

**MULTIVARIATE PHYSIOLOGICAL RESPONSES OF INDICA  
RICE (ORYZA SATIVA L. SPP. INDICA) TO SALT STRESS AS  
EFFECTIVE INDICES FOR SALT-TOLERANT SCREENING**

**THANAPHOL BORIBOONKASET**

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR  
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MAHIDOL UNIVERSITY**

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ABSTRACT

Soil salinity is an abiotic stress, which directly limits crop productivity, especially in rice. The development of salt tolerant indicators is the most important need in breeding programs. The objective of this research was to develop an effective indicator of pigment stability and PSII maintenance in salt-stressed seedlings for salt tolerant screening. Salt-tolerant [Pokkali (Pok) and Homjan (HJ)] and salt-sensitive [IR29 and Pathumthani 1 (PT1)] rice cultivars were divided into two groups, including rice seedlings with or without root systems grown under 0 (control) or 342 mM NaCl (salt stress) for 4 days. The results showed that osmolarities of rice seedlings with a root system were lower than those of seedlings without a root system, especially in salt-tolerant varieties. An increasing osmolarity was positively related to the degradation of photosynthetic pigments, resulting in the diminution of  $F_v/F_m$ ,  $\Phi_{PSII}$  and  $qP$ . In addition, chloroplast ultrastructures of salt stressed seedlings were damaged through the swelling of thylakoid and granum. Multivariate parameters of pigment degradation and chlorophyll a fluorescence diminution were fruitfully utilized to classify the salt tolerant group, HJ and Pok, and salt sensitive group, IR29 and PT1, using Hierarchical cluster analysis. It should be noted that salt-stressed seedlings with a root system were accurately evaluated for salt-tolerant identification. The effective indices were applied to classify 9 lines BC<sub>1</sub>F<sub>2</sub> population. There were classified into two classes, a salt tolerant class, (line 21) and a salt sensitive class, lines (2, 18, 20, 26, 31, 306, 409 and 598). The results indicate that the multivariate parameters can effectively identify the salt tolerant varieties in the large population of breeding programs.

KEY WORDS: RICE / ROOT SYSTEM / PSII / SALT TOLERANT SCREENING

64 P.

การตอบสนองทางสรีระวิทยาแบบสหสัมพันธ์ของข้าวอินดิคาต่อสภาวะเครียดจากความเค็มเพื่อใช้เป็นดัชนีชี้วัดที่เหมาะสมในการคัดเลือกลักษณะทนเค็ม

(MULTIVARIATE PHYSIOLOGICAL RESPONSES OF INDICA RICE (ORYZA SATIVA L. SSP. INDICA) TO SALT STRESS AS EFFECTIVE INDICES FOR SALT-TOLERANT SCREENING)

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บทคัดย่อ

ดินเค็มเป็นสภาวะเครียดทางกายภาพ ที่มีผลโดยตรงต่อการลดลงของผลผลิต โดยเฉพาะในข้าว การพัฒนาดัชนีชี้วัดความทนเค็มจึงเป็นการกำหนดเกณฑ์สำคัญในการปรับปรุงพันธุ์ข้าวทนเค็ม ในการทดลองนี้มีวัตถุประสงค์เพื่อพัฒนาดัชนีชี้วัดความทนเค็มในการคงสภาพของรงควัตถุและการทำงานในระบบการสังเคราะห์แสงระบบที่ 2 ในกล้าข้าวที่ได้รับเกลือเพื่อใช้คัดเลือกสายพันธุ์ข้าวทนเค็ม ต้นกล้าข้าวพันธุ์ทนเค็ม (Pokkali และ หอมจันทร์) และต้นกล้าข้าวพันธุ์ไม่ทนเค็ม (IR29 และ ปทุมธานี 1) ถูกแบ่งออกเป็น 2 กลุ่ม ได้แก่ กลุ่มที่มีหรือกลุ่มที่ไม่มีระบบราก เพราะเลี้ยงภายใต้สภาวะที่ได้รับเกลือโซเดียมคลอไรด์ความเข้มข้น 0 หรือ 342 มิลลิโมลาร์ เป็นเวลา 4 วัน ผลการทดลองพบว่าค่าออสโมลาริตีของต้นกล้าข้าวที่มีระบบรากมีค่าต่ำกว่ากล้าข้าวที่ไม่มีระบบรากโดยเฉพาะอย่างยิ่งในพันธุ์ทนเค็มการเพิ่มขึ้นของค่าออสโมลาริตีมีความสัมพันธ์เชิงบวกกับการสลายตัวของรงควัตถุ ซึ่งส่งผลโดยตรงต่อการลดลงของ  $F_v/F_m$ ,  $\Phi_{PSII}$  และ  $qP$  นอกจากนี้โครงสร้างของคลอโรพลาสต์ของข้าวในกลุ่มที่ได้รับเกลือจะถูกทำลาย เช่น เกิดการบวมของ thylakoid และ granum เป็นต้น การสลายตัวของรงควัตถุและการลดลงของ chlorophyll a fluorescence ถูกใช้ในการจัดจำแนกกลุ่มข้าวทนเค็ม คือ พันธุ์ Pokkali และหอมจันทร์ และกลุ่มข้าวไม่ทนเค็ม คือ พันธุ์ IR29 และ ปทุมธานี 1 โดยใช้ Hierarchical cluster analysis จากผลการทดลองสามารถสรุปได้ว่ากล้าข้าวที่มีระบบรากให้ผลการจัดกลุ่มข้าวพันธุ์ทนเค็มที่แม่นยำ ซึ่งดัชนีชี้วัดความทนเค็มที่มีประสิทธิภาพสูงถูกนำมาใช้ในการคัดเลือกประชากรข้าวพันธุ์ผสมกลับ ( $BC_1F_2$ ) จำนวน 9 สายพันธุ์ พบว่าสามารถจัดจำแนกข้าวได้เป็น 2 กลุ่มคือ ข้าวทนเค็มสายพันธุ์ 21 และข้าวไม่ทนเค็มสายพันธุ์ 2 18 20 26 31 306 409 และ 598 จากผลการทดลองชี้ให้เห็นว่าการใช้พารามิเตอร์หลายตัวแปรน่าจะนำไปใช้ในการจัดจำแนกสายพันธุ์ข้าวทนเค็มในโปรแกรมการปรับปรุงพันธุ์อย่างมีประสิทธิภาพ

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## LIST OF ABBREVIATIONS

Ø	diameter
ABA	abscisic acid
APX	ascorbate peroxidase
ATP	adenosine triphosphate
BC <sub>1</sub> F <sub>2</sub>	one way backcross F <sub>2</sub> generation
bp	basepair
°C	degree Celsius
C <sub>x+c</sub>	total carotenoid
CAM	crassulacean acid metabolism
Chl	chlorophyll
Chl <sub>a</sub>	chlorophyll a
Chl <sub>b</sub>	chlorophyll b
C <sub>x+c</sub>	total carotenoid
CI	chlorophyll index
Cl <sup>-</sup>	chloride ion
CRD	Completely Randomized Design
cv.	cultivars
DMRT	Duncan's New Multiple Range Test
DPC	diphenylcarbazide
dS m <sup>-1</sup>	decisiemens per meter
EC	Enzyme Commission Number
EC <sub>e</sub>	electrical conductivity
e.g.	exempli gratia
<i>et al.</i>	et alli (Latin), and others

## LIST OF ABBREVIATIONS (Continued)

$F_v/F_m$	maximum quantum yield of photosystem II
g	gram
$g\ l^{-1}$	gram per litre
GR	glutathione reductase
h	hour
$h^{-1}$	per hour
$h\ d^{-1}$	hour per day
$H^+$	hydrogen ion
$H_2O$	water
kDa	kilo Dalton
$H_2O_2$	hydrogen peroxide
kb	kilobase
KDML 105	Khao Dawk Mali 105
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
$mmol\ kg^{-1}$	millimole per kilogram
$mmho\ cm^{-1}$	millimho per centimeter
MPa	Mega Pascal
$\mu g$	microgram
$\mu m$	micrometre
$\mu mol\ g^{-1}\ FW$	micromole per gram fresh weight
$\mu mol\ m^{-2}\ s^{-1}$	micromole per square metre per second
mRNA	messenger ribonucleic acid
$mol\ l^{-1}$	mole per litre
MS	Murashige and Skoog medium
$Na^+$	sodium ion
NaCl	sodium chloride

**LIST OF ABBREVIATIONS (Continued)**

NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
ng	nanogram
nm	nanometer
nM	nanomolar
nmol	nanomole
NPQ	non-photochemical quenching
ns	non significant difference
OH	hydroxyl group
$P_n$	net photosynthetic rate
PSI	photosystem I
PSII	photosystem II
qP	photochemical quenching
$\Phi_{PSII}$	quantum efficiency of photosystem II
s	second
SOD	superoxide dismutase
ssp.	subspecies
var.	variety

## **CHAPTER I**

### **INTRODUCTION**

Rice is a major crop in the world as carbohydrate sources, belonging to Poaceae family. It feeds more than half of the world's population, especially in Asia. Moreover, Thailand is the top five leader of rice exporter, especially Jasmine rice, which is well known as long-grain, high cooking quality and aromatic flavor. Presently, salinity stress is one of the most serious problems worldwide in agricultural areas reducing crop productivity, especially in rice. Salt-tolerant improvement is an alternative way to eliminate this important problem. In addition, effective indices for salt tolerant identification have been still developed for utility salt-tolerant screening on the mass population of breeding program.

Root system plays a key role during plant growth and development and this organ is the first part to encounter and attach underground environment especially soil salinity or salt contamination in the soil, which are composed of osmotic and ionic stresses. Firstly, an osmotic stress causes the low water potential and a consequent loss of cell turgor in roots. Secondly, an ionic stress induced by the  $\text{Na}^+$  and  $\text{Cl}^-$  accumulates in root tissues. These stresses produce a reduction in the water uptake or water availability resulting in low photosynthetic rate. Photosynthesis is a biochemical process, which is converted light energy into biochemical energy. It is composed of two systems, including light and dark reactions. In light reaction, there are two systems; photosystem II (PSII) and photosystem I (PSI). The PSII is a site of water oxidation and electron transportation to photosystem I. Moreover, the PSI is a site of biochemical energy production namely NADPH and ATP. Chlorophyll a fluorescence technique has been generally used to reveal intrinsic functions relating to the activity of PSII. This parameter has been effectively investigated as an effective index for salt tolerant screening in many crop species.

The main selection criteria in overall agronomical characters such as seedling growth, survival and grain yield are generally used in traditional plant breeding for salt tolerance. However, indicator development is obstructed by the limitation of salt

tolerant selection using overall agronomic traits and lack of effective evaluation methods for salt tolerance among genotypes. Therefore, the phenotypic responses under salinity stress, especially in photosystem II photochemistry, which is an alternative way to develop effective indices for rice salt tolerant screening in breeding programs.

## CHAPTER II

### LITERATURE REVIEW

#### 1. Rice and the diversity of rice

Rice is a monocotyledon plant, which classified in family *Poaceae*, subfamily *Pooideae*, tribe *Oryzeae*. It has been cultivated as a major crop for about 11,500 years, and currently sustains nearly one-half of the world population (Wu *et al.*, 2004). The two rice cultivated varieties including, *Oryza sativa* and *Oryza glaberrima*. These plants are native to tropical and subtropical; in southern and southeastern Asia and in Africa. The cultivated Asian rice species, *O. sativa*, is composed of two subspecies, *indica* and *japonica* (Oka, 1988). Cultivation species are diploid ( $2n = 24$ ), whereas wild species are diploid or tetraploid. The most cultivated rice, *O. sativa* L., is an annual cereal crop with long leaves bearing an inflorescence (panicle) composed of spikelets with flower producing the seed or grain. This specie can be divided by the habitat into 3 subspecies such as *indica*, *japonica*, and *javanica*. Mostly *indica* subspecies are grown in India, Southern China, South America and Thailand. Whereas, *japonica* subspecies likely originated in China which is grown in temperate region of the world, such as Southern Europe and Japan and *javanica* subspecies are primarily grown in Indonesia, Malaysia and Philippines, located in the rice terraces of the Philippines and the mountainous regions of Madagascar (Christou, 1994). *Indica* rice is the predominant subspecies in the world because of its properties such as quality cooking and also the growing ability.

Many documents classified the rice in both species and cultivars levels. The salt tolerant and salt sensitive cultivars are announced on the database of IRRI (Akbar, 1986). The two essential parameters sufficient for expressing salt tolerance are threshold means the maximum allowable salinity without yield reduction and slope means the percent of yield reduction per unit increasing in salinity beyond the threshold. The threshold and slope of rice (*O. sativa* L.) are  $3.0 \text{ dS m}^{-1}$  and 12% per dS

$\text{m}^{-1}$  of saturated soil extract ( $\text{EC}_e$ ), respectively. Relative salt tolerance rice at 50% yield and at 50% emergence is  $3.6 \text{ dS m}^{-1}$  and  $18 \text{ dS m}^{-1}$  of  $\text{EC}_e$ , respectively. In *O. sativa* L. species are widely classified as the salt tolerant accession. Traditional cultivars are the most tolerance to abiotic stress. Cultivars Pokkali, Cherveruppu, Nona Bokra SR26B, Damodar and Getu are salt tolerant rice lines while cultivars IR28 and IR29 are salt sensitive lines. Furthermore, IRRI developed moderate salt tolerant rice lines such as IR4630-22-2-5-1-3, IR4595-4-1-13, and IR9884-54-3 which are the best combiners for salt tolerant progenies. As well as, IRRI developed new salt tolerant rice lines such as TCCP 266-2-49-B-B-3 and IR 65185-3B-8-3-2 which are vigorous growth and without lodging and increasing yield (Gregorio *et al.*, 2002).

