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THESIS

LEAF PROTEINS DIFFERENTIALLY EXPRESSED DURING SALT-STRESS RESPONSE IN STOCK PLANTED OF MULBERRY (*Morus* spp.)

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A Thesis Submitted in Partial Fulfillment of
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Salt stress is one of the major abiotic stresses affecting plant growth and productivity in Thailand, especially in the northeast. Most of the sericulture taken place in mulberry planting area is facing the saline soil problem. Mulberry is the sole source of food for domesticated silkworm (*Bombyx mori*). The quality of mulberry leaves (*Morus* spp.) directly determines both the yield and quality of the silkworm cocoon.

Screening of salt tolerant and salt susceptible mulberries was performed by adding 200 mM NaCl for 1 month into 44 varieties of 2 month-old mulberry stocks. The results showed that two local varieties, Som and Plong, were tolerant to salt soil (still survive) and the $EC_{1:5}$ values of the soil were 4.80 and 4.07 dSm^{-1}, respectively. However, BR51 (hybrid variety) and S61 (exotic variety) were salt sensitive and died before the end of the test period at the soil $EC_{1:5}$ values of 2.18 and 1.87 dSm^{-1}, respectively. Similar physiological changes and $EC_{1:5}$ values were observed in all the 4 varieties when the screening tests were repeated during the dry winter and summer. Protein expression profile of Som and S61 varieties after treated with 200 mM NaCl for 7 days were determined by 2-dimensional electrophoresis. The result was analyzed with an imagemaster 2D Elite 5.0 program. Approximately 100 protein spots were reproducibly detected on each gel. Of these, 13 protein spots were differentially expressed under salt treatment in leaves of Som and S61 varieties. After protein spots were excised, the gels were subjected to in-gel digestion and the tryptic peptides were analyzed by LC-MS/MS. The main functions of these proteins were photosynthesis and stress defense including RuBisCO, OEC, OEE1, OEE2, Rieske Fe/S protein of cytochrome b6/f complex and WAP (smHSP) protein. While the three new proteins are markedly up-regulated in tolerant variety could not be identified.

The novel proteins should be further identified and may play an important role in salt tolerance of mulberry. These proteins might be useful for marker-assisted selection in salt tolerant mulberry in the future.

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Chehasan Cheubong
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LIST OF ABBREVATIONS

^oC	=	degree Celsius
DTT	=	Dithiothreitol
DW	=	dry weight
dS/m	=	decisiemens per meter
EC_e	=	the electrical conductivity of the saturation extract
$EC_{1:5}$	=	the electrical conductivity of the soil extract (1:5 of soil: water)
kDa	=	kilo Dalton
h	=	hour
ml	=	mililiter
µg	=	microgram
µl	=	microliter
nl	=	nanoliter
µM	=	micromolar
mM	=	milimolar
mg	=	milligram
min	=	minute
pI	=	isoelectric point
rpm	=	revolution per minute
s	=	second
SDS-PAGE	=	Sodium dodesyl sulfate Polyacylamide gel Electrophoresis
Tris	=	Tris (hydroxymethyl) aminomethane
V	=	volt
w/v	=	weight by volume
w/w	=	weight by weight

LEAF PROTEIN DIFFERENTIALLY EXPRESSED DURING SALT-STRESS RESPONSE IN STOCK PLANTED OF MULBERRY (*Morus* spp.)

INTRODUCTION

Mulberry is the sole source of food for domesticated silkworm (*Bombyx mori*), belonging to Moraceae family. The quality of mulberry (*Morus* spp.) leaves directly determine both the quality and yield of the silkworm cocoon. Nearly 70% of mulberry leaf protein, which consists principally of fibroin and sericin, is utilized in the production of silk protein. Hence, improvement of mulberry varieties is necessary for successful sericulture and high quality silk industry. In addition, the mulberry plant is widely used for herbal medicine, animal fodder, eaten as a vegetable, paper production and mushroom production. It has been mainly planted in the Northeast and central of Thailand which the most people have taken up sericulture as an important agro-industry with excellent results. There are 17.8 million rais or 2.84 million ha of saline soil in the northeast of Thailand where most of sericulture takes place; i.e. 17 % of area is facing the saline soil problem.

Salt stress is an abiotic stress, which one of the major factors in limiting crop production and developmental, especially in non-halophytic plants. The most common effects of salinity on plants are loss of turgor, growth reduction, shorter stature, early senescence, respiratory changes, decreased photosynthesis, loss of cellular integrity and tissue necrosis. Salts could inhibit plant growth through osmotic stress, nutritional imbalance, and specific ion toxicity. Mulberry plants have been defined as moderate salinity tolerance group with EC_e of 4-8 dSm^{-1} and defined to non-halophytic plants. These plants, also known as glycophytes include rice (*Oryza sativa*), maize (*Zea mays*), soybean (*Glycine max*), beans (*Phaseolus vulgaris*). The plants that survived in saline condition have many mechanisms for decreasing effects of salt stress. In general, the mechanisms of salinity tolerance in plants can be categorized into three (1) tolerance to osmotic stress (2) Na^+ exclusion and (3) tissue

tolerance. These mechanisms lead to change the levels of a number of proteins, which may be structural in nature or soluble or which may exist before and after folding in the plant cell. Due to gene expression changed under stress, quantitative and qualitative altered in proteins are obvious. The proteins have been reported such as proteins involved in photosynthesis, oxidative stress, protein degradation, glycinebetaine synthesis, ATP production, cyanide detoxification and chaperone activity. Thus, the study proteins that related of salt tolerance in mulberry is of special importance, especially to understand the mechanisms and response for salt stress, also leading to be found proteins marker for screening on other plants.

Proteomics is a powerful tool of discovery science focusing on proteins. This technique had been employed to analyze, compare synthesis, turnover and modification of many proteins during development or in response to environmental changes, especially a salt stress. Currently, proteomic analysis under salt stress has been reported in any plants such as rice, potato, tomato, bean, soybean and other crop plants. In this study, the presence of screening salt tolerant and salt sensitive mulberry genotypes in Thailand by treating the plants with NaCl solution. To analyze the differential proteins expression induced by salt stress, the two varieties were subjected to a proteomic analysis based on 2D-PAGE and protein identification by LC-MS/MS,

OBJECTIVES

1. To screen salt tolerant and salt sensitive mulberries, *Morus* spp., in Thailand

2. To determine differential protein expression of salt tolerant and salt sensitive mulberries during salt stress

3. To identify proteins that related to salt stress response mechanism of mulberry

LITERATURE REVIEW

1. Mulberry (*Morus* spp.)

The mulberry belongs to the genus *Morus* of the family Moraceae. There are 24 species of *Morus* and 1 subspecies, with at least 100 known varieties. Mulberry is a fast growing deciduous woody perennial plant (Vijayan, 2009). It is found from temperate to sub-tropical regions of the northern hemisphere to the tropic of the Southern hemisphere; they can grow in a wide range of climatic, topographical and soil conditions. Presently, mulberry is growing in regions between 50^{o}N Lat. and 10^{o}S Lat. (Yokoyama, 1962); from near sea level to altitudes as high as 4000 m (Machii *et al.*, 1999).

The mulberry has simple leaves with alternate pattern. There are dimorphic forms of mulberry leaves, entire or lobed. However, the morphology of mulberry leaf is quite very according to the different conditions (Aruga, 1994). Flowers are monoecious, both male and female imperfect flowers occur in the same plant, or dioecious, male or female occurs only on the separate plants.

Species of *Morus* which have widely accepted among mulberry taxonomists and geneticists such as *Morus alba, Morus indica*, *Morus serrata*, *Morus laevigata, Morus multicaulis*, *Morus tartarica*, *Morus nigra*, *Morus Australis, Morus rotunbiloba etc.* Mulberry has different ploidy levels. Most of the genotypes available are diploids (2x, 2n=28) but exhibit higher ploidy i.e., 3x, 3n=42, 4x, 4n=56, 6x, 6n=84 and docasaploid 22x, 22n=308 (Basavaiah *et al.*, 1989). Especially, the diploid species, *M. alba* is the major variety which is being cultivated in South East Asia. However, it is rich in ploidy especially triploid varieties mulberry plant is also extensively cultivated. They are fast to grow and considered to be useful for feeding purposes (Machii, 1991).

Mulberry is the most important crop for sericulture. The silkworm (*Bombyx mori* L.) eats only mulberry leaves to make its cocoon and producing silkworm.

Mulberry leaves are rich in protein and amino acids (Machii, 1989). It is known that there is high correlation between total amount of protein in mulberry leaves consumed by the silkworm and production efficiency of cocoon shell (Machii and Katagiri, 1991). Further, it has been reported that the cost of mulberry leaf production alone covers more than 60% of the total cost of cocoon production (Das and Krishnaswami 1965). Mulberry leaf protein is the only substrate used by silkworm for biosynthesis of the silk. Nearly 70% of mulberry leaf protein is utilized in the production of silk protein (Fukuda *et al.,* 1959). Hence, improvement of mulberry varieties is necessary for successful sericulture and high quality silk.

In addition to the use in sericulture, mulberry leaf is also used as animal fodder because it has highly nutritious, palatable and digestible (70-90 %) to herbivorous like cow, sheep, goat and buffalo. The protein content in leaves and young stems varies from 15 to 28 %, depending on the variety. The mineral content in leaf is also reported to be high and no anti-nutritional factors or toxic compounds identified. It was used in traditional Chinese herb medicine for a long time (Sanchez, 2000). Recently, *M. alba* has been highlighted in various scientific investigations, exploring to validate its medicinal value worth (Butt *et al.,* 2008).

