr>
<tr>
<td>Ribosomal RNA apurinic site specific lyase</td>
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<td>Q6K5N2 (4329396)</td>
<td>Translation</td>
</tr>
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<td>B8A717</td>
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<td>Q01J22 (OsL_16860)</td>
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<tr>
<td><strong>RD31 specific proteins</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Q8H4P1 (LOC_Os07g11530.1)</td>
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<td>Os05g0530500 or Serine/threonine protein kinase or CAMK_KIN1/SNF1/Nim1_like_AMPKh.3 - CAMK includes calcium/calmodulin depedent protein kinases</td>
<td>gil113579679</td>
<td>Q0DG11 (4339410)</td>
<td>Signal transduction</td>
</tr>
<tr>
<td><strong>Proteins detected in Pathumthani and RD31 cultivar</strong></td>
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<td></td>
</tr>
<tr>
<td>armadillo/beta-catenin repeat protein-related-like</td>
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<td>Q5VR95 (4340750)</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>inactive receptor kinase At2g26730 precursor</td>
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<td>Signal transduction</td>
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<td>Q01ZB6 (OsL_30507)</td>
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<tr>
<td>Os10g0502000 or Thylakoid luminal 17.4 kDa protein</td>
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<td>Q8LNF2 (4349041)</td>
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<td>gil113534708</td>
<td>Q8S077 (4324342)</td>
<td>Response to stress</td>
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<td>Os06g0561800 or ABC transporter superfamily ABCC subgroup member 11</td>
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<td>C7J4D9 (OsL_23382)</td>
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<td>Function</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------</td>
<td>------------------------------------------</td>
<td>----------</td>
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<td></td>
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<td>Q65XL7 (4339663)</td>
<td>Signal transduction</td>
</tr>
<tr>
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<tr>
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<td></td>
</tr>
<tr>
<td>ABC1 family protein</td>
<td>gil77551374</td>
<td>Q2R2T2 (Q2R2T2)</td>
<td>Transport</td>
</tr>
<tr>
<td>CASP-like protein OsJ_01913</td>
<td>gil341958554</td>
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<td>Development</td>
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<tr>
<td>Os06g0598000 or 3-ketoacyl-CoA synthase</td>
<td>gil113596036</td>
<td>Q69X62 (4341445)</td>
<td>Lipid metabolism</td>
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<tr>
<td>Os07g0575500 or beta-hexosaminidase</td>
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<td>Q0D581 (4343697)</td>
<td>Carbohydrate metabolism</td>
</tr>
<tr>
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<td>gil113624197</td>
<td>Q6Z8M5 (4346024)</td>
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</tr>
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<td>SOS2-like protein kinase</td>
<td>gil77548730</td>
<td>Q53QG9 (OsJ_35162)</td>
<td>Signal transduction</td>
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<td>Q7XV64 (4335904)</td>
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<td>OSJNBB0066J23.23 or RALFL35 - Rapid ALkalization Factor RALF family protein precursor</td>
<td>gil21741345</td>
<td>Q7X7S5 (OsJ_14409)</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
To identify biochemical pathways relevant proteins associated with salt tolerant mechanism, a total of 51 proteins expressed only in salt tolerant cultivars, including Pathumthani, Phitsanulok02 and RD31, were analyzed for their interaction with plant hormones according to online STITCH 4.0 database (Kuhn et al., 2014). As shown in Figure 8, 5 candidate proteins including conserved hypothetical protein (OSJ13183), beta-hexosaminidase precursor (4343697), suppressor of phytochrome A (4339663), conserved hypothetical protein (LOC_Os03g40840.1) and 3-ketoacyl-CoA synthase (4341445), show interaction network with plant hormones.
Figure 8. Predicted interactions between plant hormones and rice seed proteins of salt tolerant cultivars, including Pathumthani, Phitsanulok02 and RD31, during germination under 200mM NaCl. Modes of action are shown in different color lines. Red boxes indicate identified proteins in this study. Abbreviations: conserved hypothetical protein (OSJ13183), beta-hexosaminidase precursor (4343697), suppressor of phytochrome A (4339663), conserved hypothetical protein (LOC_Os03g40840.1) and 3-ketoacyl-CoA synthase (4341445).
CHAPTER V

