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Research Article

# Shotgun Proteomic Analysis of Germinated Rice (*Oryza sativa* L.) under Salt Stress

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**Abstract**
Rice (*Oryza sativa* L.) is an important staple crop that feeds more than one half of the world's population. However, salt stress caused a dramatic decline of rice production. Proteome study of salt tolerant mechanism supplied a span-new viewpoint and valuable clue to rice tolerant improvement. In this study, the salt tolerant capacity and stress response proteins of seven Thai rice cultivars at the germination stage were determined. Pathumthani, Phitsanulok2, RD29, RD31, RD41, RD47, and Riceberry rice cultivars were germinated under 200 mm 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. Shotgun proteome 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, Phitsanulok2 and RD31. These proteins 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. This data may be used for not only improvement of rice yield under salinity stress but also enhancing salinity stress tolerance in this important crop.

**Keywords**: Proteomics, GeLC-MS, Thai rice, Salt stress, Germination

## 1 Introduction

Rice (*Oryza sativa* L.) is one of the main staple foods in Asia. Thailand is a major rice exporting country. The total area under rice is estimated to be about 11.3 million ha with average yield of 3.01 metric tons per hectare in 2015 [1]. Major rice crops are grown in northeastern region, comprising about 57% of the total, followed by the northern region at 22%; central region, 17%; and the southern region, 4% [2]. Although the total rice production in northeastern region is relatively high, the yield per unit area is quite low as compared to the national average yield. Soil salinity limits the rice plant's growth and development, resulting in yields losses of more than 50 percent [3]. Maas and Grattan (1999) reported that rice yields are reduced by 12 percent for every unit of salinity (dS/m$^2$) [4]. The adverse effects of salinity on plant growth were due to increased osmotic stress and ion toxicity that result in metabolic imbalance and oxidative stress.

Salinity affects all stages of the growth and development of the rice plant, when the rice is at the young seedling stage it becomes even more sensitive to salinity [4], [5]. Salt stress defense pathways started

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from the initial sensing of extracellular stimulation signal, then converse and amplify these physical stress signal elements into perceivable chemical signal component. Finally, intercellular signal transduction, gene expression, protein synthesis and metabolism were mediated. However, how these signaling molecular elements, tolerant 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 cultivars in Thailand including Pathumthani, Phitsanulok2, 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.

## 2 Materials and Methods

### 2.1 *Plant materials*

Seven widely cultivated Thai rice (*Oryza sativa* Indica) cultivars including Pathumthani, Phitsanulok2, 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% $H_2O_2$ for 10 min, and immersed in 1% calcium hypochlorite for 1 h. Then, the seeds were rinsed five times with sterile water (5 min each) and germinated in petri dish on filter paper in the presence of 0, 100, 150, 200, and 250 mm NaCl solution (10 mL) at 30°C for 4 days under dark condition.

### 2.2 *Protein extraction, fractionation, and in-gel digestion*

The 120 mg germinated seeds under 200 mm NaCl was weighed and milled in liquid nitrogen with a porcelain mortar. Fine powder was transferred into a 0.5 mL Eppendorf tube, mixed with 1 mL prechilled acetone and kept at –20°C for 1 h to precipitate protein. After centrifugation at 10,000 rpm for 15 min, the supernatant was discarded and the protein pellet was air dried for 1.5 h before dissolving in 0.5% sodium dodecylsulfate (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 using Bovine Serum Albumin (BSA) as standard protein [6]. The one-dimensional polyacrylamide gel electrophoresis was employed to prefractionate the protein by their molecular size [7]. The protein visualization was achieved by coomassie blue R250 staining dye method [8]. Protein bands were excised into eight sections according to their apparent molecular weights. In-gel digestion was performed according to Paemanee *et al*. [9].

### 2.3 *LC-MS/MS analysis*

Digested peptide mixtures was analyzed 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 × 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 min at a flow rate of 350 mL/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 for MS/MS analysis of peptides and then generated the spectral data for further protein quantitation and identification against database search.