Mulberry leaf is rich in gamma-aminobutylic acid, effective against high blood pressure, and alanine which effective against hangovers (Machii, 1990). The extracts of mulberry leaves are able to stop atherosclerosis as they inhibit the oxidative modification of LDL such as flavonol glycoside, quercentin 3-(6-malonylglucoside) (Katsube *et al.*, 2006). It also inhibits the tyrosinase activity that converts 1-3, 4-dihydroxyphenylalanine (DOPA) to dopachrome in the biosynthesis process of melanin (Sohn *et al.*, 2004). The leaf extracts can provide a viable treatment for Alzheimer's disease through the inhibition of amyloid β-peptide (1–42) fibril formation and attenuation of amyloid β-peptide (1–42)-induced neurotoxicity (Niidonme *et al.*, 2007). Moreover, it has been found that 1-deoxynojirimycin (DNJ), which is said to positive in lowering the blood-sugar level closely related to diabetes mellitus type II is abundant in the leaf and root. Mulberry tea is considered to be a health food. In addition, it was found that mulberry fruit has an anti-oxidative

property. Also, the bark from the root, in particular, has been used as herbal medicine to reduce high blood pressure (Machii, 1991).

Mulberry has been mainly planted in the Northeast and Central of Thailand which is the most popularly employing for sericulture as an important agro-industry with excellent results. Most of sericulture farmers are small scale farmers, who do sericulture as a secondary occupation apart from paddy field. The total planted area of mulberry in the country is around 34,880 hectares. In 2009, Thailand, which was the sixth largest producer of the silkworm fiber, had produced 1,500 tons (The Queen Sirikit Institute of Sericulture, 2009).

In Thailand, mulberry varieties were divided in to five groups

- Thai local varieties; Khunpai, Phoe, Mee, Plong, Jak, Somyai, Soi, Som, Kam, Noi, Kaew, Kaewchonnabot, Kaewsatuek Sieda, Phai etc.

- Hybrid varieties; Burirum51 (BR51; Lhunjiew no.44 x Noi), Burirum60 (BR60; Lhunjiew no.44 x Noi), Nakhon Ratchasima60 (NM60; Shujakuichi no.18 x Kaewchonnabot), Sisaket33 (SK33; open breeding of Jing), S1 (open breeding of S54) etc.

- Wild varieties; Huaipuling, Khonsarn, Raming 1, and Raming 2 etc.

- Fruit varieties; Chiangmaikinphol, Pholyaivawee, Banluang, Koraj-Ginphol, Panghong and Pholyai-Wavee etc.

- Exotic varieties; *M. acidosa*, *M. alba*, *M. australis*, *M. nigra*, Kenmochi, Kenva 2 and Lunjiew etc.

2. Saline soil

Saline soil is one of the major problems that affect plant growth and development. However, it is more widespread and acute in arid and semi-arid regions than in humid ones. Soil salinity is a condition where plant growth is reduced due to the existence of soluble salts in soil which holds water more tightly than the capability of plants (to extract water from the soil). High concentration of salt causes ion imbalance and hyperosmotic stress in plants. It is estimated that nearly 19.5% of the irrigated agricultural lands within in Thailand are considered salt affected. Furthermore, each year, there is a deterioration of 2 million ha (about 1%) of world agricultural lands to salinity, leading to reduced or no crop productivity (Flowers and Yeo, 1995).

Salt affected soils can be broadly divided into three categories base on their salinity and sodicity, namely saline soils, sodic soils and saline-sodic soils (Szabolcs, 1994; Qureshi and Barrett-Lennard, 1998).

- In saline soils, the concentration of salts has increased to the level at which crop growth is adversely affected. The soil remains permeable and has good drainage characteristics. In these soils, the electrical conductivity of the saturation extract (EC_e) are greater than 4 decisiemens per meter (dSm^{-1}), the pH usually ranges between 7.5-8.5, and the sodium adsorption ratios are less than 15.

- Sodic soils have high exchangeable sodium concentrations, which dissolve the organic matter present in the soil. In these soils, soil structure has deteriorated, permeability has decrease, and root growth is restricted. These soils have EC_e values less than 4 dSm^{-1}, pH values greater than 8.5, and sodium adsorption ratio greater than 15.

- Saline-sodic soils have the characteristics of both saline and sodic soils; EC_e values have greater than 4 dSm^{-1}, pH values normally less than 8.5 and sodium adsorption ratios are greater than 15.

Although most of the agriculturally important crops cannot grow in saline soils, it is entirely not inimical to growth of all plant species (Table 1). Some plants grow well in salt affected coastal areas, shores of backwaters lakes and marshy lands. Those plants that can survive and grow well on high concentrations of salt in the rhizosphere are called halophytes. However, some other plants cannot even tolerate a salinity caused by 10 % of seawater. Such plants are called glycophytes or non-halophytes (Cherian *et. al.*, 1999). Mulberry plants have been defined as moderate salinity tolerance group, which tolerate to soil EC_e of 4-8 dSm^{-1} (Heidi, 2008).

2.1 Saline soil in Thailand

In Thailand, salinity salt problems in many areas, which comprised 3.47 million ha. There are 17.8 million rai or about 2.85 million ha of saline soil area in the Northeast and the yield of crops is extremely decreased. Moreover, there are 3.11 million ha of area where it is easy for the spread of soil salinity. Saline soil is found almost every province in the Northeast of Thailand.

In the Northeast, saline soil area can be divided into 4 types:

1. High-level saline soil area is in the area where salty dust is found among soil texture more than 10% of the area. Plant cannot be grown and the area is left empty. It takes much money for soil improvement.

2. Middle-level saline soil is in the area where salty dust is found among soil texture 1-10% of the area. Plant can be grown but crops yield decreases.

3. Low-level saline soil is in the area where salty dust is found among soil texture less than 1% of the area. Subsurface flow is brackish or brine and its depth is 2 m. from soil surface. Such area is the paddy-field.

4. Area where it is easy for the spread of soil salinity. Nowadays, such area is the highland where crops are planted. Salty dust is not found among soil

texture but below the soil, there is salty stone. When the rain falls, water from soil surface flows pass salty stone layers and it becomes salty water flowing to the next plain area.

3. Plants under stress

Plant growth and productivity was affected by nature in the form of various abiotic and biotic stress factors. Plants are frequently exposed to many stress conditions such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. Plants also face challenges from pathogens including bacteria, fungi, and viruses as well as from herbivores (Table 2). All these stress factors are threated for plants and prevent them from reaching their full genetic potential and limit the crop productivity worldwide.

Abiotic stresses, such as salinity, drought, chemical toxicity and oxidative stress are serious threats to agriculture and result in the deterioration of the environment (Wangxia, 2003). Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Bray *et al*., 2000). The stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang *et al*., 2001). The complex plant response to abiotic stress, which involves many genes and biochemical-molecular mechanisms, is schematically represented in Figure 1.

Table 1 Effect of soil salinity on growth and plant production

EC_e (dS/m)	% Soil salinity	Levels of soil salinity	Effect on plants
2	< 0.1	Non-saline soil	Not affect on growth and yield of plants
2-4	0.1-0.2	Slightly saline soil	Affect glycophytes plants
4-8	0.2-0.4	Moderately saline soil	Affect many plant species
8-16	0.4-0.8	Strongly saline soil	Only halophytes that survive
16	> 0.8	Severely saline soil	Few salt tolerant plants can survive

Source: United States Salinity Laboratory Staff (1954)

Table 2 Salt tolerance of selected plants of agricultural importance

EC_e (dS/m)			
2-4 (Slightly saline soil)	**4-8** (Moderately saline soil)		**8-16** (Strongly saline soil)
Bean	Mulberry	Rice	Cotton
Lemon	Sunflower	Tobacco	Sugar beet
Mango	Tobacco	Wheat	Tamarind
Orange	Barley	Corn	Guava
Banana	Tomato	Grape	Coconut
Cucumber	Potato	Almond	Seablite
Pea	Carrot	Cherry	Mangrove
Onion	Watermelon	Tomato	Sweet potato

Source: Qureshi and Barrett-Lennard (1998)

Salt stress is certainly one of the most serious environmental factors limiting the productivity of crop plants (Ashraf, 1999). However, despite the advances in the increase of plant productivity and resistance to a number of pests and diseases, improvement in salt tolerance of crop plants remains elusive. This is due to the fact that salinity affects most aspects of plant physiology. Therefore, the study of salinity tolerance in plants is of special importance.

Table 3 Abiotic and biotic stresses

Abiotic stresses	Biotic stresses
1. Cold (chilling and frost)	1. Pathogens (viruses, bacteria, and fungi)
2. Heat (high temperature)	2. Insects
3. Salinity (salt)	3. Herbivores
4. Drought (water deficit condition)	4. Rodents
5. Excess water (flooding)	
6. Radiations (high intensity)	
7. Chemicals and pollutants (heavy metals)	
8. Oxidative stress (reactive oxygen species)	
9. Wind (sand and dust particles in wind)	
10. Nutrient deprivation in soil	

Source: Shilpi and Narendra (2005)