## 2. Salt stress

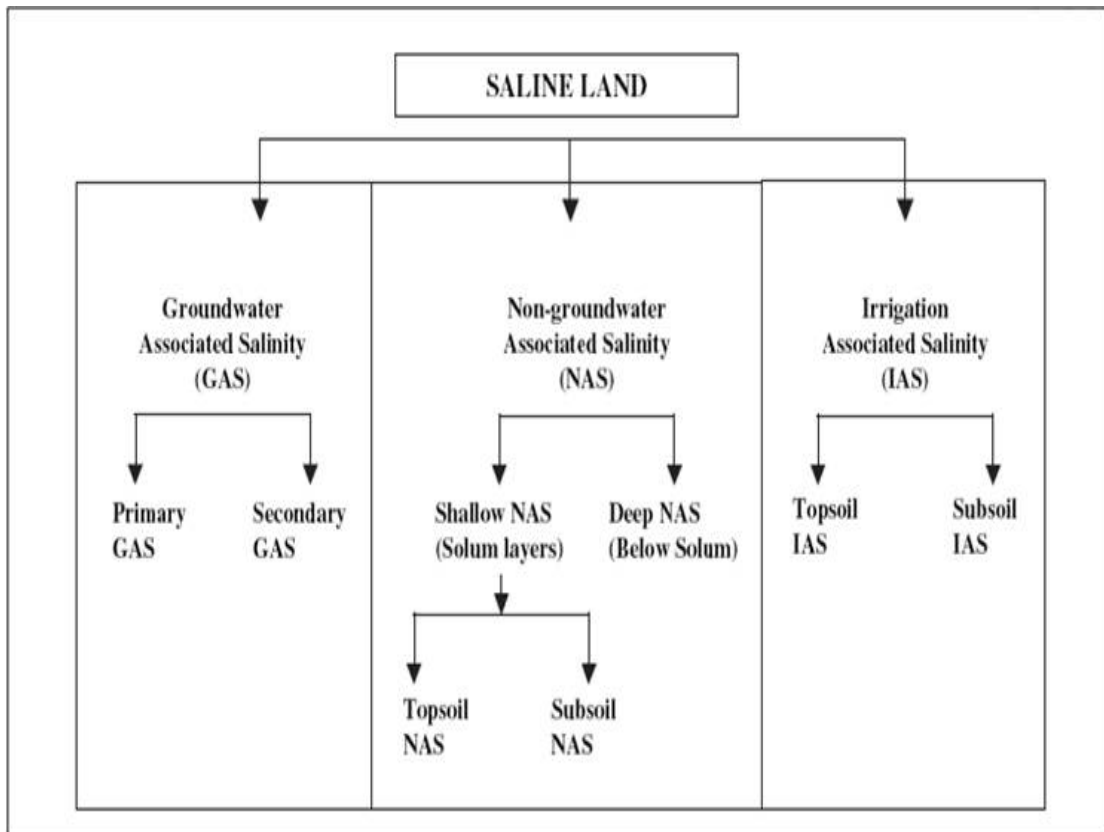
### 2.1 Global impact of salinity problem

The global distribution of saline soil in many continents (Table 1) was described by Szabolcs (1989). The total global area of salt-affected soils including saline and sodic soils is approximately 851 million hectares (Martinez-Beltran and Manzur, 2005), extending over all the continents including Africa, Asia, Australasia, and the Americas. The exact location and distribution of salt-affected soils have been studied in varying degrees of detail (Rengasamy, 2006). The dominant sources of salt are rainfall and rock weathering. Rainwater contains low amounts of salt, but over time, salt deposited by rain can accumulate in the landscape. Wind-transported (aeolian) materials from soil or lake surfaces are another source of salt. Poor quality irrigation water also contributes to salt accumulation in irrigated soils. Firstly, groundwater associated salinity (GAS), in discharge areas, water exits from groundwater to the soil surface bringing the salts dissolved in it. The driving force for upward movement of water and salts is evaporation from the soil plus plant transpiration. Generally, the water table in the landscape is at or closely to the soil surface and soil properties allow a maximum rate of water movement through the surface layers. Salt accumulation is high when the water table is less than 1.5 m below the soil surface. However, this threshold depth varies depending on soil hydraulic properties and climatic conditions. Secondly, non-groundwater associated salinity (NAS) in landscapes where the water table is deep and drainage is poor, salts which

are introduced by rain, weathering, and Aeolian deposits are stored within the soil solum. In drier climatic zones, these salt stores are usually found in the deeper solum layers. However, poor hydraulic properties of shallow solum layers lead to the accumulation of salts in topsoil and subsoil layers affecting agricultural productivity. In regions where sodic soils are predominant, this type of salinity is a common feature. Finally, irrigation associated salinity (IAS), salts introduced by irrigation water are stored within the root zone because of insufficient leaching. Poor quality irrigation water, low hydraulic conductivity of soil layers as found in heavy clay soils and sodic soils, and high evaporative conditions accelerate irrigation-induced salinity. Use of highly saline effluent water, improper drainage and soil management increase the risk of salinity in irrigated soils. In many irrigation regions, rising saline groundwater interacting with the soils in the root zone can compound the problem (Rengasamy, 2006).

**Table 1** Global distribution of saline and sodic soils (Szabolcs, 1989; Rengasamy, 2006)

Continent	Area (Million hectares)		
	Saline	Sodic	Total
North America	6.2	9.6	5.8
Central America	2.0	-	2.0
South America	69.4	59.6	129.0
Africa	53.5	27.0	80.5
South Asia	8.3	1.8	85.1
North and Central Asia	91.6	120.1	211.7
Southeast Asia	20.0	-	20.0
Europe	7.8	22.9	30.7
Australia	17.4	340.0	357.4
<b>Total</b>	351.5	581.0	932.2



**Figure 1** Major types of salinity in world soils based on salinization processes (Regasamy, 2006).

## 2.2 Effect of salt stress on rice

Salt stress can affect physiological processes from seed germination to plant development, resulting in growth and yield reduction (Ashraf, 2004). High salinity causes both hyperosmotic and hyperionic stress effects, and the consequence of these can be plant demise (Glen *et al.*, 1998; Nelson *et al.*, 1999; Yeo, 1998). Commonly, the stress is caused by  $\text{Na}^+$  and  $\text{Cl}^-$  concentration in the soil. Salt stress has three fold effects including it reduces water potential and causes ion imbalance or disturbances in ion homeostasis and toxicity (Parida and Das, 2005). The status bring about initial growth reduction, to inhibition of cell division and expansion, membrane disorganization, reactive oxygen species, metabolic toxicity and also inhibition of photosynthesis (Hasegawa *et al.*, 2000). The detrimental effects have been observed at the whole-plant level as death of plants or decrease in productivity (Abdullah *et al.*, 2001). Munns (2002) reported that during the initial phase to salinity, plants

constrained by water stress, which in turn reduced leaf expansion. During long-term exposure to salinity, plants restricted by ionic stress, which was in the lead to premature senescence of adult leaves, and thus a reduction in the photosynthetic area available to support continued growth. Reduced photosynthesis with increasing salinity attributed to either stomatal closure, leading to a reduction in intracellular CO<sub>2</sub> partial pressure, or non-stomatal factors (Bethke and Drew, 1992). The increasing evidences has showed that salinity change photosynthetic parameters, including osmotic and leaf water potential, transpiration rate, leaf temperature, and relative leaf water content. Salt also affect photosynthetic components such as enzymes, chlorophylls, and carotenoids. Changes in these parameters depend on the severity and duration of stress (Lakshmi *et al.*, 1996; Misra *et al.*, 1997) and plant species (Dubey, 1994), in addition, a considerable decrease in the fresh and dry weights of leaves, stems, and roots, and the increase of root/shoot ratio. This is attributed to the reduced numbers of seeds, spikelets, and tillers, as well as grain weight (Parida and Das, 2005). The CO<sub>2</sub> assimilation rate is reduced. As a result, chloroplasts are subjected to excess excitation energy leading to an increased rate of formation of reactive oxygen intermediates (Hare and Cress, 1997) such as peroxidase, ascorbate peroxidase (APX), superoxidase dismutase (SDS), and glutathione reductase (GR) (Vaidyanathan *et al.*, 2003). Moreover, salt stress has been shown to bring about an overall growth and productivity reduction by perturbing the functioning of vital components of photosynthesis like PSI, PSII and Rubisco (Apse and Blumwald, 2002). Rice roots, which are the first part of the plant to encounter soil salinity, also affected by NaCl. Roots of rice seedling grown in saline soils have to cope with osmotic stress and ionic stress. These stresses cause a reduction in water uptake and inhibition of root growth. Tsai *et al.* (2005) found that NaCl treatment elevated H<sub>2</sub>O<sub>2</sub> level and caused an increase of the activities of APX and GR and the expression of *OsAPX* and *OsGR* genes in rice roots. Furthermore, a number of salt stress-responsive proteins which involved in regulation of carbohydrate, nitrogen and energy metabolism, reactive oxygen species scavenging, mRNA and protein processing, and cytoskeleton stability are detected in roots of rice (*O. sativa* L. cv. Nipponbare) treated with 150 mM NaCl by two-dimensional gel electrophoresis (Yan *et al.*, 2005).

### **2.3 Salt stress defensive responses**

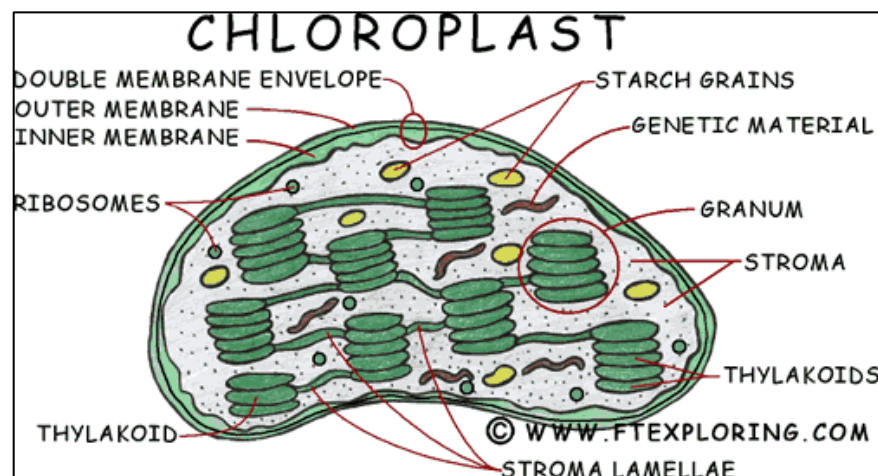
According to those suffer from salt stress, plants adapt to salt tolerance by many strategies. For biochemical ways such as, selective accumulation or exclusion of ions, control of ion uptake by roots and transporting into leaves, compartmentalization of ions at the cellular and whole-plant levels, synthesis of compatible solutes, change in photosynthetic pathway, alteration in membrane structure, induction of antioxidative enzymes, and induction of plant hormones; ABA have been investigated (Parida and Das, 2005). Rice seedlings can accumulated several compatible solutes e.g. glycinebetaine, polyamine, sugars, proline, and cyclic and non-cyclic polyols in response to salt stress. These compounds are known as compatible solutes that non toxic to rice cells even in high concentration, causing on salt tolerance (Ashraf and Harris, 2004; Dubey and Singh, 1999; Garcia *et al.*, 1997). Period of salinity stress affecting rice growth and yield components is highly dependent on plant phenology. Extensive efforts have been made in previous studies to identify differential sensitivity of rice plants to salinity at various growth stages. In common cultivated rice cultivars, young seedling and reproductive growth stages are very sensitive to root zone salinity affecting shoot dry weights and yield. Salinity during tillering stage reduces shoot dry weight as much as that one during mature stages in a field studies. When salinity is initiated at seedling stage, salinity-induced yield reduction was more than when initiated at the reproductive stage. According to, Lutts and colleagues (1995) reported that young seedling and reproductive stage are more sensitive to salinity than tillering stage. And also the studies of Heenan and colleagues (1988), salinity is initiated at the panicle stage initiation (PI) and relieved before booting and yield components are significantly reduced by salinity stress between these two stages (Zeng *et al.*, 2001).

## **3. Photosynthetic characters of rice under salt stress conditions**

### **3.1 Organization of photosynthetic electron transport system and function**

Photosynthesis is a biochemical process, which is found in algae, plant and some types of bacteria converting light energy to biochemical energy. Photosynthesis composes of 2 processes including light dependent and light independent process. The first process is the light dependent one (light reactions: Photosystem II), requires the

direct energy of light to make energy carrier molecules that are used next in the second process. The light independent process (dark reactions: Photosystem I) occurs when the products of the light reaction are used to form C-C covalent bonds of carbohydrates. The dark reactions usually occur in the dark, if the energy carriers from the light process are presented. Recent evidences suggest that a major enzyme of the dark reaction is indirectly stimulated by light, thus the term dark reaction is somewhat of a misnomer. The light reactions occur in the grana and the dark reactions take place in the stroma of the chloroplasts. The light energy is converted to biochemical energy by light-dependent reactions. The products are ATP from photophosphorelation and NADH from photoreduction. In plants and algae, light is absorbed by chlorophyll and carotenoid molecules that are bound the light-harvesting complex in the thylakoid membrane of chloroplast (Niyogi, 2000) (Figure 2).