### 2.4 *Protein quantitation and identification*

The quantitation of proteins was achieved by Differential Analysis software (DeCyderMS, GE Healthcare) [10], [11]. The analyzed MS/MS data from DeCyderMS was then sent to database searching through the Mascot software (Matrix Science, London, UK) [12]. 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 relative abundances of peptides were expressed as $\log_2$ intensities. The biological process was assigned to protein identification according to the Gene Ontology (GO) cat (http://eagl.unige.ch/GOCat/) and Uniprot (http://uniprot.org). Protein-ligand interaction was analyzed according to STITCH 4.0 database [13].

### 2.5 *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 ($p < 0.05$).

## 3 Results

### 3.1 *Seed germination assay*

At the final day of germination test, germination rate of all rice cultivars under 100, 150, and 200 mm NaCl were recorded. The percentage of successful germinated seeds was continually dropped accompany with gradually increased salt concentration (Figure 1). However, 250 mm salt stress condition caused no growth of RD29 and Riceberry. Since, 200 mm NaCl let seven rice cultivars survive till the final day of testing. While, this salt concentration could significantly reduce seeds germination. Under 200 mm salt stress condition, the germination rates of all seven rice cultivars were lower than 30%. Among them the most salt tolerant cultivar is Pathumthani, 26% seeds of this cultivar could successfully be germinated. On the contrary, RD47 was classified as the most salinity sensitive (6%). Further statistical analysis can divide rice into 3 groups based on their germination rate under salt stress (Figure 2). Pathumthani, Phitsanulok2 and RD31 were salt tolerant cultivars while RD29, RD41 and Riceberry were moderately tolerant cultivars. RD47 were classified as salt sensitive cultivarst.

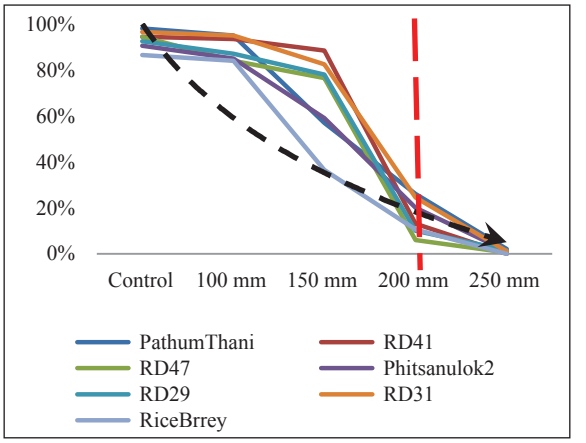


**Figure 1**: Salt tolerant ability of all 7 Thai rice seeds germinated under various concentrations of NaCl.

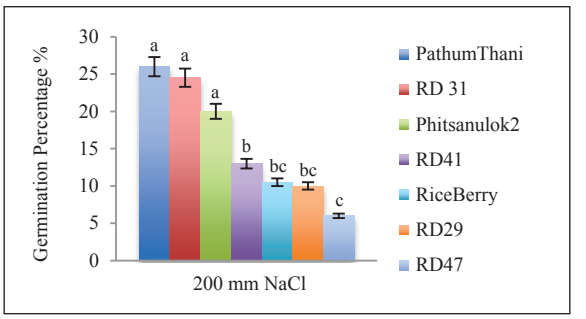


**Figure 2**: Germination percentage of seven Thai rice cultivars under 200 mm NaCl. All experiments were done in replicates.

### 3.2 *Protein profile on SDS-PAGE*

1D-SDS-PAGE analysis in Figure 3 shows the distribution of separated protein bands which are very different between treatment and control of each cultivar. Compare with protein standard marker, the protein bands in range of 23 to 50 kDa were changed significantly after 200 mm NaCl treatment.

The protein profile of Pathumthani, Phitsanulok2, RD31 and Riceberry were changed after salinity treatment higher than other cultivars.