DISCUSSIONS

1. Germination testing

In present work, the salt tolerant ability of 7 Thai rice cultivars during germinating under salt stress was investigated. The results showed that 250mM NaCl inhibited germination of Riceberry and RD29 and significant delayed the germination rate of others. Seeds of 7 Thai rice cultivars showed different germination rate when they were treated with 200mM NaCl. Germination rate of Pathumthani seeds was the highest. However, previously investigations did not classified Pathumthani as salt tolerant rice cultivar. Seedling of Pathumthani was reported as moderate salt tolerant when compared with other Thai rice (Chutipaijit et al., 2009; Wanichananan et al., 2003; Hasthasombut et al., 2011). In addition, statistic analysis result of germination test data grouped Pathumthani, RD31 and Phitsanulok02 as salt tolerance. RD29, RD41 and Riceberry were moderately tolerant cultivars while RD47 was classified as salt sensitive cultivars. The close genetic distance between these three salt tolerant varieties has been demonstrated by simple sequence repeats (SSR) approach (Worede et al., 2013).

2. Proteins associated with germination under salinity stress by Shotgun proteomics analysis

Plants can change its gene expression to face the environment-induced challenges. Numerous investigations on plant salt tolerance have been carried out at the transcriptional level. However, the amount of expressed mRNA is not always correlated with the functional product protein, due to the regulation of post-translational and post-translation modification. Thus, exploring protein expression profile is a more efficient way to get a better understanding of plant salt tolerant mechanisms. Rice is a good monocotyledon model plant. Massive published genome sequence information greatly facilitated rice proteomics investigation. Peculiarly in protein identification, which by obtained mass spectra data. LC-MS/MS is a high-throughput tandem mass spectrometry method that is available for the proteins from complex mixtures (McDonald et al., 2002).

Salt stress is the most adverse environmental barrier of agriculture productivity. As an important cash crop rice has been grown worldwide. But ample research evidence demonstrated that salt stress could suppress the development of rice. Therefore understanding the inherent resistant mechanism of rice is an issue that needs to be addressed urgently.

The seeds of 7 Thai rice cultivars were allowed to germinate in the presence of 200 mM NaCl. Four days-old germinated seeds were used as material to isolate total protein for
further analysis by shotgun proteomics. There were 1339 differentially expressed proteins were identified. These proteins distributed on many important cell components, including cell membrane, cytoplasm, peroxisome, plastid, mitochondrion and nucleus. They played role in development, protein modification, signal transduction, stress response, transport and transcription. However, function of majority proteins (34%) was not known. Three salt tolerant cultivars specifically expressed 51 proteins, 8 proteins are uniquely detected in the most salt tolerant cultivar Pathumthani, and 2 proteins in RD31. Some interesting proteins related to seed germination and salt stress were discussed.

2.1 Plant hormones related proteins

Here, 5 candidate proteins including conserved hypothetical protein (OSJ13183), beta-hexosaminidase precursor, suppressor of phythrome A (4339663), conserved hypothetical protein (LOC_Os03g40840.l) and 3-ketoacyl-CoA synthase showed interaction network with plant hormones (abscisic acid, auxin, cytokinin, ethylene and gibberellin).

Phytohormones are group small molecular organic compounds, which influence many aspect of the plant life cycle. Among them the abscisic acid (ABA) and gibberellin (GA) have long been known that regulated the growth, reproduction and stress response of plants. Research evidences revealed the plant hormone ABA can evoke suppression of germination, but this is a fully reversible reaction. The negative affect of ABA on germination is started from the initial imbibition phase of dry seeds, subsequently pronounced delayed of embryo growth via reduces availability of reserved energy and nutrients. In the contrary, GA exhibited promoting effect during seeds germination. Current research evidences suggest that GA induced expression of hydrolase can enhance the utilization of aleurone tissue storage nutrients (like protein, lipid and starch) evidently. Consequent germination promoted is the general feature of increased GA synthesis in imbibed seeds. While, the essential requirement of GA to alleviate ABA triggered dormancy was also observed from both monocot and dicot seeds. (Li et al., 2015; Schoper and Plachy, 1984; Bove et al., 2005; Bewle, 1997; Lefebvre et al., 2006; Garciarrubio et al., 1997; Millar et al., 2006; Groot and Karssen., 1987; Debeaujon and Koornneef, 2000). However, under adverse condition the response character between GA and ABA was changed. An expression pattern analysis on the multi-stress (salt, drought and freezing) of tolerant protein encoding gene GhDREB1 indicated that the enhanced abiotic stress resistant level in line with reduced GA synthesis. Over-accumulated phytohormone GA also showed inhibition function on the expressing of GhDREB1 in transgenic tobacco test system. The uniform result was detected in model plant Arabidopsis too (Shan et al., 2007; Huang et al., 2009). In the other side, ample evidence from biochemistry and molecular biology research has been proved the importance of plant hormone ABA and it consisted
ABA signaling transduction pathway for plant stress resistance (such as water deficit, low temperature, oxidant stress and high salinity) (Wan and Li., 2006; Zhang et al., 2008; Cai et al., 2015). Therefore, the antagonistic function between GA regulated growth promotion and ABA mediated stress defense response simultaneous occurring in an environmental stress influenced plant is displayed clearly.