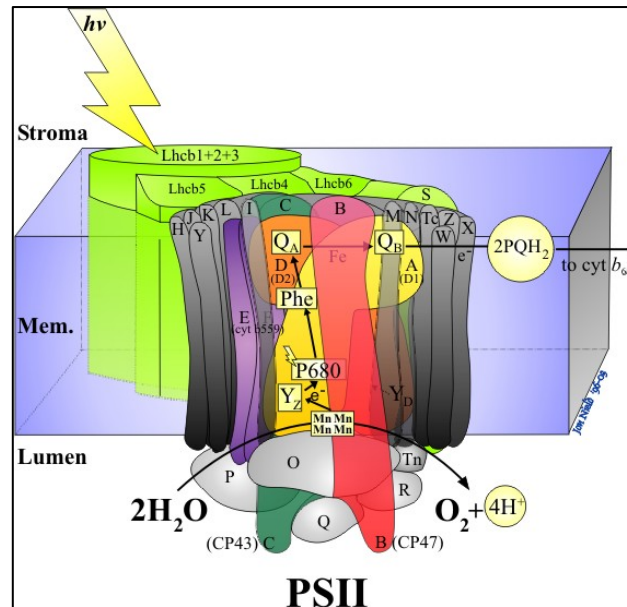


**Figure 2.** A schematic of plant chloroplast.

([www.bio.ic.ac.uk/research/barber/psIIimages/PSII.html](http://www.bio.ic.ac.uk/research/barber/psIIimages/PSII.html))

The excitation from light energy is converted to charge separation, which driving force the electron flow between photosystem II through photosystem I by cytochrome *b<sub>6</sub>f* complex. The result is the oxidation of water and input electron and proton to PS I, are used to convert CO<sub>2</sub> to biomass (Ferreira *et al.*, 2004). Photosystem II composed of more than 30 proteins subunits encoded by both nuclear and chloroplast genome (Yamamoto, 2001). The site of water oxidation in

photosynthesis light energy is captured by chlorophyll antenna and carotenoids molecule, transfer to reaction center of photosystem II (Figure 3).



**Figure 3.** A schematic diagrams showing the structure of the photosystem II complex. ([www.bio.ic.ac.uk/research/barber/psIIimages/PSII.html](http://www.bio.ic.ac.uk/research/barber/psIIimages/PSII.html))

### 3.2 Salt stress effects on photosynthesis and defense mechanisms

#### a) Photosynthetic pigments

In higher plants, chlorophyll content decreases in salt susceptible plants such as rice (Cha-um *et al.*, 2004a), tomato (Sudhir and Murthy, 2004), potato (Abdullah and Ahmed, 1990), pea (Hamada and El-Enany, 1994), and *Phaseolus vulgaris* (Seemann and Critchley, 1985). But chlorophyll content has been increased in salt tolerant plants such as pearl millet (Reddy and Vora, 1986), mustard (Singh *et al.*, 1990), and wheat (Kulshreshtha *et al.*, 1987). Content of carotenoids ( $C_{X+C}$ ) increased in rice plants under salt stress (Misra *et al.*, 1997) and decreased in black cumin (Hajar *et al.*, 1996). Similarly, various responses of chlorophyll content to salt stress has found in some cyanobacteria. In *Synechocystis* sp. PCC 6803, the chlorophyll a content at moderate (342 mM NaCl) concentration is increased while that at 684 or 1.026 mM NaCl is sharply decreased (Schubert and Hagemann, 1990; Schubert *et al.*, 1993). However, chlorophyll a content is steady in *Spirulina platensis* grown under

800 mM NaCl (Verma and Mohanty, 2000; Lu and Vonshak, 2002). In *Synechocystis* sp. PCC 6803, Schubert *et al.* (1993) found an increase in  $C_{X+C}$  content in response to high salinity (1.026 M NaCl), namely in echinenone, oscillaxanthin, and myxoxanthophyll, while  $\beta$ -carotene showed only small differences. This increased  $C_{X+C}$  content might diminish the amount of photons available for the absorption by chlorophyll a by shadowing and thus, irradiance can act as secondary stress factor. In cyanobacteria, phycobiliproteins that are attached to the stromal surface of thylakoid membranes serve as the primary light-harvesting antenna for photosystem II. The composition and function of phycobiliproteins in cyanobacteria change in response to stress conditions (Grossman *et al.*, 1993). Salt stress mainly decreases the content of phycocyanin and thereby interrupts the energy transfer from phycobiliproteins to photosystem II reaction centre (Schubert and Hagemann 1990, Schubert *et al.*, 1993; Lu *et al.*, 1999; Lu and Vonshak, 2002). Sodium stress due to the addition of NaCl,  $\text{NaNO}_3$ , and  $\text{NaHCO}_3$  caused decrease in the energy transfer from allophycocyanin to photosystem II in *S. platensis* (Verma and Mohanty, 2000).

#### **b) Photosynthetic electron transport reactions**

Salt stress increases the accumulation of NaCl in chloroplasts of higher plants or in the cytoplasm of cyanobacterial cells, affects growth rate, and is often associated with decrease in photosynthetic electron transport activities in photosynthesis (Boyer, 1976; Kirst, 1989). In higher plants, not only chlorophyll content decreased in salt susceptible plants such as salt cress (Vera-Estrella *et al.*, 2005), pea (Hamada and El-Enany, 1994) and rice (Wanichananan *et al.*, 2003; Chaum *et al.*, 2005) but also chloroplast has directly effect from salinity stress associated with induction oxidative stress (Hernandez *et al.*, 1995). Parida and co-worker (2003) reported that NaCl induced the changes in ultrastructure of halophyte chloroplast at 400 mM level by exhibited, elongated and compressed chloroplast with homogenous membrane distribution and loss of clear stomatal matrix. The results indicated that a probable loss of thylakoid membranes into grana. Moreover, salt stress inhibits photosystem II activity (Mishra *et al.*, 1991; Masojídek and Hall, 1992; Belkhodja *et al.*, 1994; Everard *et al.*, 1994, Singh and Dubey 1995; Tiwari *et al.*, 1997; Kao *et al.*, 2003; Parida *et al.*, 2003). In some studies, salt stress had no effect on photosystem II (Robinson *et al.*, 1983; Brugnoli and Björkman, 1992; Morales *et al.*, 1992). The

photosystem II inhibition under salt stress is characterized by Misra *et al.* (1999). In some reports, the photosystem II activity decreased in response to salt stress due to the dissociation of 23 kDa polypeptide extrinsically bound to photosystem II (Kuwabara and Murata, 1982; Miyao and Murata, 1984; Murata *et al.*, 1992). In cyanobacteria, various salt stress conditions stimulate the rate of respiration (Jeanjean *et al.*, 1993; Lu *et al.*, 1999) and photosystem I activity (Joset *et al.*, 1996), and impair the rate of photosynthesis (Vonshak *et al.*, 1988; 1995; Zeng and Vonshak, 1998). Salt stress inhibited photosystem II mediated oxygen evolution activity in *Synechocystis* sp. PCC 6803 (Schubert and Hagemann, 1990; Schubert *et al.*, 1993). Allakhverdiev *et al.* (2000) showed that the changes in K/N ratio inactivated both photosystem II and photosystem I in *Synechococcus* cells. Restoration of photosystem II activity by diphenylcarbazide (DPC), an artificial electron donor to photosystem II in salt treated cyanobacterial thylakoids, suggested that water splitting complex was the site of action of salt stress in *Synechococcus* cells (Allakhverdiev *et al.*, 2000). However, in some cyanobacteria the photosystem II reaction centre is the target for salt stress. Depending on the environment, both the water oxidation complex and photosystem II reaction centres (increase in the number of QB non-reducing sites) are targets for salt stress in *S. platensis* (Lu *et al.*, 1999; Lu and Vonshak, 2002). Recently, Allakhverdiev *et al.* (2002) reported for *Synechocystis* that the combination of light and salt stress inactivated photosystem II activity; particularly, salt stress inhibited the *de novo* synthesis of proteins, specifically the synthesis of D1 protein of photosystem II. In cyanobacteria and eukaryotic algae, salt stress increased electron transport activity of photosystem I (Gilmour *et al.*, 1985; Canaani, 1990). Upon a shift to high salt stress, the amount of P700 and photosystem I reaction centres increased in *Synechocystis* sp. PCC 6803 (Jeanjean *et al.*, 1993). This in turn caused increase of the cyclic electron transport around photosystem I. Fortunately, in recent years, the technique of chlorophyll a fluorescence is widely spread in plant ecophysiology studies especially in PS II photochemistry (Maxwell and Johnson, 2000). Chlorophyll fluorescence has been routinely used to monitor the photosynthetic performance of plants (Baker and Rosenqvist, 2004). More recently, the demonstrations that chlorophyll fluorescence measurements could be used to estimate, rapidly and non-invasively, the operating quantum efficiency of electron transport through PS II in leaves (Gently, 1989) and

this PS II operating efficiency is related to CO<sub>2</sub> assimilation (Gently *et al.*, 1989). Yang and Lu (2006) reported that salt stress resulted in the decrease in growth and leaf relative water content as well as net photosynthesis and the apparent quantum yield of photosynthesis. Stomatal conductance, evaporation rate, and water use efficiency decreased in salt-stressed plants. Salt stress also caused a decrease in the actual efficiency of PSII ( $\Phi_{\text{PSII}}$ ), the efficiency of excitation energy capture by open PSII reaction centers ( $F_v'/F_m'$ ), and the coefficients of photochemical quenching (qP) but caused an increase in non-photochemical quenching (NPQ). Salt stress showed no effects on the maximal efficiency of PSII photochemistry ( $F_v/F_m$ ).

### **c) Photophosphorylation and CO<sub>2</sub> fixation**

In cyanobacteria, salt stress increases the efficiency of photophosphorylation by stimulating the cyclic photosynthetic electron flow around PSI. Upon the addition of high amount of NaCl into growing medium, the activity of cyclic photophosphorylation increases in *Synechocystis* sp. PCC 6803 (Jeanjean *et al.*, 1993). The first step of photosynthetic CO<sub>2</sub> assimilation is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO; EC 4.1.1.39) in C<sub>3</sub> plants, and by phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) in C<sub>4</sub> plants. Salt stress enhances the oxygenase activity of RuBPCO while it curtails its carboxylase activity. In the halotolerant cyanobacterium *A. halophytica*, the content of RuBPCO and the rate of CO<sub>2</sub> fixation are increased in response to high salt stress (Takabe *et al.*, 1988). Echevarría *et al.* (2001) and García-Mauriño *et al.* (2003) reveal that PEPC activity is enhanced by salt stress.

### **d) Defense mechanisms**

Photosynthetic production can be influenced by a number of environmental factors such as temperature, nutrients, water, heavy metals, herbicides, radiation and salts (Muthuchelian *et al.*, 2003). The plant has to react physiologically at least to four major constraints for plant growth on saline substrates (Marschner, 1995; Volkmar *et al.*, 1998; Munns, 1993; 2002; Koyro, 2003; Rengasamy *et al.*, 2003). Control mechanisms include (a) growth rate and plant morphology, (b) resistance to water stress (reduction of the water potential), (c) regulation of CO<sub>2</sub> and H<sub>2</sub>O-exchange by stomata and (d) avoidance of ion toxicity and nutrient imbalance. For example the osmotic stress, generated by either drought or salinity, represents the

most common severe environmental stress limiting plant growth and productivity. Terrestrial plants at saline habitats are often surrounded by low water potentials in the soil solution and atmosphere. Plant water loss has to be minimized under these conditions, since biomass production depends mainly on the ability to keep a high net photosynthesis by low water loss rates. In this field of tension, biomass production of a plant has to be seen always in connection to the energy consumption and gas exchange. The reduced water potential at saline habitats creates in the plant a two-edged problem: a corresponding water and ion stress (Koyro, 2006). The uptake and accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  into the different plant organs is highly controlled (Munns, 1993; Marschner, 1995; Hasegawa *et al.*, 2000), salt-resistant species often possess special features to remove NaCl from the cytoplasm, e.g. by compartmentation in the vacuole (Mühling and Läuchli, 2002). In response to such stress conditions, many plants accumulate compatible metabolites in the cytoplasm of their cells in an attempt to combat the water problem (Bohnert *et al.*, 1995; Munns, 2002). Both effects are energy consuming in addition to the already existing metabolic costs. The metabolic regulation of biomass production, storage of metabolites and respiration (such as catabolic energy production for compartmentation) is crucial for the survival at saline habitats. (Martinez-Ballesta *et al.*, 2004).