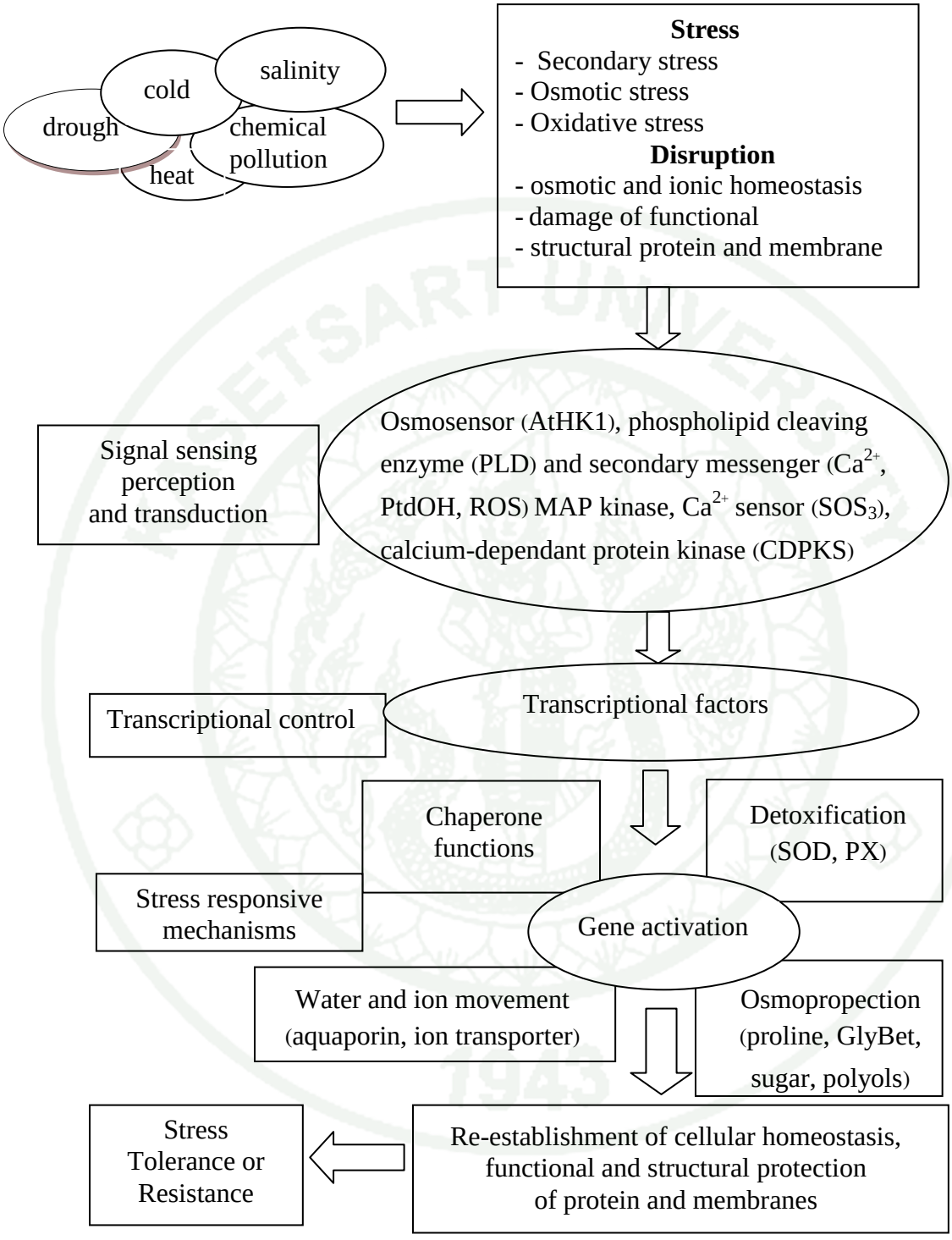

Figure 1 The complexity of the plant response to abiotic stress.

Source: Wang *et al.* (2003)

4. Salt stress in plants

More than 40 years of research on salinity have produced an uncountable number of papers and un-published results that reflect the importance of this problem in agriculture. Salt stress is an abiotic stress, which usually occurs in arid and semiarid regions, is a major environmental constraint to crop productivity (Ashraf, 1999). The stress can affect physiological processes from seed germination to plant development, resulting in growth and yield reduction (Ashraf, 2004).

High salt stress disrupts homeostasis in water potential and ion distribution. This disruption of homeostasis occurs at both the cellular and the whole plant levels. Drastic changes in ion and water homeostasis lead to molecular damage, growth arrest and even death. To achieve salt tolerance, three interconnected aspects of plant activities are important (Figure 2). First, damage must be prevented or alleviated. Second, homeostatic conditions must be re-established in the new, stressful environment. Third, growth must be resumed albeit at a reduced rate.

Salts inhibit plant growth by osmotic stress, nutritional imbalance, and specific ion toxicity (Cornillon and Palliox, 1997). Salinity reduces the ability of plants to absorb water, causing rapid reductions in growth rate (Muuns, 2002). High salt concentration in the external solution of plant cells produces several deleterious consequences.

First, salt stress causes an ionic imbalance (Zhu *et al*., 1997). When salinity results from an excess of NaCl, which is by far the most common type of salt stress, the increased intracellular concentration of Na^+ and Cl^- is deleterious to cellular systems (Serrano *et al.,* 1999). In addition, the homeostasis of not only Na^+ and Cl^- but also K^+ and Ca^{2+} are disturbed (Rodriguez and Navarro, 2000).

Second, high concentrations of salt impose a hyperosmotic shock by decreasing the chemical activity of water and causing loss of cell turgor. This negative

effect in the plant cell is thought to be similar to the effects caused by drought (Borsani, 2002).

Third, salt-induced water stress reduction of chloroplast stromal volume and generation of reactive oxygen species (ROS) are also thought to play important roles in inhibiting photosynthesis (Price and Hendry, 1991).

The effect of salt stress leads to huge losses in terms of arable land and productivity as most of the economically important crop species are very sensitive to soil salinity.

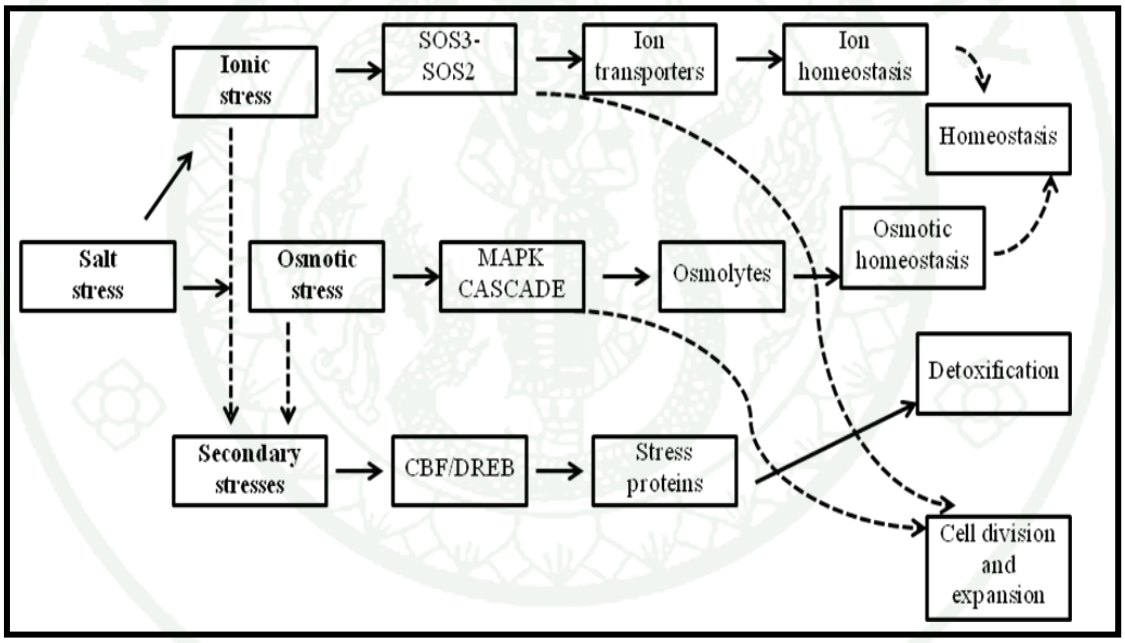

Figure 2 The three aspects of salt tolerance in plants (homeostasis, detoxification and growth control) and the pathways that interconnected them; homeostasis is broken down into ionic and osmotic homeostasis.

Source: Zhu (2001)

5. Effect of salt stress on mulberry

The most common effects of salinity on glycophytes are loss of turgor, shorter stature, decreased photosynthesis, growth reduction resulting in smaller leaves, early senescence, tissue necrosis, respiratory changes, loss of cellular integrity, and even death of the plant (Cheeseman, 1988). The accumulation of high concentrations of Na^+ or Cl^- in the leaves generally results in the formation of burning like lesions (Zhu, 2002). The nutritional deficiency may be manifested similar to those that occur in the absence of salinity. Calcium deficiency symptoms are common when Na^+/Ca^{2+} ratio is high in soil water. High salinity can also injure cells in transpiring leaves, which leads to growth inhibition (Tuteja, 2007). The salt that concentrates in the old leaves makes them die early (Munns *et al.,* 2006).

The first visible symptom of salt injury in mulberry is the appearance of yellow patches in young leaves under low to moderate salinity (Vijayan *et al.,* 2008). The yellowing of leaf may be due to degradation of chlorophyll by the increased activity of chlorophyllase (Singh *et al.*, 1998). Under higher salinity burnt like lesions appeared in the leaves (Vijayan *et al.*, 2008). Early senescence of older leaves and retardation of growth followed under higher salinity as the salt promotes senescence of leaves by increasing the production of abscisic acid (ABA) and ethylene (Zhao *et al.*, 1992). Salinity adversely affected the growth and, thus, the leaf yield of mulberry, albeit the severity of which varied depending on the tolerance level of the genotype.

The evidence has favored the effect of salt stress on mulberry

- The leaf pigments, proline, and Na^+ were increased under salinity in mulberry (Kumar *et al.*, 2003).

- The major effects of salinity on proteins by breaking electrostatic bonds and increasing hydrophobic interactions (Melander and Horvath, 1977) were much evident in mulberry as the protein concentrations in the leaves of plants grown under salinity declined significantly (Vijayan *et al.,* 2008).

- The increase in leaf thickness in response to salinity was the result of an increase in number of spongy layers rather than an increase in the size of palisade cells (Vijayan *et al.*, 2004).

- Adverse effect of salinity on the rate of photosynthesis was reported in mulberry and other woody plants (Golombek and Lüdders, 1993).

- The salinity induced cell membrane damage and increased solute leakage (Hautala *et al*., 1992).

Vijayan *et al.* (2008) reported effect of different levels of NaCl on growth and development of mulberry (*M. alba*), which selected on the basis of their performance under in vitro salinity. Salinity reduced growth and development of all genotypes. However, the putative tolerant varieties showed better performance than the putative susceptible varieties. Under low salinity (<0.5% NaCl) salt tolerant varieties showed an increase in chlorophyll and protein concentrations, while in susceptible varieties both were reduced by 3-58% at 0.5% NaCl and 50-64% at 1.00% NaCl.

