เมแทบอไลต์โพรไฟล์ของข้าว Oryza sativa L. พันธุ์ขาวดอกมะลิ 105 และพันธุ์ทนเค็ม UBN 02123-50R-B-2 ในการตอบสนองต่อความเครียดจากความเค็ม

นางสาวขวัญข้าว การภักดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2557

METABOLITE PROFILES OF ‘KDML 105’ AND SALT-TOLERANT ‘UBN 02123-50R-B-2’ RICE Oryza sativa L. IN RESPONSE TO SALINITY STRESS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry and Molecular Biology
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Thesis Title	METABOLITE PROFILES OF 'KDML 105' AND SALT-TOLERANT 'UBN 02123-50R-B-2' RICE Oryza sativa L. IN RESPONSE TO SALINITY STRESS
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ขวัญข้าว การภักดี : เมแทบอไลต์โพรไฟล์ของข้าว Oryza sativa L. พันธุ์ขาวดอกมะลิ 105 และพันธุ์ทนเค็ม UBN 02123-50R-B-2 ในการตอบสนองต่อความเครียดจากความเค็ม (METABOLITE PROFILES OF 'KDML 105' AND SALT-TOLERANT 'UBN 02123-50R-B-2' RICE Oryza sativa L. IN RESPONSE TO SALINITY STRESS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.ศุภอรรจ ศิริกันทรมาศ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.นุชนาถ วุฒิประดิษฐกุล, หน้า.

งานวิจัยนี้ได้ทำการศึกษาการตอบสนองทางเมแทบอไลต์ในข้าวสายพันธุ์ไทยสองสายพันธุ์ คือ ข้าวสายพันธุ์ไทยขาวดอกมะลิ 105 (KDML 105) ซึ่งมีคุณสมบัติไม่ทนเค็มระดับปานกลาง และข้าวเจ้าหอมทนดินเค็ม UBN02123-50R-B-2 (UBN) ผู้วิจัยพบความแตกต่างของลักษณะสรีรวิทยาอย่างชัดเจนในข้าวที่อยู่ในด้วยสารละลายโซเดียมคลอไรด์ความเข้มข้น 80 mM เป็นเวลา 12 และ 24 ชั่วโมง เมื่อศึกษาโปรไฟล์เมแทบอไลต์ของใบและรากของข้าวจำนวน 6 ซ้ำภายใต้ภาวะเครียดจากความเค็มด้วยเทคนิค GC-TOF/MS และ LC-MS/MS พบว่าเมแทบอไลต์ในวิถีไกลโคไลซิส วิถีเมแทบอลิซึมสร้างพลังงาน และเมแทบอลิซึมของกรดอะมิโน มีระดับการสะสมเพิ่มสูงขึ้นใน UBN เร็วกว่า KDML 105 ที่เวลา 12 ชั่วโมงภายใต้ภาวะเครียดจากความเค็ม ที่เวลา 24 ชั่วโมง ระดับของเมแทบอไลต์ดังกล่าวมีการลดลงใน UBN ซึ่งให้ผลตรงกันข้ามกับระดับของเมแทบอไลต์ใน KDML 105 ที่มีการสะสมเพิ่มสูงขึ้น เพื่อศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับความเครียดจากความเค็ม ได้เลือกยีนตัวแทนคือ แอล-กาแลกโตโน-1,4-แลกโตน ดีไฮโดรจีเนส กลูตาเมทดีคาร์บอกซีเลส ไพโรลีน-5-คาร์บอกซีเลท รีดักเทส และ ไรโบฟลาวินซินเทส พบว่า ยีนที่เข้ารหัสเป็นเอนไซม์กลูตาเมทดีคาร์บอกซีเลสและเอนไซม์ไพโรลีน-5-คาร์บอกซีเลท รีดักเทส ซึ่งมีความเกี่ยวข้องกับกระบวนการสังเคราะห์สารป้องกันแรงดันออสโมติก มีการแสดงออกเพิ่มสูงขึ้นอย่างมีนัยสำคัญภายใต้ความเครียดจากความเค็มที่เวลา 24 ชั่วโมง ใน UBN เมื่อเทียบกับ KDML 105 ในทางตรงกันข้ามยีนที่เข้ารหัสเป็นเอนไซม์ไรโบฟลาวินซินเทสมีการแสดงออกลดลงอย่างมีนัยสำคัญใน KDML 105 เทียบกับ UBN การวิเคราะห์รูปแบบของเมแทบอไลต์ทุติยภูมิพบระดับการสะสมที่เพิ่มขึ้นของกรดเฟอรูลิกในรากของ UBN จากผลการทดลองแสดงให้เห็นว่า UBN สามารถตอบสนองต่อความเครียดจากความเค็มได้ดีกว่า KDML 105 ผ่านการสะสมเพิ่มขึ้นของเมแทบอไลต์ปฐมภูมิ และสารป้องกันแรงดันออสโมติก ซึ่งบ่งบอกถึงความสามารถในการทนทานความเค็มที่สูงกว่าของ UBN เมื่อเปรียบเทียบกับ KDML 105

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5471922023 : MAJOR BIOCHEMISTRY AND MOLECULAR BIOLOGY
KEYWORDS: RICE / SALINITY STRESS / METABOLITE PROFILES / GC-TOF/MS / LC-MS/MS

KWANKAO KARNPAKDEE: METABOLITE PROFILES OF ‘KDML 105’ AND SALT-TOLERANT ‘UBN 02123-50R-B-2’ RICE Oryza sativa L. IN RESPONSE TO SALINITY STRESS. ADVISOR: ASST. PROF. SUPAART SIRIKANTARAMAS, Ph.D., CO-ADVISOR: ASST. PROF. NUCHANAT WUTIPRADITKUL, Ph.D., pp.

In this research, I studied metabolic responses in the two cultivars of Thai rice, moderately salt sensitive rice ‘KDML 105’ and salt-tolerant rice ‘UBN02123-50R-B-2’ (UBN). I found different physiological appearance in both cultivars when treated with 80 mM NaCl at 12 and 24 h. The metabolite profiles of six replicates of leaves and roots were then investigated using GC-TOF/MS and LC-MS/MS. The results showed that metabolites in glycolysis, energy metabolisms, and amino acid metabolisms were up-accumulated faster in UBN when compared to KDML 105 at 12h under salinity stress. At 24h, those metabolite levels in UBN were declined in contrast to those in KDML 105 that showed higher up-accumulation. To investigate stress-related gene expressions, several candidate genes, e.g. L-galactono-1,4-lactone dehydrogenase, glutamate decarboxylase, pyrroline-5-carboxylate reductase, and riboflavin synthase, were selected. I found that the genes encoding glutamate decarboxylase and pyrroline-5-carboxylate reductase, which involved in the osmoprotectant biosynthesis, were significantly higher expressed during salinity treatment at 24h in UBN than KDML 105. In contrast, the gene encoding riboflavin synthase was significantly lower expressed in KDML 105 than UBN. Secondary metabolite analyses showed the up-accumulation of ferulic acid in roots of the UBN. These results suggest that UBN can response to salinity stress much better than KDML105 through the higher up accumulation of primary metabolites and osmoprotectants, implying the higher salinity tolerance ability in the salt-tolerant rice ‘UBN02123-50R-B-2’.

Department:	Biochemistry	Student's Signature .....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature .....
		Co-Advisor's Signature .....
Academic Year:	2014	

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CHAPTER I

INTRODUCTION

Abiotic stresses such as salinity, drought, and oxidative stress have extremely affected on agricultural plants. Plants respond to survive under these stress conditions by altering series of changes in physiology, cellular level, and molecular mechanisms. Abiotic stress responses could be defined in plants as any changes such as altering homeostasis and cellular component disruption, resulting in the regulations of metabolic pathway that can be referred as acclimation (Shulaev et al. 2008). The biosynthesis and accumulation of metabolites which play important roles as signaling molecules usually define the cellular responses to stresses. As a long evolutionary period, plants grown under various environments respond differently under stress conditions.

To study the metabolic responses affected by various stresses, metabolomics has been used as a tool to identify stress-associated metabolites. Metabolomics is one of the omics studyies that is used for investigating biological processes in term of both quantitative and qualitative analysis of identified metabolites (Oikawa et al. 2008) It could potentially identify metabolites presented in various biological conditions in combination with statistical methods, and be reasonably adapted as a practical tool which has widely been applied for studying the changes in metabolite flux. In addition, metabolomics could be used to clarify the functions of stress-related genes using natural variants of studied plants in integration with genomics and transcriptomics. Many kinds of genes related to various functions are either induced or repressed by stress conditions (Shinozaki and Yamaguchi-Shinozaki 2007). As results, the changes in the accumulation levels of metabolites can be observed.

Since rice (*Oryza sativa* L.) is one of the important crops in the world as a food source and commercial plant in many countries, there are many studies for developing better rice cultivars with improving valuable traits, such as increasing grain yield and enhancing ability for stress tolerance. Rice is an annual crop with normally high growth rate in irrigated areas, rainfed lowland areas, and flood prone areas. Its

production yield has been declined by salinity stress, which is one of the major problems in rice paddy field. Sodium cations in soil with electrical conductivity (E.C.) exceed 4 dSm^{-1} are defined as saline soils. It has been estimated that 6% of saline soil occurs in the earth's surface of which 30% and 26% of land in Australia and Pakistan, respectively. Saline soil problem tends to increase to an estimated 50% of all arable land by 2050 (Ladeiro 2012). Therefore, the applications of metabolomics could be used to elaborate rice responses against salinity stresses. The outcome biological information from metabolomics could lead to either rice breeding program or development of stress-tolerant rice. In this thesis, I aimed to construct the metabolite profiles of two Thai rice 'KDML 105' and 'UBN 02123-50R-B-2' under salinity stress condition, identify stress-related metabolites and analyse the correlation between metabolites and genes. All information related to this research are reviewed in this chapter.

1. Plant response and stress

In normal condition, plant growth and development are regulated by series of genes, hormonal levels, and signal transduction molecules. To survive in changing environment, plants adapt themselves through homeostasis adjustment. There are many different types of stress-related metabolites, enzymes and transcriptional factors involving in responses to stresses.

Two main categories of stresses are biotic (e.g., pathogens, insects, and weeds) and abiotic (e.g., light, drought, high temperature, salinity, and flooding) stresses. Abiotic stresses are the main causes of plant stress that can reduce both the quantity and quality of plant products. The sequence of responses to environmental changes is stress perception, signal transduction, and response induction that are involved in acclimation responses according to type of environmental challenge, respectively (Smith et al. 2010). For example as light stress, plants develop metabolisms that are proper to the usage of light energy. High intensity of light leads to excess electron in chloroplasts, and the energy of the excited electron results to the photooxidative damage (Smith et al. 2010). Water deficits apply range responses to keep cell turgor and growth. Responses to water

deficits include in the changes of biomass dispensation, stomatal control, cell wall reinforcement, cellular osmolarity, and osmotic adjustment (Warren et al. 2011). As results from many external factors, the environmental stresses stimulate similar cellular responses such as the accumulation of osmoprotectants, the up-accumulation of antioxidant enzymes and the modification of related proteins (Vierling and Kimpel 1992).

The complexity of plant stress responses represented in figure 1 shows that the abiotic stress response is based on the expression of specific stress-related genes. The primary stresses such as drought, salinity, cold, heat, and chemical pollution are often interconnected, and cause cellular damage. The stress signals such as osmotic and ionic effects trigger the downstream signaling process and transcription controls, which is activated stress-responsive mechanisms and stress-related protein to the re-establishment of cellular homeostasis and the repairing of damaged membranes and proteins. Plant developmental processes for improving stress tolerance are regarded to the genetic manipulation of each plants (Wang et al. 2003). The ongoing explanation of the molecular control mechanisms of abiotic stress tolerance, which may result in the use of molecular tools for engineering and molecular breeding of more tolerant plants, is referred to the expression of specific stress-related genes. These genes consist three major categories: (i) those that are involved in signaling cascades and in transcriptional factors, such as MyC, MAP kinases and SOS kinase (Shinozaki and Yamaguchi-Shinozaki 1977; Munnik et al. 1999; Zhu J. K. 2001), and transcriptional factors such as HSF, and the CBF/DREB and ABF/ABAE families; ii) proteins that function directly in the membrane protections such as heat shock protein and chaperones (Vierling 1991; Ingram and Bartels 1996), osmoprotectants, and free-radical scavengers (Bohnert et al. 1995); and (iii) proteins which are involved in water and ion uptake and transport such as aquaporins and ion transporters (Maurel 1997; Blumwald 2000).

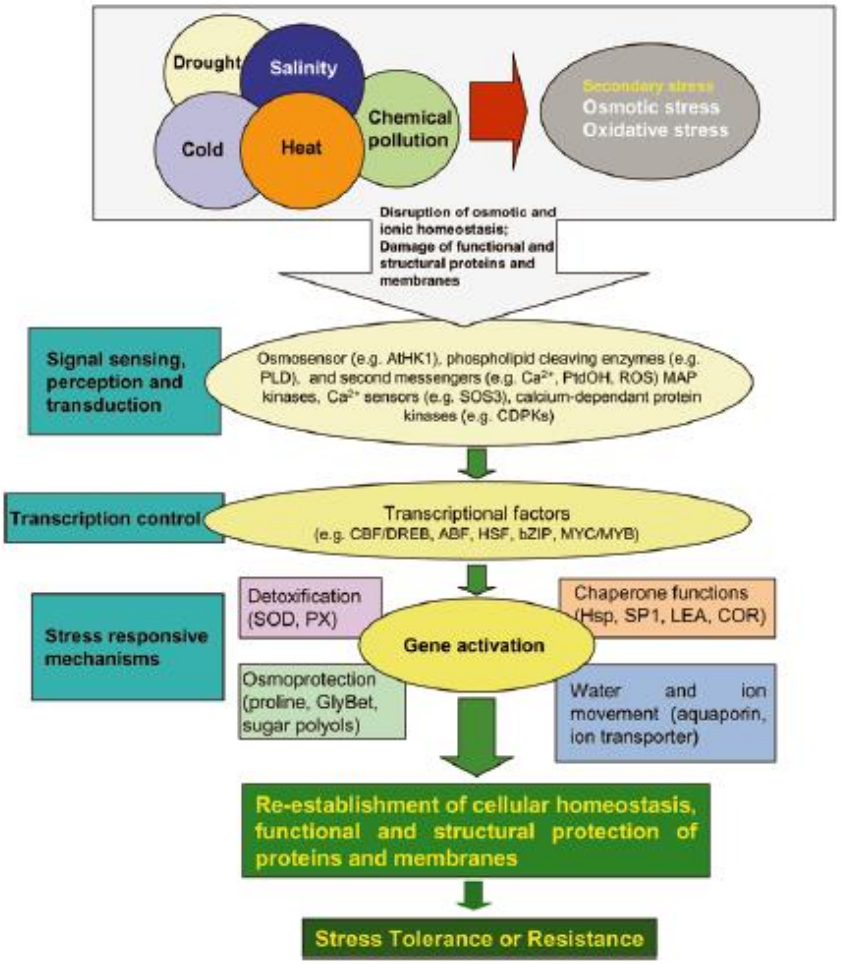

Figure 1: The complexity of the plant responses to abiotic stresses (Taken from Wang et al. (2003))

2. Salinity stress

2.1 Influences of salinity stress to plants

Salinity stress is the condition that plants expose to external exceeding salt concentration. It occurs when soluble salt are elevated in soil and water (usually NaCl). Several studies have shown that salinity stress cause to plant acclimation affecting the perturbation of plant growth. It could lead to oxidative stress, resulting in the metabolic imbalance, ion toxicity, and osmotic stress (Hussain et al. 2008). Salinity stress can affect plants on the membrane disorganization, reactive oxygen species overproduction, cellular toxicity, and the metabolic imbalance (Shuji et al. 2002). Generally, this stress starts by the high level of Na^+ and Cl^- in external environment exposed to plant roots. The high soluble salt in soil leads to decreased water potential, inhibiting water uptake which causes cell dehydration (Zhu J. K. 2001). The excessive Na^+ permeation can be toxic to root cells because the abundant of Na^+ at the root surface leads to the disruption of the K^+ uptake to the cells (Lazof and Bernstein 1999).

The Na^+ and K^+ imbalance leads to osmotic and ionic stress. In case of osmotic stress, plants maintain the intracellular water potential to be lower than the extracellular water potential in their environment. This action can either inhibit water uptake or retract water within plant cells, resulting in dehydration and reductions in cell turgor and plant growth (Shuji et al. 2002). High extracellular concentration of Na^+ and Cl^- directly exposed to root particularly brings the toxicity to root cells. The ion toxicity transfers into plant compartment along with the water stream which move from their environment to the vascular system, then transfer to shoot through xylem and intracellular space. The ion toxicity in shoot could be observed by the change in morphological symptom as shown in Figure 2. The major symptoms are leaf tip burning which represents by the white leaf tip, leaf browning and death, the interrupted plant growth, leaf rolling, spikelet sterility, and changes in flowering duration.

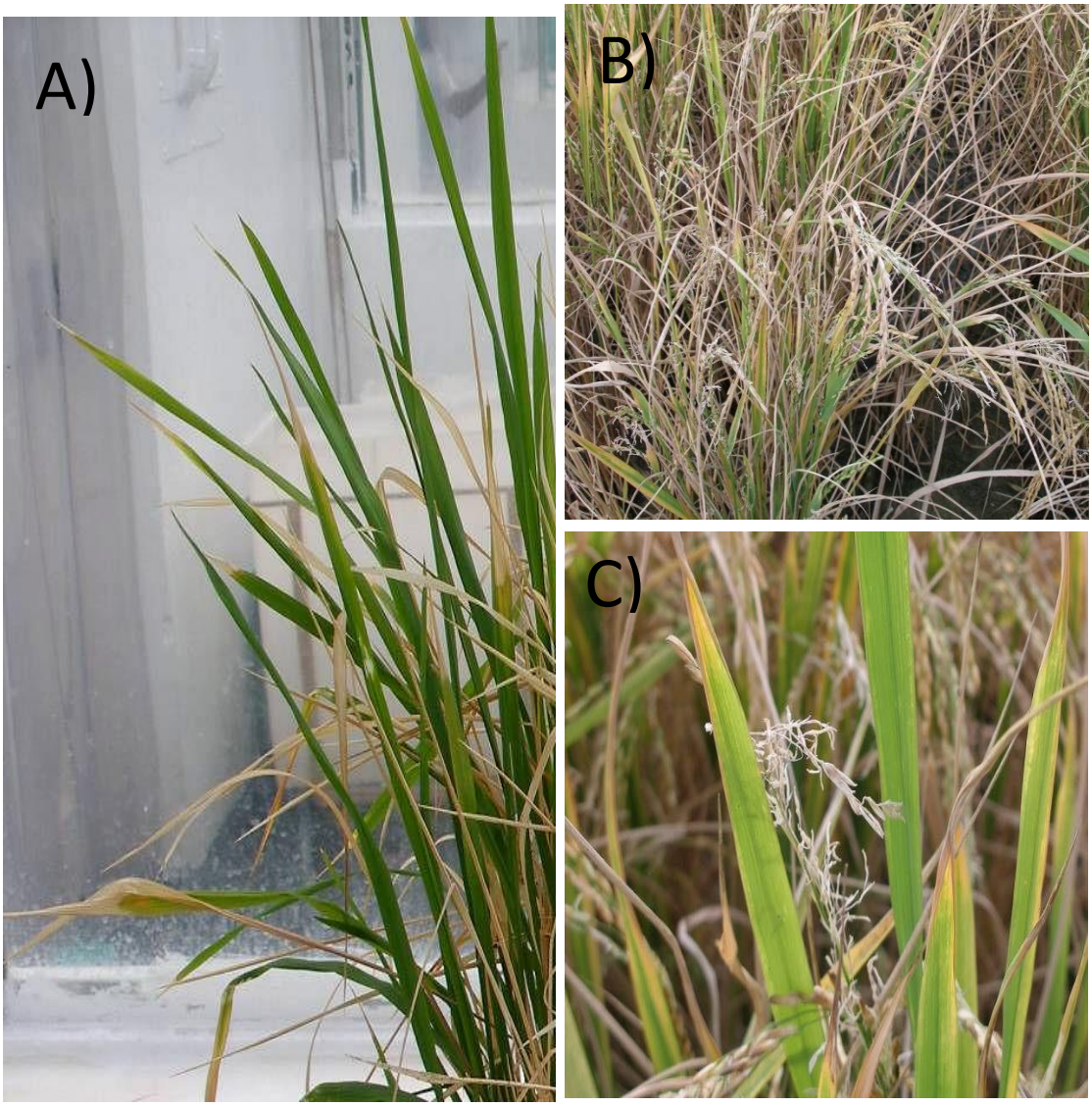

Figure 2: The morphological symptoms under salinity stress
(Taken from : http://www.knowledgebank.irri.org/ricebreedingcourse/Breeding_for_salt_tolerance.htm)

A) White leaf tip follow by tip burning

B) Leaf browning

C) Spikelet sterility

2.2 Plant responses to salinity stress

High external salt concentration conveys the K^+ and Na^+ uptake into the cell via cellular transporter causing high Na^+/K^+ ratios that are cell toxicity, consequent to the inhibition of cell growth (Smith et al. 2010). K^+ is the most abundant cation in plants, which is play crucial roles in enzyme activity, protein synthesis, osmotic homeostasis, stomatal movement, cation-anion balance, and stress response (Wang et al. 2013). The uptake of excessive Na^+ and Cl^- to the cell is part of growth inhibition under salinity stress due to the decreases in ions and vital nutrients required for plant-growing uptake. Both salinity and drought induce the similarly changes in ion uptake which leads to osmotic stress in plants (Bartels and Sunkar 2005). Under drought conditions, cell turgor recovery via osmotic stress was defined by K^+, Cl^- and Na^+ uptake, which was conducted with voltage-gate K^+ transporters at the cellular plasma membrane (Shabala and Pottosin 2010). The different concentration of ions on opposite sides of a cellular membrane suggests a voltage difference called membrane potential (Figure 3). Normally, high concentrations of sodium (Na^+) and chloride (Cl^-) ions are occurred in extracellular region, whereas high concentration of potassium (K^+) ions was in intracellular region.

In addition, ionic, osmotic and oxidative stresses are also resulted from salinity stress. The oxidative stress produces series of reactive oxygen species (ROS) in plants as hydrogen peroxide, hydroxyl radicals, and superoxide anions which can damage cellular structures, membranes, and macromolecules (Wang et al. 2003). Therefore, the oxidative stress is seriously impacted to agriculture and the reduction of crop yields.

To survive under salinity stress, plants modify various physiological and biochemical processes involving in the increase of antioxidant scavenging enzymes, the synthesis of osmoprotectants, the regulation of transcription factors, the signaling molecules transduction, and the change in metabolic pathway. The association of physiological and biological alterations could promote plant to execute the defense mechanisms against salinity stress.

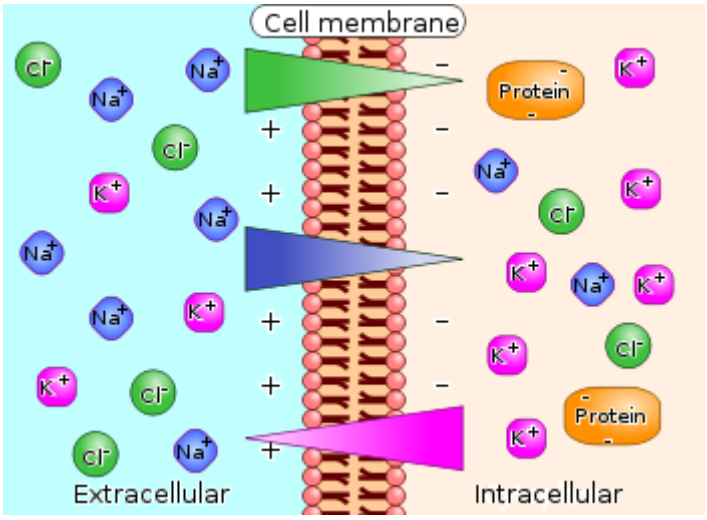

Figure 3: Differences in concentration of ions on opposite sides of a cellular membrane produce a voltage difference called the membrane potential (Taken from http://chemwiki.ucdavis.edu/Analytical_Chemistry/Electrochemistry/Case_Studies/Membrane_Potentials). High concentration of Na^+ (representd in blue circles) and Cl^- (representd in green circles) in the extra cellular regions excreted the intra cellular K^+ (representd in violet circles), which produce the osmotic imbalance.

3. Cellular defenses under salinity stress

The most severe damages from the stress frequently are present at the seedling stage and reproductive phase in rice (Kim et al. 2005), which can be seen as the reduction of grain yield and nutritional components. Germination is also inhibited by either high salt concentration or low osmotic potential of the surrounding environment.

The functional categories of salinity tolerance consist of 1) ion homeostasis which is regulated by cation transporters, tonoplast, and plasma membranes, 2) the activity of antioxidant enzymes which scavenges the reactive oxygen species, 3) the biosynthesis of osmoprotectants that protect membrane and cell structure, and 4) the changes in accumulation levels of primary and secondary metabolites.

3.1 Ion homeostasis

Ion homeostasis in cell is regulated by the ions pumps such as antiporters, symporters, and carrier proteins located on cell membrane. Plant cells do not appear to contain Na^+ATPases like animal cells, but present in the Na^+/H^+ antiporters in plasma membrane-enriched-membrane vesicles (Zhu J. K. 2001), which play a crucial role in maintenance of cellular ion homeostasis. Na^+ could enter into cell by a low affinity of K^+ transporter. In cytoplasm, the excessive Na^+ is transported into vacuole via a Na^+/H^+ antiporters in the tonoplast, and Cl^- might be followed the Na^+ into vacuole by a specific uniport channel. The Na^+/H^+ antiporters catalyze the transport of Na^+ and H^+ across cell membranes, regulating pH balance in cytoplasm and cell turgor (Serrano et al. 1999; Wang et al. 2003).

3.2 Antioxidant and detoxification enzymes

Plant salinity stress induces the formation of ROS as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^·$) and superoxide anions (O^{2-}). These ROS convey the disruption of membrane lipids, protein degradation, enzyme inhibition, and nucleic acids damage through the oxidative stress. To scavenge these toxic compounds, plants employ the antioxidant compounds such as ascorbate, glutathione, carotenoids, thioredoxins, anthocyanins, and detoxification enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase against oxidative stress (Bowler et al. 1992; Wang et al. 2003).

The high concentration of H_2O_2 in plant cell is a potential signal in plant stress, which is scavenged by series of antioxidant enzyme as mentioned above. CAT is one of the most effective enzymes that serve to rapid H_2O_2 degradation (Scandalios et al. 1997). CAT stimulates the conversion of H_2O_2 to water, which can then reduce H_2O_2 concentration in the cell. Peroxidase occurs in numerous isoforms (e.g., ascorbate peroxidase ; APX, glutathione peroxidase ; GPX, peroxiredoxin ; Prx), that are usually involved in preventing damages by ROS. The superoxide anion is converted by SOD to H_2O_2 which is consequently detoxified by CAT and various isoforms of POD. Further reaction of H_2O_2 to cell death, activation of defense genes and enhancing of detoxify proteins (Kachroo et al. 2003). These detoxification

proteins are shown in Figure 4. The oxidative stress signals will be sent to nucleus to regulate plant growth and developments against stress conditions (Chi et al. 2013). Further reactions may convey the formation of hydroxyl radicals (HO·). Antioxidant proteins protected the damages of ROS leaking from peroxisomes. H_2O_2 can easily permeate the peroxisomal membrane and various ROS transmitted to the mitochondrion play a role in the adaptive response of mitochondrial redox state, particularly the reduction state of respiratory pathways. The redox signals will be transmitted to the nucleus for regulation of plant growth and developments.

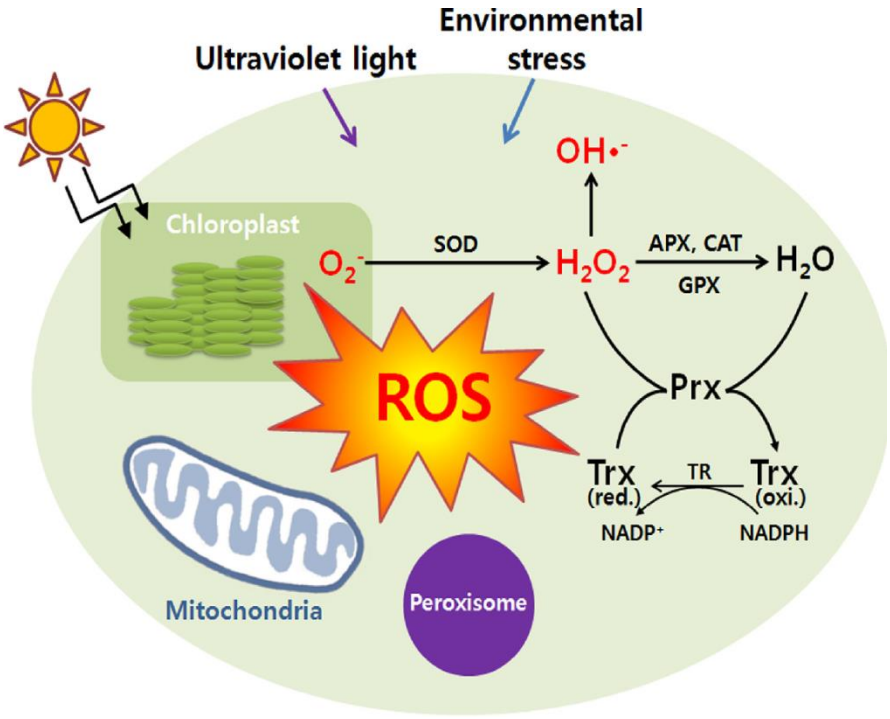

Figure 4: Cellular responses against reactive oxygen species

(Taken from Chi et al., (2013)). Reactive oxygen species (ROS) was generated by several stress conditions. The reaction started with the formation of primary ROS, superoxide anion (O^{2-}) by one electron reduction of molecular oxygen. The superoxide anion is catalyzed by SOD to form hydrogen peroxide (H_2O_2) which is scavenged by catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GPX), and peroxiredoxin (Prx). Trx is represented as the thioredoxin proteins which control several redox independence cellular reactions against abiotic stress.

3.3 Osmoprotectant biosynthesis

One of the widely acceptable responses to salinity stress is the accumulation of metabolites that acts as osmoprotectants or compatible solutes. The accumulation of osmoprotectants is related to the change in external osmolarity, resulting in the osmotic balance and the protection of cell structures. Stress associated metabolites such as carbohydrate compounds are defined as significant osmolytes which accumulate in plants (Kerepesi 1998). The reported metabolites with an osmolytes function are sugars (sucrose and fructose), sugar alcohols (inositol, glycerol, galactinol) and several amino acids. Osmoprotectants are commonly hydrophilic compounds which can replace water molecules at protein surface or membranes (Shuji et al. 2002).

Previous studies have found that the localization of osmoprotectants synthetic site is in the cytoplasm while the Na^+ and Cl^- are excluded into the vacuole, resulting in turgor maintenance under osmotic stress (Flowers et al. 1977; Rathinasabapathi 2000). High concentration of osmoprotectants can reduce the inhibition of ion effect on enzyme activity and prevent the dissociation of enzyme complexes. Osmoprotectants also serve as the scavenger of ROS and repository of carbon and nitrogen (Bohnert et al. 1995). In addition, the nitrogen containing compound was found in action as scavenger to excessive ammonium accumulation under stress (Rabe 1999).

The various synthetic reactions of osmoprotectants depend on the plant species, stress durations, and steps of related and enzymes. Structures of representative osmolytes which are commonly involved in the protection of cell structure are shown in Figure 5. The accumulation of these representative osmolytes is regulated by stress conditions, and often highly accumulated in the stress-tolerance plant cultivars. Furthermore, high accumulations of osmoprotectants do not disturb the activity of enzymes(Hasekawa and Bressan 2010).

Additionally, plant responses to abiotic stress related to the changes of accumulation levels of organic acids. The depletion of organic acids was found in salt acclimation on plants (Gong et al. 2005). The reduction of organic acid might be involved in the equalization of the inorganic anions, which might actually affected on

the uptake of anions and cations under stress (Hinsinger et al. 2003). Organic acid depletion upon salinity stress may be recruited from the TCA cycle and exclude into the synthethic pathways of amino acids and osmoprotectants as responses under stress. Several organic acids such as pipecolic acid, glutamic acid and betaines have been considered as osmoprotectants against stress.