#### **4. Root system, water relation and photosynthetic efficacy**

Roots are the organs of plants responsible for water absorption, nutrient uptake and anchorage (Pritchard, 1994). The root system of rice (*O. sativa* L.) consists of seminal roots, adventitious roots and lateral roots. The growing parts of root can be longitudinally divided into the root cap, apical meristem, the elongation zone and the maturation or differentiation zone. Lateral roots in rice are initiated by the local activation of pericycle cells from opposite protophloem poles in the differentiation zone. As lateral roots elongate, some epidermal cells known as trichoblasts extend to form root hairs. Root hairs greatly increase the absorptive surface of the root and are confined mostly to the maturation zone (Chen *et al.*, 2006). Roots play a number of important roles during plant growth and development and typically are the first and critical part of the plant to encounter soil. The inhibition of root growth by salinity is a widespread problem in agricultural practice (Greenway and Munns, 1980; Munns and

Termaat, 1986). When growing in saline soils, roots have to cope with two types of stresses. Firstly, an ionic stress induced by changes in the concentrations of  $\text{Na}^+$ ,  $\text{Cl}^-$  or both in the root-growing medium and within root tissues. Secondly, an osmotic stress comes from salt concentration in the soil that resulting in low water potential and a consequent loss of cell turgor in roots. These stresses in turn causes an inhibition of root growth and reduction in the water uptake. In shoots, high concentrations of  $\text{Na}^+$  can cause a range of osmotic and metabolic problems for plants. Leaves are more vulnerable than roots to  $\text{Na}^+$ , simply because  $\text{Na}^+$  (and  $\text{Cl}^-$ ) accumulates at higher concentrations in shoots than in roots. Roots tend to maintain fairly constant levels of NaCl over time, and can regulate NaCl levels by export to the soil or to the shoot.  $\text{Na}^+$  is transported to shoots in the rapidly moving transpiration stream in the xylem, but can only be returned to roots via the phloem. There is limited evidence of extensive recirculation of shoot  $\text{Na}^+$  to roots, suggesting that  $\text{Na}^+$  transport is largely unidirectional and results in progressive accumulation of  $\text{Na}^+$  as leaves age (Tester and Davenport, 2003). The supply of mineral ions to the leaf growing region may decline. Lower transpiration rate, coupled with reduced ion uptake by the roots, or reduced xylem loading, may cause poor supply via the xylem. So it is possible that an inadequate supply of ions to the expanding region may restrict cell division and/or expansion when plants are grown at high levels of NaCl (Berstein *et al.*, 1995). In expanding leaves, salinity has disturbed concentrations of K (Jeschke and Wolf, 1985) and P (Martinez and Lauchli, 1991). Xylem K concentration declined to about half control values in plants grown at high salt (Wolf *et al.*, 1990). An immediate response to salinity is stomatal closure. However, due to water potential difference between the atmosphere and leaf cells, and the need for carbon fixation, this is an untenable long-term strategy of tolerance (Hasegawa *et al.*, 2000). One way of controlling salt flux to the shoot is the entry of ions into xylem stream (Flowers and Yeo, 1992). The large quantities of ions in mature or older leaves accumulated under salt stress (Munns, 1993). Older leaves may restrict ion deposition in meristematic and actively growing cells and meristematic cells are less exposed to ions delivered through the transpiration stream and their small vacuolar space is not conducive to ion storage (Wyn, 1981). One of the major factors inducing leaf senescence is the decrease of chlorophyll content under saline conditions (Chen *et al.*, 1991). Leaf senescence is also correlated

with increased membrane permeability at high salt concentration. One of the major factors inducing leaf senescence is the decrease of chlorophyll content under saline conditions (Chen *et al.*, 1991). Leaf senescence is also correlated with increased membrane permeability at high salt concentration (Dhindsa *et al.*, 1981). Specific effects of salt stress on leaf senescence have been related to the accumulation of toxic ions ( $\text{Na}^+$  and  $\text{Cl}^-$ ) or to  $\text{K}^+$  and  $\text{Ca}^{2+}$  depletion (Yeo and Flowers, 1983; Yeo *et al.*, 1991). Magnesium, by comparison, has received little attention, although it could play a central role in senescence-related processes.  $\text{Mg}^{2+}$  is implicated in the regulation of protein synthesis (Flowers and Dalmond, 1992). A decrease in  $\text{Mg}^{2+}$  absorption could also be responsible for decreased chlorophyll content (Leidi *et al.*, 1991) and quenching of variable fluorescence due to increased 'spillover' of excitation energy from PSII to PSI (Krause and Weis, 1991; Lutts *et al.*, 1996). In addition, salinity induced a significant decrease in total biomass, leaf area and plant height. The concentration of  $\text{Cl}^-$  in leaves increased with increasing salinity and is higher than the corresponding concentration of  $\text{Na}^+$ . Net photosynthesis ( $P_n$ ) was reduced and the chloroplast ultrastructure was altered by salinity. Thylakoids are dilated and the number of plastoglobuli is greatly increased in both saline treatments compared with the control leaves. Moreover, a reduction in the intercellular spaces of the lagunar mesophyll was observed in the saline treatments, affecting stomatal and mesophyll conductance to  $\text{CO}_2$ . Root hydraulic resistance increased under saline conditions, affecting the water flow the root system (Navarro *et al.*, 2007). Under such conditions, modifications such as root growth increase or stomatal conductance decrease have the potential to increase crop productivity. The effect in this case is the lost photosynthesis caused by reduced stomatal  $\text{CO}_2$  uptake or shift of resources into root growth at the expense of photosynthetic and reproductive tissue (Verslues *et al.*, 2006).

In recent year, the technique of chlorophyll fluorescence has become ubiquitous in plant ecophysiology studies and has extensively been used in assessing plant responses to environmental stress (Maxwell and Johnson, 2000; Sayed, 2003). It has been known for a considerable time that changes in chlorophyll fluorescence emission from photosynthetic organism are frequently indications of changes in photosynthetic activity (Baker and Rosenqvist, 2004). For instance, Lu *et al.* (2002) investigated the salt stress effect on photosystem II photochemistry, and

photosynthetic pigment composition in halophyte plant (*Sueda salsa*). The chlorophyll fluorescence parameters, including maximum quantum yield ( $F_v/F_m$ ), quantum efficiency of photosystem II ( $\Phi_{PSII}$ ), photochemical (qP) and non-photochemical quenching (NPQ) are used. Salinity-induced senescence leaves of rice (*O. sativa* L.) cultivars differing in various salt tolerance was investigated using variable Chlorophyll a fluorescence that also proved useful in selecting salt-tolerant cultivars (Lutts *et al.*, 1996). Chlorophyll a fluorescence quenching was also used to study photosynthetic responses of rice cultivars possessing different potentials of salt tolerance. Chlorophyll a fluorescence parameters suggested that salt sensitivity in rice is associated with increased shoot sodium levels, decreased photosynthetic efficiency of PSII, and enhanced NPQ (Dionisio-Sese and Tobita, 2000).

## **5. Physiological selection criteria for salt tolerance in plants**

### **5.1 Photosynthesis and water relation**

In the past few years, plant breeders have been investigated significant achievements improving salinity tolerance in a number of agriculturally potential crops through artificial selection and conventional breeding techniques (Noble *et al.*, 1984; Allen *et al.*, 1985; Al-Khatib *et al.*, 1993; Ashraf, 1994; Shannon, 1998; Ashraf, 2002). However, it is not surprising to see that most of the selection procedures were based on differences in agronomic characters (Noble and Rogers, 1992; Ashraf, 1994). Such agronomic characters represent the combined genetic and environmental effects on plant growth and yield including also the integration of the physiological mechanisms conferring salinity tolerance (Munns, 1993). Physiological criteria should be able to supply more direct and reliable information than agronomic characters (Yeo, 1994). In previous studies, the research topic has been mainly dealing with the accumulation of compatible solutes, including proline (Lutts *et al.*, 1996), glycinebetaine (Cha-um *et al.*, 2007), trehalose (Garg *et al.*, 2002) or soluble sugars (Chinnusamy *et al.*, 2005),  $K^+/Na^+$ ,  $Ca^{2+}/Na^+$  ratios (Zeng, 2005), water relation and photosynthesis (Cha-um *et al.*, 2006)

### **5.2 Rice salt tolerant screening and cluster analysis**

The low success in rice salt tolerance breeding derived from the low selection efficiency using overall agronomic characters, lack of effective evaluation

methods for salt tolerance among genotypes, and the complexity of salinity tolerant phenotypes among genotypes. Differential salinity sensitivity at various growth stages is one of the factors, affecting salt tolerant phenotypes. Generally, vegetative growth in rice plants is very sensitive to salinity at young seedling stages and less sensitive at reproductive stages (Flowers and Yeo, 1981; Lutts *et al.*, 1995). Cluster analysis is suggested as an effective method for comparing cultivars (Jolliffe *et al.*, 1989). Cluster methods have been used to characterize plant germplasm and group genotypes into homogeneous clusters when trials were conducted under non-saline conditions at different locations (Cossa *et al.*, 1995; Franco *et al.*, 1999, 2003). Traditional Philippine upland rice cultivars have been classified by clusters analysis on growth characters under non-saline conditions (Schlösser *et al.*, 2000). The cluster method was effective in screening for salt tolerance among the genotypes of potato (Khrais *et al.*, 1998) and rice (Zeng *et al.*, 2002) when agronomic parameters were analyzed. Using cluster methods, genotypes can be assessed by multiple parameters simultaneously. There is no need to set scoring boundaries because genotypes group on the basis of variances of the characters analyzed. For instance, Zeng *et al.* (2005) reported the relationships between physiological parameters and growth performance and quantify the respective genotypic differences using multivariate analysis. Plants of thirty-one genotypes were grown in sand tanks in a greenhouse and irrigated with Yoshida nutrient solution. Two salinity treatments were imposed at 0.9 dS m<sup>-1</sup> (control) and 6.4 dS m<sup>-1</sup> with sodium chloride and calcium chloride (approximately 6:1 molar ratio). Seedlings were sampled 34 days after planting (7<sup>th</sup> to 8<sup>th</sup> leaf stage). The characters of Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, K-Na selectivity (SK,Na) and Na-Ca selectivity (SNa,Ca) were measured as physiological parameters. The characters of tiller number, leaf area, plant height and shoot dry weight were measured as growth performance. They found that under salinity stress, SK,Na increased whereas SNa,Ca decreased compared to the controls. Canonical correlation analysis indicates a strong relationship between physiological parameters and growth performance. Tiller number is a desirable parameter among the growth parameters analyzed to predict seedling growth under salinity stress. Genotypes grouped into four clusters based on ion contents and ion selectivity using Ward's minimum-variance cluster analysis. SK,Na and shoot Na<sup>+</sup> content contributed the most to the cluster formation. Similarly, genotypes grouped

into four clusters based on growth performance. Genotypes were classified into three categories based on ion cluster rankings: Category 1 with high SK,Na and low shoot  $\text{Na}^+$  content; Category 2 with intermediate SK,Na and shoot  $\text{Na}^+$  content; Category 3 with low SK,Na and high shoot  $\text{Na}^+$  content. The classification of the genotypes into Categories 1 and 3 based on their high or low SK,Na was generally consistent with their growth performance under salt stress. In contrast, ion selectivity was a less dominant mechanism controlling salt tolerance in Category 2 with intermediate SK,Na. It was concluded that ion selectivity was a relatively dominant mechanism controlling salt tolerance among rice genotypes although multiple mechanisms may be involved under moderate salt stress. The results also provided the first example of the effectiveness of cluster analysis for physiological responses to salinity stress.

## **6. Objective**

The objectives of this thesis were identified as following:

- 6.1 To study effect of salt stress on the root system relating to water relation, leading to photosynthetic pigments and photosynthetic characters of rice seedling.
- 6.2 To establish and develop an effective indicator for salt tolerant screening of rice seedlings under salt stress condition.
- 6.3 To classify the salt-tolerant and salt-sensitive pure lines in breeding program using an effective indices.

## CHAPTER II

### MATERIALS AND METHODS

#### **Plant material and growth condition**

Seeds of salt tolerance rice (*Oryza sativa* L. subspecies *indica*), including Homjan (HJ; GS.No. 4371), and Pokkali (Pok; GS.No. 17905) and salt sensitive rice (IR29; GS.No. 2818) and Pathumthani 1 (PT1) were obtained from the Pathumthani Rice Research Center (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand). In addition, nine lines from BC<sub>1</sub>F<sub>2</sub> [(PT1×HJ)×PT1] backcross population in breeding program were used as initial material.

Rice seeds were hand-dehusked, immersed in 70% ethanol for 30 sec, surface-sterilized once in 5% (v/v) Clorox<sup>®</sup> (5.25% w/v sodium hypochlorite, Clorox Co, USA) for 12 h, once soaked in 25% Clorox<sup>®</sup> for 25 min, and then rinsed thrice with sterile distilled water. Surface sterilized seeds were germinated on MS-solidified media (Murashige and Skoog, 1962). All seedlings were cultured under 25 ± 2 °C air-temperature, 60 ± 5% relative humidity (RH), and 60±10 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux (PPF) with 16 h d<sup>-1</sup> photoperiod provided by fluorescent lamps (TLD 36W/84, Cool White, Philips, Thailand). Fourteen-old seedlings were aseptically transferred to 50 ml liquid sugar-free MS media, supporting by 20 g vermiculite (photoautotrophic system) for 7 days. An amount of air-exchange in the glass vessels was adjusted to 2.32 h<sup>-1</sup>, punching a hole over the plastic cap (Ø 1 cm) and covering with a gas-permeable microporous polypropylene film (0.22 μm pore size, Nihon Millipore Ltd., Japan).

#### **Experiment I Development on effective indicator for salt tolerance screening**

HJ, Pok, IR29 and PT1 rice seedlings were divided into two groups, including with or without root systems. Sodium chloride (NaCl) salt treatments in the culture media were adjusted to 0 (control) or 342 mM (salt-stress). The osmolarity of control

culture media was adjusted to  $684 \text{ mmol kg}^{-1}$  using mannitol application for isoosmolarity among treatments. Leaves osmolarity, pigment concentration and chlorophyll *a* fluorescence including maximum quantum yield ( $F_v/F_m$ ), photon yield of photosystem II ( $\Phi_{PSII}$ ) and photochemical quenching (qP) parameters were measured after exposure to salt stress for 4 days.

### **Leaf osmolarity**

Leaf osmolarities of rice seedlings were measured, according to Lanfermeijer *et al.* (1991). A hundred milligram of leaf tissues were dissected in small pieces, transferred to 1.5 mL micro tube and then debris by glass rod. Twenty microliter of extracted solution was directly dropped on a disc filter paper in osmometer chamber (Wescor, USA) and then measured.