Zhou *et al.* (2009) studied effects of salt stress on photosynthetic characteristics of mulberry (*M. alba*) seedlings, treated with NaCl concentrations at 0.1, 0.3 0.5 and 0.57, and fresh water was used as the control. The results showed that 0.1% NaCl did not affect the photosynthetic characteristics of the seedlings, while salt concentration of ≥ 0.3%, the salinity significantly reduced net of photosynthesis rate (P_n). Under lower salt concentration treatments, P_n reduction was mainly controlled by the stomatal limits, whereas under higher salt concentration treatments, P_n decrease was mainly due to the non-stomatal limits.

6. Mechanisms of salt tolerance in plants

The response of plants to salt and other environmental stresses have been extensively investigated for many decades, still we have not been able to understand

fully the mechanism which imparts tolerance to some plants and sensitivity to others (Cheeseman, 1988) due to the complexity of the mechanism (Tuteja, 2007).

The mechanisms of salinity tolerance fall into three categories (Munns and Tester, 2008)

(1) Control of ion uptake by roots and transport into leaves

The osmotic stress immediately reduces cell expansion in root tips and young leaves, and causes stomatal closure. A reduced response to the osmotic stress would result in greater leaf growth and stomatal conductance, but the resulting increased leaf area would benefit only plants that have sufficient soil water. Greater leaf area expansion would be productive when a supply of water is ensured such as in irrigated food production systems, but could be undesirable in water-limited systems, and cause the soil water to be used up before the grain is fully matured.

(2) Selective accumulation or exclusion of ions

Na^+ exclusion by roots ensures that Na^+ does not accumulate toxic concentration within leaves. A failure in Na^+ exclusion manifests its toxic effect after days or weeks, depending on the species, and causes premature death of older leaves.

(3) Compartmentalization of ions at the cellular

Tolerance requires compartmentalization of Na^+ and Cl^- at the cellular and intracellular levels to avoid toxic concentrations within the cytoplasm, especially in mesophyll cells in the leaf. Toxicity occurs with time, after Na^+ in leaf increases to high concentration in the older leaves.

Salt tolerance is the ability of plants to grow and complete their life cycle on a substrate that contains high concentrations of soluble salt. Many factors were determined from deleterious effects of salt stress. It is not surprising that adaptation to

salinity may involve the modification of a large number of parameters. Biochemical strategies including (1) synthesis of compatible solutes, (2) change in photosynthetic pathway, (3) alteration in membrane structure, (4) induction of antioxidative enzymes, and (5) induction of plant hormones (Parida and Das, 2005).

The different parameters for salt tolerance determinants

6.1 Ion regulation and compartmentalization

Ion uptake and compartmentalization are crucial not only for normal growth but also for growth under saline conditions because the stress disturbs ion homeostasis (Adams *et al*., 1992). High level of saline ions in the apoplast alters the aqueous and ionic thermodynamic equilibria, which results in hyperosmotic stress, ionic imbalance and toxicity (Cramer *et al.*, 1986). NaCl-induced accumulation of Na^+ and Cl^- and decrease in K^+ content is commonly observed in most species exposed to salt stress (Jafari, 1998). Ion regulation is an essential factor of the mechanism of salt tolerance in plants. Plants cannot tolerate large amounts of salt in the cytoplasm and therefore, under saline conditions, they restrict the excess salts in the vacuole or compartmentalize the ions in different tissues to facilitate their metabolic functions (Reddy *et al.*, 1992; Zhu, 2003). Removal of sodium ion from the cytoplasm or compartmentalization in the vacuoles is done by a salt-inducible enzyme Na^+/H^+ antiporter (Apse *et al.*, 1999). This control mechanism is dependent on the regulation of proton pumps and antiporters operating at both plasma membrane and tonoplast. Under salt stress, plants maintain high concentrations of K^+ and low concentrations of Na^+ in the cytosol. These by regulating the expression and activity of K^+ and Na^+ transporters and of H^+ pumps that generate the driving force for transport (Zhu *et al.*, 1993). Two electrogenic H^+ pumps, the vacuolar type H^+-ATPase (V-ATPase) and the vacuolar pyrophosphatase (VPPase), coexist at membranes of the secretory pathway of plants (Dietz *et al.*, 2001).

Wang *et al.* (2001) reported that the main strategy of salt tolerance in the halophyte *Suaeda salsa* seems to be an up-regulation of V-ATPase activity, which is

required to energize the tonoplast for ion uptake into the vacuole, while V-PPase plays only a minor role.

Aharon *et al.* (2003) reported expression of all of the AtNHX members of the family, coding for vacuolar Na+/H+ antiporters, provided a recovery of the salt sensitive yeast mutant, supporting their role in Na+/H+ exchange.

Shi *et al.* (2003) reported over-expression of the *Arabidopsis thaliana* SOS1 gene, which encodes a plasma membrane Na^+/H^+ antiporter. It improves plant salt tolerance in *A. thaliana*. Transgenic plants showed substantial up-regulation of SOS1 transcript levels upon NaCl treatment, suggesting post-transcriptional control of SOS1 transcript accumulation.

6.2 Induction of antioxidative enzymes

Salt stress is complex and imposes a water deficit because of osmotic effects on a wide variety of metabolic activities (Greenway and Munns, 1980; Cheeseman, 1988). This water deficit leads to the formation of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) (Halliwell and Gutteridge, 1985), and singlet oxygen ($^1O_2$) (Elstner, 1987). These activated oxygen species are highly reactive and in the absence of any protective mechanism can damage different aspects of cell structure and function such as damage to protein, lipids and nucleic acids (Fridovich, 1986; Imlay and Linn, 1988).

As a consequence, plants evolved cellular adaptive responses like up-regulation of oxidative stress protectors and accumulation of protective solutes (Horling *et al.*, 2003). Salinity stress in plants is thought to enhance the production of many ROS such as superoxide distumase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR). All of them are the systems designed to minimize the concentrations of superoxide and hydrogen peroxide. Plant with high

levels of antioxidant defense enzymes has greater resistance to oxidative damage. Superoxide dismutase is a major scavenger of $O_2^{\bullet-}$ and its enzymatic action results in the formation of H_2O_2 and O_2. Hydrogen peroxide, thus, was eliminated by CAT and APX, including enzymatic and non- enzymatic H_2O_2 degradations (Peltzer *et al.*, 2002).

The activities of the antioxidative enzymes such as CAT, APX, POD, GR, and SOD increase under salt stress in plants and a correlation of these enzyme levels and salt tolerance exists (Kennedy and DeFillippis, 1999; Hernandez *et al.*, 2000; Benavides *et al.*, 2000).

Dionisio *et al.* (1998) determined the levels of GR, CAT, POD activities in rice (*Oryza sativa* L.) during salt stress. They were increased under salt stress at 200 mM NaCl.

Harinasut *et al.* (2003) investigated the salt induced changes in antioxidant enzymes using a local mulberry variety '*Pai*'. The amount of H_2O_2 and the activity of guaiacol specific peroxides, SOD, APX and GR were increased by 1-2 fold at 150 mM NaCl.

Sudhakar *et al.* (2001) determined the salt induced changes in the antioxidant enzyme efficacy in two high yielding varieties of mulberry (*M. alba*), confirms the relative tolerance of the S1 variety based on the low rate of lipid peroxidation and a high constitutive activity by 13-16 fold of antioxidant enzymes.

Kumer *et al.* (2008) investigated the modulations in key enzymes of nitrogen metabolism in mulberry varieties (S1, salt tolerant; ATP, susceptible) under salt stress**.** The levels of antioxidative enzymes were increased in salt tolerant mulberry strain under salt stress. In by contrast, the level of antioxidative enzymes was decreased in salt sensitive mulberry strain under salt stress and the level of protease activities was increased.

Chingkitti (2008) determined some enzyme activities in mulberry (*M. rotunbiloba*) cultures with various concentrations of sodium chloride for 1 week. After treated plant's cultures with NaCl at 300 and 500 mM, the enzymes including, glutathione-S-transferase (GST), POD, ATPases and glucosidase gave higher activities than the control sample. ATPase showed activity in leaf and root 3.5 and 5.5 times respectively, higher than in the control sample at 500 mM NaCl. Glucosidase and GST gave 3-4 times higher than in leaf at 300 and 500 mM NaCl. The neutal peroxidase in leaf gave 3.5 times higher than the control sample at 300 and 500 mM NaCl and higher than in the acidic and basidic peroxidase enzymes.

6.3 Osmolytes accumulation in plant

Osmotic accumulation of solutes by cells is a process by which water potential of a cell can be decreased without an accompanying decrease in cell turgor. It is a net increase in solute concentration that is independent to the volume changes that result from loss of water (Taiz and Zeiger 2002). Osmotic adjustment in plants subjected to salt stress can occur by the accumulation of high concentrations of either inorganic ions or organic solutes. The compatible osmolytes generally found in higher plants are organic acids, low molecular weight sugars, polyols, nitrogen containing compounds such as amino acids, amides, imino acids, ectoine (1, 4, 5, 6-tetrahydro-2-methyl-4-carboxylpyrimidine), soluble low molecular weight proteins such as LEA (late embryogenesis abundant proteins) and dehydrins and quaternary ammonium compounds (Ashraf, 2004).

Murakeozy *et al.* (2003) studied the levels of compatible osmolytes in three halophytic species (*Lepidium crassifolium, Camphorosma annua* and *Limonium gmelini* subsp. *hungaricum*) under salt stress. The investigated species were shown to accumulate both carbohydrate- and amino acid-derived osmolytes. The leaf tissues of *C. annua* preferentially stored glycine betaine and pinitol, while in *L. gmelini* β-alanine betaine, choline-O-sulphate, and pinitol were accumulated. In the leaves of *L. crassifolium* a very high amount of proline, associated with a high level of soluble carbohydrates was found.

Ashraf (1994) studied organic substances responsible for salt tolerance in, *Eruca sativa*, under salinity stress. The tolerant population accumulated significantly greater amounts of soluble sugars, proline and free amino acids in the leaves compared with the non-tolerant population.