2.2 Cell wall biosynthesis related proteins

Pectinesterase was observed in proteins isolated from seeds of salt tolerant rice. It has been indicated that the activity of the enzyme in pectin metabolism can affect seed germination. During the process of seed germination the cell wall of the radicle and of the tissues around it must expand (Müller et al., 2013).

2.3 Phytochrome related proteins

The suppressor of phytochrome A protein was detected only in proteins isolated from salt tolerant cultivars including Pathumthani, and Phitsanulok2. It was known that some photoreceptors are necessary for plant growth and development, including seed germination. For example, phytochrome B proteins, which are stable and found in green tissues (Quail, 1997) are able to regulate the hormonal signaling pathways of auxin and cytokinin (Tian et al., 2002; Fankhauser, 2002; Choi et al., 2005). Phytochromes in the seeds are necessary for controlling seed germination, especially when the seeds are subjected to light. Light activates phytochromes, as well as hormonal activities in plants (Seo et al., 2009).

2.4 ABC transporter proteins

Uniquely expression of ABC1 family protein in seeds of salt tolerant rice germinated under salt stress was also demonstrated. The ABC transporter proteins are belonging to ATP-Binding Cassette (ABC) superfamily. Their structure basically consist of two regions, a six membrane-spanning α-helices made hydrophobic transmembrane domain (TMD) and a cytosolic domain involved in ATP binding named nucleotide-binding domain (NBD). ABC transporter protein is a very large family managed broad range compounds across membrane movements that drive by the energy from ATP hydrolysis. Its members can be found both in prokaryotic and eukaryote organism. In monocot model plant Arabidopsis, 131 ABC transporter proteins were isolated and 103 members were identified as putative intrinsic membrane protein with contiguous transmembrane spanning capability. The expression of ABC transporter proteins was demonstrated to be regulated by both plant hormone (like ABA) and environmental elements. (Jasinski et al., 2003; Higgins, 1992; Sánchez-Fernández et al., 2001). Previous findings suggest the substance transfer role of ABC transporter protein
is a fundamental requirement for plant growth and development proceed normally. Exine formation was reported as the essential factor of pollen maturity. However to form exine, synthesized sporopollenin precursors must undergo a transfer procedure, which from the initial product position tapetum to final destination pollen surface. An Arabidopsis ABC transporter protein ABCG26 was demonstrated to manage sporopollenin precursors transport (Choi et al., 2011).

Germination is a vital stage for dormancy seed to start a new life cycle. In seeds, the stored substances not only bring important biological information from their parents but also supply all essential material that required for a successful germinating. The cellular organelle glyoxysome is a class of peroxisomes that exists in germinating period cotyledon and endosperm. Their function is to convert reserved fatty acids to final product sucrose through β-oxidation and glyoxylate cycle. Subsequently, support the vegetative growth. An Arabidopsis expressed single-copy gene coding peroxisomal ABC transporter family protein PED3 has been demonstrated to control fatty acids import into glyoxysome. Loss function mutat ped3-3 exhibited exogenous sucrose supply requirement to complete radicle emergence. In addition, peroxisomes produced sucrose is a powerful osmoprotectant compound that has been proved to essential for stationary phase cell of Synechocystis sp. PCC 6803 to resistant later stage salt stress (Nishimura et al., 1986; Kanai et al., 2010; Desplats et al., 2005).