### **Photosynthetic pigment concentration**

Concentrations of chlorophyll *a* ( $Chl_a$ ), chlorophyll *b* ( $Chl_b$ ), total chlorophyll (TC) and total carotenoid ( $C_{x+c}$ ) were analyzed following Shabala *et al.* (1998) and Lichtenthaler (1987) methods, respectively. One hundred milligrams of leaf material were collected from the second and third nodes of the shoot tip. The leaf samples were placed in a 25-ml glass vial (Opticlear<sup>®</sup>; KIMBLE, Vineland, New Jersey, USA), added with 10 ml of 95.5% acetone, and blended with a homogenizer (T25 basic ULTRA-TURRAX<sup>®</sup>; IKA, Kuala Lumpur, Malaysia). The glass vials were sealed with parafilm to prevent evaporation and then stored at 4 °C for 48 h. The concentrations of  $Chl_a$ ,  $Chl_b$  and  $C_{x+c}$  were measured using an UV-visible spectrophotometer (DR/4000; HACH, Loveland, Colorado, USA) at 662, 644 and 470 nm respectively. A solution of 95.5% acetone was used as a blank. The  $Chl_a$ ,  $Chl_b$ , total chlorophyll and  $C_{x+c}$  ( $\mu\text{g g}^{-1}$  FW) concentrations in the leaf tissues were calculated according to the following equations:

$$Chl_a = 9.784D_{622} - 0.99D_{644}$$

$$Chl_b = 21.42D_{644} - 4.65D_{622}$$

$$\text{Total Chlorophyll} = Chl_a + Chl_b$$

$$[C_{x+c}] = \frac{1000D_{470} - 1.90Chl_a - 63.14Chl_b}{214}$$

214

where  $D_i$  was an optical density at the wavelength *i*.

### Photosynthetic efficacy

Chlorophyll a fluorescence emission of the leaf adaxial surface was determined using a Fluorescence Monitoring System (FMS 2; Hansatech Instruments Ltd, Norfolk, UK) in the pulse amplitude modulation mode, following as previously described by Loggini et al. (1999). A leaf, light-adapted in growth chamber (EYELA, Eyselatron FLI-301 NH, Japan) and  $120 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPF provided by fluorescence lamps (TDL 36 W/84, Cool White 3350 lm; Philips, Bangkok, Thailand) for 120 min and dark adaptation for 30 min, were initially exposed to the modulated measuring beam of far-red light (LED source with typical peak at wavelength 735 nm). Original ( $F_0$ ) and maximum ( $F_m$ ) fluorescence yields were measured under weak modulated red light ( $<0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) with 1.6 s pulse of saturating light ( $>6.8 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR) and autocalculated by FMS software for Windows<sup>®</sup> (Fluorescence Monitoring System Software; Hansatech Instruments Ltd). The variable fluorescence yield ( $F_v$ ) was calculated by the equation of  $F_m - F_0$ . The ratio of variable to maximum fluorescence ( $F_v/F_m$ ) was calculated as maximum quantum yield of photosystem II photochemistry. The photon yield of photosystem II ( $\Phi_{\text{PSII}}$ ) in the light were calculated by  $\Phi_{\text{PSII}} = (F_m' - F_t)/F_m'$  after 45 s of illumination, when steady-state were achieved. In addition, photochemical quenching of photosystem II (qP) and non-photochemical quenching (NPQ) were calculated as described by Maxwell and Johnson (2000). The pigment degradation, chlorophyll a fluorescence diminution were calculated according to equation:

$$\text{Degradation (\%)} = \left[ 1 - \frac{342 \text{ mM NaCl}}{0 \text{ mM NaCl}} \right] \times 100$$

### Chloroplast ultrastructure

Electron microscopic studies according to Yamane (2003) were performed using the middle sections of fully expanded uppermost leaves of plants. Small segments were fixed in Karnovsky's fixative (mixture of 4% paraformaldehyde and 5% glutaraldehyde in 50 mmolL<sup>-1</sup> phosphate buffer, pH 7.2) and post fixed in 2% osmium tetroxide in the same buffer. Samples were dehydrated in a series of graded ethanol and propylene oxide and embedded in Epoxy aradite and polymerized at 70 °C for 24

h. Ultrathin sections (70–90 nm) were cut with a diamond knife and placed on 150 mesh copper grid. The grids were stained with 2% uranyl acetate for 20 min followed by lead citrate for 5 min. Then the sections were examined on transmission electron microscope (TEM) (TECNAI 20, Philips<sup>®</sup>, The Netherlands) at 80 kV. Photographs were illustrated at 5000 x and 11500 x magnification.

### **Experimental design**

The experiment was designed as 4×2×2 factorials in Completely Randomized Design (CRD) with four replications and five plantlets per replication. The mean values were compared by Duncan's New Multiple Range Test (DMRT) and analyzed by SPSS software (SPSS for Windows version 11, SPSS Inc., USA). The correlations between osmolarity and chlorophyll a, chlorophyll a and  $F_v/F_m$ , total chlorophyll concentration and  $\Phi_{PSII}$ , total chlorophyll concentration and qP were evaluated by Pearson's correlation coefficients. Multivariate parameters associated with significant difference in statistic analysis of varieties were input to classify group of the cluster for salt-tolerance and salt-sensitivity using Hierarchical cluster analysis in SPSS software. In addition, chloroplast ultrastructures HJ, Pok, IR 29 and PT 1 in with and without root system were observed under Transmission Electron Microscope (TECNAI 20, Philips<sup>®</sup>, The Netherlands)

### **Experiment II Classification salt-tolerant and salt-sensitive pure lines using an effective index**

Nine-lines BC<sub>1</sub>F<sub>2</sub> (PT1/HJ/PT1) population, including 2, 18, 20, 21, 26, 31, 306, 409 and 598 were used as plant materials also Pok, HJ, IR29 and PT1 were use as control. Multiple parameters from experiment I were evaluated among rice lines. The experiment was designed as 9×2 factorials in Completely Randomized Design (CRD) with four replications and five plantlets per replication. The mean values were compared by DMRT and analyzed by SPSS software (SPSS for Windows version 11, SPSS Inc., USA). Multivariate parameters associated with significant difference in statistic analysis of varieties were input to classify group of the cluster for salt-tolerance and salt-sensitivity using Hierarchical cluster analysis in SPSS software.

## CHAPTER IV

### RESULTS

#### Experiment I Development on effective index for salt tolerant-screening

##### 1 Effect of salt stress on the function of root system relating to photosynthetic pigments and photosynthetic characters of rice seedling

Salt-tolerant (Pok and HJ) and salt-sensitive (IR29 and PT1) seedlings were divided into two groups, including with or without root systems in order to investigate the function of root system in water use efficiency, relating to the water oxidation and photosynthetic characteristics of salt-stressed indica rice. The results showed that the osmolarities in salt stressed seedlings with root system were lower than those without root system (Table 2 and 3). The osmolarity in salt-stressed seedlings of Pok and HJ increased to 1.58 and 1.83 folds in with root system group and 1.78 and 1.95 folds in without root system group, respectively when compared to those control seedlings. Whereas, the osmolarity in salt-sensitive varieties, IR29 and PT1, increased to 1.97 and 2.46 folds in with root system group and 2.11 and 1.27 folds in without root system group, respectively when compared to those control seedlings. In case of PT1 variety, the osmolarity in control without root system expressed at high level ( $1043.8 \text{ mmol kg}^{-1}$ ), so the osmolarity in PT1 sensitively responded to the system of without root. In addition, osmolarity levels were converted to leaf water potential parameters in order to reveal intracellular water in leaf tissues. The results showed that the leaf water potential in Pok and HJ salt-stressed seedlings decreased to -2.43 and -2.45 MPa in with root system and -2.68 and -3.03 MPa in without root system group, respectively. While, the water potential in IR29 and PT1; salt-sensitive varieties decreased to -2.68 and -2.74 MPa in with root system group and -3.18 and -3.19 MPa in without root system group, respectively (Figure 4). In case of without root system, the water potential or water available in salt-stressed rice seedlings was limited,

especially in salt-sensitive varieties. Moreover, an increasing of osmolarity positively correlated with the reduction of leaf water potential ( $r^2 = 1$ ). It should be noted that the intracellular water in salt-tolerant varieties was maintained greater than salt-sensitive varieties, relating to an increasing of osmolarity, especially in rice seedlings with root system.

**Table 2** Leaf osmolarity, chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid concentration of salt-tolerant (Pok, HJ) and salt-sensitive (IR29, PT1) seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Varieties	Salt stress (mM)	Osmolarity (mmol kg <sup>-1</sup> )	Chlorophyll a (µg g <sup>-1</sup> FW)	Chlorophyll b (µg g <sup>-1</sup> FW)	Total chlorophyll (µg g <sup>-1</sup> FW)	Total carotenoid (µg g <sup>-1</sup> FW)
Pok	0	637.3 c	700.3 c	230.2 a	930.5 a	170.2 c
	342	1012.3 b	715.4 b	189.5 c	904.9 b	212.0 a
HJ	0	557.3 d	546.1 f	143.5 d	689.6 e	154.8 f
	342	1021.5 b	728.2 a	212.4 b	940.6 a	207.4 b
IR29	0	565.5 d	620.3 e	179.5 c	799.7 d	163.9 d
	342	1115.5 a	484.5 g	131.1 d	615.6 f	145.0 g
PT1	0	464.5 e	657.1 d	215.9 b	873.0 c	169.8 c
	342	1140.8 a	261.4 h	184.5 c	445.9 g	48.8 h

*Significant level*

Varieties	**	**	**	**	**
Salt-stress	**	**	*	**	**
Varieties x Salt-stress	**	**	**	**	**

Different letters in each column represent significant difference at  $P \leq 0.01$  by Duncan's Multiple Range Test (DMRT). Significant and highly significant levels in statistics are represented by \* and \*\*, respectively.

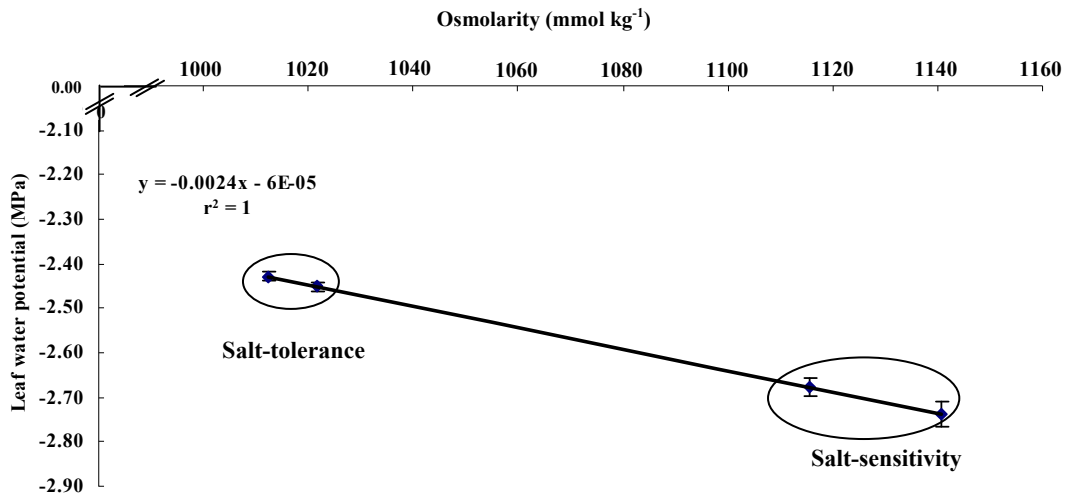
**Table 3** Leaf osmolarity, chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid concentration of salt-tolerant (Pok, HJ) and salt-sensitive (IR29, PT1) seedlings without root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Varieties	Salt stress (mM)	Osmolarity (mmol kg <sup>-1</sup> )	Chlorophyll a (µg g <sup>-1</sup> FW)	Chlorophyll b (µg g <sup>-1</sup> FW)	Total chlorophyll (µg g <sup>-1</sup> FW)	Total carotenoid (µg g <sup>-1</sup> FW)
Pok	0	625.8 e	335.8 a	115.9 c	451.7 b	76.2 a
	342	1115.3 c	332.8 a	170.0 a	502.8 a	78.4 a
HJ	0	649.0 e	189.2 e	72.2 e	261.4 d	43.8 d
	342	1264.0 b	157.0 f	54.7 f	211.7 e	53.7 c
IR29	0	628.5 e	194.1 d	67.8 e	261.9 d	54.5 c
	342	1323.8 a	128.6 g	41.3 g	169.9 f	64.4 b
PT1	0	1043.8 d	291.2 b	159.9 b	451.1 b	56.7 c
	342	1327.0 a	207.4 c	96.5 d	303.9 c	43.0 d

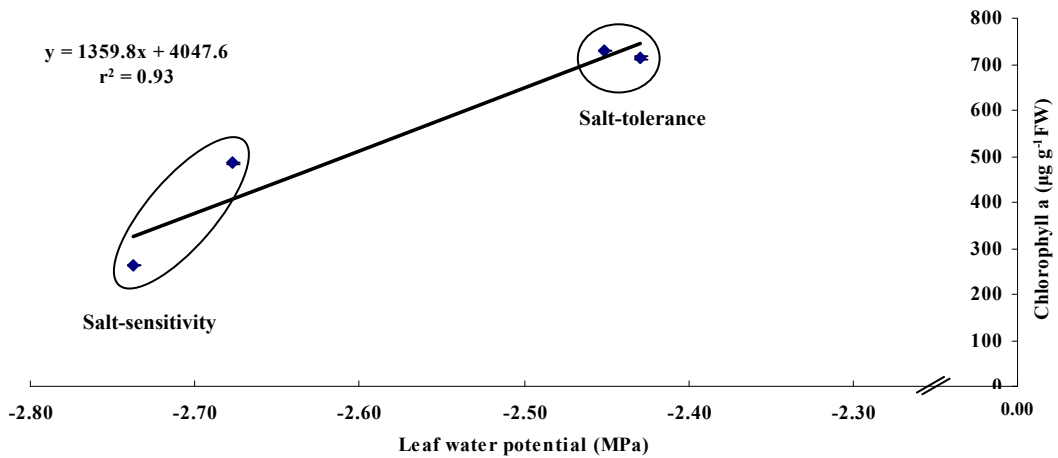
*Significant level*

Varieties	**	**	**	**	**
Salt-stress	**	**	**	**	*
Varieties x Salt-stress	**	**	**	**	**

Different letters in each column represent significant difference at  $P \leq 0.01$  by Duncan's Multiple Range Test (DMRT). Significant and highly significant levels in statistics are represented by \* and \*\*, respectively.