Mansour (2000) reported a number of nitrogen containing compounds (NCC) accumulate in plants exposed to salinity stress. Especially, amino acids, amids, imino acids, proteins, quarternary ammonium compounds (QAC) and polyamines. The specific NCC that accumulated in saline environment varies among plant species. Osmotic adjustment, protection of cellular macromolecules, storage form of nitrogen and scavenging of free radicals are proposed functions for these compounds under stress conditions.

Martino *et al.* (2003) investigated accumulation of free amino acid and glycine betaine in leaves of spinach (*Spinacia oleracea*) under salt stress. Some of free amino acids such as glycine, serine and glycine betaine were increased after 27 days salt treatment.

Proline accumulation in plants has been widely reported as a response to salinity and in larger amounts than other amino acids in salt stressed plants such as *Eruca sativa* (Jones 1981and Ashraf 1994), *Bacopa Monniera* (Ali *et al.*, 1999) and *M. alba* (Kumar *et al.*, 2003).

Park *et al.* (2005) expressed LEA protein in transgenic Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) under high salinity. The transgenic plants demonstrated growth enhanced ability under salt stress conditions. The increased tolerance was reflected by delayed development of damage symptoms caused by stress. They suggest that the genetic modification of Chinese cabbage by LEA protein gene holds considerable potentiality for crop improvement toward environment-stress tolerance.

Lal *et al.*, (2007) over-expressed *HVA1,* encodes a group 3 LEA protein and is induced by ABA and water deficit conditions and characterized from barley in mulberry.Transgenic mulberry plants were subjected to simulated salinity stress conditions to study the role of *HVA1* in conferring tolerance. The transgenic plants showed better cellular membrane stability (CMS), photosynthetic yield, less photo-oxidative damage. Under salinity stress, transgenic plants show many fold increase in proline concentration than the non-transgenic plants.

Chingkitti (2008) investigated the accumulations of selected soluble sugar in mulberry (*M. rotunbiloba*) under salt stress. At least four kinds of sugars, namely fructose, glucose, mannitol and sucrose were greatly observed in leaf but not found in root tissue.

Jyothsnakumari *et al.* (2008) investigated the expression of LEA protein (group 1, 2, 3 and 4) under different levels of salt stress in mulberry (*M. alba*) varieties (S1, a salt tolerant and ATP, a salt sensitive). Exposure of plants to NaCl resulted in higher accumulation of LEA proteins in S1 than ATP. The maximum content of LEA (group 3 and 4) was detected in S1 at 2.0% NaCl, which correlated with its salt tolerance.

Vijayan *et al.* (2008) studied morpho-biochemical changes in mulberry (*Morus* spp.) varieties during salt stress. Salinity reduced growth and development of all genotypes. However, salt tolerant varieties showed an increase in chlorophyll and protein concentrations, while in susceptible varieties both were reduced by 3–58% at 0.5% NaCl.

6.4 Growth regulation

Salt stress like many other abiotic stresses, inhibits plant growth and limits plants production. Slower growth is an adaptive feature for plant survival under salt stress. One cause of growth rate reduction under stress is inadequate

photosynthesis owing to stomatal closure and consequently limited carbon dioxide uptake (Zhu, 2001)**.** In addition, stress might inhibit cell division and expansion directly. Even mild salt stress condition could result in slower growth and significant loss of plant productivity. Some plants are probably not responsive enough and so run the risk of dying by continuing to grow when stress is already serious. Fine tuning this responsiveness could potentially improve productivity under salt stress. A cyclin dependent-protein-kinase inhibitor, might hinder cell division by reducing the activities of cyclin-dependent protein kinases inhibitor (ICK1) that help to drive the cell cycle. Salt stress might inhibit cell division by causing the accumulation of abscisic acid, which, in turn, induces *ICK1* (Wang *et al.*, 1998).

6.5 Induction of plant hormones

The plant hormones such as aminocyclopropane-1-carboxylic acid (ABA) and cytokinin plays central role in various physiological and biochemical processes related to environmental stresses (Sauter and Hartung, 2000). Under salinity ABA level increases due to the activation of genes responsible for ABA biosynthesis. Salt stress results in increased levels of ABA and ethylene production in plants (Gomez *et al.*, 1998). ABA has been found to alleviate the inhibitory effect of NaCl on photosynthesis, growth and translocation of assimilates (Popova *et al.*, 1995). ABA promotes stomatal closure by rapidly altering ion fluxes in guard cells under stress conditions. Other ABA actions involve modifications of gene expression, and the analysis of ABA-responsive promoters has revealed diversity of potential cis-acting regulatory elements (Kumar, 2005). Considering the great complexity of the mechanism of salt tolerance, it is difficult to identify any single criterion, which could be used as an effective selection criterion.

7. Screening of mulberry for salinity tolerance

The identification of suitable crop species for this purpose requires an efficient screening method, one that identifies plants tolerant to saline or alkaline soils.

Previously, screening method has been made to selected genotypes in vitro using shoot apices and axillary buds.

Hossain *et al.* (1991) screened ten mulberry varieties for salinity tolerance on MS medium containing different concentrations of NaCl (0.08–0.4%). Root formation from shoot apices and the subsequent development of the roots were used as criteria for the screening.

Vijayan *et al.* (2003) screened sixty three mulberry varieties for salinity tolerance by using axillary buds from field-grown plants, which were cultured on MS medium containing different concentrations about 0.0-1.0 % of NaCl in order to study the shoot and root growth pattern.

Vijayan *et al.* (2004) screened 6 mulberry varieties from 43 promising mulberry genotypes for salinity tolerance through *in vitro* seed germination on MS basal medium containing different concentrations 0.0-1.25 % of NaCl. There were the seeds of English black, *M. rotundiloba*, KPG-3, Kolitha-3, Mysore local and Sultanpur showed considerable tolerance to salinity.

Kashyap and Sharma (2006) selected *in vitro* salt tolerant saplings of *M. alba* (cv. Sujanpuri) were raised from nodal explants with axillary buds collected during three different periods of the year. The growth and shoot/root multiplication of the nodal explants collected between November to February and July to October were found to be better than those collected between March to June. In cultures was induced salt stress by adding different concentrations 0.1-0.4 % of NaCl.

Mogili *et al.* (2008) identified tolerant varieties under induced saline conditions using pot culture technique. Thirty promising mulberry varieties of varied genetic makeup, consisting of commercially cultivated varieties, promising germplasm accessions and triploids maintained at CSR and TI, Mysore were screened for their relative tolerance to salinity (8.0 mmho/cm) under controlled conditions. Varieties Ace 116, Ace 214 (RFS-135) and S-3 recorded high total biological yield

and leaf yield. The salinity tolerance indices based on leaf yield reduction under stress confirmed the superior tolerance of Ace 214, Acc 116 and S-3 in turn had bearing on varietal tolerance score.

Ahmad and Sharma (2010) studied the physiological and biochemical attributes in two cultivars of mulberry (local and Sujanpuri) plants under $NaHCO_3$ stress. Mulberry plants were subjected to different treatments using $NaHCO_3$. Local cultivar was found to tolerate salt stress more than Sujanpuri.

8. Proteins that related salt stress on plant

Abiotic stresses are known to induce deleterious cellular changes, especially the salt stress. Salt stress has an effect to change the levels of a number of proteins, which may be structural in nature or soluble or which may exist before and after folding in the plant cell (Srivastava *et al.*, 2004). The most crucial function of plant cell is to respond to stress by developing defense mechanisms. This mechanism is brought about by modification in the pattern of gene expression. This leads to modulation of certain metabolic and defensive pathways. Due to gene expression changed under stress, quantitative and qualitative altered in proteins are quite obvious (Qureshi, 2007).

The gene products of salt-stress response are classified in two major categories (Khan *et al.*, 2007).

1) The first group includes functional proteins, or proteins that probably function in stress tolerance. They are HSPs, LEAs, proteins involved in repair and protection from damages, such as proteinases, and plant defense-related proteins, membrane proteins such as transporters, protein synthesis-related proteins, proteins involved in synthesis of osmoprotectants (proline, sugars), senescence-related proteins, proteins involved in cellular metabolic processes, such as carbohydrate metabolism, secondary metabolism, biosynthesis of plant hormones (ABA, ethylene

and IAA), proteins regulated by plant hormones (ABA and JA), RNA-binding proteins, cytochrome P450, alcohol dehydrogenase and aldehyde dehydrogenase.

2) The second group contains regulatory proteins, such as protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response. They are various transcription factors, protein kinases such as mitogenactivated protein kinase (MAPK), mitogen-activated protein kinase kinase kinase (MAPKKK), ribosomal S6 kinase (S6K)31, calcium-dependent protein kinase (CDPK), histidine kinase (HK) and receptor-like protein kinase (RPK), protein phosphatases such as protein phosphatase 2C (PP2C), PI turnover-related proteins, such as phopholipase (PLC), phospholipase D (PLD) and calmodulin binding proteins and Ca^{+} binding proteins.

Previously, several techniques are available to perform differential analysis of protein expression. Many approaches describing the use of western analysis, enzymatic kinetics, fraction isolation, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Opiteck *et al*., 1998). Currently, the developments in sensitivity and accuracy for proteome analysis have provided new dimensions to assess the changes in protein types and their expression levels under stress.

9. Proteomics

9.1. The Proteome

The term “proteome” describes the protein complement of a genome. Many activities in living cells are performed by protein. Proteome is normally used to represent the total proteins expressed by the entire genome of a cell at a certain time and condition. Proteomes of cells are dynamic and are directly affected by environmental factors such as stress and drug treatment or by aging and disease (Kawamura, 2006). In addition, gene product from a single gene can provide multiple forms of proteins through post-transcriptional and translational modification (Wilkins

et al., 1996). Therefore, the proteins expressed in cell more than gene in genome. These proteome studies are referred as “Proteomic analysis”.