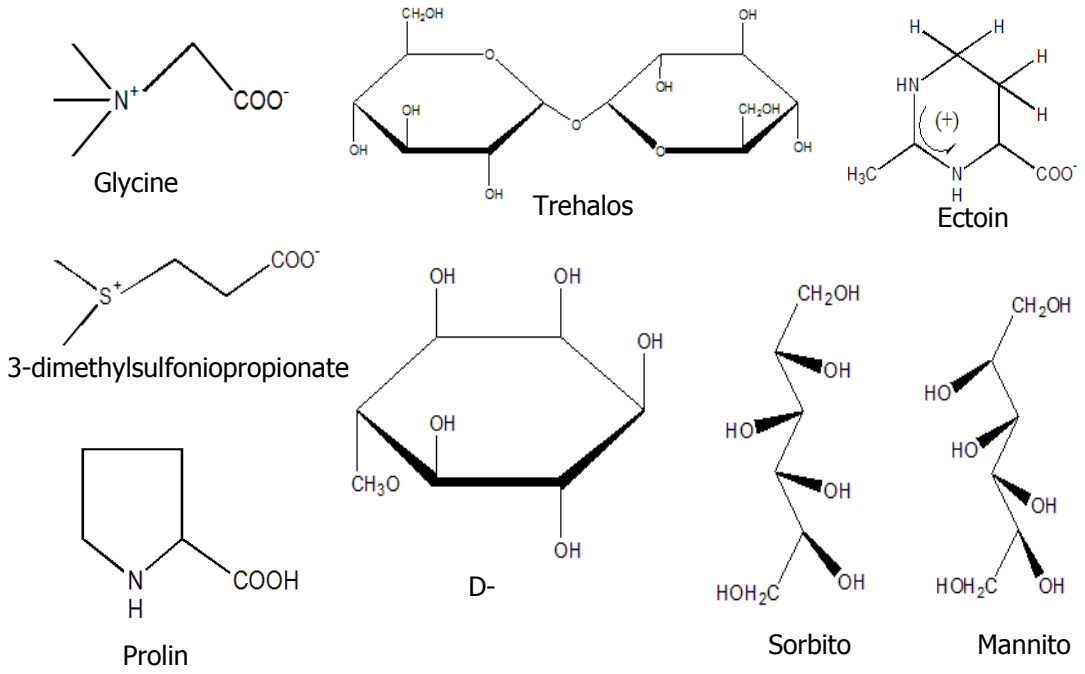

Figure 5: The common osmoprotectants or compatible solutes involving in either osmotic adjustment or cellular structure protection. (Hasegawa et al., 2000). The representative osmoprotectants have been found in the responses to salinity stress in the salt-tolerance cultivars which play various roles in action to salinity stress.

3.4 Salinity stress signals on the primary and secondary metabolites in plants

The synthesis of osmoprotectants can connect to responsive metabolic pathway under saline condition representing by the alteration in accumulation levels of related natural compounds. Osmotic stress is a consequence from salinity stress that conveys the up/down accumulation of particular primary and secondary metabolites. The representative biosynthetic pathways of proline, glycine-betaine,

pinitol, and ectoine originated from the amino acid biosynthesis pathway as glutamic acid (for proline), choline metabolism (from glycine betaine), myo-inositol (for pinitol) and aspartic acid (for ectoine) (Hasekawa and Bressan 2010). Plant polyamines have been reported to be involved in the salinity stress response in higher plants. Polyamines such as spermidine and putrescine have been considered as one of nitrogen containing compounds, which is essential for plant growth and development (Alcazar et al. 2010), and also reported as a precursor of several osmoprotectants. Spermidine and putrescine were found to enhance beta-alanine production in hairy-root cultures of red beet (Bais et al. 2000; Ramakrishna and Ravishankar 2011) as beta- alanine is found to increase in response to environmental stress.

The aspartate (Asp) family in higher plant is vital for nutritional balance. The Asp family consists of four essential amino acids as lysine (Lys), threonine (Thr), methionine (Met) and isoleucine (Ile). In the previous study, the regulatory metabolic link of the Asp-family pathway with TCA cycle was controlled by stress (Galili 2014). It is also related to glycine synthesis which is involved in plant photorespiration. The increasing Lys level in seeds of crop species was regulated by the mechanism of Asp-family pathway under cellular stress and energy regulation. Aspartic acid was also found the implication to enhance salinity stress in tomato by being the precursor to synthesize several amino acids including methionine, threonine and isoleucine (Akladious and Abbas 2013).

4. Thai rice in response to salinity stress

Rice (*Oryza sativa* L.) serves as a staple food source for the world's population. Rice has been considered as a salt-sensitive crop, especially at seedling stage and reproductive phase. As a result, high saline soil has limit rice production yields particularly in the rainfed area of South-east Asia. For several decades many approaches in order to modify rice cultivars including the enhancement of grain yield and the resistance to environment have been widely developed. The promising approaches such as genetic engineering, breeding and the study of physiological traits and molecular responses of rice in various growth conditions have been manipulated for rice production enhancement and then distributed to marginal farmers. Therefore

the developing of hybrid cultivars in order to enhance rice production yield and increase economical value have been considere.

'Khao Dawk Mali 105 (KDML 105) is consider as a famous aromatic Thai rice that universal recognized due to its aromatic, soft, and delicate cooking rice. The KDML 105 cultivation is liberally grown in rainfed lowland in the north, the north-eastern and the central part of Thailand, in which was defined as soil salinity areas. Moreover, KDML 105 is the photoperiod sensitive plant. Apart from saline condition, it also has been disturbed by many insects, agricultural animals and diseases.

Although KDML 105 production yield was restricted by several reasons as mentioned earlier, it is still be aspired due to its outstanding in soft texture and good taste. The KDML 105 seed characteristics are shown in figure 6, indicating the general size, shape and aspect of KDML 105 seeds.

Figure 6**: KDML 105 seed manner.** The picture demonstrates size of KDML 105 seed including shell covered seeds and unwrapped seeds.
(Taken from: http://www.doae.go.th/library/html/detail/rice_seed/rice_kmdl105.pdf)

Salt-tolerant rice cultivars have widely been constructed for many years ago in order to increase rice productivity and enhance stress tolerance. The salt-tolerant rice cultivars have generally been produced through breeding technique and genetic engineering. In this study, I am interested in the salt-tolerant rice 'UBN 02123-30R-B-2' (UBN). UBN was constructed from KDML 105 and the salt-tolerant cultivar IR66946-3R-196-1-1 (FL496) and IR66946-3R-230-1-1 (FL530) using breeding technique as the

breeding diagram is shown in Figure 7 (Suriya-aunroj 2005). The Simple Sequence Repeat (SSR) markers as E03_92 for aromatic QTL (MAS 1), B1.1-1, B1.1-11, (MAS 2) and RM140 for salt tolerant QTL, E03_92 for aromatic QTL and RM00 for waxy QTL (MAS 3) were applied. These three markers located on chromosome 8, chromosome 1 and chromosome 6 respectively (Figure 8). Rice plants containing SSR markers were selected and crossed back by self-pollination with KDML 105 for three generations. The final generation produced BC_2F_3 which was evaluated salt tolerance and cooking quality, and further defined as UBN rice.

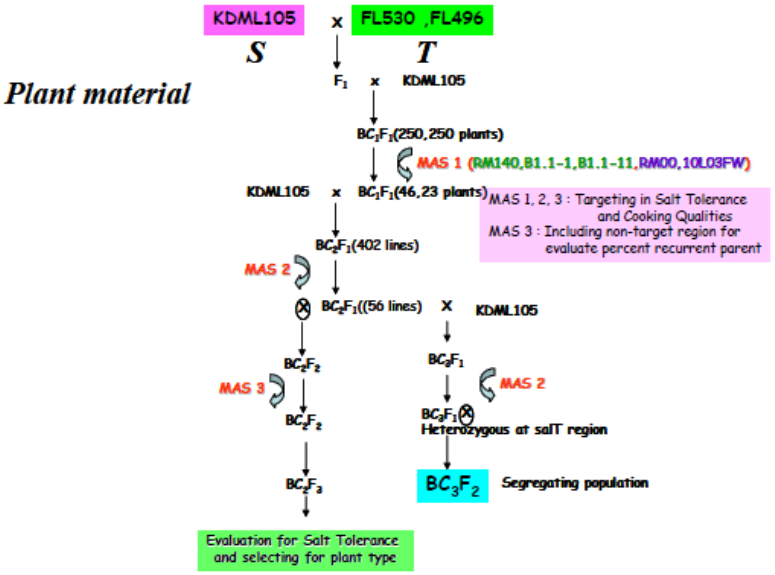

Figure 7: Diagram for BC_2F_3 and BC_3F_2 of KDML105/FL496 and KDML105/FL530 population generation. The segregating population of BC_3F_2 was further defined as UBN. (Taken from Suriyaarunroj et al.)

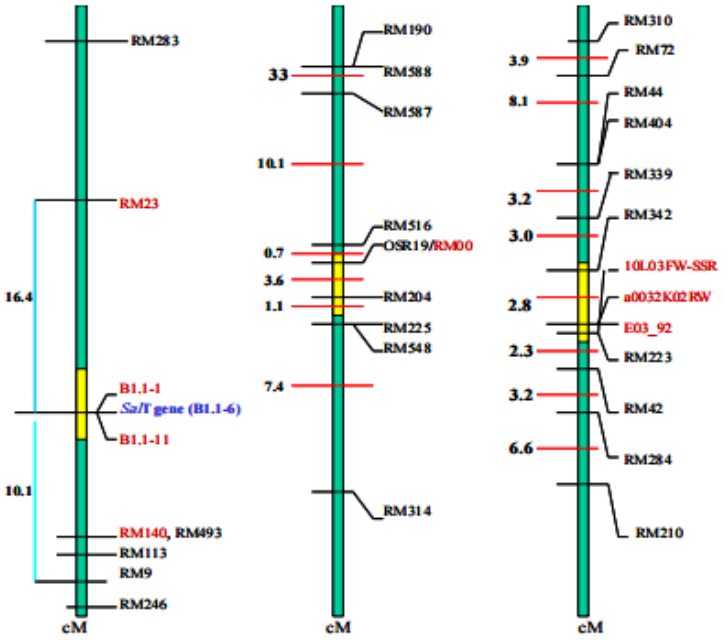

Figure 8: Map of markers flanking: a) SalT gene (MAS2) region located on chromosome 1, b) waxy gene region (MAS3) located on chromosome 6, and c) aroma gene region (MAS1) located on chromosome 8 (Taken from Suriya-arunroj, 2005)

UBN rice seeding were screened in EC 6 dS/m nutrient solution at average day/night temperature : 36/27° C. Na^+ and K^+ accumulated at 12 dS/m (12 mmol NaCl) were observed in the shoot in compared with KDML 105 and the other cultivars. The low salt injury symptom were presented in FL496 and FL530, which had higher K^+ absorption than KDML105 resulted in lower Na^+/K^+ ratio (< 0.200) compared to the well-known salt tolerant cultivar as Pokkali. As the line which had moderately low value of Na^+/K^+ ratio (0.200-0.300) termed as moderately tolerant lines, FL496 and FL530 is considered as salt-tolerance. In contrast, KDML 105, which the ratio value were 0.688, is considerably high Na^+/K^+ ratio compared to the tolerant lines. The researchers also studied the grain yield production, the reaction to diseases, insect pests and the salinity response of UBN compared to KDML 105. The results suggest that the UBN showed significantly more tolerant phenotypes than the KDML 105 in these respects - higher grain yielding and better tolerant to diseases and insects. The results of salinity tolerant screening on rice seedling is shown in Figure 9.

Figure 9: Styrofoam seedling float in hydroponic solution for salt tolerant screening. (Taken from Suriyaarunroj et al.), the result indicated the salt-tolerant on UBN seedling rice which shows leaf injury less than KDML 105. Rice seedling in red box represent KDML 105 and blue box represent UBN.

5. Metabolomics of plants under stress

The study of salinity effect to rice has been investigated on metabolic processes in several functional categories including ion uptake, ionic transport, photosynthesis, amino acid accumulation, polyamine accumulation, and reactive oxygen species (ROS) scavenges. The metabolomics provides the strategies to define the changes in accumulation of stress-related metabolites, which are plant responses to salinity stress. The different responses to salinity stress depend on the different rice cultivars which are closely related to the important traits of rice as the nutrient contents, yields and mechanisms against stresses. The study on metabolic responses to salinity stress of bayley, *Hordeum vulgare* L. cultivars, sahara and clipper, showed that two barley cultivars were different in salinity tolerance according to their specific metabolisms. Sahara was reported as salt-tolerant cultivars showing the high accumulation of hexose phosphate, TCA cycle intermediates and metabolites involved in cellular protection (Widodo et al. 2009). This study implies the possibility to use metabolomics analysis to classify the particular metabolic responses in salt-sensitive plant compared to salt-tolerant plant. The study on Zea mays seedling to salinity stress in either 0, 50 or 150 mM saline solution showed the increased levels

of alanine, glutamate, asparagine, glycine-betaine and sucrose and the decreased levels of malic acid, *trans*-aconitic acid and glucose in shoots. Correlation with salt-load shown in roots included elevated levels of alanine, gamino-*N*-butyric acid, malic acid, succinate and sucrose and depleted levels of acetoacetate and glucose (Gavaghan et al. 2011). Metabolomics was also used to study the unintended polar metabolite profiles in transgenic rice and its counterpart, revealing that this tool could provide a potential method for assessing undesirable changes in transgenic crops that enhance nutrient through the intentional modification of metabolic pathways (Kim et al. 2012).

In recent years, the term of metabolomics has been applied to investigate cellular metabolic responses to a particular abiotic stress. The global analysis of plant in each condition illustrates how cell and organisms responding to stress through the changing in metabolite levels. The deeper exploration of the regulation of plant responses to environmental stress was revealed through powerful tools. The technological development for metabolomics study has been used for basic researches in the region of plant physiology and biology (Oikawa et al. 2008). The advance technique to investigate levels of wide range metabolites are engaged with sample preparation, sample extraction, metabolite detection by analytical instrument and data processing via bioinformatic technique and statistical analyses.

For the detection of metabolites, the physiochemical properties of each metabolite are mean to be considered. The selection of the detection techniques used in analyzing metabolites depends on their hydrophobicity, acidity and volatility (Oikawa et al. 2008). Among the high resolution technique, time-of-flight gas chromatography- mass spectrometry (GC-TOF/MS) is the most extensive method which is used to the metabolites study due to the ability of GC-TOF/MS to detect hydrophilic metabolites in series of primary metabolites as amino acids, organic acids and sugars. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a powerful tool to detect the hydrophobic secondary metabolites including phenylpropanoids, alkaloids and flavonoids. The high chromatographic results is based on the separation of those compounds, indicated in the clearly information in metabolite profiling in action to stress condition.

In case of manipulation to the large scale data, the statistical method as the principal component analysis (PCA) has been applied in numerous researches as a tool to organize the biological information. The PCA strategy is to separate the large scale of different data to separated series of particular groups. For example, the used of PCA as a statistical technique to the study of the different metabolite groups in *Oryza sativa* 'Nipponbare' and *Arabidopsis thaliana* under cold and dehydration stresses, indicated the distribution of metabolites in rice plant and *A.thaliana* in subjected to seven treatment including untreated plant as control, cold and dehydration stress sample sets. The PCA score plot showed the metabolite profiles subjected to seven treatments, classified into three separation groups according to plant normal growth condition, cold stress and dehydration stress in both rice and Arabidopsis (Maruyama et al. 2014). This study implies to the usable of statistical method via principal component analysis to the classification and distribution of different metabolites.

One of the feasible roles of metabolomics is the investigation of genetic study. Metabolomics provides the possibility to identify gene functions directly to rice responses to salinity stress. Combination of metabolomics genomics studied may apply to rice breeding and the selection of the salt-tolerant cultivars by the identification of stress-related metabolite markers.

In this study, the metabolomics was applied to study salinity responses in two Thai rice cultivars, KDML 105 which represents the moderately-salt sensitive rice and the UBN 02123-50R-B-2 as the salt-tolerant rice using the high resolution technique as GC-TOF/MS for the analyses of primary metabolites and LC-MS/MS for the secondary metabolites. We also applied the principal component analysis to classify the groups of stress-related metabolites, continue with the studying of gene expression of the enzymes which is involved in the biosynthetic pathway of the stress-related metabolites. We aimed to construct the metabolite profiles in two Thai rice cultivars under normal and salinity stress conditions. Finally, we proposed to identify the stress-related metabolite markers in groups of salt-sensitive and salt-tolerant metabolite markers. The information from this study can be used to

develop rice cultivars in further study for the enhancement of salinity stress tolerant ability to Thai rice.

CHAPTER II

MATERIALS AND METHODS

I. Materials

1. Plant materials

1.1 Seeds of *Oryza sativa* L. ssp. Indica: 'KDML 105'

1.2 Seeds of *Orysa sativa* L. ssp. Indica: 'UBN 02123-50R-B-2'

2. Instruments

2.1 Equipments for plant germination and growing

- Aluminum foil
- Balance: PB303-L (Mettler Toledo, USA)
- -80˚C Freezer (Thermo Electron Corporation, USA)
- Forceps
- 100 mL glass bottles
- Growth chamber (Binder, Germany)
- Plastic net
- Plastic tray
- Magnetic stirrer and heater: C-MAG HS7 (IKA®, Germany)
- 1.5 mL microcentrifuge tubes
- pH meter (Mettler Toledo, USA)
- Ruler
- Scissors
- Shaker

2.2 Equipments for metabolomics analysis

- Grinding jars with grinding balls
- GC-TOF/MS
- Lyophilizer

- Mixer mill: MM400 (Retch)
- Speedvac concentrator: Savant SPD 2010 (Thermo Electron Corporation, USA)
- Waters Acquity UPLC™ system (Waters Co., Massachusetts, USA) fitted with a Q-ToF Premier mass spectrometer (Micromass MS)

2.3 Equipments for RT-PCR analysis

- Gel documentation apparatus: Gel DocTM (Syngene, England)
- Gel electrophoresis apparatus: Mupid®-exU (Advance Co., LTD, Japan)
- PCR: T100™ Thermal Cycle (Bio-Rad, USA)

2.4 Equipments for real-time PCR analysis

- iCycler iQ real-time system (CFX96) (Bio-Rad, USA)

2.5 Equipments for enzyme activity assay and chlorophyll content determination

- Centrifuge
- Refrigerator: Ultra low temperature freezer (New Brunswick Scientific, UK)
- Spectrophotometer : DU® 640 (Beckman Coulter, USA)

3. Chemicals and reagents

3.1 Chemicals for plant germination and growing

- Ammonium nitrate (Sigma Chemical Co., USA)
- Calcium chloride (Carlo Erba Reagenti, Italy)
- Citric acid monohydrate (Merck, Germany)
- Copper sulphate pentahydrate (Carlo Erba Reagenti, Italy)
- 70% ethanol (AnalaR NORMAPUR, Ireland)

- 1N Hydrochloric acid (Merck, Germany)
- Iron (III) chloride hexahydrate (AnalaR NORMAPUR, Ireland)
- Manganese chloride tetrahydrate (Sigma Chemical Co., USA)
- Magnesium sulphate heptahydrate (Sigma Chemical Co., USA)
- Potassium sulphate (Carlo Erba Reagenti, Italy)
- 1N sodium hydroxide (Merck, Germany)
- Sodium hypophosphate dihydrate (AnalaR NORMAPUR, Ireland)
- Zinc sulphate heptahydrate (Carlo Erba Reagenti, Italy)

3.2 Chemicals for salinity treatment

- Sodium chloride (Carlo Erba Reagenti, Italy)

3.3 Chemicals for metabolite analysis

3.3.1 GC-TOF/MS (Agilent Technologies, USA)

- n-haptane
- 20 mg/ml hydrochloride in pyridine
- 3:1:1 (v/v/v) methanol/chloroform/water
- 20 μl of methoxyamine
- 20 μl of MSTFA with 1% TMCS

3.3.2 LC-TOF/MS (Micromass MS Technologies, UK)

- 4:1 (v/v) methanol/water
- 0.1% trifluoroacetic acid in acetonitrile
- 0.1% trifluoroacetic acid in water

3.4 Chemicals for enzymatic extraction and assays

3.4.1 Extraction buffer

- Bovine serum albumin (Merck, Germany)
- 4 mM dithiothreitol (Bio Basic Canada Inc., Canada)

- 2 mM ethylenediaminetetraacetic acid (Carlo Erba Reagenti, Italy)
- 0.1 M sodium phosphate buffer (pH 7.0)

3.4.2 Guaiacol peroxidase (POX) activity assay

- 1 M H_2O_2
- 18 mM guaiacol
- 0.1 M potassium phosphate buffer (pH 7.0)

3.4.3 Catalase (CAT) activity assay

- 1M H_2O_2 20 µl
- 50 mM sodium phosphate buffer pH 7.0

3.5 Chemicals for determination of total chlorophyll content

- 80% acetone (AnalaR NORMAPUR, Ireland)

3.6 Kit

- iScript™ cDNA Synthesis Kit (BIO-RAD, USA)
- iScript™ reverse transcription supermix for RT-qPCR (BIO-RAD, USA)
- RNeasy® Plant Mini kit (Qiagen, Germany)
- SsoFast™ Evagreen® Supermix (BIO-RAD, USA)

3.7 Primers

- All oligonucleotide primers used were synthesized by Pacific Science, Thailand. (Their sequence are shown in Table 1 and 2)

3.8 Softwares

- CFX manager software (BIO-RAD, USA)
- KEGG (http://www.genome.jp/kegg/pathway.html)
- NCBI (http://www.ncbi.nlm.nih.gov/)

- Phytozome (http://www.phytozome.net/)
- SIMCA (Umetrics, Sweden)

Table 1 List of primers used for RT-PCR analysis

Name	LOC No.	Forward	Reverse	Annealing temperature (°C)
RuBisCo activase large isoform precursor	Os11g0707000	GGCGGCAGACA TCATCAAGAAG	TCGTAAACACGG GCACGAAG	53
Fructose bisphosphate aldolase, chloroplast precursor	Os11g0171300	GCTTGTCAAGA CCGCGAAAACC	CAGCCCGTTGTC CTGAGAAATG	57
Fructose-1,6-bisphosphatase	Os01g0866400	TGTTCGTCAAG GCTCTCGTCAG	CGTCCCAATTTT TCGCATTCCC	57
Catalase	Os06g51150	ATGCCACGAAG GACTTGAC	AATGACACACTT TTCCCGAC	56

Table 2 List of primers used for Real-time RT-PCR in this work

Name	LOC No.	Forward	Reverse	Annealing temperature (°C)
L-galactono-1,4-lactone dehydrogenase	Os11g04740	AGCACGCATGGAGCTTGAC	CCTTCTCCAGCAACAGACCAC	58
Glutamate decarboxylase	Os03g13300	CAGCCAACCTTCACCCTCAAC	AGCCCTTCCTTCAGCACCATC	58
Pyrroline-5-carboxylate reductase	Os01g71990	CCACAGCAGGCATATCAGCAAG	GGGGACATCAGAAAGGGGGAAG	55.8
Riboflavin synthase	Os12g35580	ACAACATCGTGCTGCCGAAC	TCGACATCGCCTCCAATCTCC	55.8

II Methods

1. Plant germination and cultivation

Seeds of two rice (*Oryza sativa* L. ssp indica) cultivars, including KDML 105 as salt-sensitive and UBN 02123-50R-B-2 as salt-tolerance rice, were rinsed with 70% ethanol for three minutes. Seed were then soaked in 2% w/v sodium hypochlorite for 20 minutes. After that, seeds were washed with sterile deionized water for several times. The sterilized seeds were immersed in water for germination. The germinated seeds were transferred into plastic nets using Yoshida nutrient solution (the composition of elements in nutrient solution are shown in table 3) (Yoshida et al. 1976), and further grew under control condition as 25 ± 2°C temperature, 70 ± 5% relative humidity (RH), and 7000 lux with 16 hr light/ 8 hr dark photoperiod provided by fluorescent lamps in the growth chamber

Table 3 The composition of elements in Yoshida nutrient solution (Yoshida et al., 1976)

Element	Reagent	Concentration of element in nutrient solution (ppm)
N	NH_4NO_3	40
P	$NaH_2PO_4 \cdot 2H_2O$	10
K	K_2SO_4	40
Ca	$CaCl_2$	40
Mg	$MgSO_4 \cdot 7H_2O$	40
Mn	$MnCl_2 \cdot 4H_2O$	0.5
Mo	$(NH_4)_6 \cdot MO_7O_{24} \cdot 4H_4O$	0.05
Zn	$ZnSO_4 \cdot 7H_2O$	0.2
Cu	$CuSO_4 \cdot 5H_2O$	0.01
Fe	$FeCl_3 \cdot 6H_2O$	0.01
	Citric acid (monohydrate)	2

2. Salinity treatment

Three hundred of two weeks old rice seedlings were transferred into Yoshida nutrient solution containing NaCl at the final concentration of either 80 mM or 160 mM. Control treatments of rice grown in Yoshida medium were carried out in parallel with the salt stress treatments. After the treatment, seedlings of each rice cultivars were observed for physiological changes and collected by separating leaves and roots at three treatment periods (0, 12 and 24 hours) and kept in liquid nitrogen immediately. All samples were kept in -80 degree celsius until analysis

3. The activity of antioxidant enzymes

3.1 Preparation of the enzyme solutions

Crude proteins were extracted from 100 mg of leaf samples by the modification method of Aebi *et al* 1974 (Aebi H. et al. 1974). The samples were homogenized with 1 ml extraction buffer (100 mM phosphate buffer pH 7.0, 1 mM

DTT and 1 mM EDTA). The extracts were then centrifuged at 15,000 rpm, 4 °C for 20 mins. The supernatant were collected for further assay.

3.2 Protein determination

The standard solution of protein determination was prepared by adding 1 mg of Bovine Serum Albumin (BSA) in distilled water 1 ml. Aliquots 100 μl of each sample was mixed in a test tube with 5 ml of 1:4 diluted Bradford dye reagent. After 15 min, the absorbance at 595 nm is recorded against the dye reagent/distilled water blank using spectrophotometer. Sample were calibrated against a standard curve of BSA with three replicates of at least five standard concentrations that are within the linear range of the assay and that also span the anticipated range of sample protein concentrations.

3.3 Guaiacol peroxidase (POX) assay

The guiacol peroxidase activity was measured according to the modification method of Slathia *el al* (Slathia et al. 2012). Reaction mixture contained 1 ml of 100 mM potassium phosphate (pH 7.0), 120 μl of 3 mM guaiacol, and the 50 μg of enzyme extract. The reaction was initiated by adding 10 μl of 0.1 mM H_2O_2. The kinetic of the formation of tetraguaiacol was measured by monitoring changes in absorbance at 436 nm for 3 minutes. The specific activity of guiacol peroxidase was calculated following equation:

$$\text{Specific activity} = \frac{\text{A436/min x total volume (ml) x dilution factor}}{\varepsilon \text{ x protein content (50 } \mu\text{g)}}$$

ε (molar extinction coefficient) = 6.39 mM^{-1} cm^{-1}

3.4 Catalase (CAT) activity

The catalase activity was measured according to the modified method of Aebi *et al* (Aebi H. et al. 1974). The enzyme solution was prepared by adding 50 μg of the enzyme solution into 1 ml of 50 mM sodium phosphate buffer pH 7.0, then 1.2 ml of

deionized water was added. The reaction was started by adding 20 μl of 1 mM H_2O_2. The kinetic of catalase was measured by monitoring the absorbance change at 240 nm for three minutes. The specific activity of catalase was calculated as the following equation:

$$\text{Specific activity} = \frac{\text{A240/min x total volume (ml) x 1,000}}{\varepsilon \text{ x protein content (50 μg)}}$$

ε (molar extinction coefficient) = 39.4 mM^{-1} cm^{-1}

4. Total chlorophyll content

The total chlorophyll content was investigated following the method of Arya *et al* (Arya and Roy 2011). Ten mg samples were homogenized with 1 ml 80% acetone and then were centrifuged at 5,000 rpm for five minutes. The supernatants were collected. The pellets were mixed with 80% acetone and then were centrifuged repeatedly. The supernatants were collected and pooled. The chlorophyll content was measured the absorption at 645 nm for chlorophyll a and 663 nm for chlorophyll b. The total chlorophyll content was calculated using the following equation:

$$\text{mg total chlorophyll / g tissue} = 20.2 \times A645 + 8.02 \times A663 \times \frac{\text{total volume}}{1{,}000 \times \text{weight of tissue (g)}}$$

5. Semi quantitative RT-PCR analysis

5.1 RNA extraction and cDNA synthesis

Total RNA was isolated using Qiagen's RNeasy Plant Mini Kit (Appendix A). RNA samples were quantified by measuring the absorbance of diluted RNA solution. The absorbance of 1.0 at 260 nm corresponds to approximately 40 μg/ml of RNA. RNA concentration was calculated using equation:

RNA concentration (ng/μl) = A_{260} x Dilution factor x 40

The calculation of ratio of A_{260} and A_{280} was used to determine the RNA quality. RNA sample with A_{260}/A_{280} between 1.7-2.0 demonstrates the acceptable quality.

Samples' cDNA fragments were then performed by iScript™ cDNA Synthesis Kit (BIO-RAD, USA) (Appendix A). The reaction mixtures were prepared following the manufacturer's instruction.

5.2 Gene expression analysis

Gene expression analysis was performed using gene specific primers as shown in Table 2.1. The *Taq* DNA polymerase was used to amplify PCR products. The cDNA was synthesized using the iScript™ cDNA Synthesis Kit (BIO-RAD, USA). The cDNAs from two weeks-old leaves were used as templates. The PCR condition for analyzing genes expression were as follows: 5 min at 95°C, 30 cycles of 30 s at annealing temperature of each gene as present in Table 2, followed by 5 min at 72°C. EF-1 alpha was used as an internal control. The PCR reaction mixtures were prepared as the following reaction

Table 4 The reaction component of RT-PCR analysis

Reaction component	volume
10x *Taq* buffer	2 μl
dNTPs (10 mM)	0.5 μl
Forward-primer (10 μM)	0.5 μl
Reward-primer (10 μM)	0.5 μl
nuclease-free water	15.2 μl
Taq DNA polymerase	0.3 μl
Template	1 μl
total volume	20 μl

6. Metabolites analysis

In order to explore the changing of metabolite profiles of KDML 105 and UBN in responses to salinity stress, the two-weeks-old of rice seedling were grown and treated under salinity stress condition. The metabolite profiles of six replicates of roots and shoots were then extracted and determined using gas chromatography time-of-flight/mass spectrometry analysis (GC/TOF-MS) and liquid chromatography quadrupole time-of-flight mass spectrometry analysis (LC-MS/MS).

6.1 Gas chromatography time-of-flight/mass spectrometry analysis (GC-TOF/MS) (modified from (Kusano et al. 2007))

6.1.1 Sample preparation and metabolite extraction

Each sample was ground using mixer mill MM400 (Retch) at a frequency of 30 Hz for 25 seconds, then 100 mg fresh weight of tissue were lyophilized. The primary metabolites were investigated by GC-TOF/MS in collaboration with the RIKEN Center for Sustainability Resource Science, Japan. The procedures of extraction and preparation were followed Kusano et al. (2007). One hundred milligrams of each sample were extracted with extraction buffer [methanol/chloroform/water (3:1:1, v/v/v)] at a concentration of 100 mg/ml and containing 10 stable isotope reference compounds. Each 1 μl injection was added 15 ng/μ of each isotope compound (Jonsson et al. 2004; Jonsson et al. 2005; Jonsson et al. 2006). The extracts were then centrifuged and transferred 200 μl of supernatant into glasses insert vials. The extracts were evaporated in an SPD2010 SpeedVac® concentrator from ThermoSavant (Thermo electron corporation, Waltham, MA, USA).

6.1.2 Derivatization

Methyl oxime derivatives were obtained by dissolving the evaporated extracts in 30 μl of methoxyamine hydrochroride-HCl (dissolved in 20 mg/ml pyridine) for 30 h at room temperature. The sample was then trimethylsilylated for 1 h by 30 μl of *N*-methyl-*N* trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) at 37 °C with shaking. After that, 30 μl of *n*-heptane was added. During the

derivatization steps all sample were performed in the vacuum glove box VSC-1000 (Sanplatec, Japan) filled with 99.9995% (G3 grade) of dry nitrogen.

6.1.3 GC-TOF/MS

The metabolite analysis were conducted by injection 1 μl of each sample in the splitless mode by an CTCCombiPALautosampler (CTC analytics, Zwingen, Switzerland) into an Agilent 6890N gas chromatograph (Agilent Technologies, Wilmingston, USA) equipped with a 30mx0.25mm inner diameter fused-silica capillary column with a chemically bound 0.25-μl film Rtx-5 Sil MS stationary phase (RESTEK, Bellefonte, USA) for metabolome analysis. Helium was used as the carrier gas with a constant flow rate of 1 ml/min. The temperature program for metabolome analysis started as 2-min isothermal step at 80 °C and this was followed by temperature ramping at 30 °C to a final temperature of 320 °C, which was maintained for 3.5 min. The normalized responses were calculated using peak area correction with the sample weight and a constant amount of the representative internal standard compound ([$^{13}C_4$]-hexadecanoic acid).