**Figure 4** Correlation between osmolarity and leaf water potential in salt-tolerant (Pok, HJ) and salt-sensitive (IR29, PT1) seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 342 mM NaCl for 4 days. Error bars represent  $\pm$  S.E.



**Figure 5** Correlation between leaf water potential and chlorophyll a in salt-tolerant (Pok, HJ) and salt-sensitive (IR29, PT1) seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 342 mM NaCl for 4 days. Error bars represent  $\pm$  S.E.

An increasing osmolarity in salt-stressed leaf tissues of rice seedlings with or without root system directly affected on leaf water potential reduction, resulting in the photosynthetic pigment degradation, especially in salt-sensitive varieties (Table 2 and 3). The reduction of leaf water potential positively correlated with the degradation of chlorophyll a pigment ( $r^2 = 0.93$ ) (Figure 5). Chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid pigments of salt-sensitive varieties in with root system were degraded to 41.1%, 20.7%, 36.0% and 41.4%, respectively. Whereas, the photosynthetic pigments of salt-tolerant varieties were damaged lower than 10% when compared to those control seedlings (Table 4). In case of salt-tolerant varieties, the concentrations of chlorophyll a and total carotenoid pigments in Pok increased during salt stress, whereas chlorophyll b and total chlorophyll pigments degraded to 17.68% and 2.75%, respectively. In contrast, the photosynthetic pigments of HJ were stabilized during salt stress (Table 4). On the other hand, the chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid pigments of IR 29 were degraded to 21.89%, 26.96%, 23.02% and 11.53%, respectively. As well as, chlorophyll a and total carotenoid contents in PT 1 were dramatically damaged to 60.22% and 71.26%, respectively (Table 4).

**Table 4** Pigment degradation, chlorophyll a fluorescence diminution of salt-tolerant (Pok, HJ) and salt-sensitive (IR29, PT1) seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Varieties	Pigment degradation (%)				Chlorophyll a fluorescence diminution (%)		
	Chl <sub>a</sub>	Chl <sub>b</sub>	TC	C <sub>x+c</sub>	F <sub>v</sub> /F <sub>m</sub>	Φ <sub>PSII</sub>	qP
<b>Pok</b>	0.00	17.68	2.75	0.00	0.81	12.57	11.53
<b>HJ</b>	0.00	0.00	0.00	0.00	3.39	0.00	0.00
<b>Average</b>	<b>0.00</b>	<b>8.84</b>	<b>1.38</b>	<b>0.00</b>	<b>0.98</b>	<b>6.29</b>	<b>5.77</b>
<b>IR29</b>	21.89	26.96	23.02	11.53	13.49	5.21	0.00
<b>PT1</b>	60.22	14.54	48.92	71.26	9.26	11.21	11.43
<b>Average</b>	<b>41.06</b>	<b>20.74</b>	<b>35.97</b>	<b>41.40</b>	<b>11.36</b>	<b>8.21</b>	<b>5.72</b>

Abbreviations: Chl<sub>a</sub>, chlorophyll a; Chl<sub>b</sub>, chlorophyll b; TC, total chlorophyll; C<sub>x+c</sub>, total carotenoid; F<sub>v</sub>/F<sub>m</sub>, maximum quantum yield of PSII; Φ<sub>PSII</sub>, quantum efficiency of PSII; qP, photochemical quenching

The degradation of photosynthetic pigments in salt-stressed rice seedlings with or without root system was directly diminished on chlorophyll a fluorescence parameters, especially in salt-sensitive varieties (Table 5 and 6). The reductions of chlorophyll a, chlorophyll b and total chlorophyll pigments showed positively correlation with the diminution of F<sub>v</sub>/F<sub>m</sub> ( $r^2 = 0.54$ ) (Figure 6), Φ<sub>PSII</sub> ( $r^2 = 0.68$ ) (Figure 7) and qP ( $r^2 = 0.69$ ) (Figure 8), respectively. The F<sub>v</sub>/F<sub>m</sub>, Φ<sub>PSII</sub> and qP of salt-sensitive varieties dropped to 11.4%, 8.2% and 5.7%, respectively. Whereas, the attenuation of chlorophyll a fluorescence parameters of salt-tolerant varieties decreased lower than 10% when compared to those control seedlings (Table 4). In case of salt-tolerant

varieties, the diminution of  $F_v/F_m$ ,  $\Phi_{PSII}$  and qP of Pok were decreased to 0.81%, 12.57% and 11.53% respectively. In contrast, the diminution of  $F_v/F_m$  in HJ increased to 3.39% while,  $\Phi_{PSII}$  and qP were stabilized (Table 2). On the other hand, the diminution of  $F_v/F_m$  and  $\Phi_{PSII}$  of IR29 decreased to 13.49% and 5.21% while, qP were maintained. As well as, the  $F_v/F_m$ ,  $\Phi_{PSII}$  and qP in salt-stressed PT1 rice were decreased to 9.26%, 11.21% and 11.43%, respectively.

Osmolarity, photosynthetic pigments and chlorophyll a fluorescence parameters in salt-stressed seedlings were evidently expressed as physiological responses. Chlorophyll b and total carotenoid pigments of HJ salt tolerance functioned as light harvesting complex. On the other hand, the light energy absorption in Pok salt tolerance was harvested using total carotenoid pigments. The chlorophyll b pigment of Pok rice seedlings was damaged during salinity stress, resulting in the diminution of  $\Phi_{PSII}$  and qP for 12.57% and 11.53%, respectively. However, chlorophyll a pigment of Pok and HJ were maintained due to the diminution of  $F_v/F_m$  referred to reaction center of PSII. In case of IR29 salt sensitivity, total carotenoid pigment was function as light harvesting complex. In contrast, chlorophyll b pigment in PT1 salt sensitivity was a major light harvesting. However, chlorophyll a pigment of IR 29 and PT1 was severely damaged during salinity stress, resulting in the diminution of  $F_v/F_m$  for 13.49% and 9.26%, respectively.

**Table 5** Maximum quantum yield ( $F_v/F_m$ ), quantum efficiency of PSII ( $\Phi_{PSII}$ ), qP and NPQ in the leaf tissue of salt-tolerant (Pok, HJ) and salt-sensitive (IR29, PT1) seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Varieties	Salt stress (mM)	$F_v/F_m$	$\Phi_{PSII}$	qP	NPQ
Pok	0	0.866 a	0.740 a	0.859 a	0.051
	342	0.859 a	0.647 b	0.760 bc	0.066
HJ	0	0.886 a	0.602 bc	0.705 bcd	0.111
	342	0.856 a	0.629 bc	0.719 bcd	0.045
IR29	0	0.875 a	0.595 bc	0.693 cd	0.071
	342	0.757 b	0.564 c	0.701 bcd	0.036
PT1	0	0.875 a	0.669 ab	0.770 b	0.109
	342	0.794 b	0.594 bc	0.682 d	0.047

*Significant level*

Varieties	**	**	**	ns
Salt-stress	**	**	**	*
Varieties x Salt-stress	**	ns	**	ns

Different letters in each column represent significant difference at  $P \leq 0.05$  and  $P \leq 0.01$  by Duncan's Multiple Range Test (DMRT). Non-significant, significant and highly significant level in statistics are represented by ns, \* and \*\*, respectively.

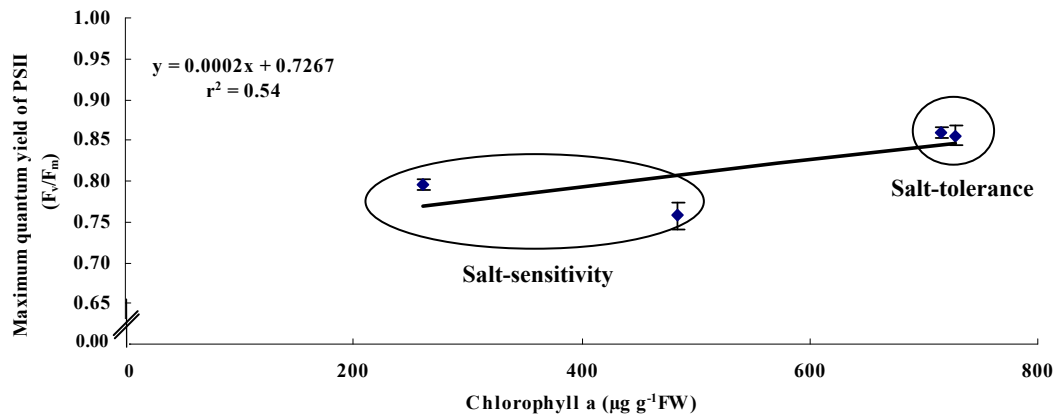
**Table 6** Maximum quantum yield ( $F_v/F_m$ ), quantum efficiency of PSII ( $\Phi_{PSII}$ ), qP and NPQ in the leaf tissue of salt-tolerant (Pok, HJ) and salt-sensitive (IR29 and PT1) seedlings without root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Varieties	Salt stress (mM)	$F_v/F_m$	$\Phi_{PSII}$	qP	NPQ
Pok	0	0.881 a	0.695 a	0.794 a	0.098 a
	342	0.831 a	0.613 ab	0.745 ab	0.031 c
HJ	0	0.861 a	0.636 ab	0.733 ab	0.060 b
	342	0.844 a	0.594 b	0.701 bc	0.062 b
IR29	0	0.869 a	0.575 bc	0.662 cd	0.054 bc
	342	0.706 b	0.484 c	0.622 d	0.043 bc
PT1	0	0.891 a	0.637 ab	0.728 b	0.046 bc
	342	0.826 a	0.571 bc	0.696 bc	0.045 bc

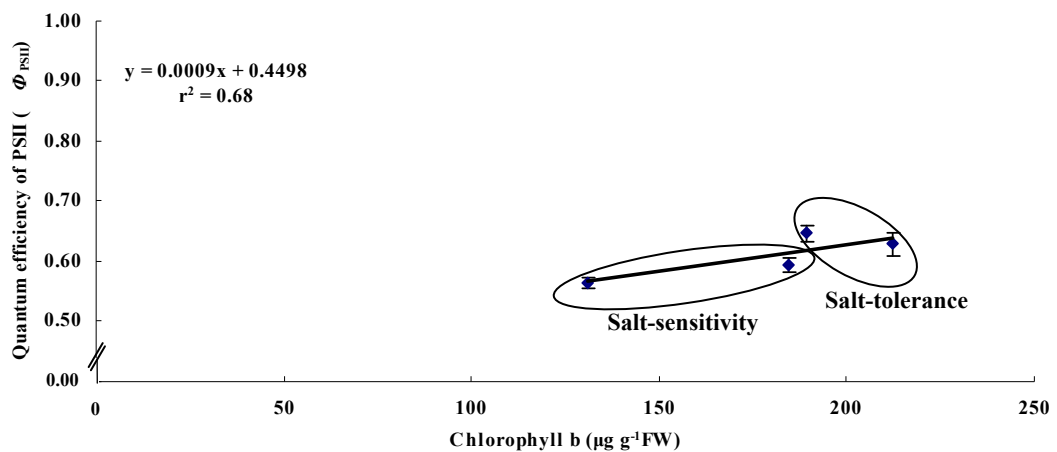
*Significant level*

Varieties	**	**	**	*
Salt-stress	**	**	*	**
Varieties x Salt-stress	**	ns	ns	**

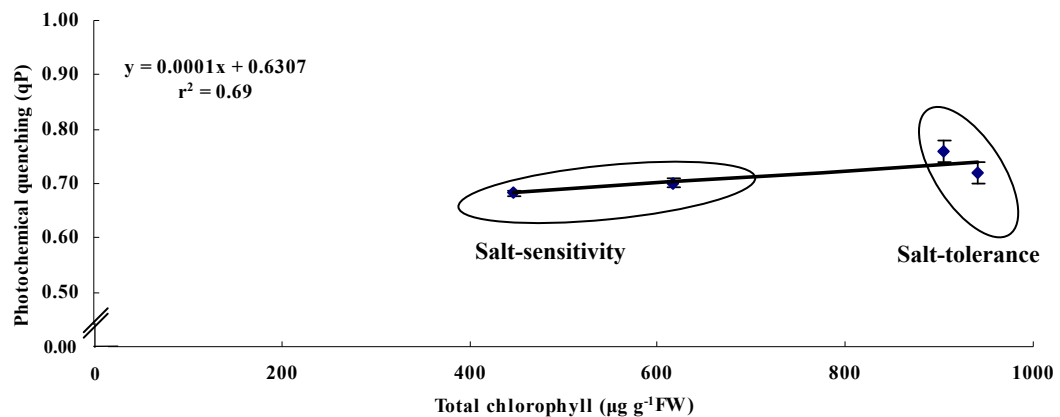
Different letters in each column represent significant difference at  $P \leq 0.05$  and  $P \leq 0.01$  by Duncan's Multiple Range Test (DMRT). Non-significant, significant and highly significant levels in statistics are represented by ns, \* and \*\*, respectively.



**Figure 6** Correlation between chlorophyll a and maximum quantum yield ( $F_v/F_m$ ) in salt-tolerant (Pok, HJ) and salt-sensitive (IR29, PT1) seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 342 mM NaCl for 4 days. Error bars represent  $\pm$  S.E.