9.2 Proteomic analysis

Proteomics is a powerful tool of discovery science focusing on proteins. Proteomics had been employed to analyze protein changes in response to environmental changes (Abbasi and Komatsu, 2004). It contributes to the studies of the expression, regulation of biological systems, functions, quantification and localization. The proteomics can be divided in to three levels (1) proteomic analysis for identification and characterization of post translational modification of proteins (2) expression proteomics for investigation of qualitative and quantitative proteins change among two or more different state of a cell (3) cell mapping proteomics for determination of protein proteins interactions and intracellular signaling circuitry (Yoojun, 2008). The application of proteomics techniques and strategies to the field of medicine extends from a way of biomarker searching for disease diagnosis, drug discovery and salt tolerant varieties plant.

Proteomics is now becoming an essential tool for research. It includes proteins extraction, preparation, separation and identification. Nowadays, there are several workflows for proteomics including 1D-Gel-LC-MS/MS, 2D-PAGE and MudPIT (Multidimensional protein identification technology) (Lee and Cooper, 2006).

9.2.1 2D-PAGE

Two-dimensional gel electrophoresis (2D-PAGE) was used for protein separation, resulting in the complex proteins separated into individual proteins. The main analytical tool of proteomics research is mass spectrometry (MS) which increase sensitivity and specificity of protein identification coupled with information from databases of protein (Figure 3). 2D-PAGE was reported by Farrell and Klose in 1975. It has been widely used to separates complex protein mixtures

extracted from cell, tissue or other biological sample. The proteins sample resolved with 2D-PAGE depends on two protein properties including isoelectric points (pI), the pH results in net charge of protein equaling to zero and molecular weights in the independent step. Because it is unlikely that two protein molecules will be similar in both properties. Thus protein that separation in 2D-PAGE is much more efficient than 1D-PAGE.

9.2.2 Protein visualization

Protein separated on 2D-PAGE was visualized by either staining with Coomassie blue dye, silver stains, fluorescent dyes, immunological detection or by radiolabelling. Each method has its own characteristics and limitation such as detection sensitivity, cost and available equipment. The Coomassie blue dye technique is widely used for staining the proteins on the 2D-PAGE gel due to its simplicity and compatibility with MS. The Coomassie blue staining requires 10-50 ng protein for detection. While the silver stains is mostly used for identification of low-abundance proteins (1-10 ng), due to its high sensitivity.

9.2.3 Protein identification

Mass spectrometry has been widely used to analyze biological samples and has evolved into a major tool for identification of proteins in proteomics research, this, due to its high sensitivity. MS allows a measurement of mass to charge ratio of ions derived from query molecules. Mass spectrometer commonly consists of three basic components including an ion source that converts molecules into gas-phase ion, a mass analyzer that separates the gas-phase ions according to mass to charge ratio, and an ion detector that measures the ratio of each ion. Identification of protein using MS approach is categorized into two strategies including peptide mass fingerprint and tandem mass spectrometry. The mass patterns are used for comparison with known libraries to confirm peptides which can be further used for protein identification (Chen, 2008).

9.2.4 Peptide Mass Fingerprinting (PMF)

PMF is an analytical technique for protein identification using data from peptide masses. A protease such as Trypsin is used to cleave a protein of interest. The interested protein is generally digested with trypsin into peptide fragment after that the accurate mass of these peptides are determined. The peptide masses provide a fingerprint of the interested protein. Since trypsin cleaves proteins at certain amino acids, the mass of tryptic peptides can be predicted theoretically for known proteins in a PMF database. The predicted peptide masses in the database are compared to the PMF of the experimentally digested peptides to identify the protein. Additionally, PMF is well suitable for protein identification for species with complete genome sequences (Pappin *et al.*, 1993).

9.3 Proteomics in plants under salt stress

Proteomics were used in plant biology research and were increased significantly since 2007, becoming a routine methodology in a number of plant laboratories worldwide. Mostly, the plant proteomic papers published during this review period used this strategy to identify and characterize proteins or plant responses to hormones, stresses development and genes involved in growth and the effect of abiotic, biotic and oxidative stresses (Jesus *et al.*, 2009).

Salt stress causes alterations in plant metabolism, including reduction in the water potential, ion imbalances and toxicity, reduction in CO_2 assimilation and susceptibility to injury and oxidative stress. Adaptation to salt stress requires alterations to cellular machinery; these result directly from modifications in gene expression. Such salt stress induced modifications can lead to the accumulation of certain metabolites and they can also lead to the appearance or disappearance of some cellular proteins or to decreases or increases in the abundances of others (Aghaei *et al.*, 2008).

Moons *et al.* (1995) studied the role of ABA and jasmonates in salt tolerance of rice using 2D-PAGE technology and they noticed that both ABA and ABA-responsive proteins, such as LEA protein. ABA concentrations were 6-30 fold higher for salt tolerant variety compared with sensitive variety. Both the salt induced endogenous ABA levels and a greater molecular response of root tissue to ABA were associated with the varietal differences in tolerance.

Askari *et al.* (2006) investigated the effects of salinity levels on proteome of *Suaeda aegyptiaca* leaves identified 27 spot proteins including proteins involved in photosynthesis, oxidative stress tolerance, protein degradation, glycinebetaine synthesis, ATP production, cyanide detoxification, and chaperone activities.

Parker *et al.* (2006) studied the effect of salt stress in the rice leaf lamina and found an increase in eight proteins, including Ribulose-1, 5-bisphosphate carboxylase (RuBisCo) and ferritin occurred by 24 h of exposure to 50 mM NaCl and continued to increase during the following 6 days. Only one protein, a putative phosphoglycerate kinase, was found to increase in expression within 24 h and did not increase over a longer period of exposure to salt. There were also proteins that showed no change 24 h after exposure to salt, but had increased (superoxide dismutase) or decreased (S-adenosyl-L-methionine synthetase) after 7 days salt treatment.

Kumari *et al.* (2007) studied effect of salinity levels on growth and proteomic changes in 2 varieties mulberry (*M. alba*) with contrasting salt tolerance. Salt stress results in differential changes in protein level qualitatively and quantitatively in both the cultivars. More than 150 protein spots were detected in leaves of both varieties. Salt stress causes to much decrease total polypeptides profiles in the sensitive variety than tolerant variety.

Veeranagamallaiah *et al.* (2008) studied the effect of salt stress, which was imposed by serving the Hoagland half strength nutrient solution with 100, 150 and 200 mM NaCl for 7 days in foxtail millet (*Setaria italica* L. cv. Prasad) seedings

identified 29 spot proteins including proteins involved in signal transduction, photosynthesis, cell wall biogenesis, stress related and several metabolisms like energy, lipid, nitrogen, carbohydrate and nucleotide metabolisms.

Jellouli *et al.* (2008) investigate the salt stress responsive proteins in grapevine (*Vitis vinifera*), subjected to a supply of 100 mM NaCl over 15 days using hydroponic condition and they found an 48 proteins displaying a differential expression pattern including 32 up-regulated, 9 down-regulated and 7 new protein spots induced after salt treatment.

Aghaei *et al.* (2008) investigate the salt stress-responsive proteins in potato shoots, subjected to a supply of 100 mM NaCl over 4 weeks. They could be identified 47 spot proteins including proteins involved in photosynthesis, protein-synthesis, osmotine-like proteins, TSI-1 protein, heat-shock proteins, protein inhibitors, calreticulin. These results suggest that up-regulation of defense-associated proteins may confer relative salt tolerance to potato plants.

Yang *et al.* (2009) studied the effect of salt stress, which subjected to a supply of 100 mM NaCl over 25 days in the leaves of *Populus cathayana* Rehder identified 38 spot proteins that play a role in numerous cellular functions, including signal transduction, mRNA processing and the regulation of the cell cycle.

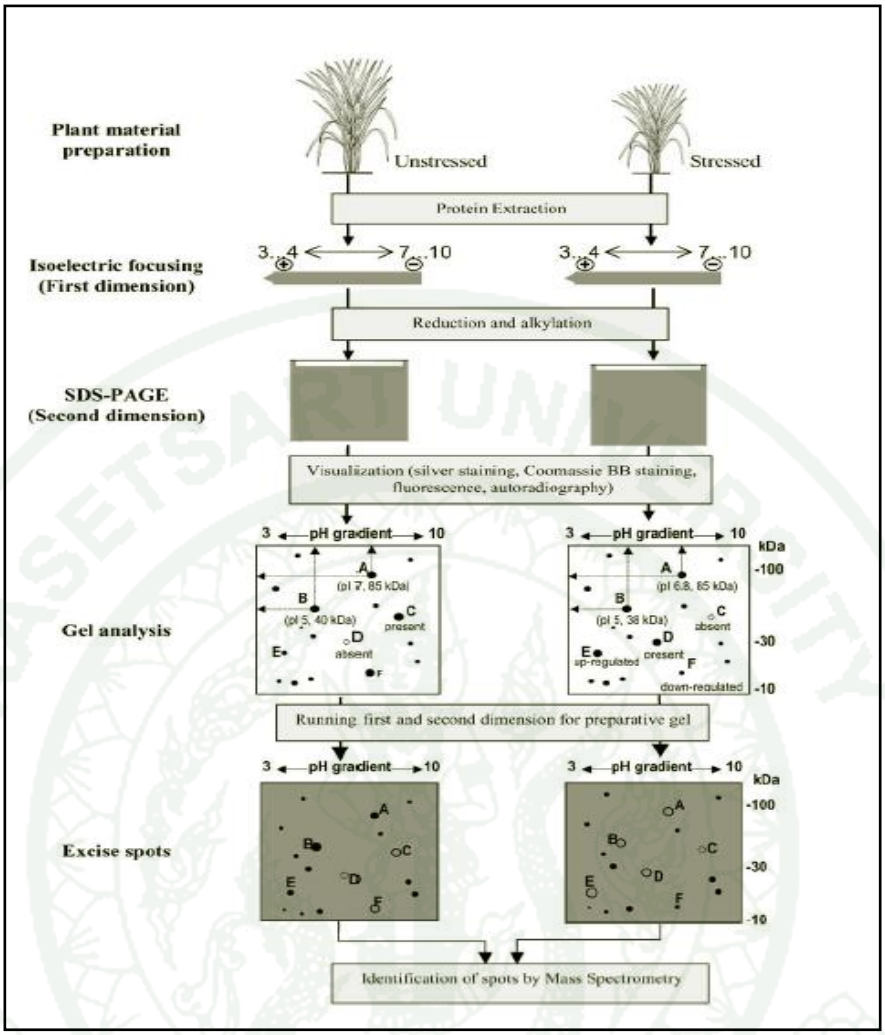

Figure 3 Demonstration process of the stressing plant system and protein extraction, focusing, SDS-PAGE resolution, visualization, digestion and protein identification using advanced mass spectrometry and bioinformatic tools.