### 3.3 *LC-MS/MS*

By shotgun proteomics analysis, 1339 different expressed

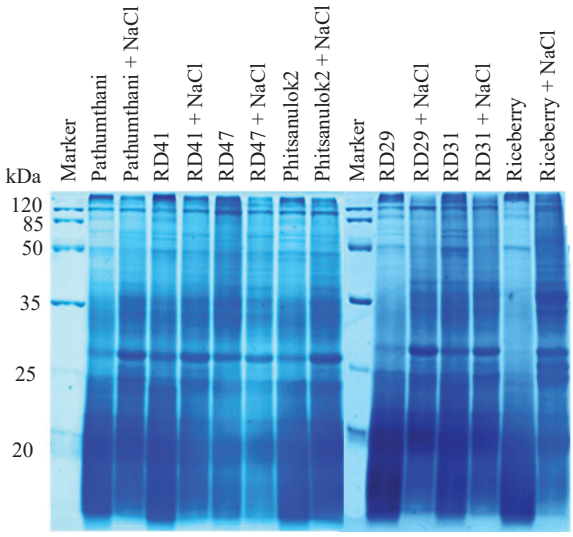


**Figure 3**: Total seed protein profile of 7 Thai rice cultivars germinated under 200 mm NaCl on Coomassie stained 12.5%SDS-PAGE.

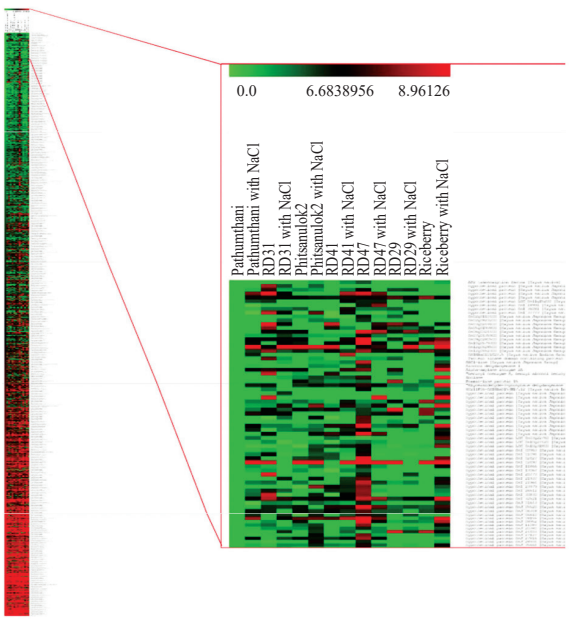


**Figure 4**: Heatmap depicting the level of expression (absent in green, lowest in dark green, and highest in red) of 7 Thai rice cultivars germinated under 200 mm NaCl.

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 different expressed proteins and 44% of them were up-regulated expressed. However, 1288 different expressed proteins were observed in the salt sensitive cultivar RD47 and just 27% of these proteins were up-regulated.

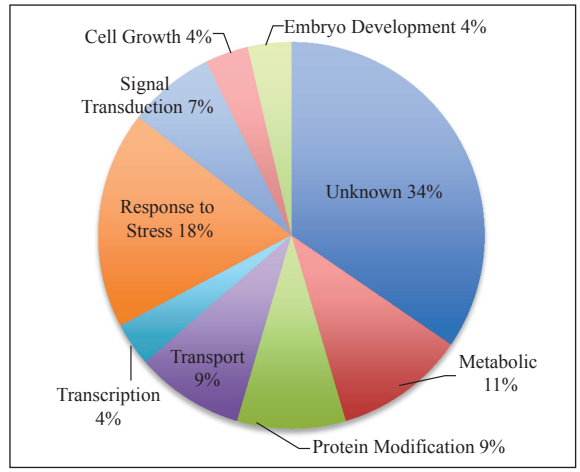


**Figure 5**: Distribution of Gene Ontology (GO) terms in Biological process category of 1339 differentially expressed proteins in seeds of 7 Thai rice cultivars germinated under 200 mm NaCl.

### 3.4 *Ontology of identified proteins*

Function prediction result showed that these salt stress response proteins involved in many biological processes (e.g. protein modification, transport, development, signal transduction and transcription). These identified 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 proteomes contained no function (Figure 5).

### 3.5 *Proteins detected only in germinated seeds of salt tolerant cultivars*

The specific seed proteins present in each rice cultivar were analysed via an online interactive Venn diagram viewer program or jvenn [14]. The result showed that three salt tolerant cultivars expressed 51 specific proteins (Table 1). Among them, 8 proteins were unique 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 6).