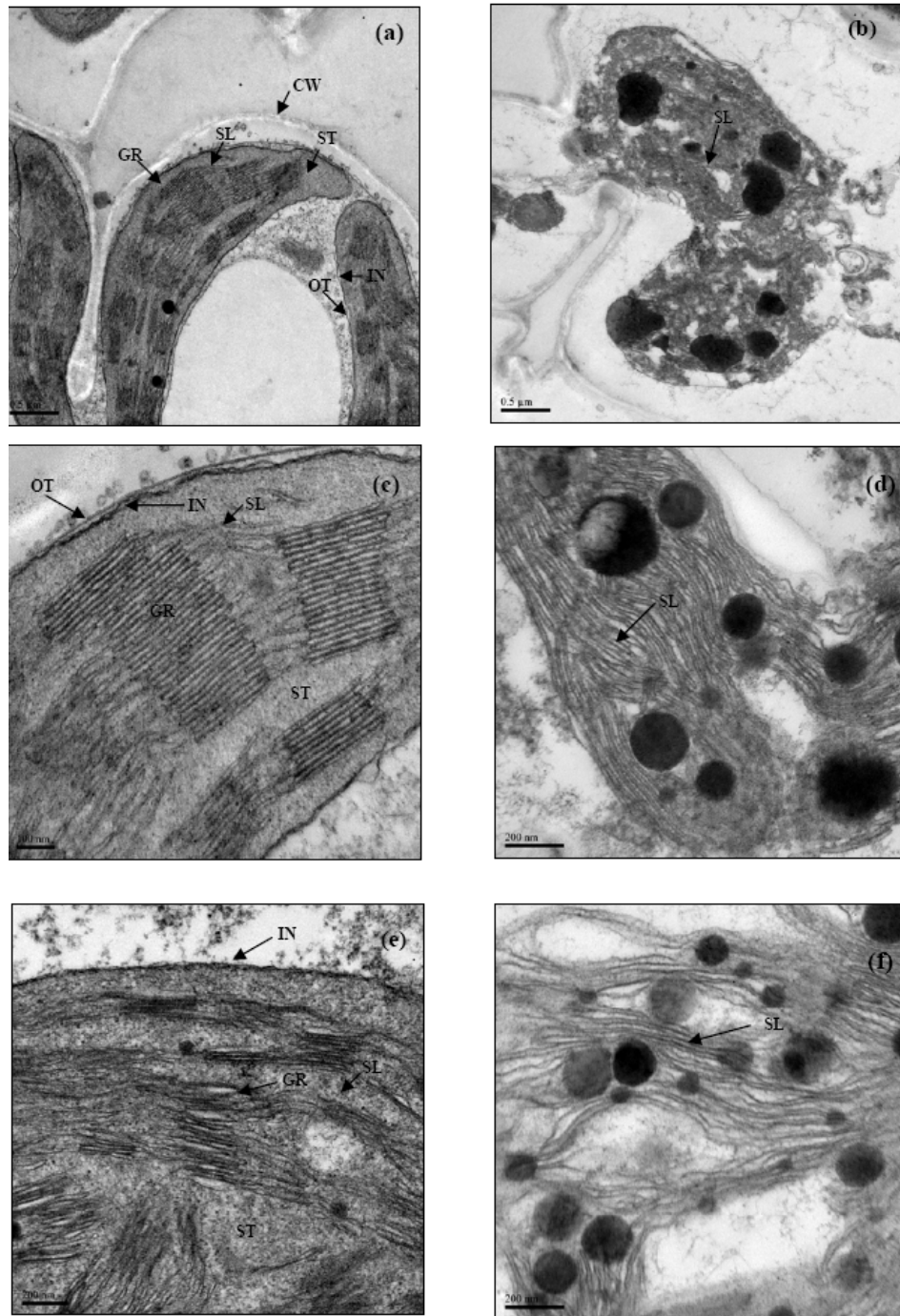
**Figure 7** Correlation between chlorophyll b and quantum efficiency of PSII ( $\Phi_{PSII}$ ) in salt-tolerant (Pok, HJ) and salt-sensitive (IR29, PT1) seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 342 mM NaCl for 4 days. Error bars represent  $\pm$  S.E.



**Figure 8** Correlation between total chlorophyll and photochemical quenching (qP) in salt-tolerant (Pok, HJ) and salt-sensitive (IR29, PT1) seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 342 mM NaCl for 4 days. Error bars represent  $\pm$  S.E.

## 2. Chloroplast ultrastructure changes

The distinct granal and stomatal thylakoids arrangement and clear stroma matrix showed in rice seedlings grown under 0 mM NaCl or control condition (Figure 9a, c), whereas the thylakoids in salt stress (342 mM NaCl) treated leaves were swollen (Figure 9d, f). In addition, the granal thylakoids, the stroma matrix and the stroma lamellae disappeared (Figure 9b, d). The chloroplast envelope, including outer and inner membrane in salt-sensitive varieties of both with and without root system was damaged (Table 7). However, the results showed that the chloroplast envelope of HJ seedling without root system was sensitively injured when exposed to 342 mM NaCl similar to the salt sensitive varieties.



**Figure 9** The ultrastructure of chloroplast in leaf tissue of rice with (a, b, c) and without (d, e, f) root systems grown *in vitro* photoautotrophic condition and exposed to 0 (a, c, e) or 342 (b, d, f) mM NaCl for 4 days. CW, cell wall; GR, granum; IN, inner membrane; OT, outer membrane; SL, stroma lamellae; ST, stroma. Bar = 50 μm in (a), (b) and 200 nm in (c)-(f).

**Table 7** The characterization of chloroplast ultrastructure under TEM micrograph (X 11500), including outer membrane, inner membrane, thylakoid, granum, stroma and stroma lamellae in the leaf tissue of salt-tolerant (Pok, HJ) and salt-sensitive (IR29, PT1) seedlings with or without root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 0 or 342 mM NaCl for 4 days.

System	Varieties	Salt stress (mM)	Changes in ultrastructure of chloroplast						
			Outer mb.	Inner mb.	Thylakoid	Granum	Stroma lamellae		
With root	Pok	0	+	+	+	+	+	+	
		342	+	+	+	-	+	+	
	HJ	0	+	+	+	+	+	+	
		342	+	+	+	-	+	+	
	IR 29	0	+	+	+	+	+	+	
		342	-	-	+	-	-	+	
	PT 1	0	+	+	+	+	+	+	
		342	-	-	+	-	+	+	
	Without root	Pok	0	+	+	+	+	+	+
			342	+	+	+	-	+	+
		HJ	0	+	+	+	+	+	+
			342	-	-	+	-	+	+
IR 29		0	+	+	+	+	+	+	
		342	-	-	+	-	-	+	
PT 1		0	+	+	+	+	+	+	
		342	-	-	+	-	-	+	

+ APPEARED

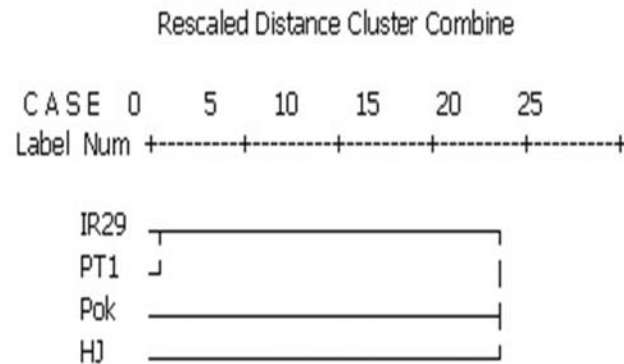
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### 3. Cluster analysis using multivariate parameter cluster analysis

Chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid of salt-sensitive varieties grown under 342 mM NaCl decreased to 21.89%, 26.93%, 23.02%, and 11.53%, respectively in IR 29 and decreased to 60.22%, 14.54%, 48.92%, 71.26% in PT 1, respectively when compared to those control condition (0 mM NaCl) (Table 4). Whereas, the photosynthetic pigments in Pok and HJ salt-tolerant varieties were unaffected. The leaf water potential positively related to the chlorophyll a degradation (Figure 5), resulting in diminution of  $F_v/F_m$  (Figure 6), In addition to, chlorophyll b concentrations showed correlation with  $\Phi_{PSII}$  (Figure 7) and total chlorophyll concentrations were correlated with qP (Figure 8). Multivariate parameters of pigment degradation and chlorophyll a fluorescence diminution were input to classify the salt tolerant and salt sensitive groups using the Hierarchical cluster analysis. The results showed that IR29 and PT1 were evidently identified as salt sensitive group, whereas Pok and HJ were clearly categorized as salt tolerant group, respectively (Figure 9). It should be indicated that the percentages of pigment degradation and chlorophyll a fluorescence diminution in rice seedlings exposure to 342 mM NaCl for 4 days were accurately evaluated for salt-tolerant identification. It should be played as effective indices for salt-tolerant screening in a large population of breeding program.

\*\*\*\*\*HIERARCHICAL CLUSTER ANALYSIS\*\*\*\*\*

Dendrogram using Average Linkage (Between Groups)



**Figure 10** Cluster analysis of salt-tolerant (Pokkali, HJ) and salt-sensitive (IR29, PT1) seedlings with root system, using multivariate parameters of pigment degradation and chlorophyll a fluorescence diminution by Hierarchical cluster analysis of SPSS software.

**Experiment II Salt-tolerant classification using multivariate parameters cluster analysis**

Chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid concentrations in all backcross (BC<sub>1</sub>F<sub>2</sub>) population grown under 342 mM NaCl decreased (Table 8), relating to those the diminution of chlorophyll a parameters (F<sub>v</sub>/F<sub>m</sub>, Φ<sub>PSII</sub> and qP) (Table 9). The reduction of photosynthetic pigments and the diminution of chlorophyll a fluorescence parameters (Table 10) were subjected into the Hierarchical cluster analysis in SPSS software. In addition to Pok and HJ were used as positively control as well as IR29 and PT1 were used as negatively control. The results revealed that there were classified into two classes, including salt tolerant (HJ, Pok and 21) and salt sensitive (IR29, PT1, 2, 18, 20, 26, 31, 306, 409 and 598) groups (Figure 11). The results indicated that the pigment degradation and the chlorophyll a fluorescence diminution were clearly identified the salt tolerant varieties in the backcross population of breeding programs.

**Table 8** Chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid concentrations of BC<sub>1</sub>F<sub>2</sub> population of Thai rice lines 2, 18, 20, 21, 26, 31, 306, 409 and 598 seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Lines	Salt stress (mM)	Chlorophyll a (µg g <sup>-1</sup> FW)	Chlorophyll b (µg g <sup>-1</sup> FW)	Total chlorophyll (µg g <sup>-1</sup> FW)	Total carotenoid (µg g <sup>-1</sup> FW)
2	0	191.7 i	53.7 e	245.4 h	99.6 f
	342	143.3 m	41.1 gh	184.4 k	87.8 j
18	0	347.1 c	111.0 a	458.1 b	114.9 d
	342	104.3 q	34.5 j	138.8 n	65.5 n
20	0	237.7 f	69.8 d	307.5 f	96.9 g
	342	138.4 n	43.1 g	182.0 k	91.5 i
21	0	350.5 b	101.8 b	452.5 c	146.2 c
	342	310.7 d	83.0 c	393.7 d	163.8 b
26	0	132.0 p	39.4 hi	171.4 l	79.8 l
	342	109.0 o	36.2 ij	145.2 m	69.1m
31	0	188.2 j	54.3 e	242.5 h	80.6 l
	342	104.3 q	30.8 k	135.1 n	85.3 k
306	0	214.3 h	72.5 d	286.8 g	91.2 i
	342	182.0 k	48.4 f	230.4 i	101.5 e
409	0	277.7 e	70.4 d	348.1 e	147.8 c
	342	235.2 g	73.6 d	308.8 f	102.2 e
598	0	432.9 a	113.7 a	546.6 a	170.4 a
	342	173.3 l	47.6 f	221.1 j	94.3 h

<i>Significant level</i>					
<b>Rice varieties</b>		**	**	**	**
<b>Salt stress</b>		**	**	**	**
<b>Rice varieties x Salt stress</b>		**	**	**	**

Different letters in each column represent significant difference at  $P \leq 0.05$  and  $P \leq 0.01$  by Duncan's Multiple Range Test (DMRT). Non-significant, significant and highly significant levels in statistics are represented by ns, \* and \*\*, respectively.

**Table 9** Maximum quantum yield ( $F_v/F_m$ ), quantum efficiency of PSII ( $\Phi_{PSII}$ ), qP and NPQ in the leaf tissue BC<sub>1</sub>F<sub>2</sub> population of Thai rice lines 2, 18, 20, 21, 26, 31, 306, 409 and 598 seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 0 or 342 mM NaCl for 4 days.