6.1.4 Mass spectrometry and Data processing

The Leco ChromaTOF optimized for Pegasus 4D software version 2.32 (Leco, St. Joseph, MI, USA) was used in order to compare the response of the metabolite peaks. All data-pretreatment procedures such as data normalization, baseline correction, and the subsequent data treatments were performed using custom scripts according to the study of Jonsson *et al* 2005. Mass spectrum were then matched agaianst reference mass spectra by using the National Institute of Standards and Technology (NIST) mass spectral search program for the NIST/EPA/NIH mass spectral library (version 2.0) and the custom mass spectral search software written in JAVA (http://www.metabolome.jp/). An in-house metabolite library of Platform for RIKEN Metabolomics and the library of the Golm Metabolome Database (GMD) at CSB.DB were provided for analysis of mass spectra obtained. The identification and annotation of extracted MS spectra were done according to their RI and comparison with the reference mass spectra in the libraries.

6.2 Liquid chromatography tandem mass spectrometry analysis (LC-MS/MS) (modified from (Matsuda et al. 2012; Saika et al. 2012; Yang et al. 2014)

6.2.1 Sample preparation and metabolite extraction

The extraction was performed by adding 80% MeOH containing 0.5 mg/L of lidocaine and 0.5 mg/L of 10-camphorsulphonic acid as internal standards into 50 μL/mg of dry sample. Extraction was performed using a mixer mill (MM300, Retsch) with zirconia beads for 10 min at 20 Hz. Extracts were then centrifuged at 15,000 g and applied to an Oasis HLB μ-elution plate (Waters) equilibrated with 80% MeOH, including 0.1% acetic acid for filtration. After that, the extracts were evaporated by SPD2010 SpeedVac before dissolved in 200 μL of water containing internal standard. To remove insoluble residue, Ultrafree-MC filter with 0.2 μm pore size was conducted (Millipore, Germany).

6.1.3 LC-MS/MS

Three microliters of filtrated samples were subsequent subject to metabolome analysis by liquid chromatography coupled with electrospray quadrupole time-of-flight tandem mass spectrometry (LC-ESI-Q-TOF-MS) through Acquity BEH ODS column (LC, Waters Acquity UPLC system; Q-TOF-MS, Waters Q-TOF Premier). Metabolome analysis and data processing were performed as described previously (Matsuda et al., 2009, 2010). Briefly, the metabolome data were obtained in the positive and negative ion mode (m/z 100–2000; dwell time 0.45 sec; inter-scan delay 0.05 sec), from which a data matrix was generated using MetAlign (De Vos et al., 2007; Lommen, 2009).

6.3 The processing of metabolite profile data

The normalized MS data from GC-TOF/MS and LC-MS/MS analysis were imported to SIMCA software version 13.0.3.32 (UMETRIC). Peaks were identified or annotated based on retention indices (RIs) and the reference mass spectra comparison to the Golm Metabolome Database (GMD) and the RKEN Center for

Sustainability Resource Science in-house spectral library. The metabolite profiles were constructed using the Principal component (PCA) analysis and the Partial Least Squares Discriminant (PLS-DA) *Analysis*. The change of metabolite levels was performed by the calculation the ratio of salinity treatment and control sample sets. The Student's T test (T-test) ($P<0.05$) were combined to investigate the significantly changes in metabolite levels.

7. Real-time RT-PCR amplification

Metabolites with changes in accumulation levels were subsequently performed the synthesized-gene expression via Real-time RT-PCR using SsoFast™ EvaGreen® Supermix (BIO-RAD, USA). The PCR condition for analyzing genes expression were as follows: 5 min at 95°C, 30 cycles of 30 s at annealing temperature of each gene as present in Table 2.2, followed by 5 min at 72°C and increment temperature as 60-95°C with the increment of 0.5 °C. The number of PCR cycles was 40 cycles. EF-1 alpha was used as an internal control. The PCR reaction mixtures were prepared as the following reaction

Table 5 The reaction component of real-time PCR analysis

Reaction component	volume
SsoFast EvaGreen Supermix	5 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl
nuclease-free water	2 µl
cDNA template (5 ng RNA-equivalent/µl)	2 µl
total volume	10 µl

8. The metabolite map construction

The accumulated folding of each metabolite were used to constructed construct the plant metabolite map by the Pathvisio software (www.pathvisio.org). The significant changes of metabolite levels were identified on metabolite maps representing by specific colors. The levels of gene expressions were also drawn into metabolite maps to display the expression of stress-related genes in parallel with the changing of metabolite levels.

CHAPTER III

RESULTS

3.1 Visible physiological changes of two rice cultivars under salinity stress conditions

As a preliminary study, rice plants were studied for the effect of salt concentrations on physiological changes. Two salt concentrations of 80 mM and 160 mM NaCl have been applied to the two-week-old rice plants for 24 hr. KDML 105 showed wilted leaves when treated with both NaCl concentrations (Figure 10). However, at the concentration of 160 mM NaCl, the physiological change of KDML 105 was much more severe when compared with that treatd with 80 mM NaCl. For UBN, the plants treated with 160 mM NaCl also showed leaf injury but it was clearly different when compared to that treated with 80 mM NaCl. At 80 mM NaCl, there were apparently differences in physiological changes on leave between KDML 105 and UBN.

Figure 10 The visible physiological changes of rice under salinity stresses. Rice seedlings were separated into three groups as control, 80 mM NaCl and 160 mM NaCl. The picture was taken after salinity treatment for 24 hours.

3.2 The activities of antioxidant enzymes

The physiological changes of two rice cultivars hinted the initial information about rice seedlings under salinity stress. In order to gain more information, the antioxidant enzyme activities, catalase (CAT) and peroxidase (POD), were studied in both KDML 105 and UBN. Two weeks-old rice seedlings were treated with 80 mM NaCl for 12 hours and 24 hours. The rice seedlings in nutritional medium without NaCl were used as control. After salinity stress, the levels of CAT activity in rice seedlings were increased in both cultivars. The CAT activities of stressed rice plants at 24 hours were higher than in 12 hours as shown in Figure 11. CAT activity of UBN was higher than KDML 105 at 24 hours under salinity stress. These results implied a better ability of UBN to scavenge H_2O_2 by CAT when compared to KDML 105.

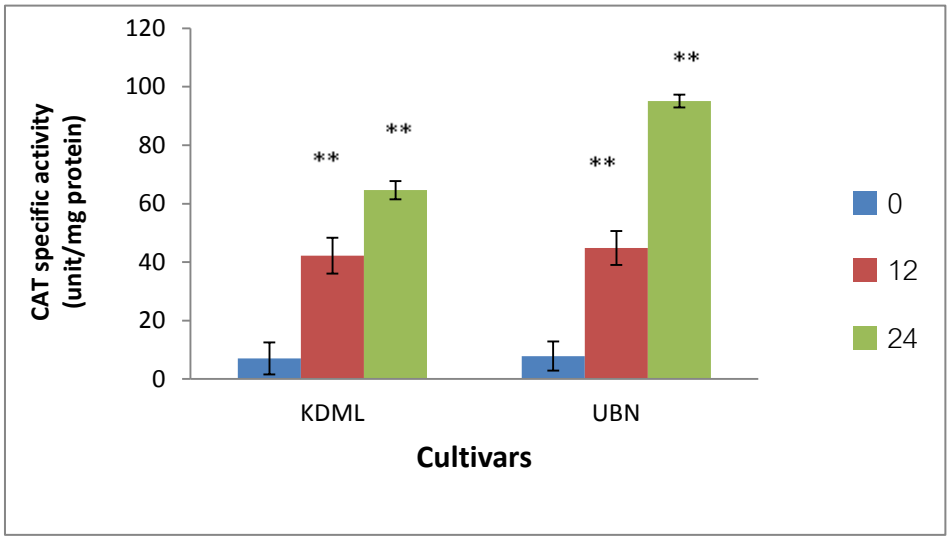

Figure 11 The catalase activity (CAT) of KDML 105 and UBN at 12 hours and 24 hours of salinity treatment (160 mM NaCl, n=3). Samples with salinity stress were compared to non-salinity treatment as control. Bars represent standard deviation of each group while asterisks represent the bar with significantly difference with ** $P < 0.01$, compared to control in each cultivar.

POD activity of two rice cultivars was also determined with the same sample sets used in the CAT activity experiment. Leaf samples were investigated for POD activity at 12 hours and 24 hours of salt treatment. The changes in POD activity of rice seedlings presented at 12 hours of salinity stress and slightly decreased at 24 hours. Both rice cultivars showed the similar results.. The higher POD activity in UBN

compared to KDML 105 at 12 hours demonstrated that these cultivars might rapidly defend cellular damage through the activity of POD.

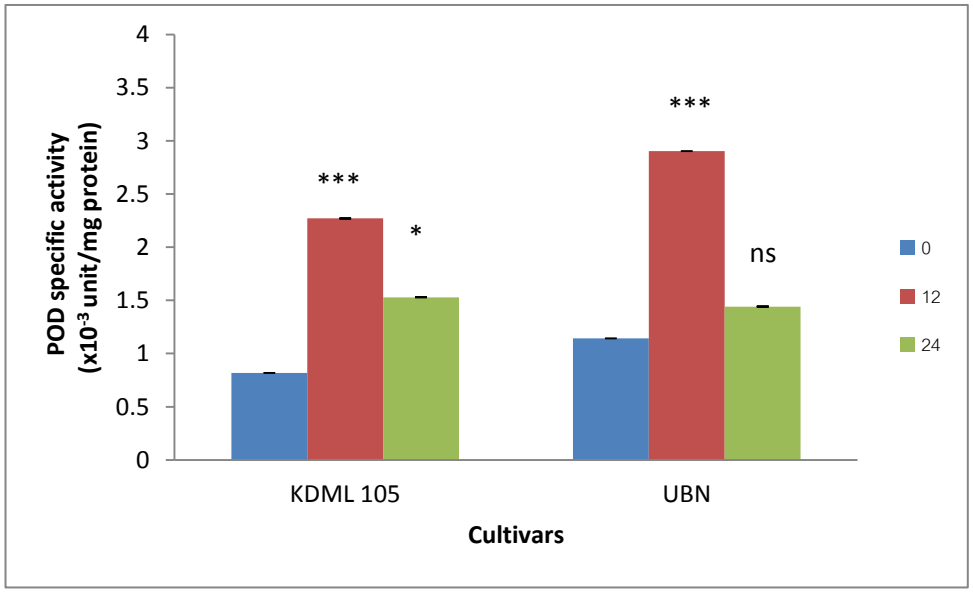

Figure 12 The peroxidase activity (POD) of KDML 105 and UBN at 12 hours and 24 hours of salinity treatment (160 mM NaCl, n=3). Sample with salinity stress was compared with non-salinity treatment as control. Bars represent standard deviation of each group while asterisks represent the bar with significantly difference with *** $P < 0.001$, * $P < 0.05$, and ns : no significant changed compared to control in each cultivar.

3.3 The total chlorophyll content

Since the study of physiological changes in rice have shown that rice were affected by salinity stress particularly in aerial parts. The study of total chlorophyll content was then performed. The increase in total chlorophyll content was found in both KDML 105 and UBN since 12 hours of salinity stress. At 24 hours, the total chlorophyll content in KDML 105 was decreased whereas there was no any effect in the level of total chlorophyll in UBN. In addition, the results from chlorophyll a and b measurement indicated the decreases of chlorophyll a and b content in KDML 105, whereas chlorophyll a and b in UBN were not changed.

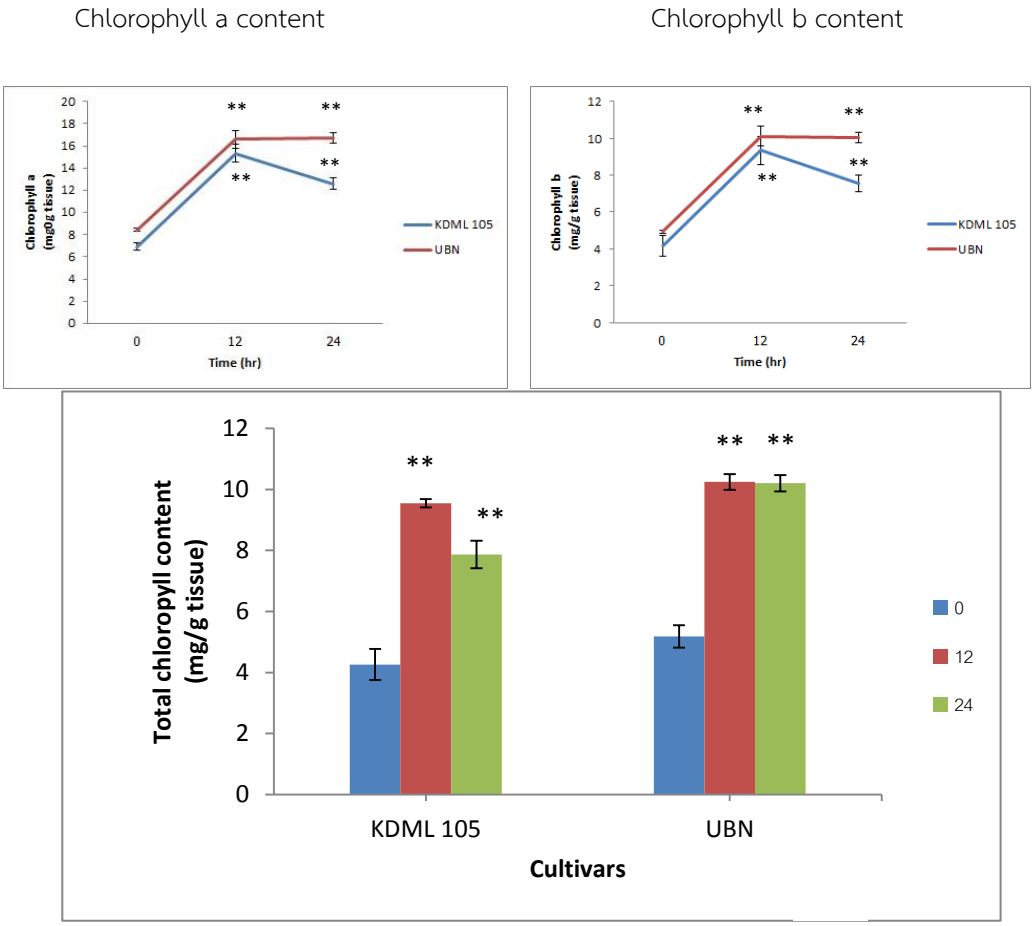

Figure 13 Total chlorophyll contents of KDML 105 and UBN at 12 hours and 24 hours after salinity treatment (160 mM NaCl, n=3). Samples with salinity stress were compared with non-salinity treatment as control. Bars represent standard deviation of each group while asterisks represent the bar with significantly difference with ** $P < 0.01$, compared to control in each cultivar.

3.4 Gene expression analysis of KDML 105 and UBN using semi-quantitative RT-PCR

For the study of salinity stress effect on gene expression as indicators prior to metabolomics, semi-quantitative RT-PCR was conducted to compare expression levels of stress-related genes in KDML 105 and UBN. The primers were designed from stress-related genes according to the previous study (Kim et al. 2005). They are genes encoding for RuBisCo, fructose-1,6-bisphosphatase, fructose-1,6-aldolase, and catalase. We used EF-1 alpha as internal control gene. The results indicated that all

stress-related genes were higher expressed in UBN cultivars at 24 hours (Figure 14). The result illustrated the higher expression of RuBisCo after salinity stress in both KDML 105 and UBN leaves. Interestingly, the expression of RuBisCo was slightly increased since 12 hours after salinity stress and was higher expressed at 24 hours. UBN showed higher expression of RuBisCo than KDML 105. The expression of fructose-1,6-bisphosphatase was highly presented in UBN leaf at 24 hours. The expression of fructose-1,6-bisphosphate aldolase was slightly increased in KDML 105 leaf at 12 hours and decreased at 24 hours while the increases in this gene expression in UBN was clearly detected at 24 hours after salinity stress. The expression of catalase was only increased in UBN leaf after 24 hours. These results demonstrated that there were differently gene expression patterns between KDML 105 and UBN caused by salinity stress which could imply that UBN have better stress tolerance capacity than KDML 105.

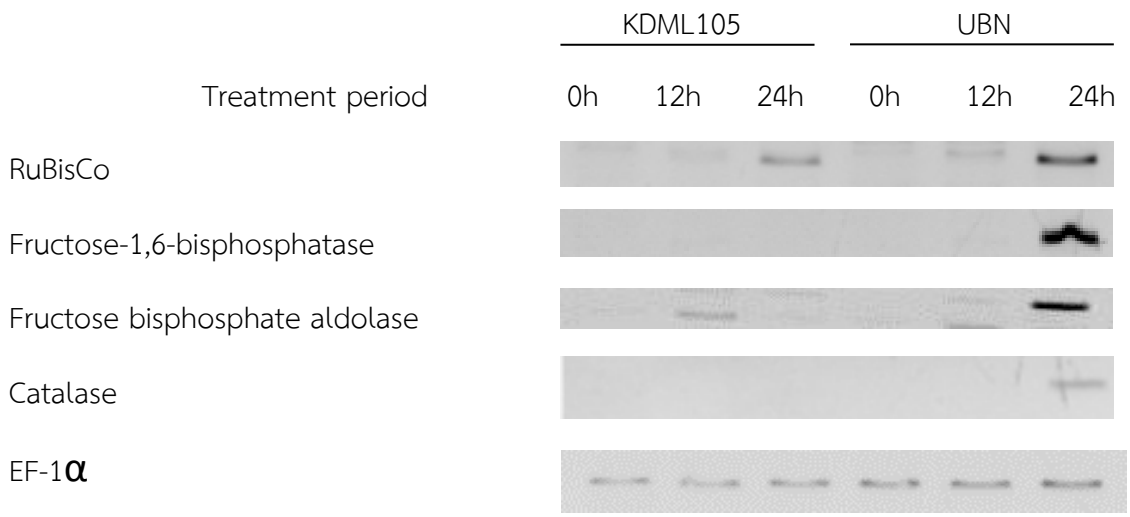

Figure 14 Expression of stress-related genes analyzed by semi-quantitative RT-PCR. The expression cycles were 30 cycles, using EF-1α as an internal standard.

3.5 The metabolite profiles of KDML 105 and UBN under salinity stress analyzed by GC-TOF/MS

The GC-TOF/MS analysis identified overall 239 metabolites according to the retention times and specific mass fragments, of which 101 metabolites could be annotated by comparing with the reference compounds in the databases (see chapter 2). The difference in metabolite profiles of two rice cultivars was evaluated and represented as the fold changes of accumulation. The normalized data was calculated in ratio of the salinity treatment condition by the control condition of each time point. The comparison of altered metabolite levels of untreated plants and salinity-treated plants could specify the significantly changes in metabolite levels of four growth condition. There were 40 and 25 metabolites changed in leaves at 12 and 24 hours, and 60 and 17 metabolite changed in roots at 12 and 24 hours respectively.

To illustrate different accumulated metabolites between KDML 105 and UBN under normal condition, the Partial Least Squares Discriminant Analysis (PLS-DA) models were constructed. Clear separations between the clusters of six replications of KDML 105 and UBN were found (Figure 15). Each circle represents the metabolite profile of a single biological replicate and each replicate with the same treatment was grouped together representing the similar metabolite profiles. The clusters from two components, leaves (A) and roots (B), demonstrated discriminations of each biological replicate between two cultivars in normal growth condition with similar pattern in both leaf and root tissues. The number of significance as R^2X displayed the fraction of the total variation of the X block that can be explained by each component. $R^2X[1]$ represents the variables of GC-TOF/MS responses and $R^2X[2]$ represents sample variation explained by the model. PLS-DA plots explained number of significance as follows: $R^2X[1]$ variant as 0.239, $R^2X[2]$ as 0.119 in leaves and $R^2X[1]$ as 0.227 and $R^2X[2]$ as 0.116 in roots. The confidence region of score plot was calculated based on the Hotelling's T2 ellipse which considered that significance level exceeded 95% is outliers. The results indicated that metabolite profiles between KDML 105 and UBN were different.

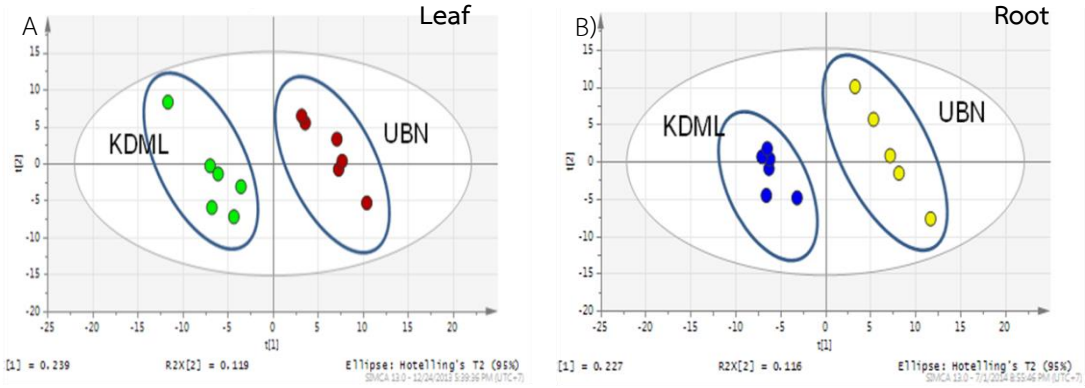

Figure 15 PLS-DA score plots of metabolites from the different tissues, leaves (A) and roots (B) of KDML 105 and UBN under normal condition. Small circles illustrate metabolite profiles of a single biological replicate.

The PLS-DA score plots of metabolite profiles in two cultivars were performed for analyses of the different metabolites in KDML 105 and UBN in control and salt stress conditions at 12 and 24 hours. The metabolite clusters of four sample sets was grouped according to control and stress conditions (Figure 16) in leaves (A) and roots (B), of control KDML 105, treated KDML 105, control UBN and treated UBN at two treatment periods of 12 and 24 hours. Each circle represents the metabolite profile of a single biological replicate. PLS-DA indicates the metabolite profiles of control plants and salinity treated plants which were separated into four groups according to plant treatment conditions. Metabolite shifts in leaves of KDML 105 between control and stress condition were higher than in UBN by the consideration of the distinct clusters. In contrast, metabolite shifts in roots of UBN were occurred more than in KDML 105. The separately metabolite shifts suggested different rice responses to salinity stress due to the different cultivars and various stress durations. PLS-DA models revealed clear differences in the metabolic composition between KDML 105 and UBN regarding to control and salinity treated groups. The number of significant from GC-TOF/MS analyses after salinity stress as follows: leaves at 12 hours; $R^2X[1]$ variant as 0.189, $R^2X[2]$ as 0.0995, leaves at 24 hours; $R^2X[1]$ as 0.255 and $R^2X[2]$ as 0.0889, roots at 12 hours; $R^2X[1]$ as 0.181 and $R^2X[2]$ as 0.159 and roots

at 24 hours; $R^2X[1]$ as 0.155 and $R^2X[2]$ as 0.152. The significance levels of Hotelling's T2 ellipse of each metabolite were 95% which illustrated the plausibility of each score plot

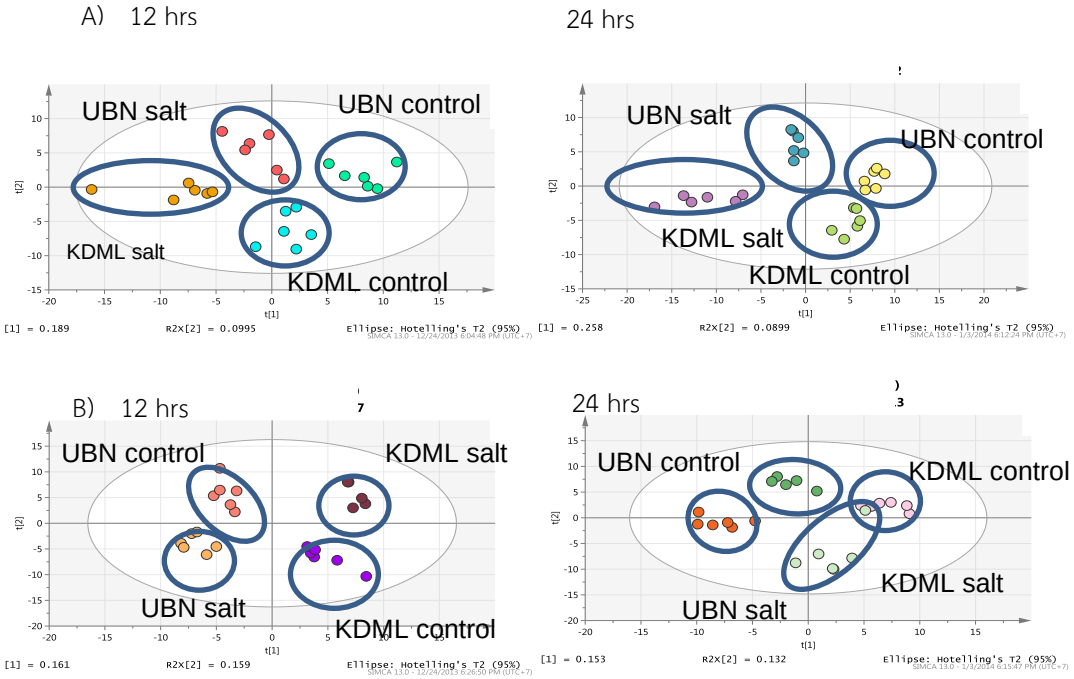

Figure 16 PLS-DA score plots of metabolite groups in control and salinity treatment conditions at 12 and 24 hr. The pictures display results in leaves (A) and roots (B) tissue of KDML 105 and UBN. PLS-DA score plots indicate the separation of metabolite groups according to four conditions as KDML 105 control, KDML 105 salt stress, UBN control and UBN salt stress.

3.5.1 Overview metabolic changes

The initial information for the understanding of the metabolic responses to salinity stress comparing between two different cultivars was illustrated as the simplified metabolic maps in leaves (Figure 17) and roots (Figure 8). The red and blue colors represent the up-accumulated and down-accumulated metabolites, respectively. The gray color indicates the metabolites with no significantly change and the white square represented the metabolites that could not be detected by GC-TOF/MS. Rice cultivars and stress durations of each metabolite are illustrated in the square box as presented in the figures.

According to the metabolic maps, KDML 105 and UBN leaves showed metabolic differences caused by the variable of cultivars and stress duration. Our metabolome analyses revealed a large number of metabolites which were affected as results from salinity stress. In leaves, glucose was increased at 12 hours under stress in UBN and at 24 hours in KDML 105. In TCA cycles, isocitrate, 2-oxoglutarate and malate were increased in both cultivars with the different accumulation levels according to variation of stress periods whereas citrate was increased only in UBN leaves. Fumarate and succinate were reduced. Sugars and polyols such as fructose, raffinose, ribose and inositol were significantly increased in both cultivars under stress. Amino acids such as branched chain amino acids (leucine, valine and isoleucine), beta-alanine, lysine, methionine, phenylalanine, threonine, tryptophan, and tyrosine were up-accumulated at 12 hours of salinity stress and still increased until 24 hours. Asparagine and aspartate were differently levels due to the duration of stress. Stress-related metabolites such as polyamines were also up accumulated. The reduction of shikimate and quinate levels were observed. Moreover, the accumulation levels of galacturonic acid were decreased only in UBN. In roots, the decreases of metabolites subjected to glycolysis such as glucose, glucose-6-phosphate and fructose-6-phosphate were observed. The changes of fructose, raffinose, sucrose and ribose were differently obtained as subsequently results by various stress durations. Metabolites involved in TCA cycles including citrate, isocitrate, 2-oxoglutarate and malate showed significantly increased while fumarate was only reduced in accumulation levels. The branched chain amino acids showed

significantly increased particularly at 24 hours, amino acids in this group were also increased in both cultivars. Salinity stress had affected the accumulation of several amino acids such as asparagine, aspartate, beta-alanine, lysine, methionine, phenylalanine, threonine, tryptophan and tyrosine by revealing the changes in their accumulation levels. Stress-related metabolites such as inositol and polyamines were up-accumulated especially amino acids in glutamate family, which demonstrated the differently changes in accumulation levels between KDML 105 and UBN. The results suggested that rice responses in the changes of metabolite accumulations after salinity stress was depended on the different cultivars and stress durations. Interestingly, 4-aminobutyric acid, galacturonic acid and raffinose were solely changed only in UBN in both 12 and 24 hours, while there were no changes of these metabolites in KDML. The results suggested the difference of salinity stress tolerance between KDML 105 and UBN in which UBN have the ability to tolerant by the changes in accumulation levels of these metabolites.

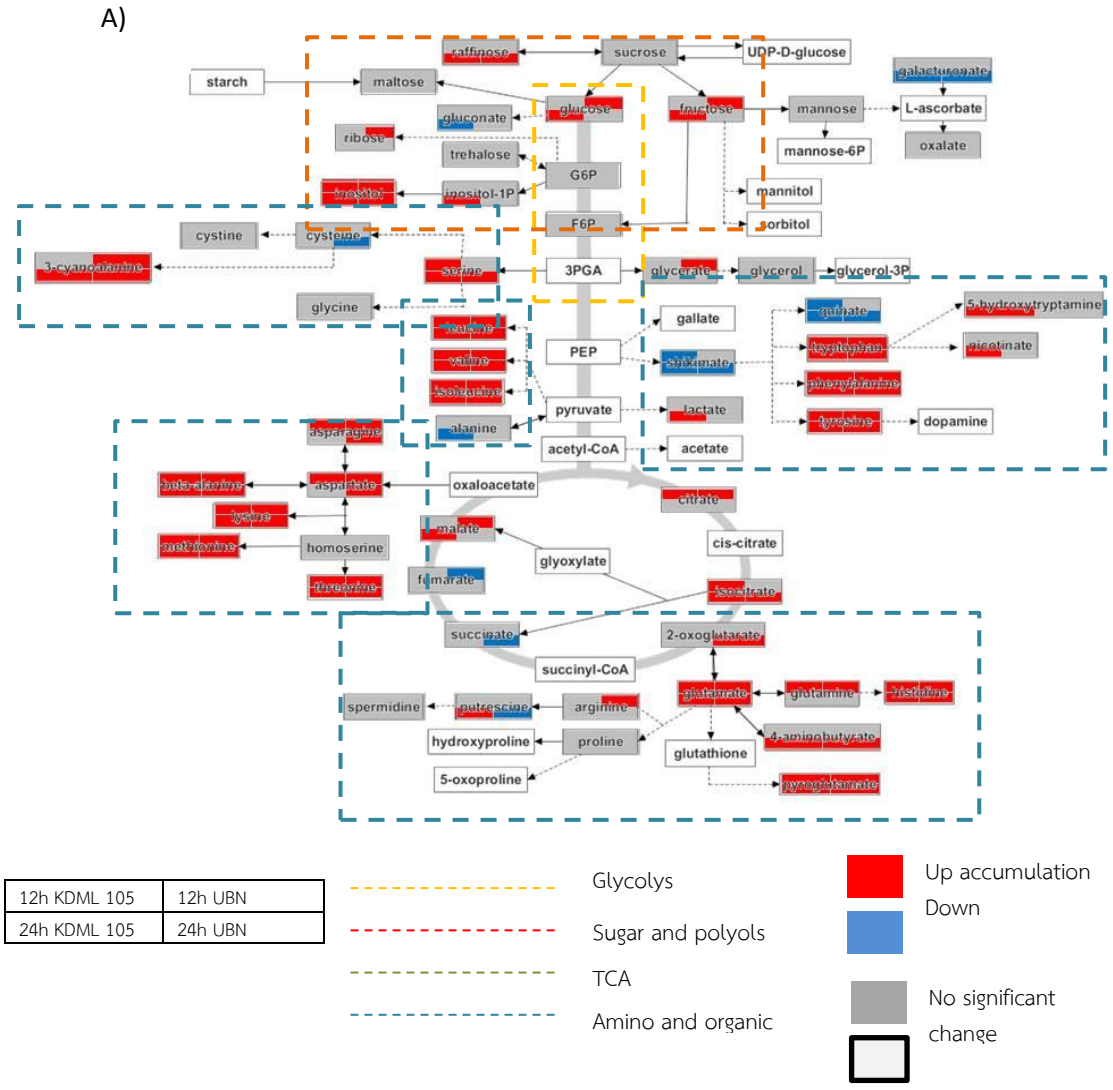

Figure 17 Simplified metabolic maps of rice under salinity stress in leaves (A) and roots (B). The accumulation profiles were shown in compared with non-stress rice as control. Red color represented the up-accumulation, blue represented the down-accumulation, grey represented non-significant change metabolites and white represented the undetectable metabolites

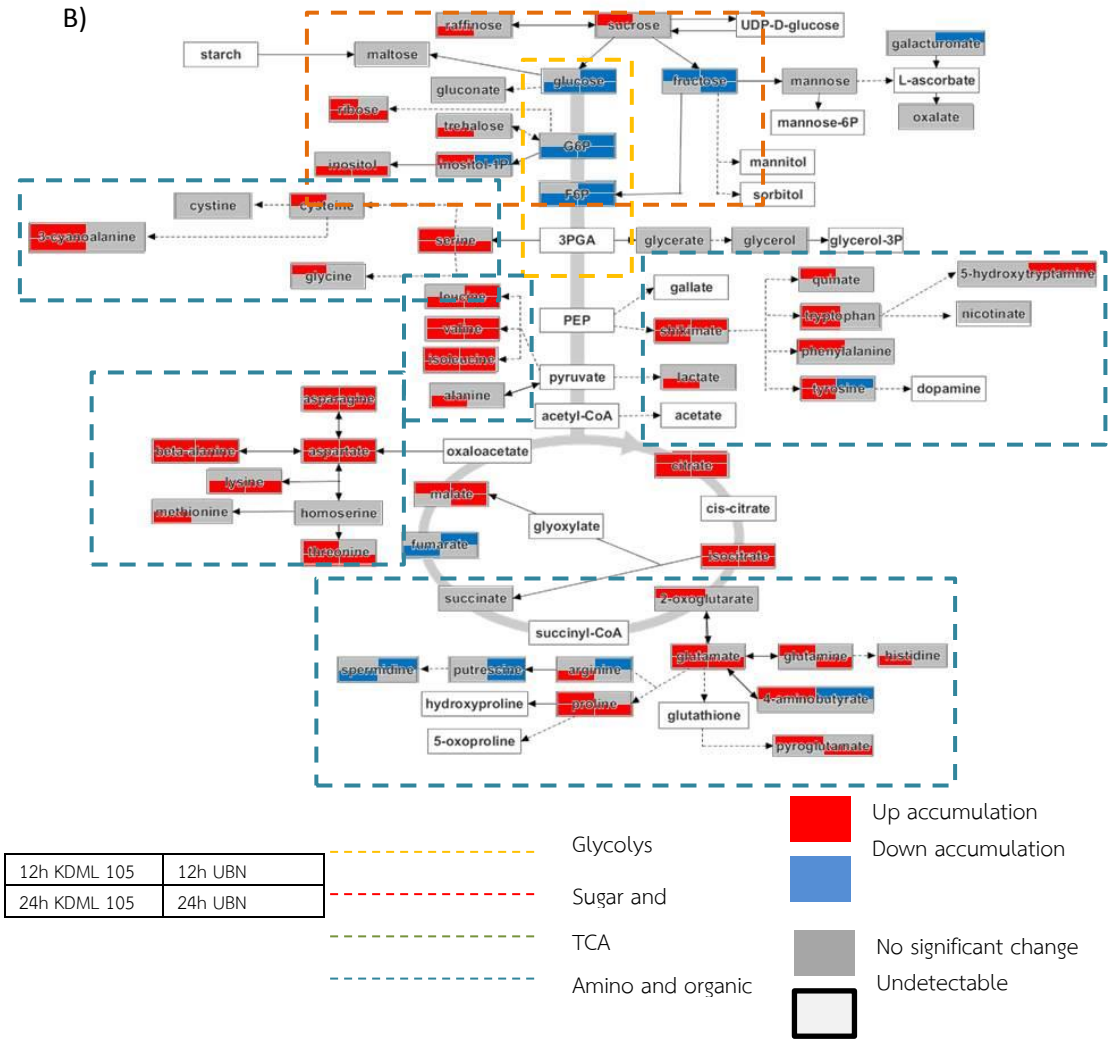

Figure 18 (Continued) Simplified metabolic maps of rice under salinity stress in leaves (A) and roots (B). The accumulation profiles were shown in compared with non-stress rice as control. Red color represented the up-accumulation, blue represented the down-accumulation, grey represented non-significant change metabolites and white represented the undetectable metabolites

3.5.2 Detailed changes

In this study, the category of metabolites was divided into five groups as metabolites involved in glycolysis, TCA cycles, sugar and polyols, amino acids and others, that altered more than two fold in their accumulation levels, were considered in this part.