**Table 1**: Identified proteins observed only in salt tolerant cultivars including PathumThani, Phitsanulok2 and RD31

| Protein Name | NCBI Accession Number | Function |
|---|---|---|
| Pathumthani specific proteins | | |
| AP2 domain containing protein | gi\|108706208 | Response to ethylene |
| hypothetical protein OsI_04642 | gi\|218189468 | Unknown |
| hypothetical protein OsI_09152 | gi\|218191676 | Unknown |
| hypothetical protein OsI_34642 | gi\|125532949 | Unknown |
| Os01g0227500 or Cytochrome P450 family protein | gi\|113532003 | Secondary metabolism |
| OSIGBa0101C23.5 or fibroin heavy chain precursor | gi\|116310245 | Unknown |
| Os01g0634600 or Pectinesterase | gi\|255673491 | Cell wall metabolism |
| Ribosomal RNA apurinic site specific lyase | gi\|47848269 | Translation |
| RD31 specific proteins | | |
| latency associated nuclear antigen | gi\|23495789 | Unknown |
| Os05g0530500 or Serine/threonine protein kinase or CAMK_KIN1/SNF1/Nim1_like_AMPKh.3 - CAMK includes calcium/calmodulin dependent protein kinases | gi\|113579679 | Signal transduction |
| Proteins detected in Pathumthani and RD31 cultivar | | |
| armadillo/beta-catenin repeat protein-related-like | gi\|55295990 | Signal transduction |
| Cytochrome P450 71C4 | gi\|108864365 | Secondary metabolism |
| Os10g0111000 | gi\|255679169 | Unknown |
| hypothetical protein OsI_02743 | gi\|125526737 | Unknown |
| inactive receptor kinase At2g26730 precursor | gi\|38175491 | Signal transduction |
| hypothetical protein OsI_07091 or glutelin | gi\|218190679 | Unknown |
| RD31 and Phitsanulok2 | | |
| benzoyl coenzyme A, benzyl alcohol benzoyl transferase | gi\|53792630 | Secondary metabolism |
| hypothetical protein | gi\|41053024 | Unknown |
| Os01g0914100 or Lipid transfer protein or LTPL38 - Protease inhibitor/seed storage/LTP family protein precursor | gi\|113534708 | Response to stress |
| Os06g0561800 or ABC transporter superfamily ABCC subgroup member 11 | gi\|255677144 | Transport |
| Os09g0559200 or RuvA domain 2-like containing protein | gi\|113632150 | DNA repair |
| Os10g0502000 or Thylakoid lumenal 17.4 kDa protein | gi\|113639598 | Photosynthesis |
| Proteins detected in Pathumthani and Phitsanulok2 cultivar | | |
| Plasma membrane proton-ATPase gene OSA3 | gi\|4416349 | Transport |
| Os09g0515100 or Bromodomain protein 103-like | gi\|113631897 | Transcription |
| Os05g0571000 or suppressor of phythochrome A | gi\|113579921 | Signal transduction |
| hypothetical protein OsJ_14967 or aspartic proteinase nepenthesin-2 precursor | gi\|125590542 | Unknown |
| hypothetical protein OsI_25891 | gi\|125558226 | Unknown |
| hypothetical protein OsI_12401 | gi\|218193222 | Unknown |
| hypothetical protein OsI_05957 | gi\|218190116 | Unknown |
| hypothetical protein OsI_07094 | gi\|125539333 | Unknown |
| Hypothetical protein | gi\|108707721 | Unknown |
| hypothetical protein OsI_02742 | gi\|125526736 | Unknown |
| bZIP transcription factor domain containing protein | gi\|125528055 | Unknown |
| Proteins detected in Pathumthani, RD31 and Phitsanulok2 cultivar | | |
| ABC1 family protein | gi\|77551374 | Transport |
| hypothetical protein OsI_18253 | gi\|218195997 | Unknown |
| CASP-like protein OsI_01913 | gi\|341958554 | Development |
| hypothetical protein LOC_Os03g40840 | gi\|108709730 | Unknown |
| hypothetical protein OsI_34684 | gi\|218185030 | Unknown |
| hypothetical protein OsI_35251 | gi\|218185315 | Unknown |
| hypothetical protein OsI_39084 | gi\|125537374 | Unknown |
| hypothetical protein OsJ_01266 or transposon protein | gi\|222618182 | Unknown |
| hypothetical protein OsJ_03124 | gi\|222619110 | Unknown |
| hypothetical protein OsJ_07808 or F-box family protein | gi\|222623383 | Unknown |
| hypothetical protein OsJ_13183 | gi\|222626082 | Unknown |
| hypothetical protein | gi\|47847850 | Unknown |
| Os04g0436000 | gi\|255675489 | Unknown |
| Os06g0598800 or 3-ketoacyl-CoA synthase | gi\|113596036 | Lipid metabolism |
| Os07g0575500 or beta-hexosaminidase | gi\|255677908 | Carbohydrate metabolism |
| Os08g0514000 or lectin-like protein kinase | gi\|113624197 | Signal transduction |
| OSJNBb0066J23.23 or RALFL35 - Rapid Alkalinization Factor RALF family protein precursor | gi\|21741345 | Unknown |
| SOS2-like protein kinase | gi\|77548730 | Signal transduction |