Source: Qureshi *et al.* (2007)

There are many reports on the use of proteomics or 2D-PAGE to study salt tolerance in crop plants but only a study of salt tolerance in mulberry was reported (Kumari *et al.* 2007). In this study, therefore, we report the differential protein expression in salt tolerant and salt susceptible mulberry varieties under salt stress as detected by 2D-PAGE, tryptic digestion and LC-MS/MS analysis.

MATERIALS AND METHODS

Materials

1. Plant materials and soil

Forty- four stocks of mulberry varieties were collected from Queen Sirikit Sericulture Center (Sisaket)* and Kasetsart University (main campus)**, (Kampangsaen campus)*** of Thailand. In this study 4 groups of mulberry was used.

1.1 Thai local varieties: Yaiburirum***, Khunpai*, Phoe***, Mee* Plong*, Jak*, Somyai*, Hangplalod***, Kaewkrasang***, Baiphoe*, Chiangkam*, Baimon*, Kreu***, Soi***, Kam*, Som***, Kaewchonnabot*, Noi**, Kaew***, Sieda*, Paiubon*, Kaewsatuek*, Yauk*, Yaisisaket*, Maelukon***, Keekai***, Tadum***, Tadang*, Dang* and KKN-1 ***

1.2 Hybrid varieties: Burirum51*** (BR51), Burirum 60*** (BR60), Sisaket33* (SK33) and S1*

1.3 Fruit varieties: Chiangmaikinphol** and Pholyaivawee**

1.4 Exotic varieties: Tonkin*, *M. alba***, Kenva-2*, S14*, S61*, *M. Nigra***, Lhunjiew** and KNN2**

Top ten and Kampu soils (1:1 proportion) were used for cultivating all of mulberries sample.

2. Chemicals and reagents

The chemicals and reagents used in this study were analytical grade and commercial grade

2.1 General chemicals

Absolute ethanol (Merck, Germany)
Acetic acid (Sigma, USA)
Acetone (Merck, Germany)
Acetonitrile (Merck, Germany)
Hydrochloric acid (Merck, Germany)
Liquid nitrogen (TIG, Thailand)
2-mercaptoethanol (Merck, Germany)
Methanol (Merck, Germany)
Polyvinylpyrrolidone MW = 40,000 (Sigma, USA)
Sodium chloride (Merck, Germany)
Trichloroacetic acid (Sigma, USA)

2.2 Chemicals for protein determination

Bradford reagent (Pierce, USA)
Protein standard, BSA (Pierce, USA)

2.3 Chemicals for proteomics

Acrylamide (BIO-RAD, USA)
Ammonium bicarbonate (BIO-RAD, USA)
Ammonium persulfate (BIO-RAD, USA)
Bromophenol blue (BIO-RAD, USA)
Commassie Brilliant Blue R-250 (USB, USA)
CHAPS (Amersham Biosciences, USA)
Dithiothreitol, DTT (Amersham Biosciences, USA)
Iodoacetamide (GE Healthcare, USA)
IPG Buffer, pH 3-10 L (GE Healthcare, USA)
Low molecular weight markers (GE Healthcare, USA)
N, N'-methylene-bis-acrylamide, Bis (BIO-RAD, USA)

Sodium dodecsyl sulfate, SDS (BIO-RAD, USA)
Tetramethylethylenediamine, TEMED (BIO-RAD, USA)
Thiourea (GE Healthcare, USA)
Tris-base (BIO-RAD, USA)
Urea (Amersham Biosciences, USA)

3. Enzyme for protein work

Trypsin (Sigma, USA)

4. Glass ware

Immobiline DryStip Cover Fluid (Amersham Biosciences, USA)
Immobiline DryStip, pH 3-10 L, 18 cm (Amersham Biosciences, USA)
Strip Holder Cleaning Solution (Amersham Biosciences, USA)
Falcon
Re-swelling tray (Amersham, Biosciences)
ZORBAX Bio-SCX series II, Agilent Technologies
GS-710 imaging densitometer (Bio-Rad, USA)

5. Software

ImageMaster 2D Platinum 7.0 (Amersham Biosciences, USA)
MASCOT program

6. General equipments

Autoclave: Model HA-300M
Autometicpipette: Pipetteman, (Gilson, France)
A -20°C Freezer (Brandt, France)
A -80°C Freezer (Thermo Fisher Scientific, USA)
Balance (Satorious, Germany)

Centrifuge, refrigerated centrifuge: Model BR 4i (JOUAN SA, USA)

Centrifuge, microcentrifuge: Model Spectrafuge 16M

Conductivity meter

Hot plate (Thermo Fisher Scientific, U.S.A.)

Incubator: Model IPR 150.XX2.C

Incubator water bath: Model INNOVA 3100 (New Brunswick Scientific, USA)

Isoelectric Focusing System: Model Ettan IPGphor III (Amersham Biosciences, USA)

Magnetic stirrer (Thermo Fisher Scientific, USA)

Microplate reader: Model VERSA max (Molecular Devices, Canada)

pH meter: Denver Instrument model 215

Vertical electrophoresis system (Amersham Biosciences, USA)

Vortex mixer

7. Equipments for proteomics

Electrophoresis power supply: Model Power Pac HC (Amersham Biosciences, USA)

ImageScanner: Model ImageScanner II (Amersham Biosciences, USA)

LC MS/MS model LTQ (Agilent, USA)

Vacuum centrifuge (Bio-Rad, USA)

Methods

1. Plant preparation

The cuttings of mulberry stock varieties with 3-4 active buds were grown in plastic pod (10 cm wide x 15 cm high), which filled 600 g of soil (Top ten and Kampu soils 1:1 w/w)**.** The pots were watered daily and were kept in the home-made green house 1.5x4 m under natural photoperiod of 12–13 h and temperature of 32 ± 4°C. Two month-old plants were subjected to salt stress treatments.

2. Screening of mulberry and growth characteristics

Pretreatment experiment was performed by treating mulberry stock with 100 ml tab water daily for a week. After that screening of salt tolerance was carried out by treating the mulberry plants alternately with 100 ml of 200 mM NaCl for one day followed by running tap water for 2 days for over the period of 1 month. The samples of control were watered daily with 100 ml of tap water. The $EC_{1:5}$ values of the soil were measured at 0, 15 and 30 days. The growth characteristics of all plants were observed after adding sodium chloride solution for 0 day, 15 days and 30 days. The screening tests were repeated in the selected 2 tolerant and 2 susceptible varieties in dry (winter) and summer season for compared with rainy season. All experiments were carried out in triplicate.

Soil $EC_{1:5}$ was determined by suspended air dry soil 5 g with deionized water 25 ml, shaked for 12 hours, left sample for 12 hour and then measured $EC_{1:5}$ of the solution above the settled soil by conductivity meter.

3. Protein extraction for 2D-PAGE

Salt tolerant mulberry varieties, Som, and salt susceptible varieties, S61, were treated with 100 ml of 200 mM NaCl, and watered daily. The each sample was mixed together by used three replicate and then cleaned and kept at -80°C. Five hundred

milligram of leaves were kept after salt treatment for 0 (control) and 7 (stress) days and ground in liquid N_2 using a mortar and pestle. A portion of fine leaf powder was placed in 10 ml pre-chilled Falcon tubes and re-suspended in 5 ml of cold trichloroacetic acid solution (Appendix) and mixed in Vortex mixer for 15 min. The proteins were precipitated for 60 min at -20 °C and centrifuged at 10,000 rpm for 15 min at 1°C. The pellets were washed twice with cold acetone containing 0.07 % β-mercaptoethanol 5 ml, kept at -20 °C for 1 h and centrifuged at 10,000 rpm for 15 min at 1°C then dried in vacuum, weighed and re-suspended in lysis buffer (Appendix). The samples were mixed by converting the tube, incubated at room temperature for 1 h and sonicated for 5 min. The supernatant was collected by centrifugation at 10,000 rpm for 15 min at 1°C. (Raharjo *et al.*, 2004).The concentration of proteins was determined by Lowry's method using BSA as a standard (Lowry *et al.*, 1951).

4. 2D-PAGE

IPG strip (18 cm, pH 3.0-10.0, linear) was rehydrated overnight with 330 µl of rehydration buffer (Appendix) in a re-swelling tray at room temperature. For analytical and preparative gels, 200 mg of protein were loaded. The first dimensional separation was performed in Ettan IPGphor III Isoelectric Focusing Unit at 20 °C. The running condition was as follows: 500 V for 1 h, followed by 1000 V for 1 h, and then 8000 V gradient and finally 72000 volt × hours (Vh). The focused strips were equilibrated twice for 15 min in 10 ml of equilibration solution at room temperature. The first equilibration was performed in 15 ml rehydration buffer containing 1% DTT for 30 min at room temperature.

The second equilibration was performed in 15 ml rehydration buffer containing 2.5% iodoacetamide for 30 min at room temperature. For SDS-PAGE, the equilibrated strips were positioned on the stacking gel and sealed with 1% agarose solution. Separation in the second dimension was performed by SDS-PAGE on a vertical slab of acrylamide (Appendix) using the Ettan Dalt Six with 2 V/gel 1 h and then 17 V/gel about 4 h at 20 °C, until bromophenol blue reached the bottom of the

gel. Analytical gels were stained with Coomassie Brilliant Blue (CBB) R-250. Triplicate replications of each sample were performed.