All accumulation levels of both cultivars under salinity stress were also represented in tables 6-9, as leaves and roots respectively.

Glycolysis

The changes in metabolite levels in glycolysis of leaves and roots in two cultivars demonstrated in Figure 19 and 20, respectively (Table 6-9). In leaves, there was only glucose that showed significantly increased under salinity stress in both 12 and 24 hours salinity treatment. At 12 hours, UBN leaves indicated the higher accumulation of glucose more than KDML 105.

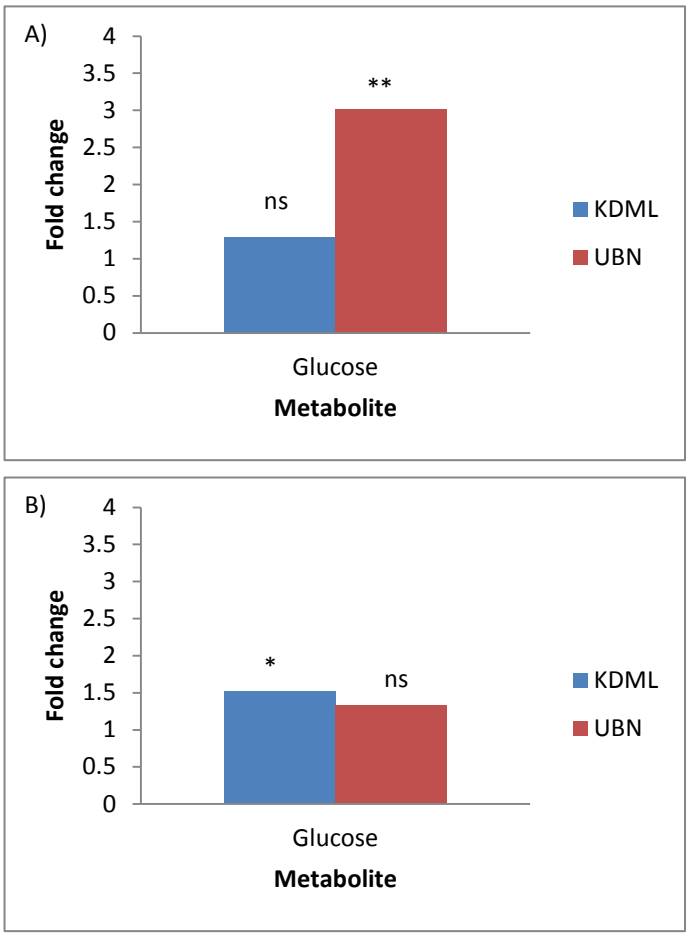

Figure 19 The changes of glucose levels in leaves of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test ($P<0.05$) as ** $P < 0.01$ and * $P< 0.05$, ns represents non-significantly changed in metabolite level when compared to their respective control.

In roots, metabolites which significantly changed in accumulation levels were glucose, glucose-6-phosphate and fructose-6-phosphate (Figure 20). The results showed that those metabolites were significantly decreased in accumulation levels in both KDML 105 and UBN. The decreased levels of these metabolites revealed the responses to glycolysis under saline treatment.

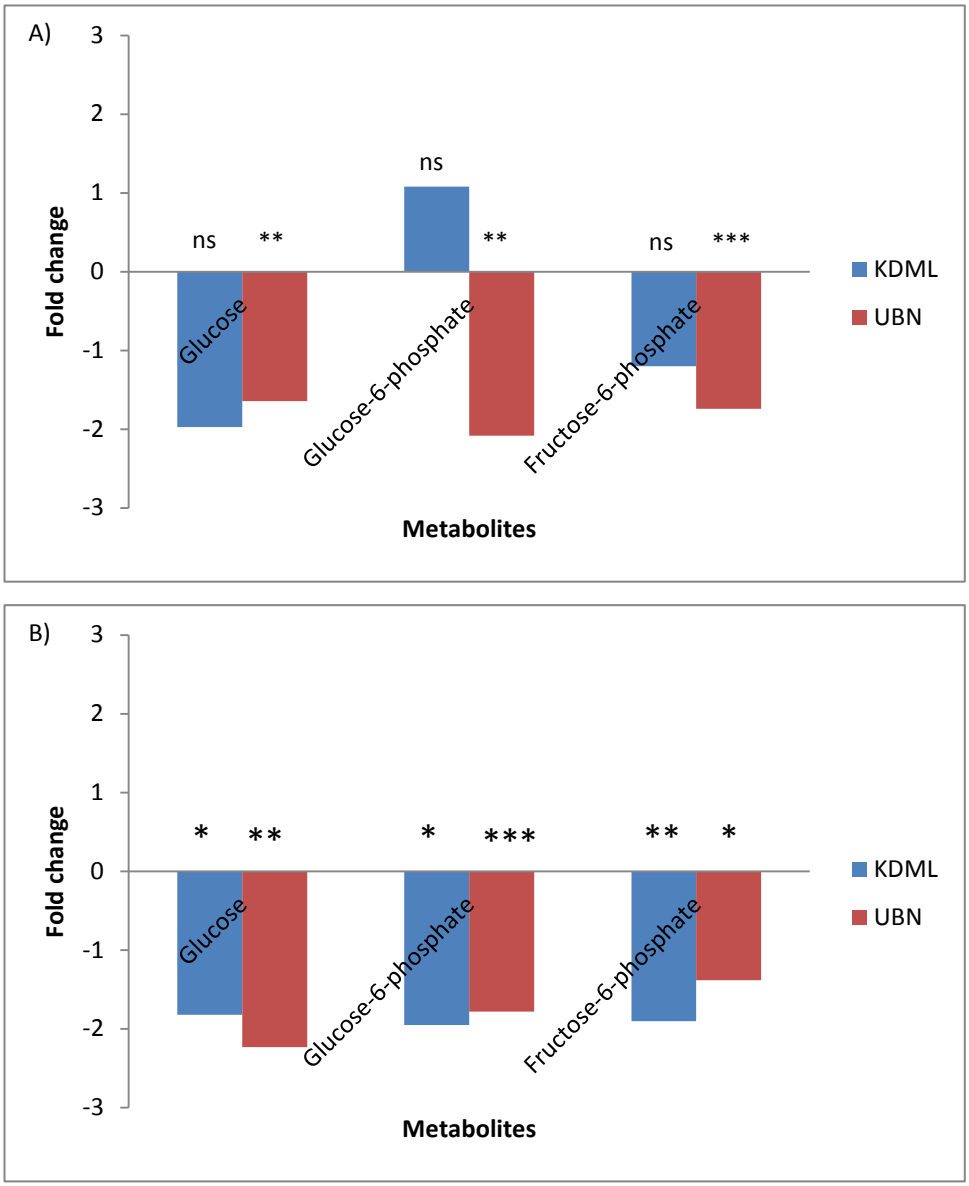

Figure 20 The changes of glucose levels in roots of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test ($P<0.05$) as *** $P < 0.001$, ** $P < 0.01$ and * $P< 0.05$, ns represents non-significantly changed in metabolite level when compared to their respective control.

TCA cycle

The intermediates involved in TCA cycles were calculated for the changes in accumulation levels (Figures 21-22) (Tables 6-9), both leaves and roots were found the up accumulation of 2-oxoglutaric, citric, isocitric and malic acids. In leaves, there was only 2-oxoglutaric which accumulated more than two fold in both cultivars.

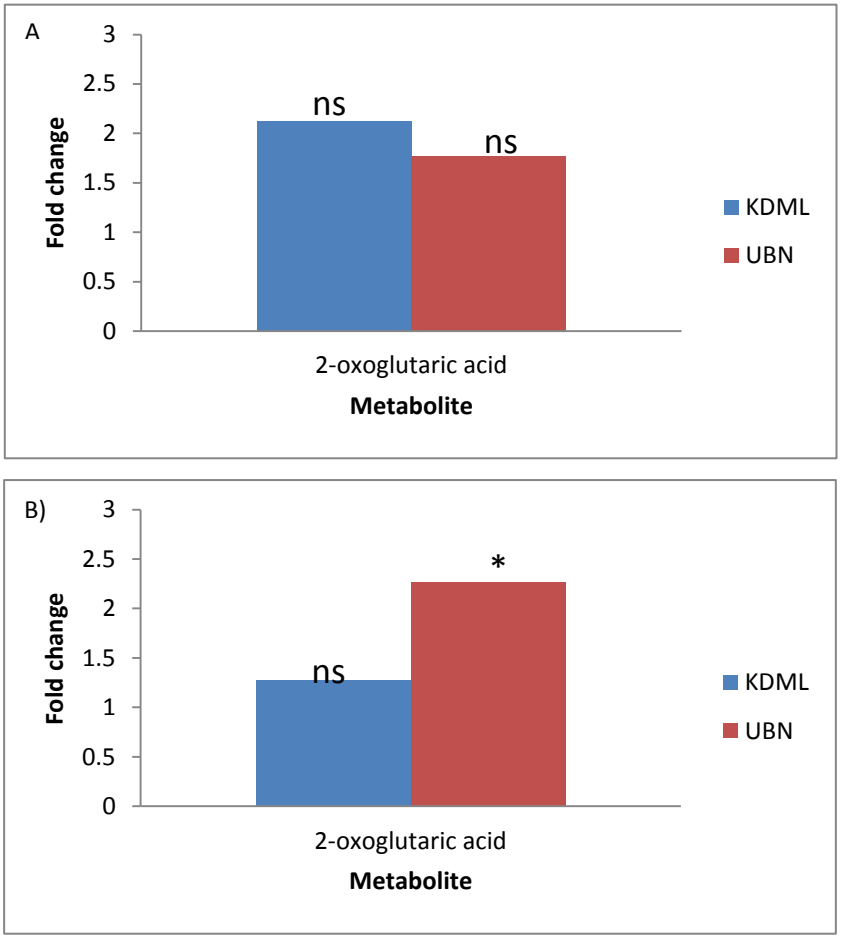

Figure 21 The changes of TCA cycles metabolites in leaves of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test ($P<0.05$) as * $P< 0.05$ and ns represents non-significantly changed in metabolite level when compared to their respective control.

In roots, the accumulation of citric and isocitric acids after salinity stress at 12 hours was found. The accumulation levels of citric and isocitric acids tended of to decrease at 24 hours. The results also showed the up-accumulation of malic acid in roots at 12 hours and 24 hours under stress. Moreover, the levels of citric, isocitric and malic acids in UBN were higher than in KDML 105 at both 12 and 24 hours.

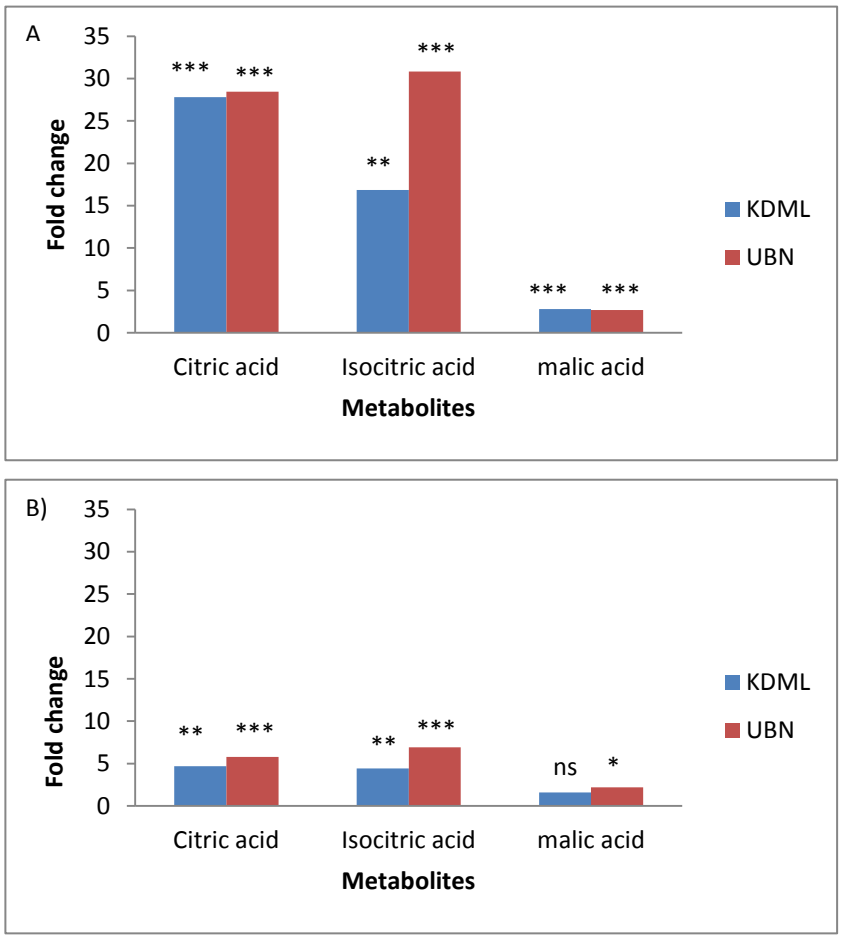

Figure 22 The changes of TCA cycles metabolites in roots of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test ($P<0.05$) as *** $P < 0.001$, ** $P < 0.01$ and * $P< 0.05$, ns represents non-significantly changed in metabolite level when compared to their respective control.

Sugars and polyols

The metabolite levels of sugar and polyols of leaves showed the trend of up accumulation at 12 hours after stress (Figure 23) (Tables 6-7). The alteration of metabolite levels at 24 hours showed the similar tendency with those of 12 hours. At 24 hours, the metabolic fold changes of sugars and polyols in KDML 105 were higher than those in UBN. The results demonstrated that KDML 105 leaves were affected by salinity stress since there were many metabolites which increased in their accumulation levels. Stress-related metabolites such as arabinose, fructose, raffinose and ribose in KDML 105 leaves, which considered as osmoprotectants, were higher up-accumulated than in UBN at 24 hours as a results from salinity stress.

In roots, the decreases in fructose were found in both cultivars while sucrose was only decreased in UBN (Figure 24) (Tables 8-9). The increases of galactinol, raffinose and trehalose were found with difference in the accumulation levels regarding to these two different cultivars and stress periods. The high accumulation of raffinose was found in KDML 105 roots at 12 hours which were higher than UBN, in contrast, it was lower than in UBN roots at 24 hours.

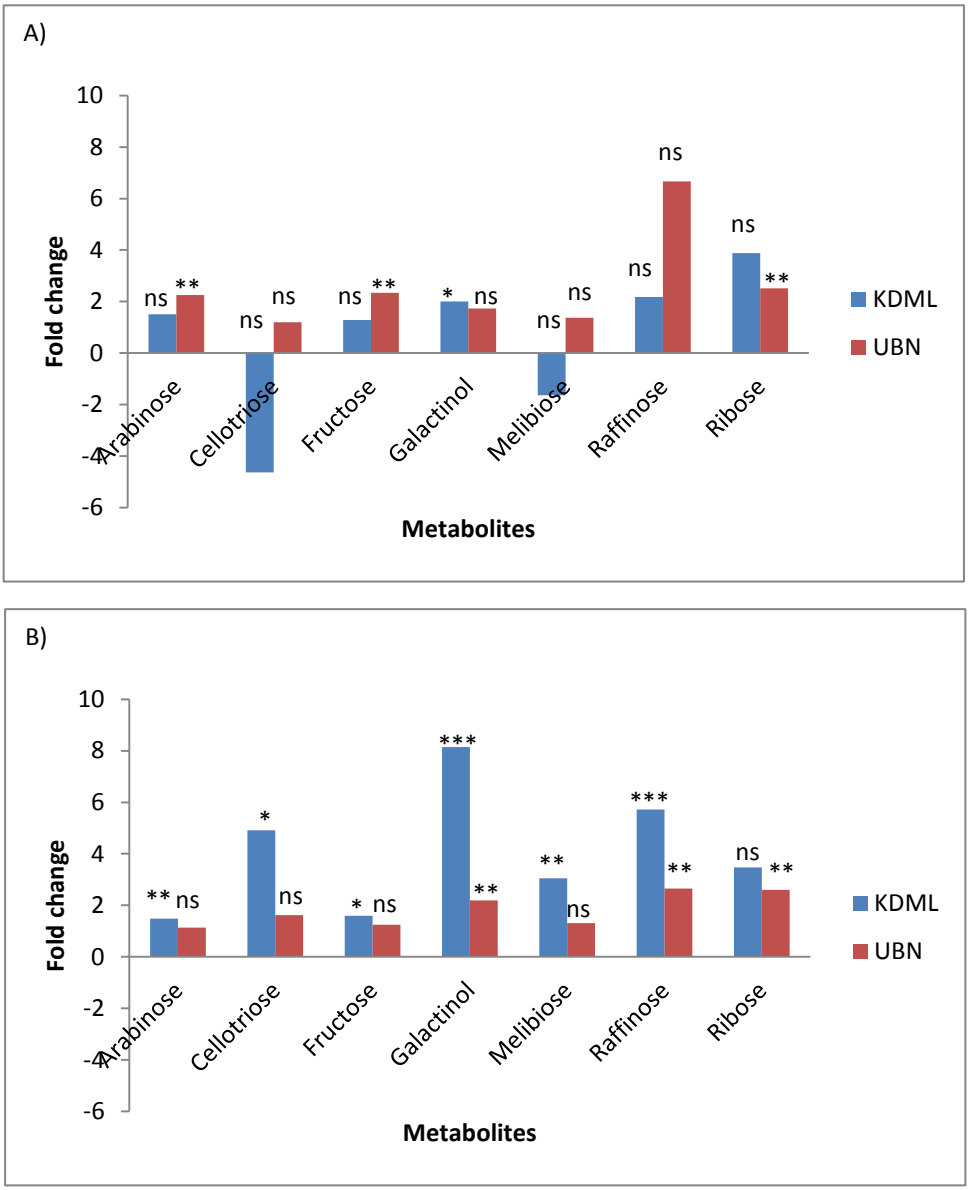

Figure 23 The changes of sugars and polyols in leaves of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test (P<0.05) as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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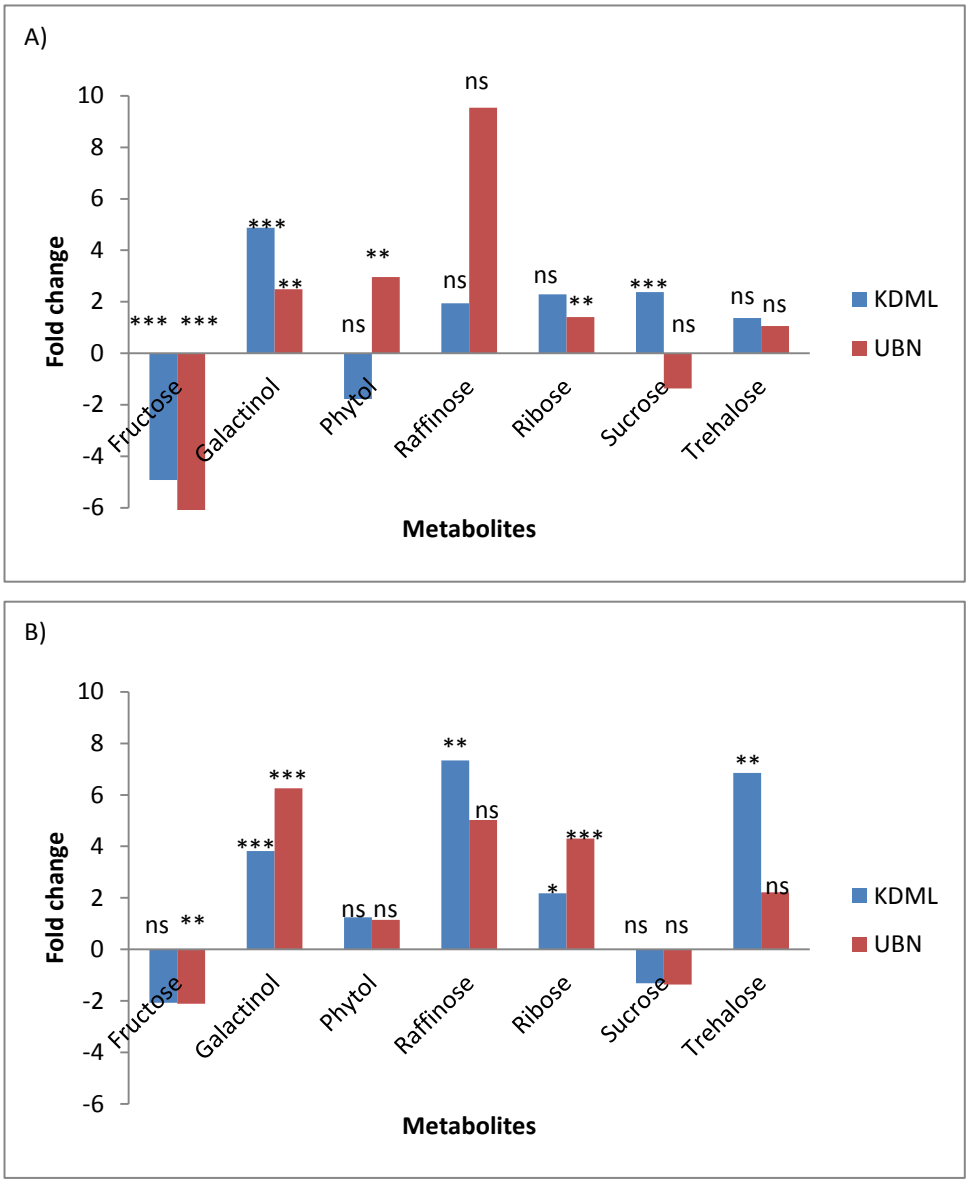

Figure 24 The changes of sugars and polyols in roots of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test (P<0.05) as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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Amino acids

The changes in accumulation levels of amino acids were reported in five groups as members for five families including (1) aspartate, (2) alanine and branched chain amino acids, (3) glutamate, (4) serine and (5) shikimate families.

Aspartate family

Amino acids in aspartate family are aspartic acid, asparagine, beta-alanine, lysine, methionine and threonine. In leaves, levels of amino acids in this family are increased in both KDML 105 and UBN. At 12 hours after salt stress, almost amino acids in aspartate family in UBN leaves were higher than those of KDML 105. At 24 hours salinity stress, amino acids in KDML 105 leaves were accumulated higher than in UBN leaves (Figure 25) (Tables 6-7). In roots, amino acids in aspartate family of both KDML 105 and UBN roots were significantly increased. Beta-alanine was highly up-accumulated at both 12 hours and 24 hours. Aspartic acid and asparagine were also higher accumulated in UBN more than in KDML 105 (Figure 26).

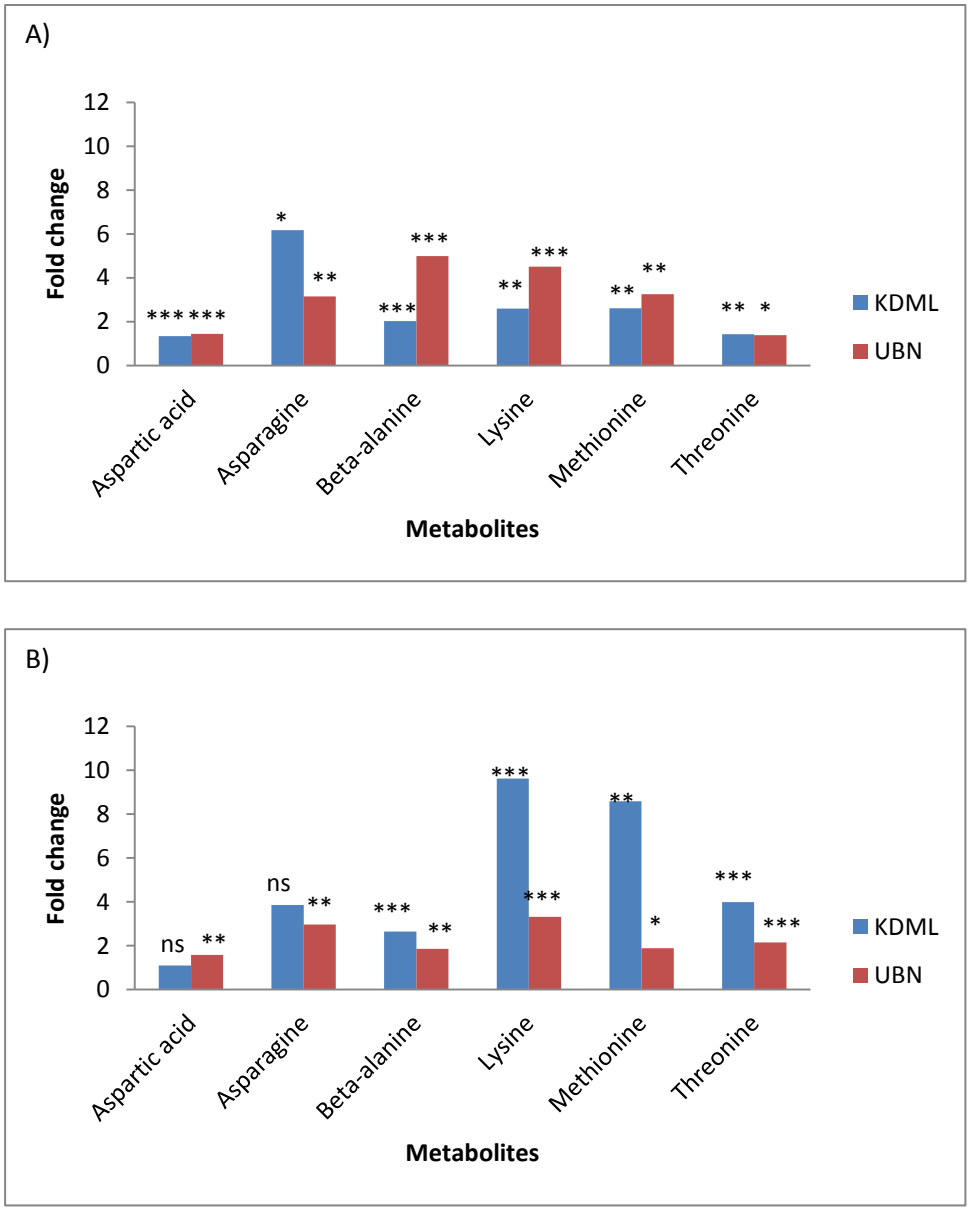

Figure 25 The amino acids in aspartate family in leaves of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test (P<0.05) as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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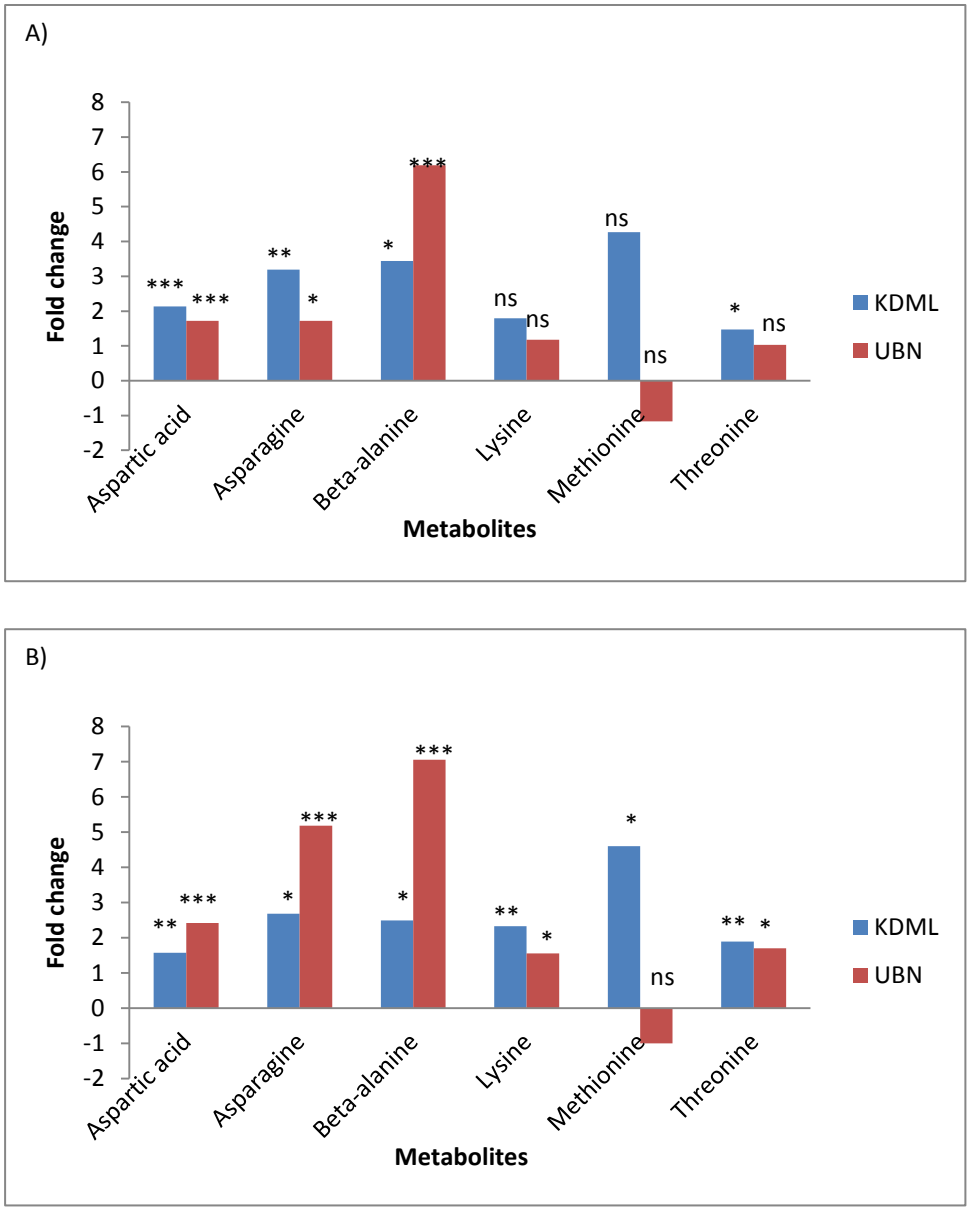

Figure 26 The amino acids in aspartate family in roots of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test (P<0.05) as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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Glutamate family

Amino acids and metabolites in glutamate family were 4-aminobutyric acid, arginine, glutamate, glutamine, histidine, ornithine, proline, putrescine, pyroglutamate and spermidine. In leaves, the levels of metabolites were increased in KDML 105 and UBN at both 12 hours and 24 hours of salinity stress (Figure 27) (Tables 6-7). At 12 hours, the accumulation levels of 4-aminobutyric acid, arginine, glutamine, histidine, ornithine and proline in UBN were slightly higher than that in KDML 105. At 24 hours, the levels of 4-aminobutyric acid, histidine and ornithine in KDML 105 were highly up accumulated comparing with UBN. 4-aminobutyric acid has been reported in experimental observations which initial produced in responses to abiotic stress.