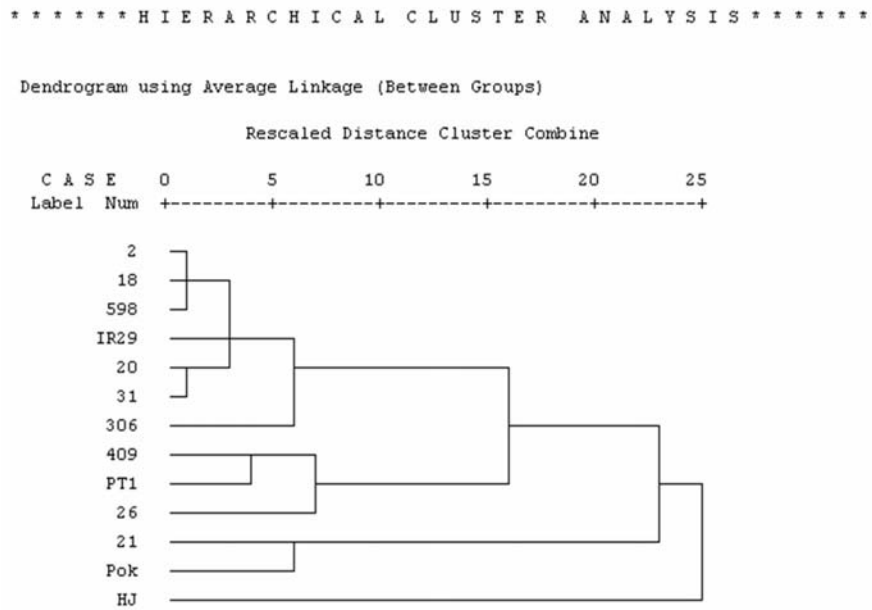
Lines	Salt stress (mM)	$F_v/F_m$	$\Phi_{PSII}$	qP
2	0	0.843 abcd	0.631 bcdef	0.707 bcd
	342	0.806 cd	0.547 fgh	0.690 cd
18	0	0.917 a	0.755 a	0.830 a
	342	0.831 bcd	0.543 fgh	0.675 d
20	0	0.909 ab	0.645 bcde	0.702 cd
	342	0.830 bcd	0.583 cdefgh	0.690 cd
21	0	0.914 a	0.712 ab	0.784 ab
	342	0.786 d	0.520 h	0.659 d
26	0	0.917 a	0.649 bcd	0.725 bcd
	342	0.830 bcd	0.560 defgh	0.673 d
31	0	0.880 abc	0.657 bc	0.759 abc
	342	0.778 d	0.532 gh	0.670 d
306	0	0.907 ab	0.626 bcdefg	0.716 bcd
	342	0.820 cd	0.545 fgh	0.659 d
409	0	0.909 ab	0.631 bcdef	0.700 cd
	342	0.832 bcd	0.556 efgh	0.666 d
598	0	0.923 a	0.590 cdefgh	0.656 d
	342	0.832 bcd	0.548 fgh	0.653 d
<b>Significant level</b>				
<b>Rice varieties</b>		*	*	**
<b>Salt stress</b>		**	**	**
<b>Rice varieties x Salt stress</b>		ns	**	**

Different letters in each column represent significant difference at  $P \leq 0.05$  and  $P \leq 0.01$  by Duncan's Multiple Range Test (DMRT). Non-significant, significant and highly significant levels in statistics are represented by ns, \* and \*\*, respectively.

**Table 10** Pigment degradation, chlorophyll a fluorescence diminution of rice seedlings with root systems grown *in vitro* photoautotrophic condition and subsequently exposed to 0 or 342 mM NaCl for 4 days.

Lines	Pigment degradation (%)			Chlorophyll a fluorescence diminution (%)			
	Chl <sub>a</sub>	Chl <sub>b</sub>	TC	C <sub>x+c</sub>	F <sub>v</sub> /F <sub>m</sub>	Φ <sub>PSII</sub>	qP
2	25.22 e	23.53 f	24.85 e	11.78 e	4.38 i	13.31 e	2.49 g
18	69.96 a	68.92 a	69.71 a	43.00 b	9.35 f	28.10 a	18.71 a
20	41.76 d	38.21 d	40.95 d	5.50 f	8.73 g	9.65 h	1.75 h
21	11.38 i	18.46 g	12.95 h	0.00 g	13.95 a	26.93 b	15.98 b
26	17.46 f	8.08 h	15.31 g	13.35 d	9.51 e	13.67 d	7.20 e
31	44.56 c	43.29 c	44.28 c	0.00 g	11.57 b	18.94 c	11.64 c
306	15.10 h	33.30 e	19.71 f	0.00 g	9.56 d	12.97 f	7.97 d
409	15.30 g	0.00 i	11.29 i	30.85 c	8.48 h	11.98 g	4.91 f
598	59.97 b	59.97 b	59.56 b	44.67 a	9.82 c	7.17 i	0.37 i

Abbreviations: Chl<sub>a</sub>, chlorophyll a; Chl<sub>b</sub>, chlorophyll b; TC, total chlorophyll; C<sub>x+c</sub>, total carotenoid; F<sub>v</sub>/F<sub>m</sub>, maximum quantum yield of PSII; Φ<sub>PSII</sub>, quantum efficiency of PSII; qP, photochemical quenching. Different letters in each column represent significant difference at P ≤ 0.05 and P ≤ 0.01 by Duncan's Multiple Range Test (DMRT).



**Figure 11** Cluster analysis of BC<sub>1</sub>F<sub>2</sub> (Line 2, 18, 20, 21, 26, 31, 306, 409, 598), Pok and HJ (positive control), IR29 and PT1 (negative control) seedlings with root system, using multivariate parameters of pigment degradation and chlorophyll a fluorescence diminution by Hierarchical cluster analysis of SPSS software.

## CHAPTER V

### DISCUSSION

#### Experiment I Development on effective index for salt tolerant screening

##### 1. Effect of salt stress on the function of root system relating to photosynthetic pigments and photosynthetic characters of rice seedlings

In present study, the results showed that the osmolarity in salt-stressed rice seedlings with or without root system was higher than those control seedlings. It means that the water available in salt stress condition was limited, relating to low water potential parameter (Figure 4). Salt contamination in the saline soil is well known to function as water limitation and ion toxicities (Joly, 1989; Pault and Federman, 1991; Hasegawa *et al.*, 2000; Fricke *et al.*, 2006). In addition, the results revealed that the osmolarity in salt-tolerant varieties was maintained lower level than salt-sensitive varieties in either with or without root system. It should be noted that low osmolarity maintenance or high water use efficiency of rice seedlings grown under salt stress is associated with salt tolerant ability (Suriya-arunroj *et al.*, 2004). There are several research topics to mention on water potential reduction, relating to an increasing salinity. For example, leaf water potential in maize genotypes, BR3123, BR5504 and BR5011, decreased to -0.49, -0.51 and -0.62 MPa, respectively (Neto *et al.*, 2004). In sorghum varieties, the water potential in Serena and Seredo varieties decreased to -1.60 and -1.56 MPa at 250 mM NaCl, respectively (Netendo *et al.*, 2004). In the present study, the results indicated that leaf water potential reduction was positively correlated to the degradation of photosynthetic pigment such as chlorophyll a pigment ( $r^2 = 0.93$ ) (Figure 5). It should be noted that the degradation of photosynthetic pigments depending on the intracellular water available in leaf tissues. In previous studies, the photosynthetic pigments in plant species cultivated under salinity stress condition were degraded. In Serena and Seredo sorghum, the

chlorophyll a pigments in salt stressed leaves (250 mM NaCl) reduced for 70% and 58%, respectively. In addition, the chlorophyll b concentrations decreased to 68% and 69% (Netendo *et al.*, 2004). Moreover, in rice seedlings (cv. Taipei 309), chlorophyll a, chlorophyll b and total chlorophyll concentrations in salt-stressed leaves (150 mM NaCl) reduced to 29%, 28% and 28% respectively when compared to those control seedlings (Bahji *et al.*, 2002). In addition, total chlorophyll and total carotenoid concentrations of jasmine rice seedlings treated with 342 mM NaCl for 4 days sharply decreased to 82% and 85.7%, respectively when compared to those control seedlings (Cha-um *et al.*, 2004a). In the present study, the results revealed that the photosynthetic pigments in salt-tolerant varieties grow under salt stress conditions were maintained, whereas those parameters in salt-sensitive varieties were damaged (Table 4).

The degradation of photosynthetic pigments was positively correlated with the diminution of chlorophyll a fluorescence parameters during salt stress (Table 4).  $F_v/F_m$ ,  $\Phi_{PSII}$  and qP of salt-sensitive varieties also significantly decreased, relating to the degradation of chlorophyll a, chlorophyll b and total chlorophyll concentrations, while the  $F_v/F_m$ ,  $\Phi_{PSII}$  and qP of salt-tolerant varieties slightly decreased in salinity stress. Likewise, in sorghum, the  $F_v/F_m$  and qP significantly decreased, but NPQ is increased substantially under saline conditions. However,  $F_v/F_m$  in salt-tolerant cultivars was almost not affected by salt stress, whereas the  $F_v/F_m$  in sensitive cultivars NPQ increased (Dionisio-Sese and Tobita, 2000). It might be possibly demonstrated that the salt sensitivity in cereals might be associated with reductions in PSII photochemical efficiency.

## 2. Chloroplast ultrastructure changes

Abiotic stresses such as salinity and water stress well established as a direct effect to the ultrastructure of rice chloroplast. The ultrastructure of chloroplast in rice seedlings was strongly damaged by salt stress induced -1.0 MPa water potential. The thylakoid in salt-stressed leaves swelled and a slight destruction of the chloroplast envelope was observed (Yamane *et al.*, 2003). Similarly, the thylakoid of *Arbutus unedo* plants are dilated and a number of plastoglobuli in both 52 and 105 mM NaCl treatments was higher than in the control group (Navarro *et al.*, 2007). In present study, outer and inner membrane of chloroplast envelope of IR29, PT1 seedlings with

and without root system and HJ seedlings without root system were injured during 4 days of salt exposed (Table 7). It should be noted that the chloroplast envelope, thylakoid and stoma in salt-tolerant rice with root system were maintained, whereas those parameters in salt-sensitive varieties were damaged during salinity stress.

### **3. Cluster analysis using multivariate parameter cluster analysis**

In previous studies, the responses of plant to salt stress represented by the difference of chlorophyll a fluorescence parameters and the degradation of photosynthetic pigments. In addition, there are many criterias to develop the salt-tolerant indices such as growth, chlorophyll a fluorescence parameters and pigments degradation for salt-tolerant classification in breeding program such as in rice (Cham *et al.*, 2006), barley (Belkhodja *et al.*, 1994; 1999) and wheat (Khrishnaraj *et al.*, 1993). However, a single character in most previous studies was generally applied to evaluate as salt-tolerant cultivars. In this study, the physiological parameters, pigment degradation and chlorophyll a fluorescence diminution, were alternatively played as effective salt-tolerant indicators using multivariate parameters cluster analysis. The results showed that Pok and HJ seedlings were identified as salt tolerant class, whereas IR29 and PT1 were classified as salt sensitive class.

### **Experiment II Salt-tolerant classification using multivariate parameters cluster analysis**

The salt tolerant classification of backcross ( $BC_1F_2$ ) populations was based on physiological characters, including the pigment degradation and the chlorophyll a fluorescence diminution (Table 10). The results showed that line HJ, Pok and 21 was identified as salt tolerant varieties, whereas line IR29, PT1, 2, 18, 20, 26, 31, 306, 409 and 598 were identified as salt sensitive varieties (Figure 11). In addition, the cluster analysis in breeding program has been investigated for mass population screening. For example, in wheat genotypes were classified in three clusters, including salt tolerance (Kharchia, Sakha 8 and Sakha 93), moderate salt tolerance (Sakha 69, Drysdale, Thassos and Gemmeza 7) and salt sensitivity (Giza 168, Triso, Westonia and Sakha 61) by Ward's minimum variance cluster analysis using multi-parameters such as growth performance, yield components and biomass (El-Hendawy *et al.*, 2005). As well as, in rice genotypes were classified to into four clusters, high salt tolerance

(IR63352-AC202), moderate salt tolerance (Daeyabyeo, GZ5385-29-3-3, GZ5121-5-2-1, Nonabokra, IR29, IR63731-1-1-4-3-2, S-102, Pokkali, IR4630-22-2-2-5-1-3, IR50184-3B18-2B-1 and IR51490-AC10), moderate salt sensitivity (AC26, GZ5310-20-3-2, Agami, GZ1368-5-4, GZ5385-29-3-2, Sakha 101, IR70074-AC14 and IR70074-AC1) and high salt sensitivity (IR61920-3B-15-2-2, GZ178, GZ5310-20-3-3, GZ177, M-205, GZ5385-3-2-3-1, GZ5310-20-2-1, M-104, GZ5291-7-1-2, M-202 and L-205) by Ward's minimum variance cluster analysis based on growth performances, including tiller number, leaf area and shoot dry weight (Zeng, 2005). Therefore, multivariate parameters cluster analysis, including the degradation of photosynthetic pigments and the diminution of chlorophyll a fluorescence parameters should be applied to screen the salt tolerant varieties in breeding program.

## **CHAPTER VI**

### **CONCLUSION**

Osmolarity in salt-tolerant varieties with or without root systems grown under salt stress were maintained lower than those in salt-sensitive varieties. An increasing of osmolarity was related to low water available into root system or water loss from leaf tissue when exposed to salinity stress and positive related to the degradation of photosynthetic pigments, including chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid. The water available in leaf tissue associated with the chloroplast envelope, thylakoid and stroma in salt-tolerant rice with root system could be maintained, whereas the defensive systems to protect the chloroplast envelope salt-sensitive varieties were lacked during salinity stress. In addition, multivariate parameters of pigment degradation and chlorophyll a fluorescence diminution were successfully utilized to classify the salt tolerant group, Pok and HJ, and salt sensitive group, IR29 and PT1, using the Hierarchical cluster analysis. It was indicated that the percentages of pigment degradation and chlorophyll a fluorescence diminution were played as effective indices in multivariate parameters to identify the salt tolerant rice. The effective indices were applied to classify 9 lines BC<sub>1</sub>F<sub>2</sub> population. There were grouped into two classes, including salt tolerant class, lines 21 and salt sensitive class, lines 2, 18, 20, 26, 31, 306, 409 and 598. The results indicated that the multivariate parameters should be further identified the salt tolerant varieties in the large population of breeding programs.

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