Phytohormone auxin has been conformed governed root system development. The emergence level of auxin directly impacted both root elongation growth and lateral root formation. Many molecular evidences supported the ABC transporter proteins exist a strong relationship with roots development. In an Arabidopsis expression analysis, the multidrug resistance-like ABC transporter protein MRD1 was revealed to contain auxin acropetal transport regulator property, knockout MRD1 caused auxin acropetal transport decline pursuantly impaired MRD1 managed nascent lateral root production (Wu et al., 2007; Gaedeke et al., 2001; Santelia et al., 2005).

However, auxin is not the unique phytohormone that ties to ABC transporter protein to exert its hormonal function. ABA also an important phytohormone involved extensive biological activities of plant, such as reproduction, senescence and abiotic stress response. Preceding investigations have been identified that ABA is primarily produced in vascular tissue. An ABC transporter family protein AtABCG25 obtained from Arabidopsis was reported as an export that controlled ABA molecules transport from initial vascular tissue to distant action site. The AtABCG25 overexpression line exhibited higher ABA concentration in guard cell resulted reducing of leaf water loss (Lee et al., 2011; Nakashima et al., 2009; Jia et al., 2002). As the constituent of ABA intercellular signaling pathway, several plant
environmental stress researches have been suggested ABC transporter proteins contribute to multiple abiotic stress (like heavy metal, drought, Salinity and low temperature) induced response and resistance mechanism. Up-regulation expression of ABC transporter proteins caused increasing of plant stress resistance (Moons, 2003; Klein et al., 2004; Smart and Fleming., 1996). The present investigation was documented that the membrane stabilization effect is a marked contribution of expressed ABC transporter proteins for abiotic stress tolerance improvement. In wheat, the experiments that carried out in a range method have been proved the cell membrane stability can be used as a measure index for water stress tolerance capacity. In addition, a research on osmotic stress triggered ABC transporter protein response in Lactococcus lactis revealed the osmotic stress induciable ABC transporter protein OpuA able to activate by both nonionic and ionic compounds created transmembrane osmotic gradient and activated OpuA constituted defense system can protected L. lactis against hyperosmotic stress. Overexpression of ABC transporter OpuA promoted membrane reconstitution. These finding supplied molecular evidence to ABC transporter protein regulated cell membrane stability under stress condition (Blum and Ebercon, 1981; Bajji et al., 2002; van der Heide and Poolman, 2000).

2.5 Lipid transfer protein (LTP)

Lipid transfer protein specifically expressed in germinated seeds of salt tolerant cultivars in this study. The cultivar specific response protein Os01g091400, was annotated as plant lipid-transfer protein by Pfam (Robert et al., 2014). LTPs are group multigene code small molecular mass (~7 to 10kDa) protein, they are ubiquitous exist in higher plants. The main function of LTPs is transport broad range of lipids (eg. phospholipids and galactolipids) between cellular membranes (Vignolsa et al., 1997; Charvolin et al., 1999; Watanabe and Yamada, 1986). The inhibition activity of microbial and fungal pathogens is a common capability of LTPs. In addition, the LTPs also revealed the regulation role in plant reproduction and seeds development (Cammue et al., 1995; Jia et al., 2010; Zhang et al., 2010; Wang et al., 2015).

Since plant LTPs are able to bind ubiquitous to lipids, thus plenty enough evidence attested they are participated to various abiotic stresses response and defense mechanisms. A recent study on rice LTP1-type protein encode gene LTP10 found that its expression was highly regulated by salt stress stimulation during development. However, the expression analysis trial of a wheat lipid transporter protein (TdLTP4) under salt and drought stress condition indicated that this gene was abiotic stress responsible and its transcriptional level positively was correlated to its tolerant capacity. Overexpression of TdLTP4 gene can significantly improve transgenic Arabidopsis growth status under NaCl treatment.
environment. While, the other wheat LTPs encoding gene *TaLTP3* was also demonstrated to be induced by multiple abiotic stresses including salt, drought and heat stress. Further investigation in transgenic *Arabidopsis* expression system showed that overexpression of *TaLTP3* gene lead to thermo-tolerance and oxidative stress resistance capacity marked increase.

Chilling is one of the most harmful abiotic stresses affected large crop yield reduction and death. However, a plant lipid transporter family protein WAX9 that purified from acclimated cabbage leaf was reported as a cryoprotectant to help plant avoid the freeze-thaw damage (Wang et al., 2009; Moraes et al., 2015; Safi et al., 2015; Wang et al., 2014; Hincha, 2002).