In roots, metabolite levels in this group were up accumulated including arginine, glutamate, glutamine, histidine, ornithine, proline and pyroglutamate at 12 hours and 24 hours after stress. Putrescine showed significantly down-accumulation. The levels of putrescine in UBN were lower than that of KDML 105 (Figure 28) (Tables 8-9). Spermidine was down accumulated at 12 hours of KDML 105 roots and 24 hours of UBN roots under stress. Putrescine and spermidine were very responsive in external condition, therefore the decreases in their accumulation levels might play roles to salinity tolerance in both cultivars.

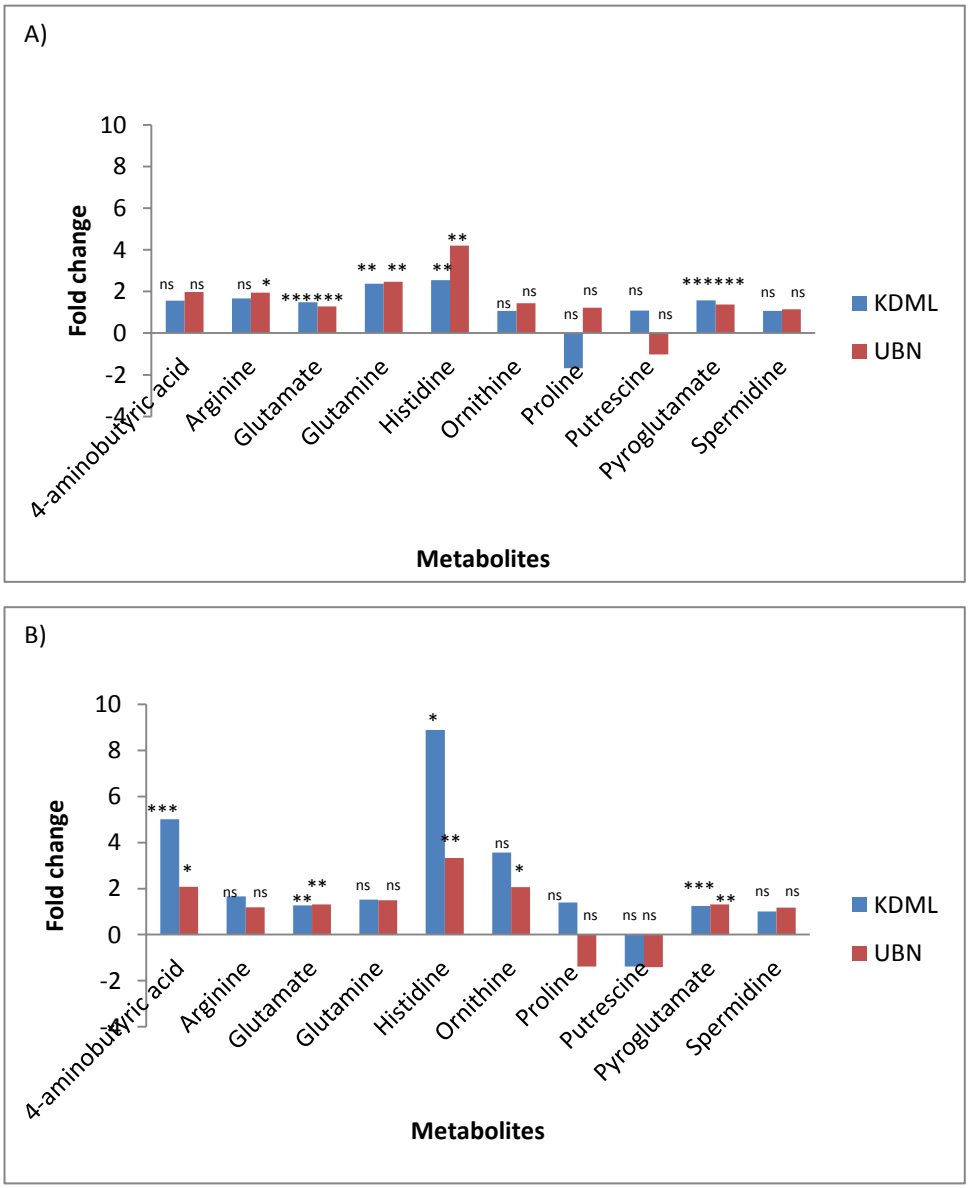

Figure 27 The amino acids in glutamate family in leaves of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test (P<0.05) as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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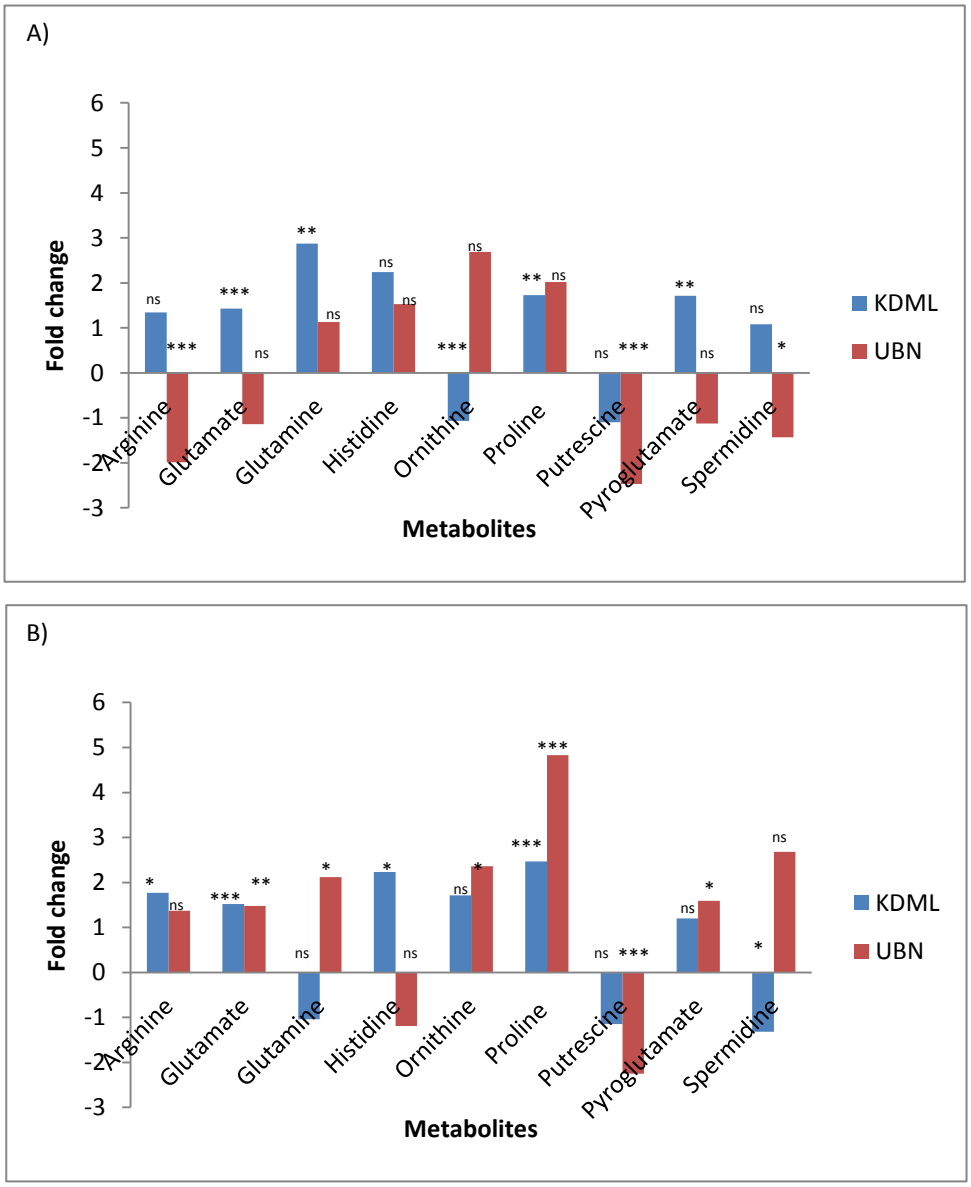

Figure 28 The amino acids in glutamate family in roots of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test (P<0.05) as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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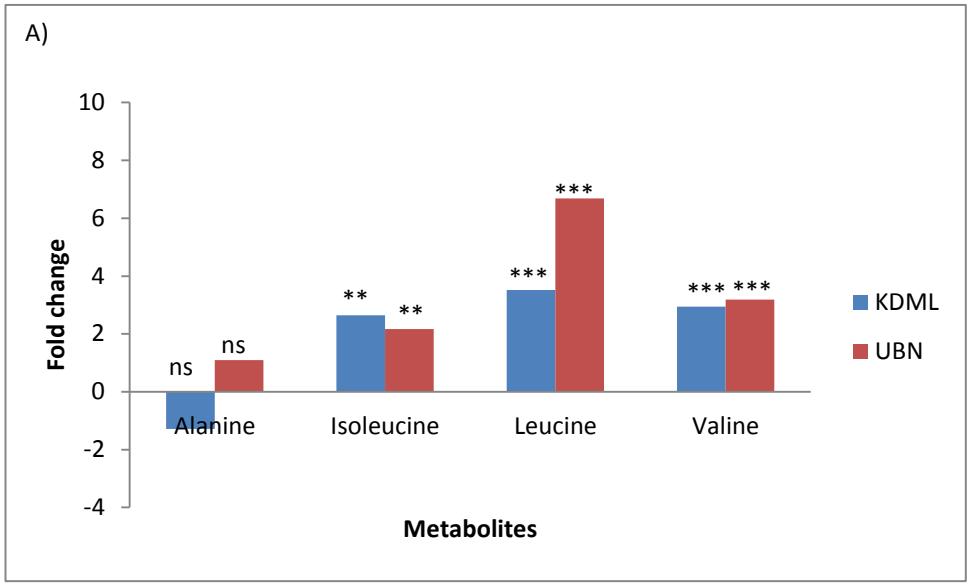
Alanine and Branched chain amino acids

Amino acids which were derived from pyruvate are alanine and branched chain amino acids including isoleucine, leucine and valine. In leaves, alanine, isoleucine, leucine and valine were up-accumulated in leaves of both cultivars. At 24 hours, branched chain amino acids in KDML 105 were largely higher than in UBN (Figure 29). Metabolites in branched chain amino acids in roots were up-accumulated at both 12 hours and 24 hours. Alanine was decreased at 12 hours and increased at 24 hours (Figure 30). At 24 hours, the levels of isoleucine, leucine and valine in KDML 105 was higher than in UBN.



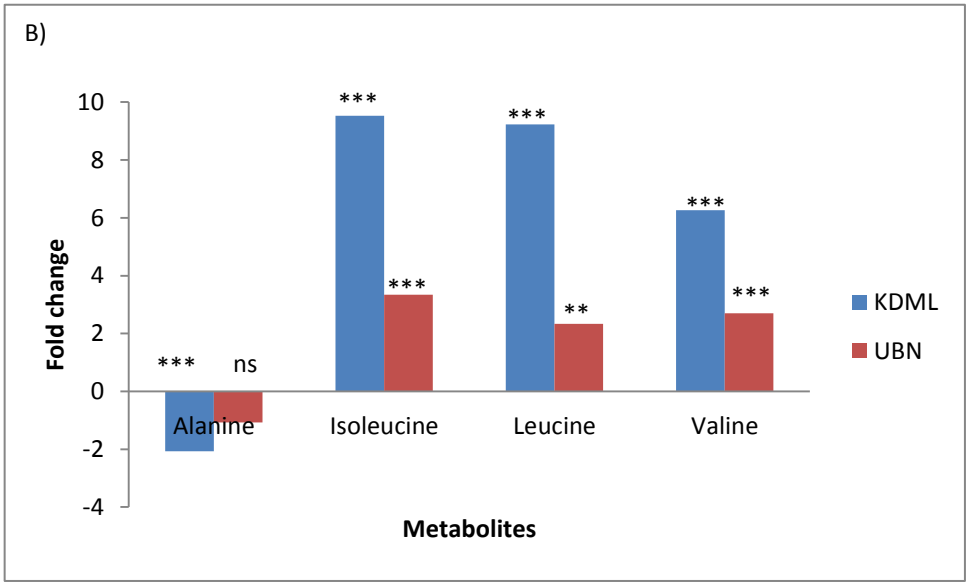


Figure 29 Alanine and branch chain amino acids in leaves of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test (P<0.05) as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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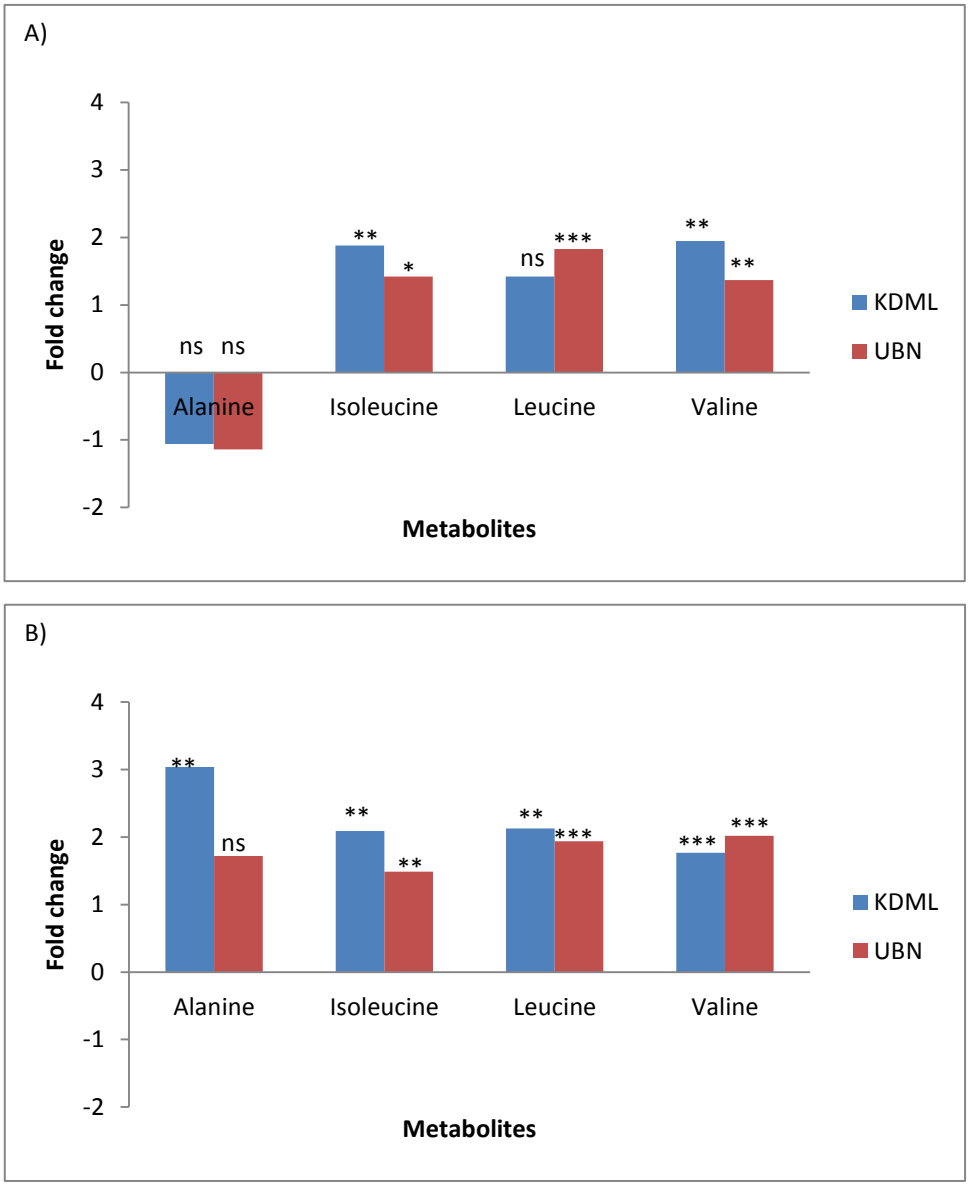

Figure 30 Alanine and branch chain amino acids in roots of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test (P<0.05) as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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Serine family

Metabolites in serine family consist of 3-cyanoalanine, cysteine, glycine and serine. In leaves, 3-cyanoalanine and serine were increased in both KDML 105 and UBN. Cysteine was increased in KDML 105 and decreased in UBN leaves. There were similar results at 12 hours and 24 hours under salt stress (Figure 31) (Tables 6-7). In roots, at 12 hours, glycine, 3-cyanoalanine and cysteine were increased in KDML 105 and decreased in UBN. These results showed the clear different responses to salinity stress between two cultivars. The levels of serine were increased in two cultivars at both 12 and 24 hours (Figure 32) (Tables 8-9). At 24 hours, glycine and cysteine were increased in KDML 105 and decreased in UBN. Serine and 3-cyanoalanine were up-accumulated in both KDML 105 and UBN.

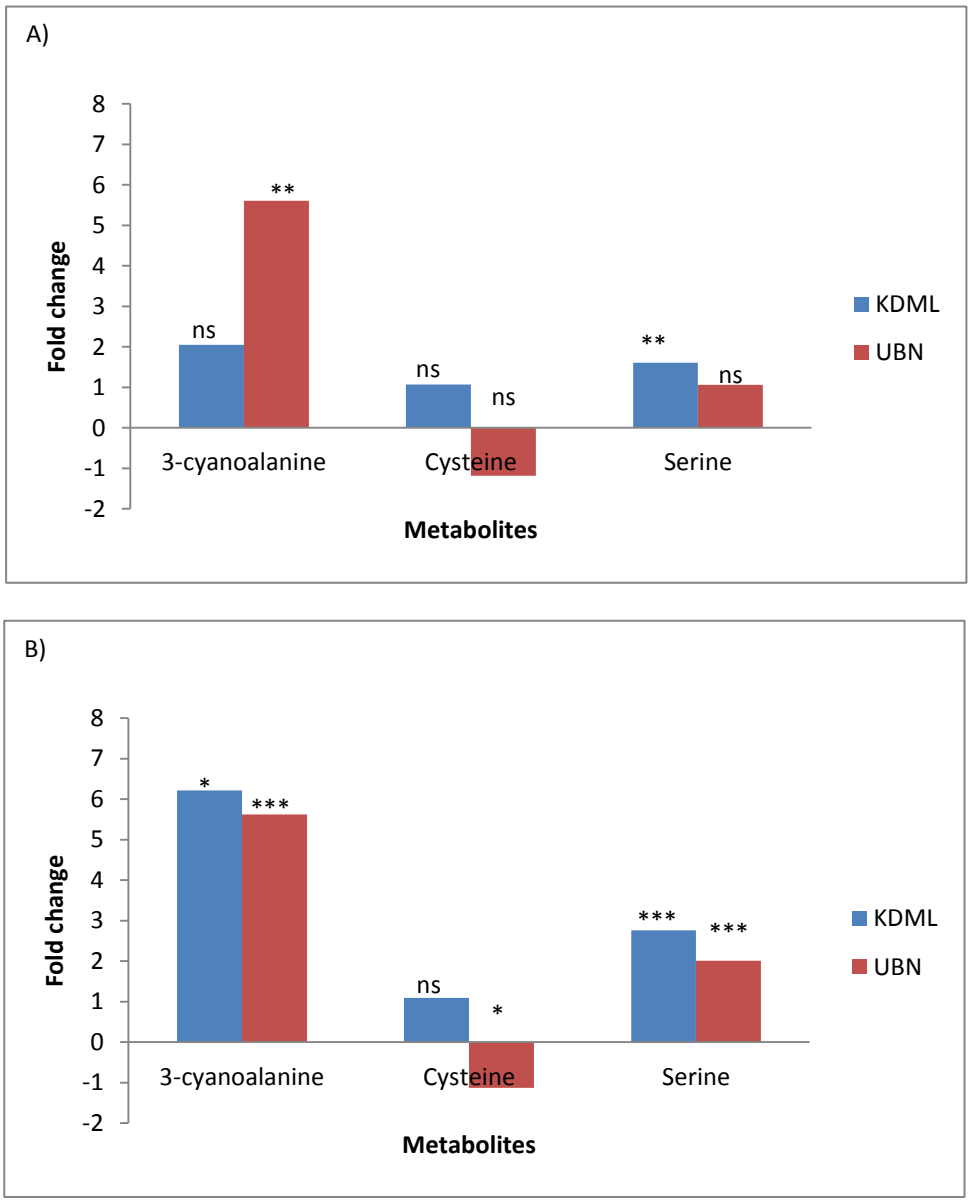

Figure 31 Amino acids in serine family in leaves of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test ($P<0.05$) as *** $P < 0.001$, ** $P < 0.01$ and * $P< 0.05$, ns represents non-significantly changed in metabolite level when compared to their respective control.

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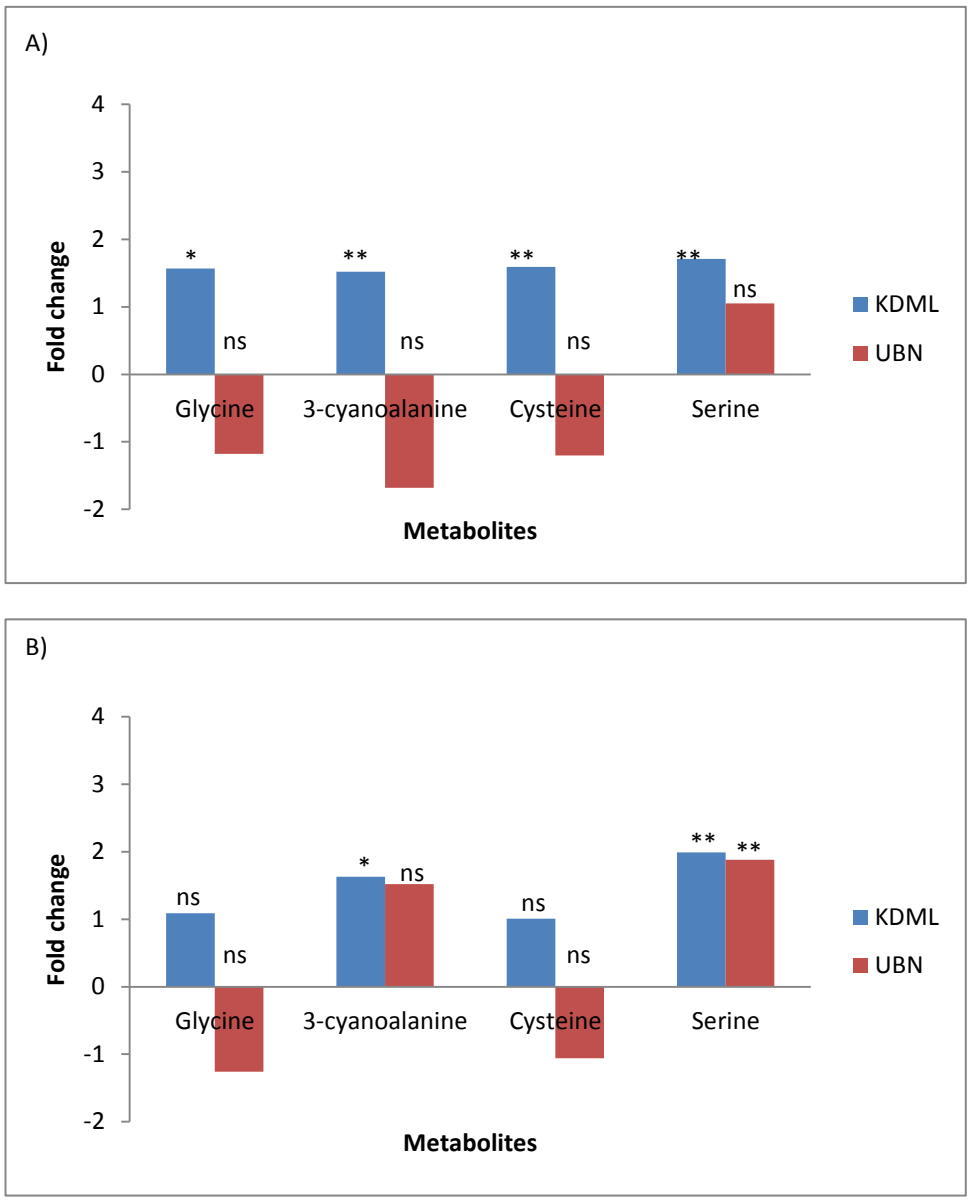

Figure 32 Amino acids in serine family in roots of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test ($P<0.05$) as *** $P < 0.001$, ** $P < 0.01$ and * $P< 0.05$, ns represents non-significantly changed in metabolite level when compared to their respective control.

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Shikimate family

Amino acids and metabolites affiliated in shikimate family namely 5-hydoxytryptamine, nicotinate, phenylalanine, quinic acid, shikimic acid, tryptophan and tyrosine. At 12 hours, the metabolic changes in leaves showed that nicotinate, phenylalanine, tryptophan and tyrosine were up-accumulated whereas other metabolites such as quinic acid and shikimic acid were down accumulated in both KDML 105 and UBN (Figure 33) (Tables 6-7). 5-hydoxytryptamine were decreased in KDML 105 but increased in UBN. At 24 hours, there were nicotinate, phenylalanine, tryptophan and tyrosine which significantly up accumulated, with the contrary results of 5-hydoxytryptamine, quinic acid and shikimic acid which were down accumulated.

In roots, the increases of 5-hydoxytryptamine, nicotinate quinic acid, shikimic acid and tryptophan were occurred in both cultivars at 12 hours. Phenylalanine and tyrosine were increased in KDML 105, nevertheless, their accumulation levels were decreased in UBN that might cause by the different responses in distinct species. At 24 hours, the two cultivars indicated the increases of phenylalanine, quinic acid, and tyrosine. Tryptophan was highly accumulated in KDML 105 at 24 hours (Figure 34) (Tables 8-9). 5-hydoxytryptamine and nicotinate were decreased in KDML 105 but increased in UBN. The levels of phenylalanine, quinic acid and tyrosine in KDML 105 were higher than in UBN which possibly described the differently tolerant to salinity stress between KDML 105 and UBN. Moreover, the higher amounts of these metabolites in KDML 105 likely explained sensitively responses to salinity stress of this cultivars.

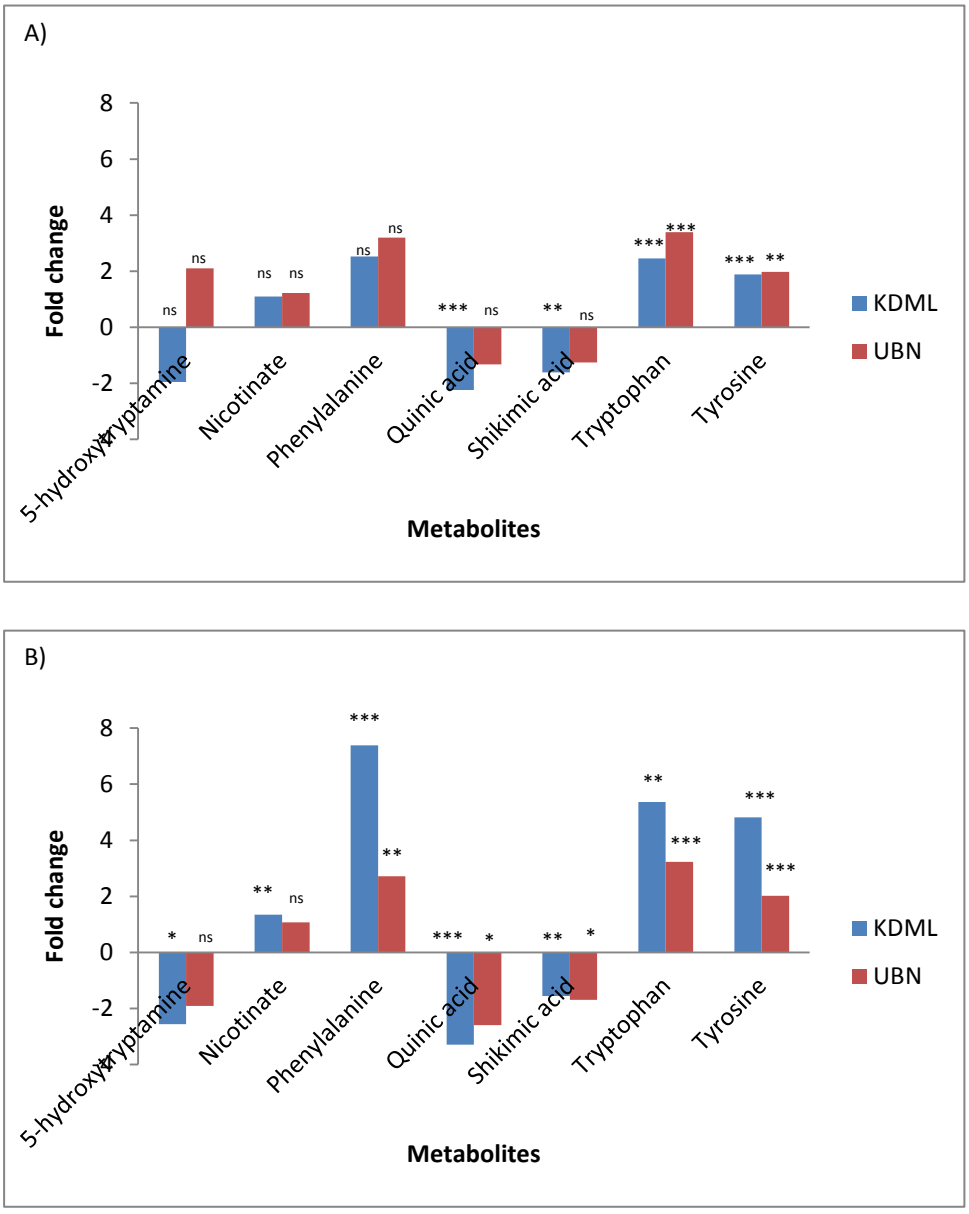

Figure 33 Amino acids in shikimate family in leaves of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test (P<0.05) as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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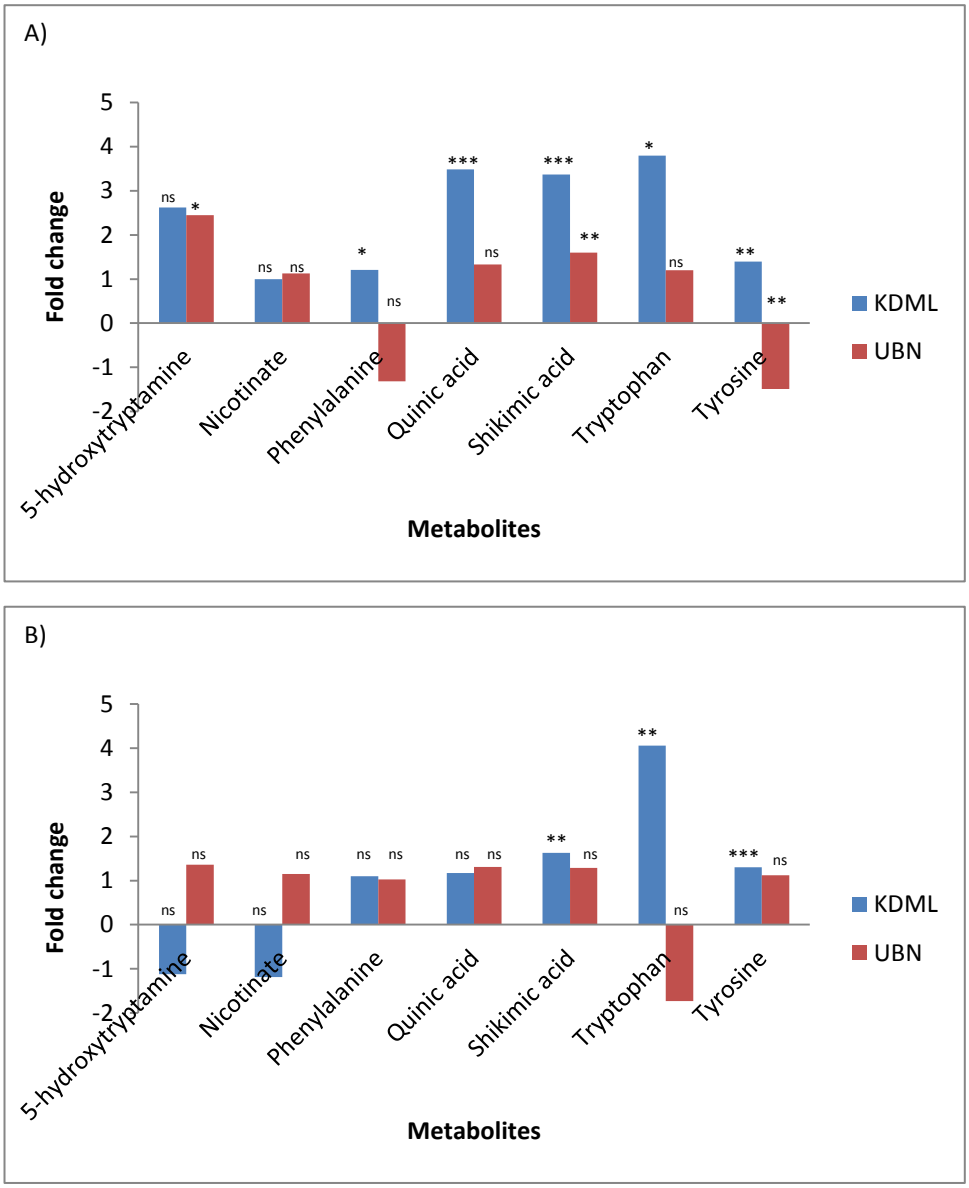

Figure 34 Amino acids in shikimate family in roots of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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Metabolites in the other group

Metabolites and organic acids in others group that were affected cause by salinity stress represent in Figures 35-36 (Tables 6-9). In leaves, galacturonic acid and suberic acid were significantly decreased at both 12 hours and 24 hours. Glutaric acid, lactic acid and lumichrome were up accumulated. In roots, at 12 hour, metabolites in UBN showed the tendency to decrease while metabolites in KDML 105 were up accumulated. Galacturonic acid confers improved tolerance by the synthesis of ascorbic acid, a stress-scavenged metabolite. Therefore, the higher reduction of galacturonic acid in KDML 105 indicated that this cultivar have been affected by salinity stress which consequently to ascorbic acid production. Differential accumulation of metabolites in these group may related to cultivar-specific response against salinity stress.