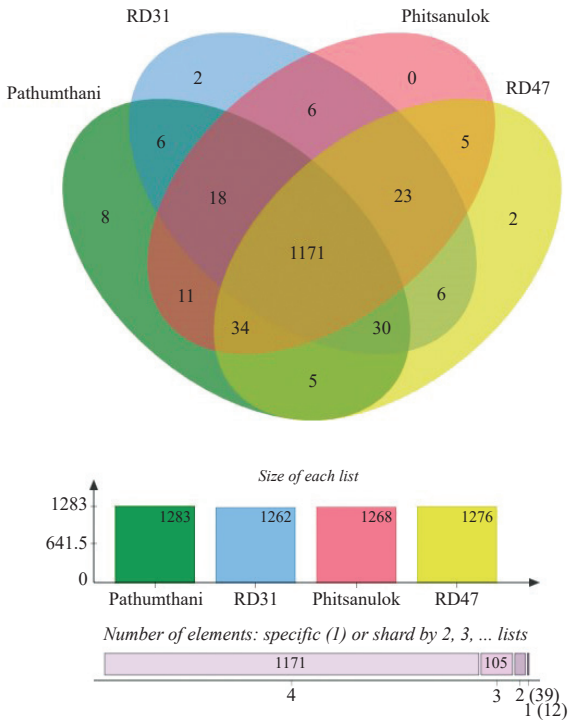


**Figure 6**: Venn diagram showing the number of expressed proteins detected in germinated seeds of 4 Thai rice cultivars analyzed by shotgun proteomics. Pathumthani, Phitsanulok2 and RD31 were salt tolerant cultivar while RD47 was a salt sensitive cultivar.

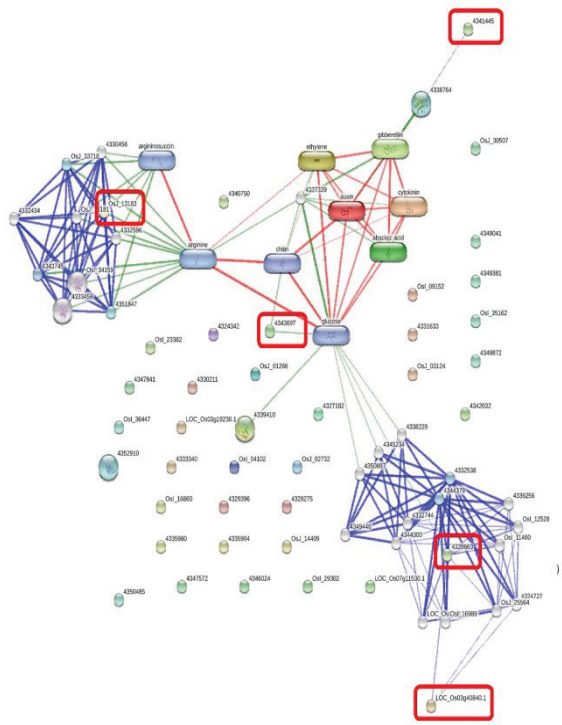

**Figure 7**: Predicted interactions between plant hormones and rice seeds proteins detected only in salt tolerant cultivars, including Pathumthani, Phitsanulok2 and RD31, during germination under 200 mm NaCl. Modes of action are shown in different color lines. Red boxes indicate identified proteins 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).