Cuticular resistance is a widely used avoidance strategy of plant that aimed to limit water loss from leaves. The water stress defense effectiveness of this strategy has been demonstrated in rice. Further investigation of epicuticular wax, which the critical constituent of cuticular resistance mechanism showed that removing of epicuticular wax from rice leaf resulted in a sharply decline of cuticular resistance capacity. Meanwhile, salt, heat, cold and ABA inducible *Thellungiella salsuginea* lipid transporter protein *TsnsLTP4* was proved to control epicuticular wax deposition. Transgenic analysis of *TsnsLTP4* code gene in *Arabidopsis* expression system also revealed the gene expression level positively paralleled with cuticular wax deposition and salt tolerant capacity. All these evidences suggested that the LTPs involved in plant stress response and defense mechanism deeply (Shepherd and Griffiths., 2006; O’TOOLE et al., 1979; Sun et al., 2014)

2.6 SOS2

Salt Overly Sensitive (SOS) signal pathway that make plants able to sense and respond to extracellular high concentration salt induced stimulation. Former researches showed that SOS pathway is activated at the early phase of salt stress to help plants avoid damage via its ionic balance re-establishment function. More recent molecular biology investigation revealed that plant SOS signal pathway consisted by three subunit elements (SOS1, SOS2, SOS3) (Ji et al., 2013). SOS1 is a Sodium/hydrogen exchanger family protein, also known as Na/H antiporter. Na/H antiporters usually contain 10-12 transmembrane protein domains and exist in the hydrophobic region of various membranes (such as plasma membrane, mitochondria membrane and tonoplast) (Numata M et al., 1998; Orlowski and Grinstein., 1997). Na/H antiporters played critical role on cellular PH volume maintaining and Na⁺ transport. Overexpressed Na/H antiporter localized on both plasma membrane and tonoplast could improve the resistant capacity of *Arabidopsis* by maintaining a low Na⁺ concentration in the cytosol (Mager et al., 2011; Shi et al., 2002; Maris et al., 1999).
SOS2 (also known as CBL-interacting serine/threonine-protein kinase) contains a NAF domain that is necessary for the interaction with calcium signal sensor protein SOS3/calcineurin B-like calcium sensor protein (CBL) to form CBL-CIPK protein complex. The generated SOS2/SOS3 protein complex consequently will bind to their target protein SOS1 by phosphorylation to activate SOS pathway mediated Na⁺ transport mechanism. SOS pathway was regulated by both SOS2 and SOS3 (Guo et al., 2001; Hashimoto et al., 2012; Qiu et al., 2002). In present work, CBL-interacting protein kinases (CIPK) family protein SOS2-like protein kinase that contained the same functional NAF domain with SOS2 was detected only in germinated seeds of salt tolerant rice. The function of SOS2-like protein kinase on CBL/CIPK signaling pathway and its multiple-stress (include salt stress) signal inducible character has been proved in rice (Kyung-Nam Kim et al., 2003).

3. Proposed defense mechanism of salt stress

In this study, at least 5 proteins (conserved hypothetical protein (OSJ13183), beta-hexosaminidase precursor (4343697), suppressor of phytchrome A (4339663), conserved hypothetical protein (LOC_Os03g40840.1 and 3-ketoacyl-CoA synthase (4341445)) in seeds of salt tolerant rice showed interaction with phytohormones which are known to regulate many stress resistant responses of plant. The transcription level of Abscisic acid (ABA) is closely related to gibberellin (GA) level (Cadenas et al., 2001; Wang et al., 2015). Increased auxin concentration could catalyze hypocotyl growth. While, continuing increased concentration in the tip of radicle was also observed in germinating stage seeds. But the cellular efflux of auxin strongly depends on ABA responsible ABC transporter protein. Signal cross-talk investigation showed that auxin could reduce ABA trigged germination inhibition (Liu et al., 2007; Markus Geisler et al., 2005; Akbari G et al., 2007; Wu et al., 2007; Monroe-Augustus et al., 2003). Taken together, altered ABA pathway by salt stress might trigger ABC transporter protein on plasma membrane to boost auxin efflux and promote radicle growth of rice seeds.