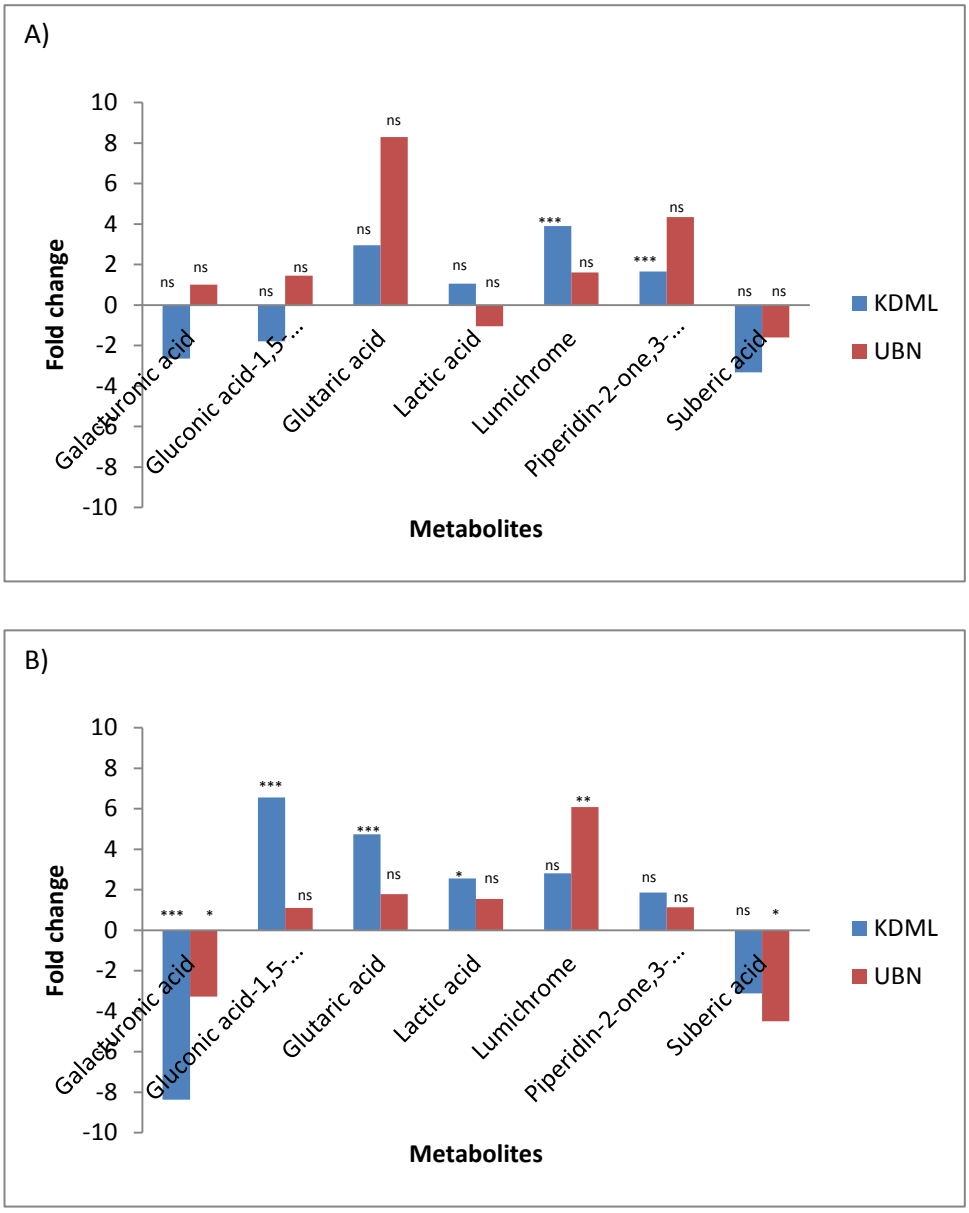

Figure 35 Metabolites in the other group in leaves of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test (P<0.05) as *** P < 0.001, ** P < 0.01 and * P< 0.05, ns represents non-significantly changed in metabolite level when compared to their respective control.

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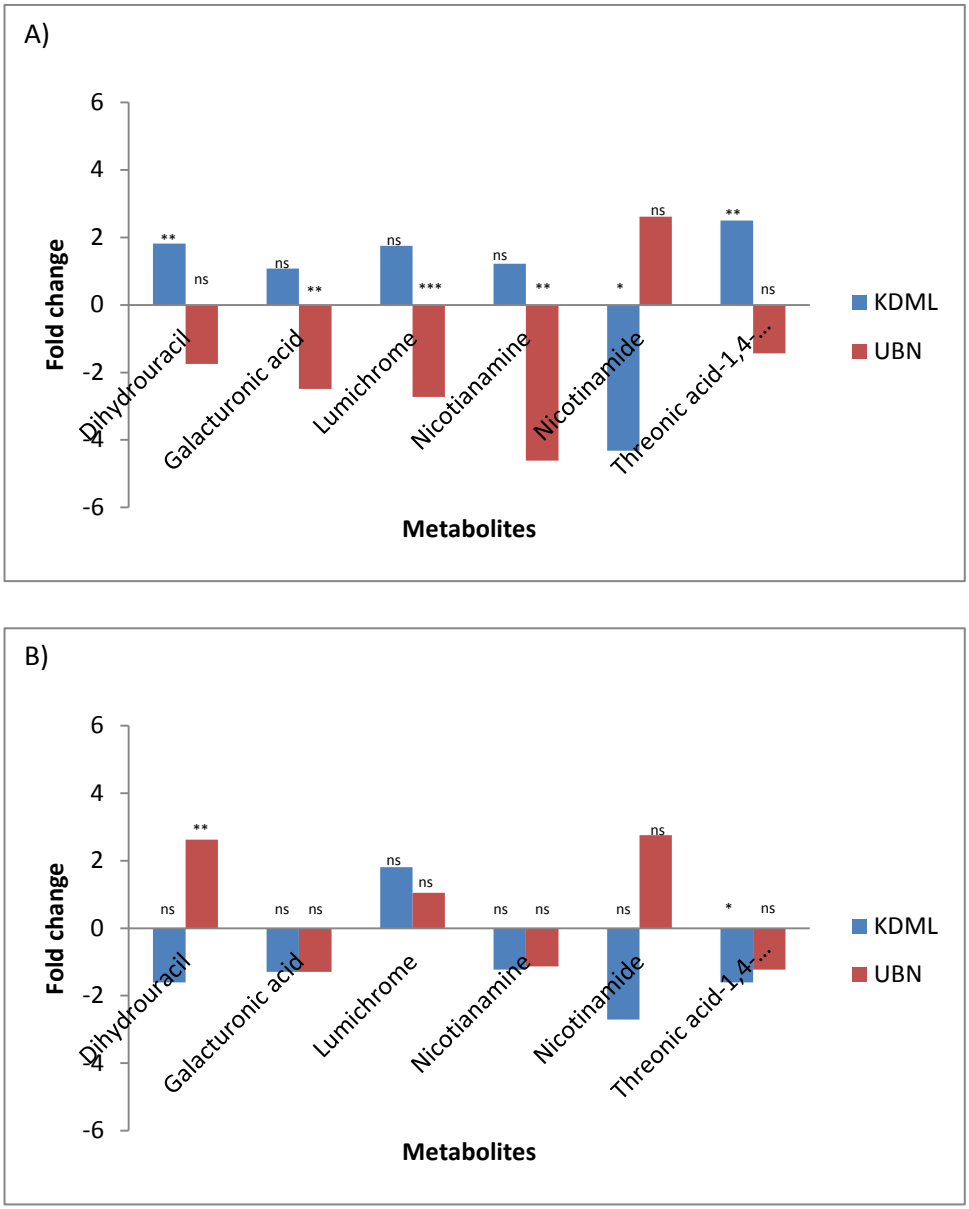

Figure 36 Metabolites in the other group in roots of KDML 105 and UBN after 12 hours (A) and 24 hours (B) of salinity stress. Asterisks above the bars represent P value of Student's T test ($P<0.05$) as *** $P < 0.001$, ** $P < 0.01$ and * $P< 0.05$, ns represents non-significantly changed in metabolite level when compared to their respective control.

Table 6 Accumulation fold changes of salinity-treated rice KDML 105 and UBN in leaves compared to control condition. (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change		24h Fold change	
		KDML	UBN	KDML	UBN
Glycolysis	Glucose	ns	3.01**	1.51*	ns
TCA cycle	2-oxoglutaric acid	ns	ns	ns	2.26
Sugar and polyols	Arabinose	ns	2.25**	1.48**	ns
	Cellotriose	ns	ns	4.92*	ns
	Fructose	ns	2.34**	1.60*	ns
	Galactinol	2.01*	ns	8.14***	2.19**
	Melibiose	ns	ns	3.05**	ns
	Raffinose	ns	ns	5.72***	2.65**
	Ribose	ns	2.51**	ns	2.60**
Amino acids and derivatives	Alanine	ns	ns	-2.07***	ns
	4-aminobutyric acid	ns	ns	5.61***	2.08*
	Asparagine	6.17*	3.16**	ns	2.97**
	Beta-alanine	2.03**	4.99***	2.64***	1.86**
	3-cyanoalanine	ns	5.60**	6.22*	5.62***
	Glutamine	2.37**	2.47**	ns	ns
	Histidine	2.55**	4.20***	8.89***	3.33**
	5-hydroxytryptamine	ns	ns	-2.56*	ns
	Isoleucine	2.64**	2.17**	9.53***	3.34***
	Leucine	3.52***	6.68***	9.23***	2.33**
	Lysine	2.60**	4.51**	9.62***	3.31***
	Methionine	2.62**	3.25**	8.59**	1.89*
	Phenylalanine	2.52***	3.20**	7.39***	2.71**
	Serine	1.61*	ns	2.76***	2.01***
	Threonine	1.43*	1.38*	3.99***	2.14***
	Tryptophan	2.45***	3.39***	5.37**	3.23***
	Tyrosine	1.88***	1.97**	4.82***	2.02***
	Valine	2.94***	3.19***	6.26***	2.70***

Table 7 (Continued) Accumulation fold changes of salinity-treated rice KDML 105 and UBN in leaves compared to control condition. (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change		24h Fold change	
		KDML	UBN	KDML	UBN
Others	Galacturonic acid	ns	ns	-8.37***	-3.27*
	Gluconic acid-1,5-lactone	ns	ns	6.56***	ns
	Glutaric acid	ns	ns	4.74***	ns
	Lactic acid	ns	ns	2.56*	ns
	Lumichrome	ns	ns	ns	6.09**
	Ornithine	ns	ns	ns	2.07*
	Piperidin-2-one, 3-amino-	ns	4.35***	ns	ns
	Quinic acid	-2.24***	ns	-3.30***	-2.60*
	Suberic acid	ns	ns	ns	-4.49**
	Threonic acid	-1.38**	2.77***	1.95***	1.56***
	Thronic acid-1,4-lactone	ns	-3.08**	-1.56***	ns

Table 8 Accumulation fold changes of salinity-treated rice KDML 105 and UBN in roots compared to control condition. (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change		24h Fold change	
		KDML	UBN	KDML	UBN
Glycolysis	Glucose	ns	-1.64**	-1.82**	-2.23**
	Glucose-6-phosphate	ns	-2.08**	-1.95*	-1.77***
TCA cycle	Citric acid	27.79***	28.43***	4.71**	5.77***
	Isocitric acid	16.86***	30.81***	4.43**	6.92***
	Malic acid	2.81***	2.70***	ns	2.21*
Sugar and polyols	Fructose	-4.92***	-7.58***	ns	-2.11**
	Galactinol	4.87***	2.49**	3.82***	6.26***
	Phytol	ns	2.96**	ns	ns
	Raffinose	ns	ns	7.33**	ns
	Ribose	2.29*	ns	2.18*	4.30***
	Sucrose	2.38***	ns	ns	ns
	Trehalose	ns	ns	6.85**	ns
Amino acids and derivatives	Alanine	ns	ns	3.04**	ns
	4-aminobutyric acid	2.94*	-2.36***	ns	ns
	Asparagine	3.19**	1.72*	2.68*	5.18***
	Aspartic acid	2.13***	1.72***	1.57**	2.42***
	Beta-alanine	3.44*	6.19***	2.49*	7.06***
	5-hydroxytryptamine	ns	2.45*	ns	ns
	Glutamine	2.87**	ns	ns	2.12*
	Histidine	ns	ns	2.23*	ns
	Isoleucine	1.88**	1.42*	2.09**	1.49**
	Leucine	ns	1.83***	2.13**	1.94***
	Lysine	ns	ns	2.33**	1.55**
	Methionine	ns	ns	4.60*	ns
	Proline	1.73**	ns	2.46***	4.83***

Table 9 (Continued) Accumulation fold changes of salinity-treated rice KDML 105 and UBN in roots compared to control condition. (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change		24h Fold change	
		KDML	UBN	KDML	UBN
Amino acids and derivatives	Tryptophan	3.80*	ns	4.06**	ns
	Valine	1.95**	1.37**	1.77***	2.02***
Others	Dihydrouracil	1.82**	ns	ns	2.63**
	Galacturonic acid	ns	-2.49**	ns	ns
	Lumichrome	ns	-2.72***	ns	ns
	Nicotianamine	ns	-4.61**	ns	ns
	Nicotinamide	-4.32*	ns	ns	ns
	Ornithine	ns	2.69***	ns	2.36*
	Putrescine	ns	-2.47***	ns	-2.25**
	Quinic acid	3.49***	ns	ns	ns
	Shikimic acid	3.37***	1.60**	1.63**	ns
	Threonic acid-1,4-lactone	2.50**	ns	-1.60*	ns

3.5.3 Hierarchical clustering analysis (HCA) of metabolites analyzed by GC/TOF-MS

Hierarchical clustering analyses of the metabolite profiles of salt-sensitive cultivar and salt-tolerant were performed for analyzing data taken from GC/TOF-MS. The hierarchical cluster was related to stress-associated metabolites and marked signal from our short-duration stress. The groups of metabolites in each yellow box represented the metabolic changes caused by saline treatment containing sugar, amino acids and organic acids in leaves and roots of KDML 105 and UBN (Figure 37). The red color represents the up-accumulation in metabolite levels, the green were the level of metabolites present in normal growth condition. The color intensity demonstrated the increases and decreases accumulation levels, with could describe the more intense of the color, the higher or lower of those metabolite accumulation. Each metabolite levels represent in the hierarchical cluster was the comparison of each biological replicate compared to their control. Several metabolites including sugar, amino acids and organic acids were up accumulation as represented in the more intensive red color, similarly, the intense green color displayed down accumulation of each group. The clustering pattern from HCA analysis provided the outline of salinity stress-associated metabolic alteration in primary metabolites. The detailed of metabolite changes in individual categories have been described previously in this chapter.

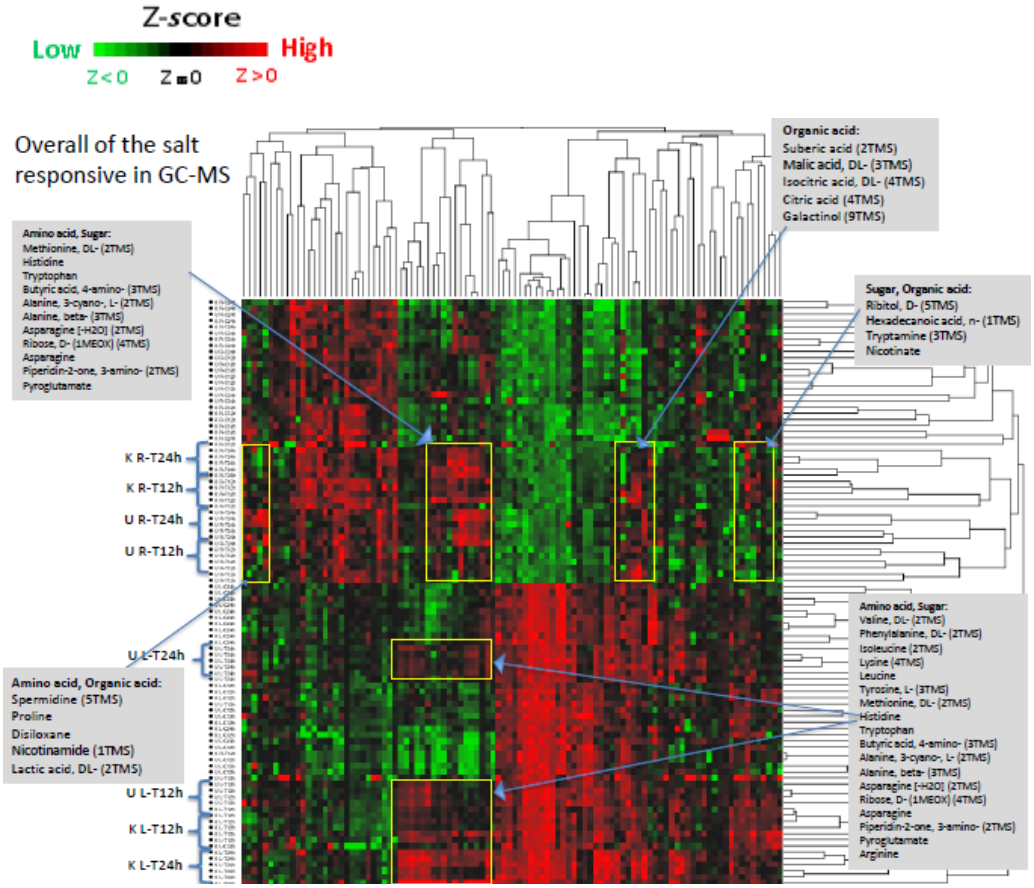

Figure 37 Hierarchical clustering analysis (HCA) of the metabolic changed analyzed by of KDML 105 and UBN under salinity stress condition. The red color represents the up-accumulation in metabolite levels, the green were the level of metabolites present in normal growth condition.

3.6 The expression of genes involved in stress-related metabolites biosynthesis

In order to study gene expression of stress-related metabolites in salt stress, metabolites which changed in accumulation levels more than two folds had been considered. In this study, we focused on the changes of metabolite levels in leaves samples at 24 hours of 160 mM NaCl salinity stress due to the clearly observed in physiological changes (see Figure 10). Galacturonic acid had been considered because it was eminently decreased in metabolites levels at 24 hours of salinity stress. 4-aminobutyric acid and lumichrome were also selected to study gene expressions since the up accumulation in metabolic levels. It was not found any significantly changed in proline levels in leaves tissue, however, we also selected proline as it was largely reported as an important role against stresses.

Two weeks old rice seedlings were studied responses to salinity stress by transfer to nutritional medium which added 80 mM NaCl. Gene expression levels of metabolite candidates in leaves after 24 hours salinity treatment were measured by Real-time PCR. Data were analyzed the statistical manners with Student's T-Test at $P < 0.05$.

The stress-related genes that were selected in this study including L-galactono-1,4-lactone dehydrogenase, glutamate decarboxylase, pyrroline-5-carboxylate reductase and riboflavin synthase, which are responsive in the metabolic pathway of galacturonic acid, 4-aminobutyric acid, proline and lumichrome respectively (Figure 38). The results illustrated that L-galactono-1,4-lactone dehydrogenase (Os11g04740) was decreased in expression levels compared to control condition. Glutamate decarboxylase (Os03g13300) was highly expression in UBN leaves after salt stress. Pyrroline-5-carboxylate reductase (Os01g71990) was significantly increased in expression level on UBN leaves and Riboflavin synthase (Os12g35580) was significantly increased in KDML 105 leaves. Differentiate expressions were relative to the different cultivars and time points.

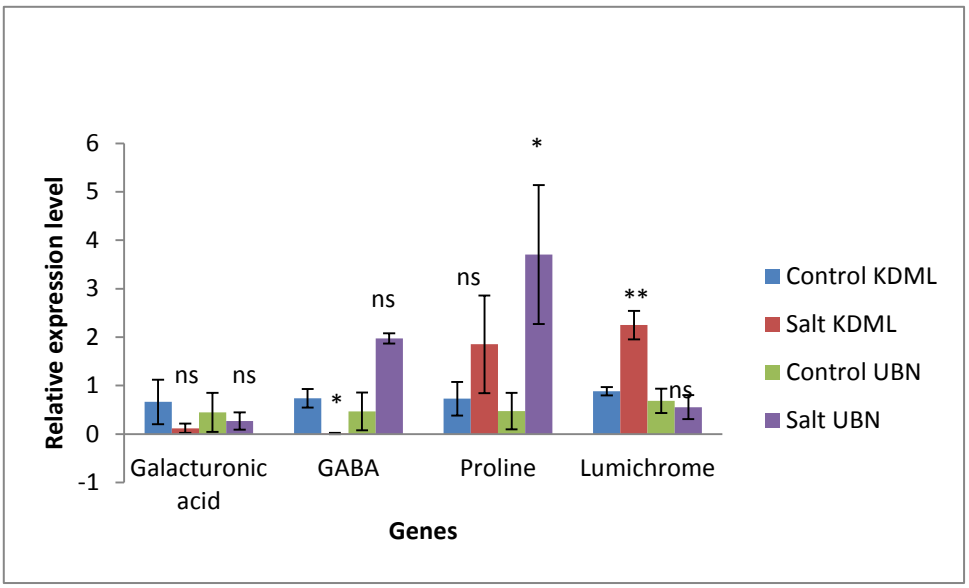

Figure 38 Relative gene expressions of KDML 105 and UBN leaves under salinity stress (160 mM NaCl) compared with control condition of each sample. Bars represent standard deviation of each gene while asterisks represent the bar with significantly difference with ** $P < 0.01$. * $P < 0.05$, ns : non-significantly changes.

3.7 Correlation of gene expressions and stress responsive metabolites

The further identification of gene expressions and metabolic responses to salinity stress were performed. The stress-related genes and metabolites were put into the relative metabolic map reference from KEGG database. Squares and circles in relative metabolic map represented as metabolites and genes. Red and blue colors represent metabolites and genes which significantly up and down regulation respectively, while grey represented non-significantly changes. White squares occurred in the map demonstrated as undetectable metabolites.

Galacturonic acid

The metabolite levels of galacturonic acid in KDML 105 (decreased 8.37-folds) and UBN (decreased 3.27-folds) leaves were down accumulation (Figure 39). L-galactono-1,4-lactone dehydrogenase (Os11g04740) is the gene involved in the synthesis of ascorbic acid using galactono-1,4-lactone, which derived from galacturonic acid as a substrate. In KDML 105, it was found that glutaric acid and glutaric acid-1,5-lactone were significantly up accumulation, but there were non-significantly changed in metabolites levels in UBN leaves. The decreases of L-galactono-1,4-lactone dehydrogenase was correlated with the down accumulation of galacturonic acid. Although ascorbic acid and L-galactono-1,4-lactone could not be detected by GC/TOF-MS in our analysis, however, the decreases of galacturonic acid possibly suggested the uses of these metabolite to form ascorbic acid in responses to salinity stress.

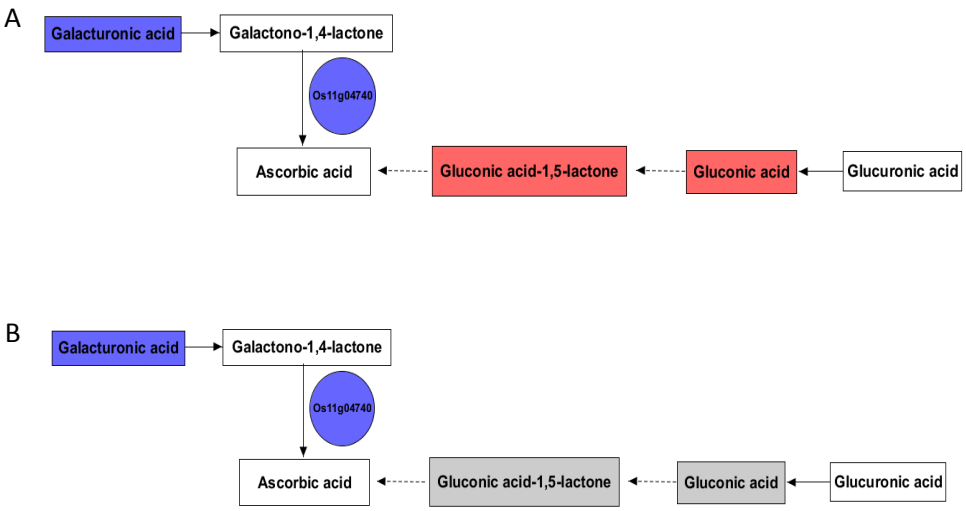

Figure 39 Gene-metabolite correlation of ascorbic acid in KDML 105 (A) and UBN (B) leaves at 24 hours after salinity treatment (160 mM NaCl). Squares and circles represented as metabolites and genes respectively. Red and blue color labelled the up and down regulation, respectively.

4-aminobutyric acid

The relative metabolic map showed the up accumulation of 4-aminobutyric acid in both KDML 105 (increased 5.61-folds) and UBN (increased 2.08-folds). Glutamate decarboxylase (Os03g13300), which responsive in the synthesis of 4-aminobutyric acid, indicated the down regulation in KDML 105 and up regulation in UBN (Figure 40). The different in gene expression between KDML 105 and UBN might suggest the variable time-dependent regulation between these distinct cultivars. The expression of glutamate decarboxylase in KDML 105 might rapidly express at the initial state of salinity stress. Succinic acid which can be alternatively generated by 4-aminobutyric acid was significantly decreased in UBN leaves after 24 hours of salt stress.

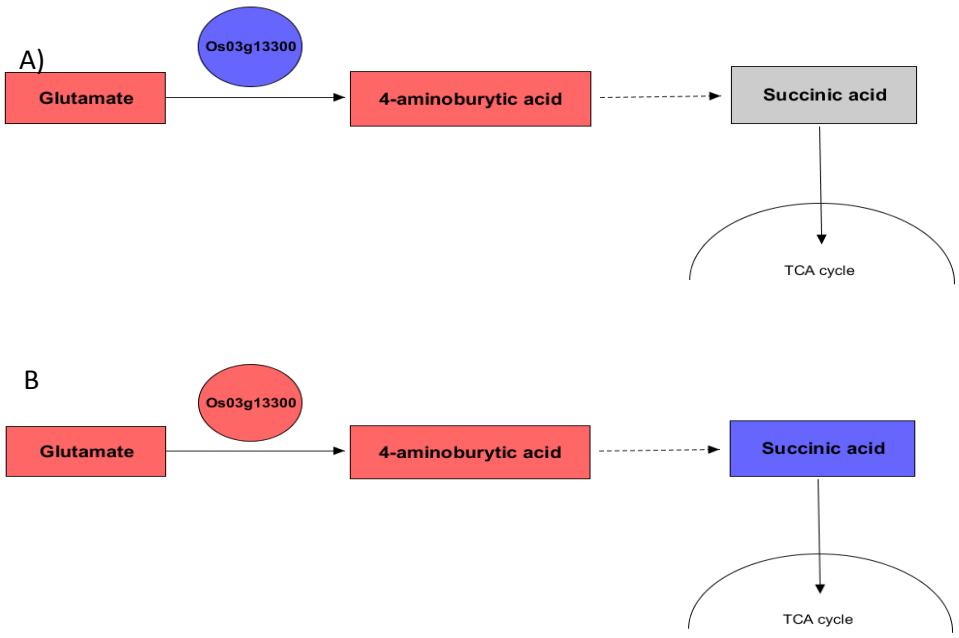

Figure 40 Gene-metabolite correlation of 4-aminobutyric acid in KDML 105 (A) and UBN (B) leaves at 24 hours after salinity treatment (160 mM NaCl). Squares and circles represented as metabolites and genes respectively. Red and blue color labelled the up and down regulation respectively.

Proline

Even though proline was not significantly changed in metabolite levels of both KDML 105 and UBN leaves, there were occurred the increases of Pyrroline-5-carboxylate reductase (Os01g71990) in KDML 105 and UBN leaves under salt stress (Figure 41). These results were not only showed the alteration in metabolic levels of proline but other related metabolites in glutamate family and polyamines. Putrescine was up accumulated in KDML 105 but decreased in UBN leaves. Glutamate was similarly increased in both KDML 105 and UBN. Ornithine was only up accumulation in UBN with non-significantly changed in KDML 105.

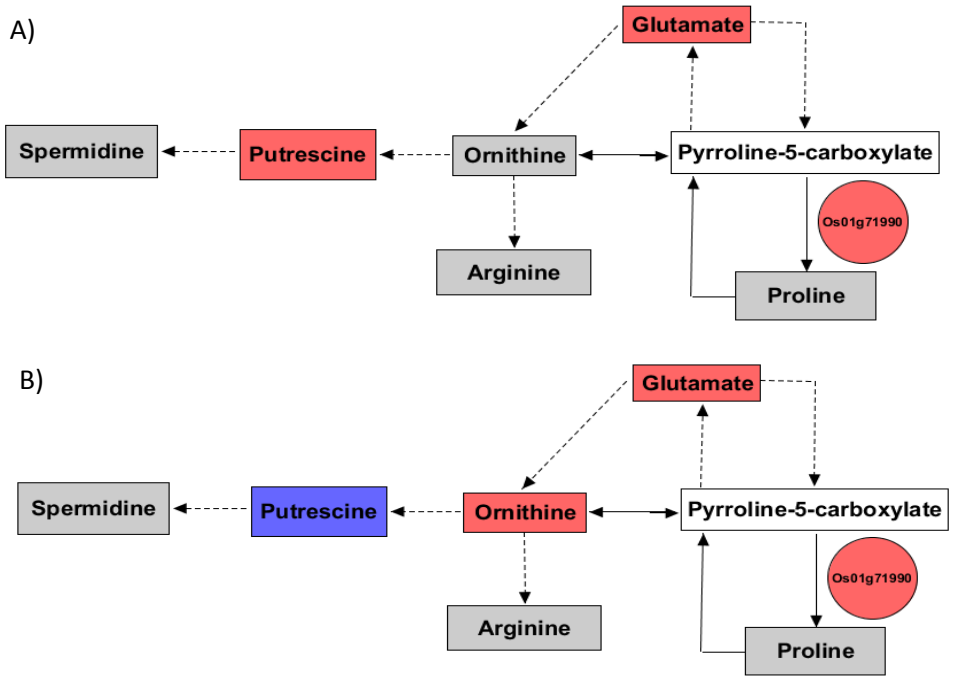

Figure 41 Gene-metabolite correlation of proline in KDML 105 (A) and UBN (B) leaves at 24 hours after salinity treatment (160 mM NaCl). Squares and circles represented as metabolites and genes respectively. Red and blue color labelled the up and down regulation, respectively.