To identify biochemical pathways relevant proteins associated with salt tolerant mechanism, a total of 54 proteins expressed only in salt tolerant cultivars, including Pathumthani, Phitsanulok2 and RD31, were analyzed for their interactions with plant hormones according to online STITCH 4.0 database.

As shown in Figure 7, 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), show interaction network with plant hormones.

## 4 Discussion

### 4.1 *Germination under salinity stress*

In present work, the salt tolerant ability of 7 Thai rice cultivars during germinating under salt stress was investigated. The results showed that 250 mm 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 200 mm NaCl. Germination rate of Pathumthani seeds was the highest. However, previously investigations did not classify Pathumthani as salt tolerant rice cultivar. Seedling of PathumThani 60 was reported as moderate salt tolerant when compared with other Thai rice [15]–[17]. In addition, statistic analysis result of germination test data grouped Pathumthani, RD31 and Phitsanulok2

as salt tolerance. RD29, RD41 and Riceberry were moderately tolerant cultivars while RD47 was classified as salt sensitive cultivars. The closely genetic distance between these three salt tolerant cultivars has been demonstrated by Simple Sequence Repeats (SSR) approach [18].

### 4.2 *Proteins associated with germination under salinity stress by Shotgun proteomics analysis*

Plants can change its gene expression to face the environment-induced challenge. 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 efficiency way, which 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 [19].

Salt stress is the most adverse environmental barrier of agriculture productivity. As an important cash crop, rice has been grown worldwide. But 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 are 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.

### 4.3 *Proposed defence mechanism of salt stress*

In this study, at least 5 proteins 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 [20], [21]. 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 [22]–[25]. 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*. [26]. 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 [27], [28]. SOS1 or Na/K antipoter located in plasma membrane and tonoplast decreased Na+ concentration in cytosol [29], [30]. The extrusion and long distance transport function of sodium ions by Na/H antiport (SOS1) was proved [31], [32]. However, SOS1 required SOS2 and SOS3 to generate protein complex before perform its duties [33]–[35]. 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 during 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 8).

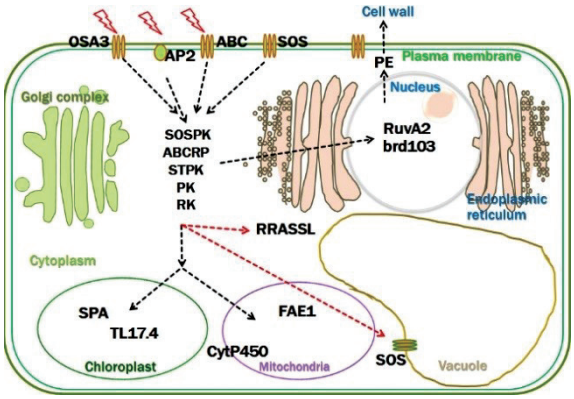


**Figure 8**: Proposed germination pathway of rice seeds under 200 mm NaCl. OSA3=Plasma membrane proton-ATPase gene OSA3, AP2=AP2 domain protein, RuvA2=RuvA domain 2, brd103=Bromodomain protein 103, SOSPK= SOS protein kinase, ABCRP=armadillo/beta-catenin repeat protein, STPK=Serine/threonine protein 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.

## 5 Conclusions

Pathumthani, RD31 and Phitsanulok2 cultivars were classified as salt tolerance. RD29, RD41 and Riceberry were moderately tolerance while RD47 was classified as salt sensitive. 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 were demonstrated. During the process of seed germination, cell wall of the rice radicle and of the tissues around it will be regulated by pectinesterase. Phytochrome in salinity stressed seed can regulate the hormonal signaling pathways and trigger ABC transporter protein on plasma membrane to promote radicle growth. Lower sodium ion concentration in cytoplasm of rice seed will finally be controlled by SOS pathway either in plasma membrane and/or tonoplast.

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