The osmotic stress sensing capability of ABC transporter has demonstrated by van der Heide et al., 2001. Once external signal detected and transported inside cell, the secondary messengers will be generated to guide the activation of corresponding resistance response. Stress resistant protein SOS2-like protein kinase mediated calcium ion in rice seeds and took action under high salty condition (Guo et al., 2001; Shimada et al., 2006). SOS1 or Na/K antipoter located in plasma membrane and tonoplast decreased Na⁺ concentration in cytosol (Miller et al., 2010; Blumwald 2000). The extrusion and long distance transport function of sodium ions by Na/H antiport (SOS1) was proved (Razzaque et al., 2013; Shi et al., 2002; Raquel et al., 2009). However, SOS1 required SOS2 and SOS3 to generate protein
complex before perform its duties (Chen et al., 2007; Fukuda et al., 2004; Qiu et al., 2002). It can be assumed that SOS pathway either in plasma membrane and/or tonoplast might be activated to lower sodium ion concentration in cytoplasm of rice seed.

Taken together, during seed germination, cell wall of the radicle and of the tissues around it will be expanded by pectinesterase. Phytochrome in seed and salt stress can regulate the hormonal signaling pathways of auxin and cytokinin. Altered ABA pathway by salt stress might trigger ABC transporter protein on plasma membrane to boost auxin efflux and promote radicle growth of rice seeds. SOS pathway either in plasma membrane and/or tonoplast will be activated to lower sodium ion concentration in cytoplasm of rice seed (Figure 9).
Figure 9. Proposed germination pathway of rice seeds under 200mM NaCl. OSA3=Plasma membrane proton-ATPase gene OSA3, AP2=AP2 domain protein, RuvA2=RuvA domain 2, brd103=Bromodomain protein 103, SOSPK=SOS protein kinase, ABCRP=armadillo/beta-catenin repeat protein, STPK=Serine/threonine protein kinase, PK=Protein kinase, RK=receptor kinase, FAE1=Fatty acid elongase 1, SPA=Suppressor of phytochrome A, TL17.4=Thylakoid lumenal 17.4 kDa, RRASSL=Ribosomal RNA apurinic site specific lyase, PE=Pectinesterase
CHAPTER VI

CONCLUSION

Pathumthani, RD31 and Phitsanulok02 cultivars were classified as salt tolerance. RD29, RD41 and Riceberry were moderately tolerance while RD47 was classified as salt sensitive.

There are 1339 differentially expressed proteins identified in seeds of 7 Thai rice cultivars germinated under 200 mM NaCl for 4 days. These proteins distributed on many important cell component, including cell membrane, cytoplasm, peroxisome, plastid, mitochondrion and nucleus. They played role in development, protein modification, signal transduction, stress response, transport and transcription. However, function of majority proteins (34%) was not known. Three salt tolerant cultivars specifically expressed 51 proteins, 8 proteins are uniquely detected in the most salt tolerant cultivar Pathumthani, and 2 proteins in RD31. Interactions between plant hormones and 5 candidate proteins including conserved hypothetical protein (OSJ13183), beta-hexosaminidase precursor (4343697), suppressor of phythochrome A (4339663), conserved hypothetical protein (LOC_Os03g40840.1 and 3-ketoacyl-CoA synthase (4341445) affect seed germination.

During the process of seed germination, cell wall of the rice radicle and of the tissues around it will be expanded by pectinesterase. Phytochrome in seed and salt stress can regulate the hormonal signaling pathways and trigger ABC transporter protein on plasma membrane to promote radicle growth. SOS pathway either in plasma membrane and/or tonoplast will be activated to lower sodium ion concentration in cytoplasm of rice seed.
REFERENCES


Bridging the rice yield gap in the Asia-Pacific region. RAP Publication (FAO), no. 2000/16


IRRI Seed Quality Training Manual


APPENDIX

1. Germination testing result

Table 1. Germination testing results:

<table>
<thead>
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<th>Sample</th>
<th>Pathumthani</th>
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<th>Phitsanulok2</th>
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<th>RD31</th>
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Repetition 2

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<th>RD31</th>
<th>Riceberry</th>
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</tr>
</tbody>
</table>
2. Protein concentration measurement used standard curve of standard protein (BSA)

Fig. 1 Lowry method used BSA standard curve

BSA concentration (mg/ml)

\[ y = 0.0177x + 0.0048 \]

\[ R^2 = 0.98896 \]
3. SDS-PAGE marker

Fig. 2 protein molecular weight maker loaded in 12% Tris-glycine gel
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