Lumichrome

Lumichrome can generally be derived from ribose and synthesized through riboflavin metabolism. In this study, lumichrome and ribose were only up accumulation in UBN leaves (increased 6.09-folds) with non-significantly changed in KDML 105 (Figure 42). Gene which responsible for the synthesis of lumichrome is Riboflavin synthase (Os12g35580), which was significantly up-regulation in KDML 105 leaves but decreased in expression level in UBN. Lumicrome was compensatory stimuli in whole-plant carbon assimilation and caused promote plant growth (Khan et al., 2008). The up accumulation in metabolic level of lumichrome was promising involved in salinity tolerant in UBN.

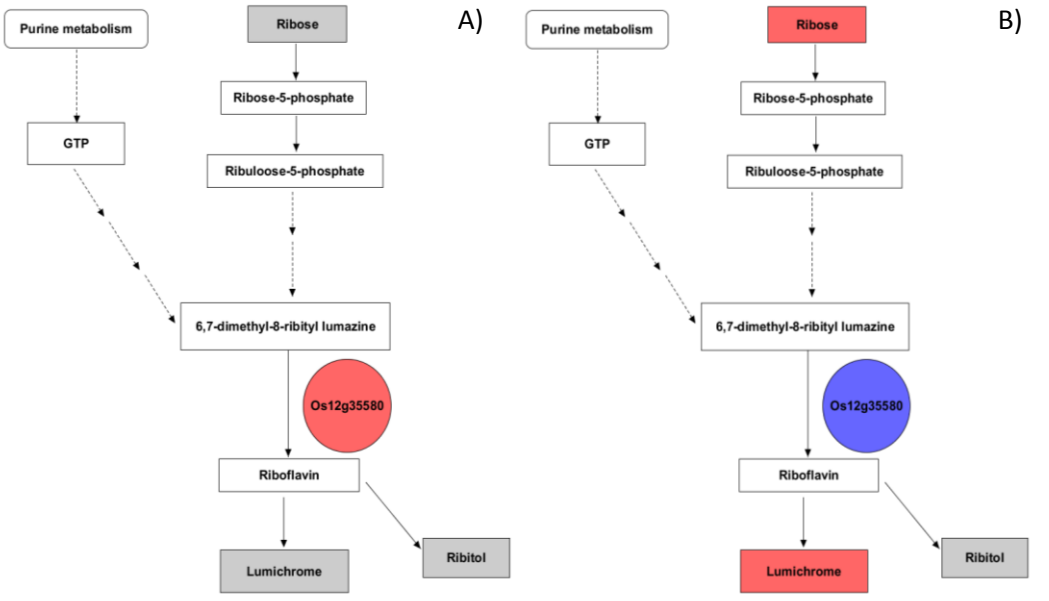

Figure 42 Gene-metabolite correlation of lumichrome in KDML 105 (A) and UBN (B) leaves at 24 hours after salinity treatment (160 mM NaCl). Squares and circles represented as metabolites and genes respectively. Red and blue color labelled the up and down regulation, respectively.

3.8 The metabolite profiles of KDML 105 and UBN under salinity stress analyzed by LC-MS/MS

LC-MS/MS was used in this study to identify the secondary metabolites with responses to salinity stress. The PCA score plots of LC-MS/MS analyses have been constructed similarly with the data from GC-TOF/MS. The models were constructed according to PLS-DA (Figure 43). The metabolite profiles in each cultivar were group together. The PLS-DA score plot of the analyzed LC-MS in normal condition *indicated* the separated clusters from different tissues as leaves (A) and roots (B) because the naturally difference from distinct cultivars. However, the clusters were not well-separated in the secondary metabolite profiles of the two cultivars that might be preliminary explain that the secondary metabolites in KDML 105 and UBN were not apparently different. The number of significant in LC-MS/MS analyses as follows: $R^2X[1]$ variant as 0.138, $R^2X[2]$ as 0.452 in leaves and $R^2X[1]$ as 0.356 and $R^2X[2]$ as 0.32 in roots. The 95% of Hotelling's T^2 range indicated the validation of metabolite profiles.

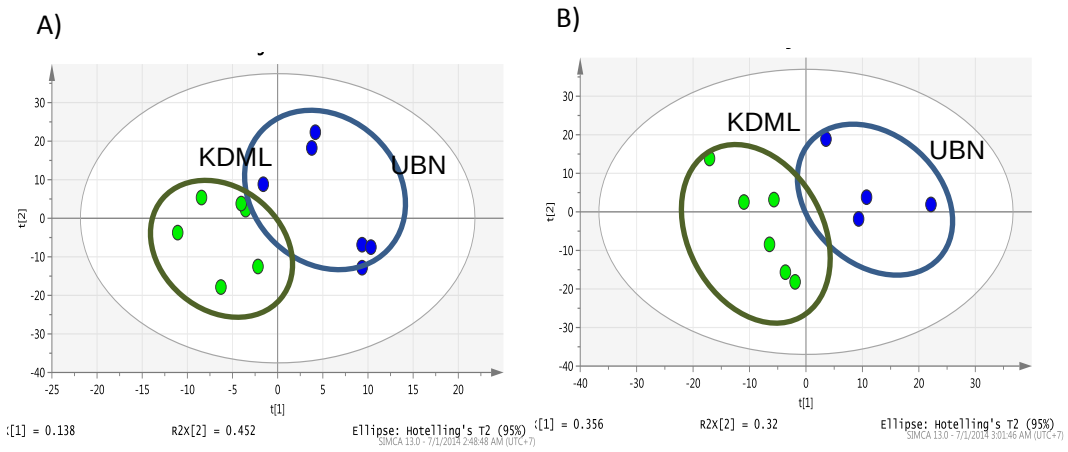

Figure 43 PLS-DA score plots of metabolites from the different tissues, leaves (A) and roots (B) of KDML 105 and UBN under normal condition. Small circles illustrate metabolite profiles of a single biological replicate.

To study the effect of salinity stress on the alteration levels of secondary metabolite, we also construct the PLS-DA score plots from the data of LC-MS/MS in

both leaves (Figure 44) and roots (Figure 45) tissues under salinity treatment. The results indicated that after salinity treatment, the levels of secondary metabolites were changed which could be observed by the metabolite shifts between control and salinity-treated group of each cultivar. In leaves, the metabolite profiles were not clearly indicated the difference in ranking at 12 hours. At 24 hours, the clear separation of metabolite profiles between control and salinity-treated groups of both cultivars were occurred. The metabolite profiles of control condition in KDML 105 and UBN were not well-separated indicating the common secondary metabolites in both cultivars in normal growth condition. The number of significant from LC-MS/MS analyses in leaves after salinity stress as follows: $R^2X[1]$ as 0.249 and $R^2X[2]$ as 0.269 at 12 hours, and $R^2X[1]$ as 0.115 and $R^2X[2]$ as 0.343 at 24 hours after salinity stress. The Hotelling's T2 ellipse were 95% in both treatment periods.

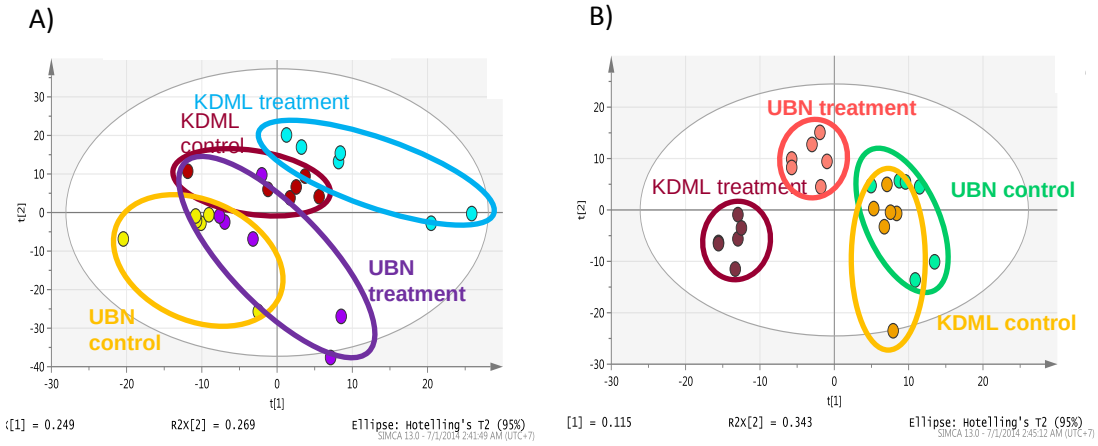

Figure 44 PLS-DA score plots of metabolites from leaves at 12 hours (A) and 24 hours (B) of KDML 105 and UBN under stress condition (160 mM NaCl). Small circles illustrate metabolite profiles of a single biological replicate.

In root, three clusters of metabolite profiles containing KDML 105 control, KDML 105 treatment and UBN treatment were group together whereas the cluster of UBN treatment was separated from the others. The results suggested the changes in accumulation levels of secondary metabolites in UBN caused by salinity stress. The similar pattern of metabolic responses in KDML 105 control, KDML 105 treatment

and UBN treatment indicated that secondary metabolites in these clusters were not affected by salinity stress. At 24 hours, all clusters were not clear separated suggesting salinity stress did not stimuli the changes in secondary metabolites. At 12 hours, the number of significant represented $R^2X[1]$ as 0.237 and $R^2X[2]$ as 0.366, and, $R^2X[1]$ as 0.381 and $R^2X[2]$ as 0.268 at 24 hours with The significance levels of Hotelling's T2 ellipse were 95% in both durations.

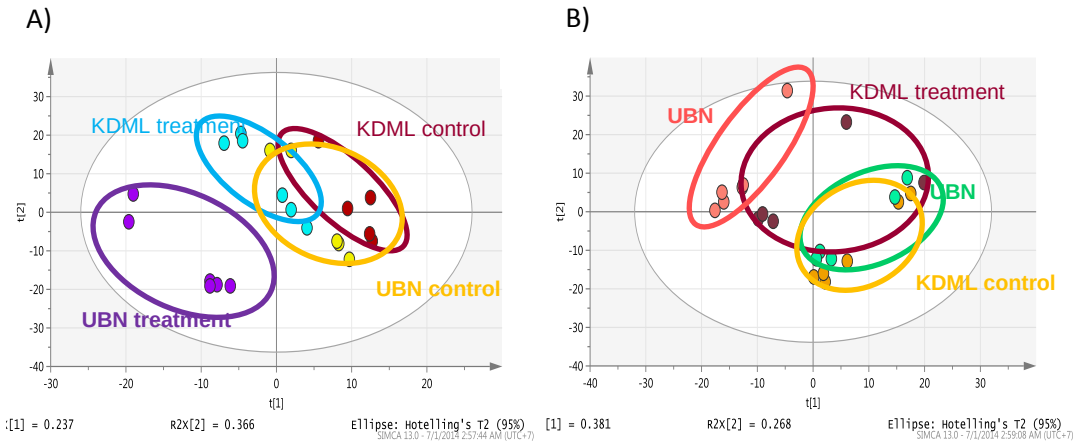

Figure 45 PLS-DA score plots of metabolites from roots at 12 hours (A) and 24 hours (B) of KDML 105 and UBN under stress condition (160 mM NaCl). Small circles illustrate metabolite profiles of a single biological replicate.

The significantly metabolic changes were represented in Tables 10 and 11. The different metabolites in both leaves and roots under normal condition revealed the accumulation of ferulic acid UBN roots in normal condition. There was no significantly difference in metabolite levels in root between KDML 105 and UBN presented in positive detection mode.

Table 10 Metabolite ratios of KDML 105 and UBN leaves under control condition. (P : ***<0.001, **< 0.01, *<0.05)

Detection mode	Metabolite	Retention time	m/z	Fold Change
Negative mode	Tricin 7-O-beta-D-glucopyranoside	4.5058	491.1186	1.09*
	Schaftoside, Neoschaftoside	3.5164	563.1396	1.12**
	Neocarlinoside, Carlinoside	3.2221	579.1354	1.45**
	Isoscoparin 2''-(6-(E)-ferulylglucoside)	4.1396	799.2076	1.24*
Positive mode	Neocarlinoside, Carlinoside	3.2215	581.1511	1.24*

Table 11 Metabolite ratios of KDML 105 and UBN roots under control condition detected by LC-MS. (P : ***<0.001, **< 0.01, *<0.05)

Detection mode	Metabolite	Retention time	m/z	Fold Change
Negative mode	Ferulic acid	3.2277	193.0506	2.01**

The significantly changes in metabolite levels between KDML 105 and UBN at 12 hours salinity treatment were shown in Tables 12-14 as leaves and roots respectively. The results suggested the up-accumulation of ferulic acid in both leaves and roots tissue. The other metabolites such as glucoside, glucopyranoside and oryzafuean were found significantly with down accumulation in leaves. In roots, ferulic acid, phytocassane and neocarlinoside were up-accumulated. After salinity stress for 24 hours, there was any significantly changed in metabolic levels of annotated metabolite in root tissue of both KDML 105 and UBN. In leaves, differential metabolites were occurred regarding to the different cultivars. However, the changes in metabolite levels were less than two folds suggesting essential secondary metabolites were not affected by salinity treatment.

Table 12 Metabolite ratios of KDML 105 and UBN at 12 hours in leaves under salinity stress condition detected by LC-MS. (P : ***<0.001, **< 0.01, *<0.05)

Detection mode	Metabolite	Retention time	m/z	Fold Change	
				KDML	UBN
Negative mode	Ferulic acid	3.2277	193.0506	ns	1.58***
	Schaftoside, Neoschaftoside	3.5164	563.1396	ns	-1.10*
	Isoscoparin 2''-(6-(E)-ferulylglucoside)	4.1396	799.2076	ns	-1.19**
Positive mode	Oryzafuran	3.7153	317.067	ns	-1.22**
	Tricin 7-O-beta-D-glucopyranoside	4.2145	493.1347	ns	-1.77**
	Isoorientin 2''-O-glucopyranoside;	3.3997	611.1615	ns	-1.36**
	Neocarlinoside, Carlinoside	3.2999	581.1504	ns	-1.15*
	Isoscoparin 2''-(6-(E)-p-coumaroylglucoside)	4.2125	771.2153	ns	-1.72**

Table 13 (Continued) Metabolite ratios of KDML 105 and UBN at 12 hours in roots under salinity stress condition detected by LC-MS. (P : ***<0.001, **< 0.01, *<0.05)

Detection mode	Metabolite	Retention time	m/z	Fold Change	
				KDML	UBN
Negative mode	Ferulic acid	3.1956	193.0515	ns	1.80**
Positive mode	Oryzalexin C	4.4104	301.2178	ns	1.21**
	Ineketone, Phytocassane C	4.4115	319.2276	ns	1.16**
	Phytocassane B	4.4431	335.2216	ns	1.15**
	Momilactone B	4.2158	331.1902	ns	1.13*
	Schaftoside, Neoschaftoside	3.5354	565.1553	ns	1.13*
	Neocarlinoside, Carlinoside	3.2999	581.1504	ns	1.15**

Table 14 Metabolite ratios of KDML 105 and UBN at 24 hours in leave under salinity stress condition detected by LC-MS. (P : ***<0.001, **< 0.01, *<0.05)

Detection mode	Metabolite	Retention time	m/z	Fold Change	
				KDML	UBN
Negative mode	3,4-Dihydroxybenzoic acid	4.2916	153.0191	-1.65*	ns
	Neocarlinoside, Carlinoside	3.131	579.1351	-1.43**	ns
	Isoorientin 2''-O-glucopyranoside	3.3993	609.1454	-1.58***	ns
Positive mode	Isoorientin 2''-O-glucopyranoside	3.3997	611.1615	-1.57***	-1.55*
	Neocarlinoside, Carlinoside	3.2215	581.1511	ns	-1.28**
	Tricin 7-rutinoside	4.2917	639.1934	ns	-1.16*
	Isoscoparin 2''-O-glucoside	3.7985	625.177	-1.25**	-1.29*

CHAPTER IV

DISCUSSION

This study aimed to construct the metabolite profiles of salt-sensitive and the salt-tolerant rice cultivars. We conducted metabolomics to integrate the information of metabolic responses related to salinity stress. The analyses led to identify carbohydrates, amino acids, organic acid and the intermediate metabolites in energy metabolism which were generally affected under salt stress. The results could give the understanding on stress defense mechanisms in salt-tolerant cultivars and could be used to identify salt-tolerant metabolite markers for the further crop development.

4.1 Visible physiological changes of rice under different salt concentrations

In preliminary study, we observed the effect of salinity stress to the visible physiological changes between the moderately salt-sensitive cultivar 'KDML 105' and salt-tolerant cultivar 'UBN 02123-50R-B-2'. It was found that the salt concentration at 80 mM NaCl induced wilted leaves on KDML 105 with the slightly injury in UBN at 24 hours of salinity treatment. For salt concentration at 160 mM NaCl, the leaves of two rice seedling cultivars were severely damaged and could not be distinguished their physiological changes. The results suggested that the salt concentration at 80 mM NaCl was suitable to apply for further studying metabolic changes.

4.2 The activities of antioxidant enzymes

Since the preliminary results from physiological changes hinted us the initial information that UBN might be tolerant to salinity stress more than KDML 105. Therefore, we conducted the antioxidant activity measurements to gain more information about rice responses to salinity stress. The antioxidant activities of catalase (CAT) and peroxidase (POD) were conducted. As present in Figure 11, CAT

specific activity in KDML 105 and UBN showed significantly increased under salt stress. At 24 hours, the CAT specific activity was higher than 12 hours under salt treatment. The results suggested that the longer duration of salt treatment could provide the higher catalase activity enhanced in rice seedlings. As CAT is the main enzyme which converts H_2O_2 to H_2O and O_2 (Scandalios et al. 1997), it was elucidated that CAT activity contributes to the cellular protection against oxidative damage under salt stress. In the previous study, it was reported that CAT activity in stressed seedlings of salt tolerant (Pokkali) was significantly higher than salt-sensitive cultivar (Pusa Basmati 1) and it also activated under salinity condition in both cultivars (Vaidyanathan et al. 2003). Thus, the higher CAT activity in UBN could partly allow the better detoxification of H_2O_2 leading to the salinity tolerance in this cultivar at 24 hours.

The POD activity of KDML 105 and UBN indicates the increased POD activity subjected to the cellular response against ROS signaling. In particular, POD activity was higher in UBN leaves more than in KDML 105 at 12 hours. The exogenous NaCl could enhance cellular POD activity to scavenge the formation of ROS. From the previous study, it showed the increase in CAT and POD activities in rice seedlings of salt-tolerant cultivar in nutrient solutions supplemented with NaCl to achieve electrical conductivity of 7 and 14 dS m^{-1} as treatment solutions for 20 days (Mishra et al. 2013). The decrease in POD activity under salinity stress could imply a recovery in leaf lipid peroxidation in which the scavenging mechanisms of reactive oxygen intermediates would adjust the balance among SOD, CAT and POD. POD possibly be dependable as a signal for the stress adjustment, while CAT might be induced for the removal of reactive oxygen intermediates during stress. Additionally, the major scavenging components for reactive oxygen intermediates found in most plants found are SOD, the ascorbate–glutathione cycle, glutathione peroxidase (GPX), and CAT (Mittler 2002). When high levels of ROS are formed, those oxidative stress cause to the proliferation of peroxisome (Lopez-Huertas et al. 2000). The massive population of peroxisome might be efficient to scavenge ROS due to the high level of CAT in peroxisome, thus, the role of POD might be deducted.

Cellular responses against salinity stress start with the formation of superoxide anion (O^{2-}), which is catalyzed by SOD to formed H_2O_2. H_2O_2 were then detoxified by CAT, POD and GPX to water. Peroxiredoxin (Prx) is the shunting reaction which could convert H_2O_2 to H_2O through redox regulating protein such as thioredoxin (Trx) (Chi et al. 2013). During the reactions, reducing agents and other soluble reducing equivalents of ferredoxin (Fd), and Trx are generated (Shurmann and Bhuchanan 2008). Enhanced levels of GPX would be associated with increased in oxidative stress tolerance (Meloni et al. 2003). Therefore, at 24 hours of salinity stress, rice might alter itself to form those soluble reducing equivalents or to detoxify ROS through the ascorbate–glutathione cycle or GPX reaction as results from the decrease in POD activities. The reduction of POD activities suggested that rice might alter the suitable antioxidative reaction to eliminate ROS during salinity stress.

The higher POD activity at 12 hours was suggested that UBN was considered having an ability as salt tolerance when compared to KDML 105. The improved activities of CAT and POD in salt-tolerant cultivar help the early detoxification of ROS, and the levels of activities are maintained even under high concentration of NaCl (Vaidyanathan et al. 2003).

4.3 The total chlorophyll content

Under saline stress, ROS can be formed by converting excitation energy from chlorophyll, resulting to the formation of single oxygen species (Asada 1999). According to Figure 13, the total chlorophyll content in KDML 105 and UBN were increased under salinity stress. The total chlorophyll content in salt-tolerant UBN rice was higher than in KDML 105 at 24 hours under salinity stress. The results were similar to the previous study from Meloni *et al* 2003, who found the increase in total chlorophyll content in cotton Guazuncho that is the salt-sensitive cultivars and in salt-tolerant cultivars, Pora. Under salinity stress with 50, 100 and 200 mM NaCl, total chlorophyll content in Guazuncho showed significantly decreased. The total chlorophyll content is related to antioxidant enzyme activities, the adjustment of plant defenses system to detoxify ROS aided in enhancing total chlorophyll content and improved photochemical efficiency of PSII (Al-aghabary et al. 2007). At 24 hours

under salt stress, Total chlorophyll content in KDML 105 leaves was decreased under 24 hours of salinity stress which indicated that external salt concentration could affected to photosynthesis apparatus in the salt-sensitive cultivar. The reduction of total chlorophyll content in KDML 105 could imply to the reduction of leaf photosynthesis rate owing to the effect of salinity stress, the depreciation of chlorophyll pigments and the instability of the pigment protein complex (Meloni et al. 2003; Jaleel et al. 2007). On the other hand, the chlorophyll reduction led to the reproduction of chlorophyll to endure under salinity stress.

Moreover, chlorophyll in plant leaves is directly related to their nutritional state. The amount of chlorophyll pigment has been used as an evaluation indicator of the nutritional state for several types growth condition (Simão et al. 2013). Thus, the steady content of chlorophyll pigment in UBN could be elucidated that these cultivars can provide the stability of nutritional contents under stress condition.

4.4 Gene expression analysis of KDML 105 and UBN using semi-quantitative RT-PCR

The primers for semi quantitative RT-PCR analysis in this study were selected from the candidate genes of Kim *et al* (2005), which studied the expression of protein involving to salinity stress in rice. The expression pattern of fructose-1,6-bisphosphatase, fructose bisphosphate aldolase, and catalase were highly expression in UBN leaves at 24 hours salinity treatment (Figure 14). Rubisco were high expression in both KDML 105 and UBN leaves. The expression patterns were slightly increased since 12 hours salinity stress and were higher expressed at 24 hours, indicating the expression of Rubisco was enhanced regarding to the stress durations.

Salinity stress could affect plant photosynthetic apparatus. Photosynthesis machinery in plants could be damaged in long term stress. From the results of semi-quantitative RT-PCR, the highly expression of Rubisco at 24 hours in both KDML 105 and UBN was corresponded to the study of Kim *el al* ((Kim et al. 2005), which reported that the up-regulation of Rubisco large and small chains in rice leaves under saline condition was observed. The result indicated that the maintenance of catalytic activity of Rubisco supports the perception of photorespiration which might be

stimulated as an activity of CO_2 assimilation under salinity stress in rice seedlings. Additionally, Sivakumar *et al* (Sivakumar et al. 2000) also reported the improvement of oxygenase activity of Rubisco under salt stress that accompanied with the enhanced regulation of Rubisco in this study. Salinity stress leads to stomatal closure, which could reduce CO_2 availability and interrupt carbon fixation in leaves, that causes to the lacking of carbon generated via photosynthesis (Chiba et al. 2003). During leaf senescence by salinity stress and in dark cycles, the degraded of Rubisco always present due to the translocation into growing organs and it were then reproduced, therefore, the increased expression levels of Rubisco in leaves might also found to compensate the losing Rubisco back to leave tissues (Vernon et al. 1982).

The expression of fructose bisphosphate aldolase and fructose-1,6-bisphosphatase in UBN was up-regulated under salinity stress. These two enzymes was also up-regulated in maize under salt stress as fructose bisphosphate aldolase catalyzes the reaction of fructose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate and fructose-1,6-bisphosphatase converts fructose-1,6-bisphosphate to fructose-6-phosphate (Zörb et al. 2004). Regarding to the study of Kim *et al* 2005 was also found the up accumulation of fructose bisphosphate aldolase and fructose-1,6-bisphosphatase in rice leaves, it could be assumed that salt stress induced changes in enzymes involved in Calvin cycles and transferred to fructose-6-phosphate (Haake et al. 1998). Carbohydrates were synthesized and transferred as energy compound into other tissues among rice during stress condition (Chaves et al. 2008). The up accumulation of fructose bisphosphate aldolase was caused by salinity stress that stimulated oxidative stress leading to an anaerobic condition. Plant acclimations to oxidative damage have related to the ability to synthesize ATP in glycolysis (Andrews et al. 1994). The enhanced expression of aldolases suggested that these proteins may play a role in rice seedlings responses to anaerobic condition caused by oxidative stress, which generated from salinity stress (Abbasi and Komatsu 2004). Fructose bisphosphate aldolase showed the different expression patterns as the slightly increases in expression levels at 12 hours in KDML 105, and up expression in UBN at 24 hours. The results suggested that the

difference of plant cultivars affected to the different expression patterns of this gene under stress condition.

The highly specific activity of CAT was observed in UBN leaves at 24 hours, according to the result from Figure 11, correspondingly, we also found the higher expression level of CAT gene in the same condition as shown in Figure 14. Increased transcript accumulation of CAT provided that it could catalyze the breakdown of H_2O_2 in responses to salinity stress as explain in the study of Savouré et al, in which NaCl could induce CAT transcript and correlated to catalase activity in *N. plumbaginifolia* (Savoure et al. 1999) (Savouré 1999). However, the expression of CAT could not be found at 12 hours of salinity stress. Since there are several isoforms of CAT while the CAT primer used in this study was designed by only one isoform. Different CAT isoforms regulated by different controlled genes (Cavalcanti et al. 2004). The high activity of CAT in the Figure 11 was provided by total isoforms of CAT. Thus, the low of CAT expression at 12 hours might cause by the different expression and regulation in each isoform of CAT.

4.5 The metabolite profiles of KDML 105 and UBN under salinity stress analyzed by GC-TOF/MS

4.5.1 Overview metabolic changes

The PLS-DA score plot of metabolite profiles in control condition compared with salinity treatment indicated the different clusters of metabolite profiles regarding to four experimentally conditions such as KDML 105 control, KDML 105 salinity stress condition, UBN control and UBN salinity stress condition with two treatment periods as 12 and 24 hours (Figure 16). The differentiated classes of control plants and salinity treated plants were found. The shifts in metabolite groups might initially be described the modulating in metabolic responses to salinity stress as the separated distance in metabolite clusters revealed the changes in metabolite levels of control and salinity-treated plants. The results of PLS-DA score plots

indicated that the metabolite profiles of rice exposed to control and salinity treatment were classified according to four different conditions.

Several studies had performed PCA score plot for the classification of different metabolites responsive in several stresses. In the study of metabolite profiles in cold and drought stresses in rice, represented that the PCA score plot could distinguish seven treatments of differently metabolites referring to their control and stress conditions. The seven treatments were classified into three groups according to growth conditions such as cold, dehydration and untreated as control (Maruyama, 2014). The PLS-DA were also analyzed the effect of saline concentration in roots and leaves of maize, which could establish the separated groups of control, 50 mM NaCl and 150 mM NaCl (Gavaghan et al. 2011). The previous researches have assured the uses of principal component analysis as a pre-processing method that can help to interpret trends of metabolic changes before moving into the next stage of analysis. In this study, we certainly evaluated rice adjustment to salinity stress that rice able be altered metabolic levels toward the shift of metabolite groups in PLS-DA score plot. The Hotelling's T2 tests were significant at 0.05 level (95%) indicating significantly different of metabolite profiles in PLS-DA plot.

In salinity stress condition, stress responsive metabolites were adjusted to enhance mechanisms against stress. At 12 hours of salt stress, leaves of KDML 105 and UBN demonstrated the up-accumulation of amino acids in aspartate, branched chain amino acids (BCAAs), glutamate, shikimate and serine families. The intermediate metabolites involved in TCA cycle and some sugars were significantly increased in UBN leaves. In roots, we found the down-accumulation of amino acids regarding in glutamate family, shikimate family, some sugars and intermediate metabolites in glycolysis. The overview of metabolite maps indicated the different metabolite levels between control and salinity treated-plants as the higher up accumulation of primary metabolites, which was induced by salinity stress. The increases in primary metabolites in rice under salinity stress might imply a part of the indicators on general stresses and cell damage.

4.5.2 Detailed changes

Metabolite profiles in this study indicated that rice exposed to salinity stress improved the changes of metabolite levels in rice plants. The representative metabolites which presented their accumulation fold changes more than two folds have been selected for further study. The metabolites were clustered into five groups based on the involvement in glycolysis, TCA cycles, sugar and polyols, amino acids and others. In glycolysis, there was only glucose that showed significantly increased in both 12 and 24 hours of leaf tissues. In roots, glucose, glucose-6-phosphate and fructose-6-phosphate were significantly decreased. The decreases of intermediates in glycolysis such as glucose, glucose-6-phosephate or fructose-6-phosphate and pentose phosphate such as fructose and fructose-6-phosphate always appeared with the increases in organic acids content under stress (Morcuende et al. 2007). Thus, it possibly suggested that the reduction of metabolites in glycolysis causing to biosynthesis of organic acids. In wheat roots, increases in sucrose and decreases in glucose-6-phosphate were possibly from subsequently increased flux of sucrose (Xue et al. 2008).

The up-accumulation of intermediates in TCA cycle such as citric acid, isocitric acid and malic acid possibly suggested in the flow of carbon from glycolysis through the TCA cycle and might involve in the respiration activity for maintaining growth under stress. The concentrations of citrate, isocitrate and 2-oxoglutarate increased in shoots were considered as the results from salinity stress. Despite the increase levels of intermediates in TCA cycle remained unclear, malate have potential to balance charge and osmotic pressure and act as the interplant signal transduction for signaling in drought and salinity stress for growth development and adaptation (Zhu J. K. 2001). The up accumulations of malate in this study were resemble proposed to the study of effect of salinity stress in maize in which malate proposes to adapt osmotic pressure in responses to salinity stress (Gavaghan et al. 2011). The increases of citric acid may have roles as CO_2 reservoir in CAM plants such as *Bryophyllum calycinum*, *Clusia minor*, *Clusia rosea*, *Notonia petrea*, and *Cissus quadrangularis*, which were reported the high concentrations under stress (Bucio et al. 2000). The exchanged pathway of citric and isocitric acid were incorporated to the cross-linked

metabolic processes and might provide carbon skeletons for nitrogen assimilation. Therefore, the highly accumulation of citric and isocitric acid in UBN might support the execution of the glyoxylate cycle and play an essential role in the Krebs cycle and energy metabolism in whole plants (Popova and Pinheiro de Carvalho 1998).

As the major respiratory carbon sources driving energy supply for cells, sugar are considered as important signaling messengers during stress under adverse growth conditions (Radomiljac et al. 2013). In this study, there were the higher levels of sugars and polyols in two rice tissues. As the cellular damages in root and leaf tissues caused to the inhibition of carbon assimilation and uptake, the increased of sucrose in leaves were correlated with the accumulation of fructose and another sugars which suggested to the starch degradation and sucrose metabolism as the alternative carbon sources (Maruyama et al. 2014). Raffinose accumulation acts as one of important osmoprotectants. The increases of galactinol levels elucidated as precursor for biosynthesis of raffinose as the formation of raffinose and myo-inositol by galactinol and sucrose catalyzed by raffinose synthase (Kaplan et al. 2007). Therefore, the increases of raffinose level in UBN might cause by its role as osmoprotectants for protection on cellular components. After salinity stress, the high accumulation levels of many metabolites were found in KDML 105 and UBN leaves which we focused on the significantly accumulation in salt-tolerance cultivar for the further selection of metabolite markers which related to salinity tolerant. The results suggested that the up-accumulation of sugars (arabinose, fructose, raffinose, and ribose) amino acids (proline, alanine, beta-alanine, 3-cyanoalanine, proline, and etc.) played important roles as the osmoprotectant in order to stabilize the homeostasis on both inner and outer of the cell during salinity stress treatment (Smith et al. 2010). The down-accumulation of fructose, glucose and glucose-6-phosphate could be described resulting from the uses of energy for growth performance and reproduction under salinity stress (Widodo et al. 2009). In addition, these metabolites might be changed to be precursor of the down-stream metabolisms against salinity stress.

Amino acids involved in aspartate family are aspartic acid, asparagine, beta-alanine, lysine, methionine and threonine. As aspartate has been considered as an

important amino acid since it leads to the synthesis of four essential amino acids including lysine, threonine, methionine and isoleucine, therefore, metabolic link of the aspartate pathway with energy and stress regulation can be used to improve Lys level in some organs of crop species (Galili 2014). Beta-alanine involved in the pantothenate (vitamin B5) synthetic process. At present, despite knowledge regarding to the regulatory relationship in this pathway in plants is limited, the increase of beta-alanine in KDML 105 might involve in the pantothenate biosynthesis under salinity stress in plants which can be used as initial information for further study on roles of beta-alanine.

The up-accumulation of glutamine and glutamate was reported both as signaling molecules of ammonium transport that they have been defined as the main responsible in nitrate and ammonium uptakes in plants (Davenport 2002). The up-accumulation of glutamine under salinity stress in leaves and roots might implicated a high internal N concentration that could generated the imbalance of C:N in rice (Kusano et al. 2011). The primarily production of 4-aminobutyric acid was reported in experimental as a response to abiotic stresses (Bouche and Fromm 2004). Therefore, the high increases of 4-aminobutyric acid in KDML 105 indicated that KDML 105 was sensitive to salinity stress since the initial stage of stress responses. Arginine, glutamine, histidine and ornithine respond to salinity stress in term of accumulation of N-containing metabolites in rice. It was shown that polyamine metabolism is involved in several cases of stress tolerance (Bouchereau et al. 1999; Khorshidi and Hamedi 2014). Thus, the changes in accumulation levels of proline, putrescine and spermidine could imply the role of these metabolites to salinity tolerance in rice seedling. The high accumulation of proline and spermidine in UBN roots suggested that UBN had the tolerant ability better than KDML 105 among saline condition. Proline is also considered as an osmoprotectant, the highly accumulation of proline in UBN roots showed potentially to protect cellular damages in roots while exposed to NaCl solution. Proline biosynthesis in plant could proceed either through the glutamate or the ornithine pathway depending to environmental and nitrogen storage condition. The rapidly accumulation and degradation of proline can naturally

be observed during osmotic stress according to cellular exigency to maintenance turgor pressure in cellular oxidative stress (Trovato et al. 2008).

Amino acids in branched chain amino acids (BCAAs) consist of isoleucine, leucine and valine. Since they also were derived from pyruvate as same as alanine, alanine was grouped together with metabolites in this group. Metabolites in branched chain amino acids in KDML 105 and UBN were up accumulated in both 12 hours and 24 hours. In young tissues, BCAAs are normally synthesized (Gonda et al. 2010), they also have been considered as the energy sources in carbon starvation since BCAAs are synthesized from pyruvate and 2-oxo acids (Binder 2010). BCAAs were up accumulated in rice seedling which could imply to plant adaptations to salinity stress related to the changes in carbon and energy sources. The levels of BCAAs in KDML 105 leaves were higher than those in UBN, it was suggested that KDML 105 provided the alteration in energy balances as a result from salinity stress.

Metabolites in serine family consist of 3-cyanoalanine, cysteine, glycine and serine. Cysteine and 3-cyanoalanine can be correlated into asparagine which is an important amino acid in plants (Machingura and Ebbs 2010). The down accumulation of cysteine and 3-cyanoalanine in KDML 105 roots demonstrated that cysteine might be used as a precursor to synthesize asparagine in order to transport nitrogen compound from roots to shoots, and maintained N:C balance under salinity stress in contrary with the results from UBN roots. 3-cyanoalanine is converted by a nitrilase to asparagine, aspartate and ammonia, this pathway provides the assimilation of cyanide into primary metabolism and is available in all higher plants thus far investigated (Zidenga 2011). The enhancement of nitrogen assimilation and transport in KDML 105 revealed that this cultivar was affected by salinity stress consequently to the contribution of essential compounds. The different metabolic responses demonstrated species-specific difference between KDML 105 and UBN especially after saline treatment.

The decreases of shikimic acid in both KDML 105 and UBN could describe that it was substrate in early state leading to the synthesis of phenylalanine and tryptophan (Morcuende et al. 2007), subsequently to the up accumulation of

phenylalanine and tryptophan in leaves. One role for shikimic acid is it conveys the contribution to osmotic pressure. Shikimic acid was studied in *E. pauciflora* and found its tendency to increase in leaves under stress. The other role for shikimic acid is being a precursor to aromatic amino acids such as phenylalanine, tryptophan and tyrosine (Liu et al. 2014). Thus, the reduction of shikimic acid led to the increases in accumulation levels of aromatic amino acids due to its role as a precursor in biosynthesis pathways.

The metabolite levels of amino acid suggested that the difference in altering metabolism was occurred in KDML 105 and UBN under stress condition. The high accumulation of amino acids in leave of KDML 105 after salinity stress might be caused by the low levels of these amino acids presented in normal condition whereas UBN were slightly changed in accumulation of amino acids caused by the high accumulation levels under normal condition. At 12 hours of salinity stress, we found the higher up-accumulated of primary metabolites in UBN than in KDML 105, and those metabolites were then reduced their accumulation levels at 24 hours. In contrary, the metabolite levels in KDML 105 showed the higher up-accumulated than UBN at 24 hours. It could summarize that primary metabolites were up-accumulated faster in UBN when compared to KDML 105 under salinity stress. This may suggest that the UBN, the salt-tolerance cultivar might have the better salinity tolerance than KDML 105. At 24 hours, it was found that the global metabolic changes of KDML 105 were higher than 12 hours since it attempted to adapt itself during salinity stress for longer periods. Several amino acids at 24 hours in UBN leaves showed significantly decreased, indicated that UBN might alter for the cellular stability under stress.

4.5.3 Hierarchical clustering analysis (HCA) of metabolites analyzed by GC/TOF-MS

The heat map showed that metabolites were classified according to groups of metabolites such as sugars, amino acids and organic acids. The results suggested that during salinity stress, control and salinity treated plants were accumulated differently

metabolites based on various metabolic functions. The most notable metabolic phenotypes were observed in the salinity treated plants, indicated as the high accumulation of several metabolites (red mosaic). Overall responsive metabolites under salinity stress detected by HCA analyses revealed the different accumulation of primary metabolites responsible for their stress-associated functions between control and salinity stress conditions which their detailed changes in accumulation levels had been described earlier. At 12 hours, the up accumulation of primary metabolites in UBN leaf was occurred as indicated in the deep red mosaics, the recovery stage of UBN leaf was then found as the lower intensity of red mosaics. Some of green mosaics, which represented metabolite levels in normal condition) were found in UBN leaf at 24 hours. The results illustrated tend to recover of metabolic processes involved in primary metabolisms of UBN in salinity stress.

4.6 Expression patterns of stress-related metabolites in responses to salt stress using Real-time PCR

The stress-related genes that were selected in this study including L-galactono-1,4-lactone dehydrogenase, glutamate decarboxylase, pyrroline-5-carboxylate reductase and riboflavin synthase transcripts, which are involved in the metabolic pathway of galacturonic acid, 4-aminobutyric acid, proline and lumichrome respectively. The gene encoding L-galactono-1,4-lactone dehydrogenase were down regulated in both cultivars under salinity stress. The results also showed higher expression levels of pyrroline-5-carboxylate reductase and riboflavin synthase, which synthesized proline and lumichrome, in UBN when compared to KDML 105. Proline (synthesized by Pyrroline-5-carboxylate reductase), which gene were high expression levels but metabolite levels was not found significantly changed. It might possibly imply that proline had been used as osmoprotectants during salinity stress and there was less of protein accumulated in leaf tissues. Thus, we could not identify proline accumulation in leaves due to it might already transport to other parts on rice tissue.

The gene encoding glutamate decarboxylase, which are used for the generation of 4-aminobutyric acid was significantly down-regulated in KDML 105 leaves while 4-aminobutyric acid was still up accumulation, that caused by there are not only metabolisms through glutamate decarboxylase that can synthesize 4-aminobutyric acid. Riboflavin synthase, which synthesized lumichrome was significantly lower expressed in KDML 105 than UBN. The correlation of metabolites, gene expressions, and the alternative pathways were considered.

4.7 Correlation of gene expressions and stress responsive metabolites

The correlation of stress-related metabolites and genes involved in biosynthesis of metabolites was presented. According to accumulation level of galacturonic acid indicated that it related to biosynthetic pathway of ascorbic acid. The down regulation of L-galactono-1,4-lactone dehydrogenase transcripts and galacturonic acid levels in both KDML 105 and UBN suggested that it might involve in the initial step of an ascorbic acid biosynthesis. Beside the synthetic of ascorbic acid through galacturonic acid pathway, the increases of gluconic acid and gluconic acid-1,5-lactone provided the alternative pathway for the synthesis ascorbic acid under long term stress condition. In this case, it was probably possible that after 24 hours of salt stress galacturonic acid was down regulated. Thus, gluconic acid and gluconic acid-1,5-lactone were regulated in alternative pathway for the synthesis ascorbic acid instead the synthetic through galacturonic acid pathway.

The down regulation of glutamate decarboxylase was occurred in KDML 105, whereas it was up regulated in UBN leaves. 4-aminobutyric acid was higher expressed in UBN than KDML 105 under salt stress. It might suggest that at 24 hours of salt stress, UBN could maintain the regulation of glutamate decarboxylase to synthesis 4-aminobutyric acid better than in KDML 105. 4-aminobutyric acid is one of the important osmoprotectants and it could be a precursor for synthesis intermediate metabolite in TCA cycle. The lower regulation of glutamate decarboxylase in KDML 105 might describe the pre-respond against salinity stress in KDML 105 at an initial stage and its regulation of glutamate decarboxylase was declined at 24 hours. The

decreases of succinic acid in UBN suggested the changes in TCA cycle that might cause by the continual responses against salinity stress in UBN.

The relative maps of proline biosynthesis in KDML 105 and UBN represented the up regulation of Pyrroline-5-carboxylate reductase in both cultivars**.** Proline was not only act as an osmoprotectants, it was also occurred as feedback inhibitor of glutamate and precursor for metabolites formation in glutamate and polyamine groups. In this study, there was no significant change in proline levels under stress in both cultivars at 24 hours. Nevertheless, at 24 hours ornithine and putrescine were changed their accumulation levels that might be used proline as a precursor. The increases in expression of Pyrroline-5-carboxylate reductase ewithout the significantly changes in proline levels in both cultivars might suggest the time-dependent for biosynthesis of this metabolites, in which proline was synthesizing by Pyrroline-5-carboxylate reductase but the translation or post-translational modification was not completed due to the time-dependent at all expression stages. On the other hand, the rapidly decreases of proline concentration was occurred due to the exceeding of proline concentration could cause to the production of ROS, induced cellular apoptosis and cell death. In this case, proline might rapidly up and down-accumulated before 24 hours since rice would attempt to retain cellular equilibrium under salinity stress.

According to the biosynthesis of lumichrome, it was suggested the biosynthesis of lumichrome through riboflavin pathway. Riboflavin synthase (Os12g35580) was significantly up regulated in KDML 105 with no significant change in its accumulation level, whereas it was found the up regulation of riboflavin synthase with the increases of lumichrome levels in UBN. The results suggested that the high accumulation of lumichrome in UBN was possibly feedback inhibitor of its own synthesis pathway. The carbon assimilation in plant can be stimulated by the accumulation of Lumichrome, which was reported to promote root respiration and plant growth (Khan et al. 2008). However, the clearly functions of this metabolites during salinity stress have not yet been clear investigated. The higher express of riboflavin synthase in UBN than KDML 105 could suggest that UBN was responded against salinity stress through the alteration of this pathway in which lumichrome was

successfully post-translated and accumulated in leaf tissue, while in KDML 105, these metabolite was not changed caused by either its unsuccessful synthesized or it was not simulated during salinity stress.

4.8 The metabolite profiles of KDML 105 and UBN under salinity stress analyzed by LC-MS/MS

The metabolite profiles which constructed by LC-MS/MS technique did not shown the significant changes in accumulation levels exceed two folds in both leaf and root tissues. PLA-DA analysis of secondary metabolites in two cultivars at control condition illustrated the unclear separated metabolites in both leaves and roots. At 12 hours, metabolite profiles in leaves were also showed ambiguous separations in control plant and salinity-treated plants. Despite metabolite profiles of salinity treated group of UBN leaves at 24 hours were separated from other clusters, metabolite profiles of KDML 105 control, KDML 105 treatment and UBN control were also clustered in the same region. The separation of metabolite clusters in PLS-DA score plot were corresponded to the accumulated fold changes of secondary metabolites in which there was only ferulic acid which were up accumulated in UBN root (1.80-folds). At 24 hours, the separation of metabolite cluster in leaves and roots of two cultivars remained un-separated. The results suggested that in two weeks old rice seedlings in this study, the secondary metabolites were not much affected by salinity stress. However, we found the changes in accumulation levels of metabolites in groups of glucoside, glucopyranoside, carlinoside and oryzafuean in leaves and roots of UBN after salinity stress, although their accumulation fold changes did not exceed two folds.

The secondary metabolite grouped in glucosides is regularly revealed the roles as the enhancement of water solubility and reduction of chemical reactivity and can be found in both storage and transport forms (Chong 2002). Some of glucosides such as 4-*O*-β-D glucoside has been considered as transports forms of lignin monomers and related to specific mechanisms against pathogen attack (Whetten et al. 1998). Carlinoside and neocarlinoside were grouped into the eight C-

glucosylflavonoids in rice. From the results in our study, it was indicated that secondary metabolites might not be affected by salinity stress. On the other hands, secondary metabolites were reported as the involvement in biosynthesis of plant signal transduction under normal and stress conditions (Wasternack and Hause 2013), plant responses to biotic stress (Whetten et al. 1998) and plants resistance against the disadvantageous effects of predators and herbivores (Mazid et al. 2011). A secondary metabolite is defined as a compound in which its biosynthesis was restricted to the selected plant groups, and normally has a role to prevent plants from insects, predators, herbivores and other biotic stresses (Pichersky and Gang 2000). Thus, further experiment for clearly investigation and understanding of secondary metabolites in rice under salinity stress is needed to be performed.

Rice, in responses to salinity stress, the indispensability of metabolic control is the regulation of primary metabolites. As in all organisms, the regulation of primary metabolites is a major factor in survival to various environmental systems. The regulatory mechanism naturally involved to regulate the synthetic and execution of hundreds of enzymes in primary metabolites. It could allow rice and other plant species to limit nutrition and resources necessary for growth and reproduction. Some important mechanisms such as the substrate induction, feedback inhibition and ATP regulation are required. Under salinity stress, rice both KDML 105 and UBN induced the major changes in regulatory mechanisms of glycolysis and TCA cycles in order to govern carbon skeleton and energy charge through ATP, NADH and NADPH. The imperative of those regulations are a dynamic process based on the concentration of effective molecules in each plants (Drew 1977). Additionally, primary metabolisms under salinity stress in rice related to the synthetic of several amino acids, especially the production of several different growth substrates including carbon or nitrogen containing compounds. As the results shown the altering in accumulation levels of amino acids in aspartate, BCAAs, and glutamate families in which for the recruitment of the appropriate substrates and therefore probably involved in the energy state of cells. Some of primary metabolites grouped in osmoprotectants such as proline, 4-aminobutyric acid, and sugars (arabinose, fructose, raffinose, and ribose) were also up

accumulation for protecting cellular membranes and organelles against cellular oxidative stress.

The response for stimulatory additives of secondary metabolites generally resembles to the enzymatic induction. Secondary metabolite was reported as the effect from its precursor and as the inducer of enzyme biosynthetic for the specific roles in severely stresses (Drew 1977). In this study, the salinity stress in 80 mM NaCl for 24 hours only induced the enhancement in primary metabolites more than secondary metabolites due to this stress condition was not harsh enough to induce the accumulation of secondary metabolites. The study of secondary metabolite accumulations in *Catharanthus roseus* reported that the salinity treated plants in 100 mM NaCl for 30, 45, 60, 75 and 90 days after sowing provided the higher alkaloid content for each condition of higher NaCl treated plants (Jaleel et al. 2007) (Jaleel, 2008). It could be summarized that salinity treated plant with high concentration of salt solution could increase its production of secondary metabolites. However, the detailed explorations of secondary metabolites in response to salinity stress have not been yet specified for the suitability of plants on the comprehensive modification in diversified saline condition.

CHAPTER V

CONCLUSIONS

1. From the preliminary studies of physiological changes, antioxidant enzyme activities, total chlorophyll content, and gene expression by semi-quantitative RT-PCR in KDML 105 and UBN, could summarize that UBN have the primarily responses against salinity stress better than KDML 105.
2. PLS-DA score plot and loading plot analysis in KDML 105 and UBN showed the shifts in metabolite groups under salinity stress that were different among two cultivars.
3. Primary metabolites were up-accumulated in UBN at 12 hours, and those metabolites were then down-accumulated at 24 hours under salinity stress. In contrast, primary metabolites in KDML 105 showed the up accumulation at 24 hours. The results suggested that UBN responded to salinity stress faster than KDML 105.
4. The expression of genes involved in the biosynthesis of galacturonic acid, 4-aminobutyric acid, proline and lumichrome were affected by salt stress. The genes encoding glutamate decarboxylase and pyrroline-5-carboxylate reductase, which involved in the osmoprotectant biosynthesis, were significantly higher expressed during salinity treatment at 24h in UBN than KDML 105. While the gene encoding riboflavin synthase was significantly lower expressed in KDML 105 than UBN. The results indicated the better in transcripts of osmoprotectants in UBN compared to KDML 105.
5. The secondary metabolites of two-weeks old rice seedlings were not changed in their accumulation levels exceed two folds. It could be summarized that the stress duration for 24 hours was not affected the changes in secondary metabolites.

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APPENDIX

APPENDIX A

Protocols

1. mRNA extraction by RNeasy Plant Mini Kit

1. Disrupt a maximum of 100 mg plant material by immediately place tissue in liquid nitrogen. Grind thoroughly. Decant tissue powder and liquid nitrogen into RNA-free microcentrifuge tube.
2. Add 450 µl buffer RLT to a maximum of 100 mg tissue powder. Vortex vigorously.
3. Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube. Centrifuge for 2 min at full speed. Transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet.
4. Add 0.5 volume of ethanol (96-100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 5.
5. Transfer the sample (usually 650 µl), with any precipitate, to an RNeasy Mini spin column (pink) in a 2 ml collection tube (supplied). Close the lid, and centrifuge for 15 s at ≥ 8000 x g (≥ 10,000 rpm). Discard the flow-through.
6. Add 700 µl buffer RW1 to the RNeasy spin column. Close the lid, and centrifuge for 15 s at 8000 x g. Discard the flow-through.
7. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 15 s at 8000 x g. Discard the flow-through.
8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 2 s at 8000 x g. Discard the flow-through.

   Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied). Centrifuge at full speed for 1 min to dry the membrane.
9. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30-50 µl RNeasy-free water directly to the spin column membrane. Close the lid, and centrifuge for 1 min at ≥ 8000 x g to elute the RNA.

10. If the expected RNA yield is › 30 µg, repeat step 9 using another 30-50 µl of RNase-free water. Alternatively, use the eluate from step 9 (if high RNA concentration is required). Reuse the collection tube from step 9.

2. cDNA synthesis by iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad)

1. prepare the following reaction mixture in 0.2 ml tube, *** do not add iScript RT supermix until step 3**

Reagent		
iScript RT supermix	4	µl
nuclease-free water	x	µl
RNA template (up to 1 µg total RNA)	x	µl
Total volume	20	µl

2. incubate the reaction mixture without iScript RT supermix at 65 ˚C for 5 minutes, chill on ice immediately for 1 minute
3. Add iScript RT supermix and incubate at the following temperature program in thermal cycler:

   Priming 25 ˚C 5 minutes

   Reverse transcription 42 ˚C 30 minutes

   RT inactivation 85 ˚C 5 minutes

   Hold 4 ˚C
4. Place cDNA at –20 ℃ for long-term storage

3. Preparation for agarose electrophoresis

1. Buffer used in agarose gel electrophoresis

5X Loading buffer

- Bromophenol blue 10 mg
- Sucrose 1 g
- Gel Red 15 µl
- Dissolve in DI water 5 ml

10X TBE buffer

- Tris-base 108 g
- Boric acid 55 g
- 0.5 M EDTA (pH 8.0) 40 ml
- Dissolve in DI water 1 L

APPENDIX B

Metabolite changes in salinity stress

Table 15 Metabolite changes of salinity-treated rice KDML 105 and UBN in leaves compared to control condition. (12 hours) (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change	
		KDML	UBN
Glycolysis	Glucose	ns	3.01**
TCA cycle	Citrate	1.34*	1.29**
	Isocitric acid	1.38*	ns
	Malic acid	ns	1.26*
sugar and polyols	Arabinose	ns	2.25**
	Fructose	ns	2.34**
	Galactinol	2.01*	ns
	Inositol	1.31*	1.32**
	Ribose	ns	2.51**
Amino acids	Arginine	ns	1.94*
	Asparagine	6.18*	3.16**
	Aspartic acid	1.34***	1.44***
	Beta-alanine	2.03***	4.99***
	3-cyanoalanine	ns	5.60**
	Glutamate	1.49***	1.29***
	Glutamine	2.37**	2.47**
	Histidine	2.55**	4.30**
	Isoleucine	2.64**	2.17**
	Leucine	3.52***	6.68***
	Lysine	2.60**	4.51***
	Methionine	2.62**	3.25**
	Phenylalanine	2.52***	3.20**

Table 16 (Continued) Metabolite changes of salinity-treated rice KDML 105 and UBN in leaves compared to control condition. (12 hours) (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change	
		KDML	UBN
Amino acids	Serine	1.61**	ns
	Threonine	1.42**	1.38*
	Tryptophan	2.45***	3.37***
	Tyrosine	1.86***	1.97**
	Valine	2.94***	3.19***
Others	Dihydrouracil	ns	1.63**
	Fumaric acid	ns	-1.70**
	Glyceric acid	ns	1.44**
	Hexadecanoic acid	1.12*	ns
	Lumichrome	3.90***	ns
	Malic acid	ns	1.26*
	(3R)-3-Methyl-1,4-bis(trimethylsilyl)piperazine-2,5-dione	1.30***	ns
	Nicotinamide	-2.39*	ns
	Octadecadienoic acid, 9,12-(Z,Z)-, n-	-1.50*	1.60*
	Pentasiloxane, dodecamethyl	-1.50***	ns
	Piperidin-2-one, 3-amino-	4.35***	ns
	Pyroglutamate	1.57***	1.38***
	Quinic acid	-2.24***	ns
	Shikimic acid	-1.61**	ns
	Tetrasiloxane, decamethyl-	1.60**	ns
	Threonic acid	-1.38**	-2.77***
	Threonic acid-1,4-lactone	ns	-3.08**
	Tocopherol,alpha-	ns	-1.69***

Table 17 Metabolite changes of salinity-treated rice KDML 105 and UBN in leaves compared to control condition. (24 hours) (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	24h Fold change	
		KDML	UBN
Glycolysis	Glucose	1.51*	ns
TCA cycle	Isocitric acid	1.45***	1.37*
	Malic acid	1.67***	ns
	Succinic acid	ns	-1.40**
Sugar and polyols	Arabinose	1.48**	ns
	Beta-sitosterol	ns	-1.25**
	Campesterol	-1.42**	-1.30**
	Cellotriose	4.92*	ns
	Fructose	1.60*	ns
	Galactinol	8.14***	2.19**
	Inositol	1.29**	1.33**
	Inositol-1-phosphate	1.14**	ns
	Melibiose	3.05**	ns
	Phytol	ns	-1.33*
	Raffinose	5.72***	2.65**
	Ribose	ns	2.51**
	Stigmasterol	-1.14*	-1.20*
Amino acids	Alanine	-2.07***	ns
	4-aminobutyric acid	5.61***	2.08*
	Asparagine	ns	2.97**
	Aspartic acid	ns	1.60**
	Beta-alanine	2.64***	1.86**
	3-cyanoalanine	6.22**	5.62***
	Cysteine	ns	-1.13*
	Glutamate	1.27**	1.32**

Table 18 (Continued) Metabolite changes of salinity-treated rice KDML 105 and UBN in leaves compared to control condition. (24 hours) (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	24h Fold change	
		KDML	UBN
Amino acids	Histidine	8.89*	3.33**
	5-hydroxytryptamine	-2.56*	ns
	Isoleucine	9.53***	3.34***
	Leucine	9.23***	2.33**
	Lysine	9.62***	3.31***
	Methionine	8.59**	1.89*
	Phenylalanine	7.39***	2.01**
	Threonine	3.99***	2.14***
	Tryptophan	5.37**	3.23***
	Tyrosine	4.82***	2.02***
	Serine	2.76***	2.01***
	Valine	6.26***	2.70***
Others	Allantoin	ns	1.53**
	Galacturonic acid	-8.37***	-3.27*
	Gluconic acid	1.72**	ns
	Gluconic acid-1,5-lactone	6.56***	ns
	Glutaric acid	4.74***	ns
	Glyceric acid	-1.32*	ns
	Hexadecanoic acid	1.13***	1.14***
	Hydroxylamine	1.39**	ns
	Lactic acid	2.56*	ns
	Lumichrome	ns	6.09**
	(3R)-3-Methyl-1,4-bis(trimethylsilyl)piperazine-2,5-dione	1.37***	1.33**
	Nicotianamine	ns	1.30*
	Nicotinate	1.34**	ns

Table 19 (Continued) Metabolite changes of salinity-treated rice KDML 105 and UBN in leaves compared to control condition. (24 hours) (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	24h Fold change	
		KDML	UBN
Others	Octadecatrienoic acid, 9,12,15-(Z,Z,Z)-, n-	1.41*	ns
	Ornithine	ns	2.07*
	2-oxoglutaric acid	ns	2.26*
	Pentasiloxane, dodecamethyl	-1.63**	1.67***
	Putrescine	-1.39***	-1.41*
	Pyroglutamate	1.25***	1.31**
	Quinic acid	-3.30***	-2.60*
	Shikimic acid	-1.57**	-1.70*
	Suberic acid	ns	-4.49*
	Tetrasiloxane, decamethyl-	1.49***	1.37*
	Threonic acid	1.95***	-1.56***
	Threonic acid-1,4-lactone	-1.56***	ns

Table 20 Metabolite changes of salinity-treated rice KDML 105 and UBN in roots compared to control condition. (12 hours) (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change	
		KDML	UBN
Glycolysis	Fructose-6-phosphate	ns	-1.90***
	Glucose-6-phosphate	ns	-2.08**
	Glucose	ns	-1.64**
TCA cycle	Citrate	27.79***	28.43***
	Isocitric acid	16.86***	30.81***
	Fumarate	ns	-1.51**
	Malic acid	2.81***	2.70***
Sugar and polyols	Arabinose	ns	-1.57**
	Campesterol	ns	1.29*
	Fructose	-4.92***	-7.58***
	Galactinol	4.87***	2.49**
	Phytol	ns	2.96**
	Ribose	2.29*	ns
	Sucrose	2.38***	ns
Amino acids	Arginine	ns	-1.99***
	4-aminobutyric acid	2.94*	-2.36***
	Asparagine	3.19**	1.72*
	Aspartic acid	2.13***	1.72***
	Beta-alanine	3.44*	6.19***
	3-cyanoalanine	1.52**	ns
	Cysteine	1.58**	ns
	5-hydroxytryptamine	ns	2.45*
	Glutamate	1.43***	ns
	Glutamine	2.87**	ns
	Glycine	1.57*	ns

Table 21 (Continued) Metabolite changes of salinity-treated rice KDML 105 and UBN in roots compared to control condition. (12 hours) (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change	
		KDML	UBN
Amino acids	Isoleucine	1.88**	1.42**
	Leucine	ns	1.83***
	Phenylalanine	1.21*	ns
	Proline	1.73**	ns
	Serine	1.71**	ns
	Threonine	1.47*	ns
	Tryptophan	3.80*	ns
	Tyrosine	1.40**	-1.49**
	Valine	1.95**	1.37**
Others	Caffeic acid, trans-	ns	-1.32*
	Dihydrouracil	1.82**	ns
	Galacturonic acid	ns	-2.49**
	Glycolic acid	ns	-1.61**
	Hexadecanoic acid	1.15*	-1.17***
	Inositol-1-phosphate	1.57**	-1.51**
	Lumichrome	ns	-2.72***
	(3R)-3-Methyl-1,4-bis(trimethylsilyl)piperazine-2,5-dione	1.68**	ns
	Nicotianamine	ns	-4.61**
	Nicotinamide	-4.32*	ns
	Ornithine	ns	2.69***
	2-oxoglutaric acid	1.38*	ns
	Pentasiloxane, dodecamethyl	ns	-1.52***
	Piperidin-2-one, 3-amino-	ns	-1.57**
	Putrescine	ns	-2.47***
	Pyroglutamate	1.71**	ns

Table 22 (Continued) Metabolite changes of salinity-treated rice KDML 105 and UBN in roots compared to control condition. (12 hours) (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change	
		KDML	UBN
Others	Quinic acid	3.49***	ns
	Shikimic acid	3.37***	1.60**
	Spermidine	ns	-1.43*
	Tetracosanoic acid, n-	ns	-1.42***
	Tetrasiloxane, decamethyl	1.66***	-1.93***
	Threonic acid-1,4-lactone	2.50**	ns

Table 23 Metabolite changes of salinity-treated rice KDML 105 and UBN in roots compared to control condition. (24 hours) (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change	
		KDML	UBN
Glycolysis	F6P	-1.74**	-1.38*
	G6P	-1.95*	-1.77***
	Glucose	-1.82*	-2.22**
TCA cycle	Citrate	4.12**	5.77***
	Isocitric acid	4.43**	6.92***
	Fumarate	-1.31*	ns
	Malic acid	ns	2.21*
Sugar and polyols	Beta-stigmasterol	-1.21*	ns
	Beta-sitosterol	-1.33**	ns
	Fructose	ns	-2.11**
	Galactinol	3.82***	6.26***
	Inositol	1.63***	1.29**
	Raffinose	7.34**	ns
	Ribose	2.28*	4.30***
	Trehalose	6.85**	ns
Amino acids	Alanine	3.04**	ns
	Arginine	1.77*	ns
	Asparagine	2.68*	5.18***
	Aspartic acid	1.57**	2.41***
	Beta-alanine	2.49*	7.06***
	3-cyanoalanine	1.63*	ns
	Glutamate	1.58***	1.47**
	Glutamine	ns	2.12*
	Histidine	2.23*	ns
	Isoleucine	2.09**	1.49**

Table 24 (Continued) Metabolite changes of salinity-treated rice KDML 105 and UBN in roots compared to control condition. (24 hours) (P : ***<0.001, **< 0.01, *<0.05, ns : non significantly changes)

Categories	Metabolites	12h Fold change	
		KDML	UBN
Amino acids	Leucine	2.13**	1.94***
	Lysine	2.33**	1.55*
	Methionine	4.60*	ns
	Proline	2.47***	4.83***
	Serine	1.99**	1.88**
	Threonine	1.89**	1.70*
	Tryptophan	4.06**	ns
	Tyrosine	1.30**	ns
	Valine	1.77***	2.02***
Others	Dihydrouracil	ns	2.63**
	Hydroxylamine	1.18*	ns
	Lactic acid	1.94**	ns
	(3R)-3-Methyl-1,4-bis(trimethylsilyl)piperazine-2,5-dione	1.86**	ns
	Ornithine	ns	2.36*
	Pentasiloxane, dodecamethyl	-1.65**	ns
	Piperidin-2-one, 3-amino-	1.73*	1.90**
	Putrescine	ns	-2.25**
	Pyroglutamate	ns	1.59*
	Ribitol	ns	27.36*
	Shikimate	1.63**	ns
	Spermidine	-1.32*	ns
	Tetracosanoic acid, n-	-1.30*	ns
	Threonic acid-1,4-lactone	-1.60*	ns

VITA

Miss Kwankao Karnpakdee was born on May 24th, 1988. She is a first child of her family and grew up in Sing Buri. When she was eleven years old, she started her high school study at Princess Chulabhorn's Collage where is a boarding school in Pathumthani. She graduated in Bachelor degree in Industrial Chemistry from King Mongkut's University of Technology North Bangkok. She continued her study for the degree of Master of Science at the Department of Biochemistry, Chulalongkorn University since 2011. During those years, she studied the metabolomics of two rice cultivars and participated in related project such as the study of metabolomics in brassica spouts. She got the supporting funds from the National Research Council of Thailand and the Thai Kem Khang scholarship. She plans to pursue her PhD. In the field of bioenergy production from crops.