5. Image record and data analysis

The 2D-PAGE images were scanned using a GS-710 imaging densitometer (Bio-Rad, USA). ImageMaster 2D Platinum 7.0 software was used for matching and analysis of protein spots on 2D gels. Protein spots in all gels were detected with the same parameters. A matchset was created to compare the gels between stress and control of each sample. The gels were compared between salt tolerant and salt susceptible mulberry varieties of each sample treatments. Landmarks were used to align and position the protein spots of all the members in the matchset. Gels were then normalized: the intensity of every spot was expressed as the ratio of the total intensity of the gel image. The gels were then normalized the intensity of every spot was expressed as the ratio of the total intensity of the gel image. The abundance of each protein spot was estimated by the percentage volume. In quantitative analyses, 1.3-fold abundance ratio to control was set as the threshold for evaluating the up-regulated or down-regulated protein spots and 1.8-fold for identifying the highly changed protein spots in stress. Molecular weights of proteins were determined by co-electrophoresis of standard protein markers and p*I*s were determined by migration of the protein spots on IPG strips.

6. Sample preparation for MS analysis

The gels were cut into 10 pieces of an approximately 5x5 mm. After de-staining of the proteins, each gel piece was independently dehydrated with acetonitrile, dried in a vacuum centrifuge, rehydrated with 10mM DTT in 25mM NH_4HCO_3 for 1 h at 56°C, and washed with water. The proteins in these gel pieces were then *S*-alkylated with 55mM iodoacetamide in 25mM NH_4HCO_3 for 45 min in the dark at room temperature. Gel slices were washed, dried and subjected to in-gel digestion with trypsin (final concentration of 10 ng/ml) in 50mM NH_4HCO_3 overnight at 37°C. Peptides in the gels were then twice extracted with a solution containing 5%

formic acid and 50% acetonitrile. The solutions containing the peptides were pooled, dried and resuspended with 0.1% formic acid. The peptides obtained from separate gel pieces were independently subjected to an integrated microfluidic device (HPLC-Chip for a nano scale LC) that was coupled to an ion trap MS (LC/MSD Trap XCT Ultra, Agilent Technologies, Palo Alto, CA, USA).

7. Mass spectrometry

Pools of sample peptides were analyzed with HPLC-Chip-MS that has an increased sensitivity (at least 5-fold) compared with conventional nanoLC/MS/MS (Hardouin *et al.* 2006). Peptides were injected into the HPLC-Chip, consisting of a 40 nl enrichment column and a 43mm x 75 µm separation column packed with ZORBAX 300SB-C18 (particle size 5 mm). Peptides that were initially trapped within the enrichment column were fractionated using the separation column with a linear gradient of acetonitrile (2–60% for 30 min in the presence of 0.1% formic acid) at a flow rate of 300 nl min^{-1}. Eluates were then analyzed with an electron spray ionization ion trap MS that was connected online to the HPLC-Chip. MS analysis was performed in a positive mode with a capillary voltage of 1,950 V, scan range of 350-2,000 m/z, and 4.0 l min^{-1} of dry gas (300°C). Data-dependent MS/MS analysis was performed using the five most intense ions in each cycle, followed by dynamic exclusion of these ions from further selection for 1 min. The MS/MS fragmentation amplitude was set to 1.2 V with a smart fragmentation of 30-200%. MS and MS/MS spectra were processed using Data Analysis software (version 3.3, Agilent Technologies). The peak list files thus obtained were used in database searches.

8. Database analysis

Precursor and fragmented ion spectra were subjected to searches against the National Center for Biotechnology Information (NCBI) non-redundant database (downloaded on February 20, 2010) using MASCOT as a search tool (Pappin *et al.*, 1993). For the identification of proteins, search parameters were set as follows: Viridiplantae (Green plant) fixed modification, carbamidomethylation at cysteine;

variable modification, oxidation at methionine; precursor mass tolerance, 1.2 Da; fragmented mass tolerance, 0.6 Da; digestion enzyme, trypsin; allowed miss cleavage, 1. Default settings were used for other parameters.

RESULTS AND DISCUSSION

Results

1. Screening of salt tolerant and susceptible mulberry varieties in Thailand

Forty four mulberry varieties were divided into 4 groups, namely local, hybrid, fruit and exotic varieties. After treating all strains with 200 mM sodium chloride solution for one month, plant growth characteristics and soil $EC_{1:5}$ measurement were recorded (Appendix Table 1). At the beginning of treatment, the soil $EC_{1:5}$ was 0.18 ± 0.04 dSm^{-1}. After treating of salt stress for 15 days, the $EC_{1:5}$ in all varieties was about 2.43 ± 0.53 dSm^{-1}, which increased for 14 time when comparing with the control samples. Whereas at the end of treatment (30 days), $EC_{1:5}$ of all samples was ranged from 1.87 to 5.69 dSm^{-1} with the average of 3.12 ± 0.92 dSm^{-1}. The $EC_{1:5}$ value of Thai local, hybrid, fruit and exotic varieties were about 3.25 ± 0.63, 3.04 ± 0.68, 2.71 ± 0.60 and 2.76 ± 0.64 dSm^{-1}, respectively. The $EC_{1:5}$ values of Thai local varieties were slightly higher than the other groups. The $EC_{1:5}$ of S61, BR51, Baimon and Somyai varieties was decreased from 1.87 to 2.25 dSm^{-1}. Kaewchonnabot and S61 varieties gave the highest and lowest $EC_{1:5}$ of 5.69 ± 0.47 and 1.87 ± 0.15 dSm^{-1}, respectively (Figure 4).

The growth characteristic of mulberry under salt stress was changed depending on varieties and times. In the control condition (soil with no extra NaCl), all of varieties survived. In the first week after salt treatment, each variety showed slightly different in growth characteristics when compared with its own control. After two weeks the growth characteristic of all varieties changed to non fresh and wilting e.g. the color of leaves of BR51 and S1 varieties was changed to yellow patch and S61, S1, and BR51 died after 3 weeks of salt treatment (Figure 5). Therefore, these varieties can divided in to three groups: (1) all of treated plants were died (2) some treated plants were died, wilting or survive (3) all of treated plants were survived (Appendix Table 2).

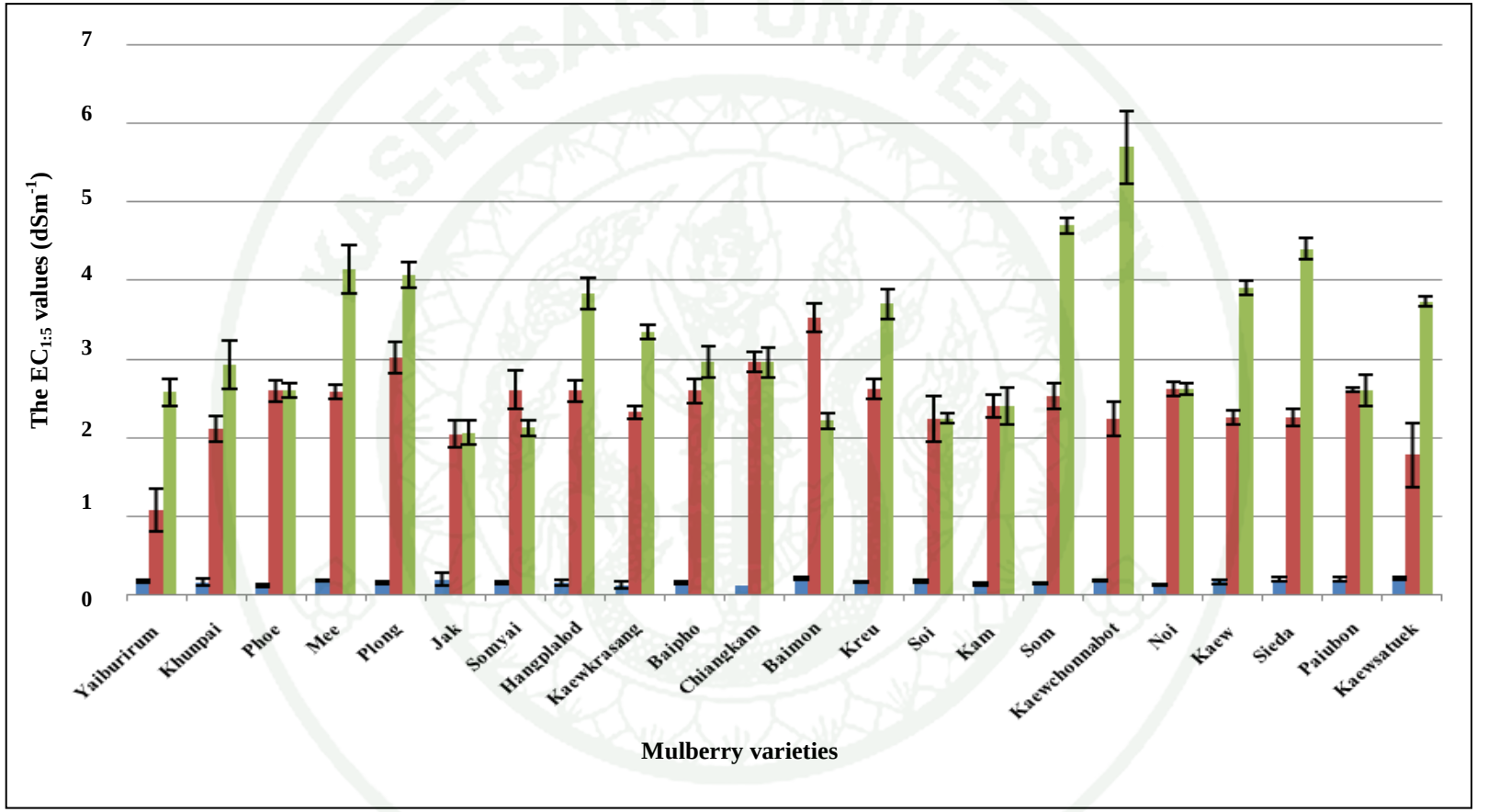

Figure 4 The $EC_{1:5}$ values in soil extract of mulberry varieties after treated with 200 mM NaCl for 0, 15 and 30 days. The data represents by means ± SE of three replication. (; 0 day, ; 15 days, ; 30 days)

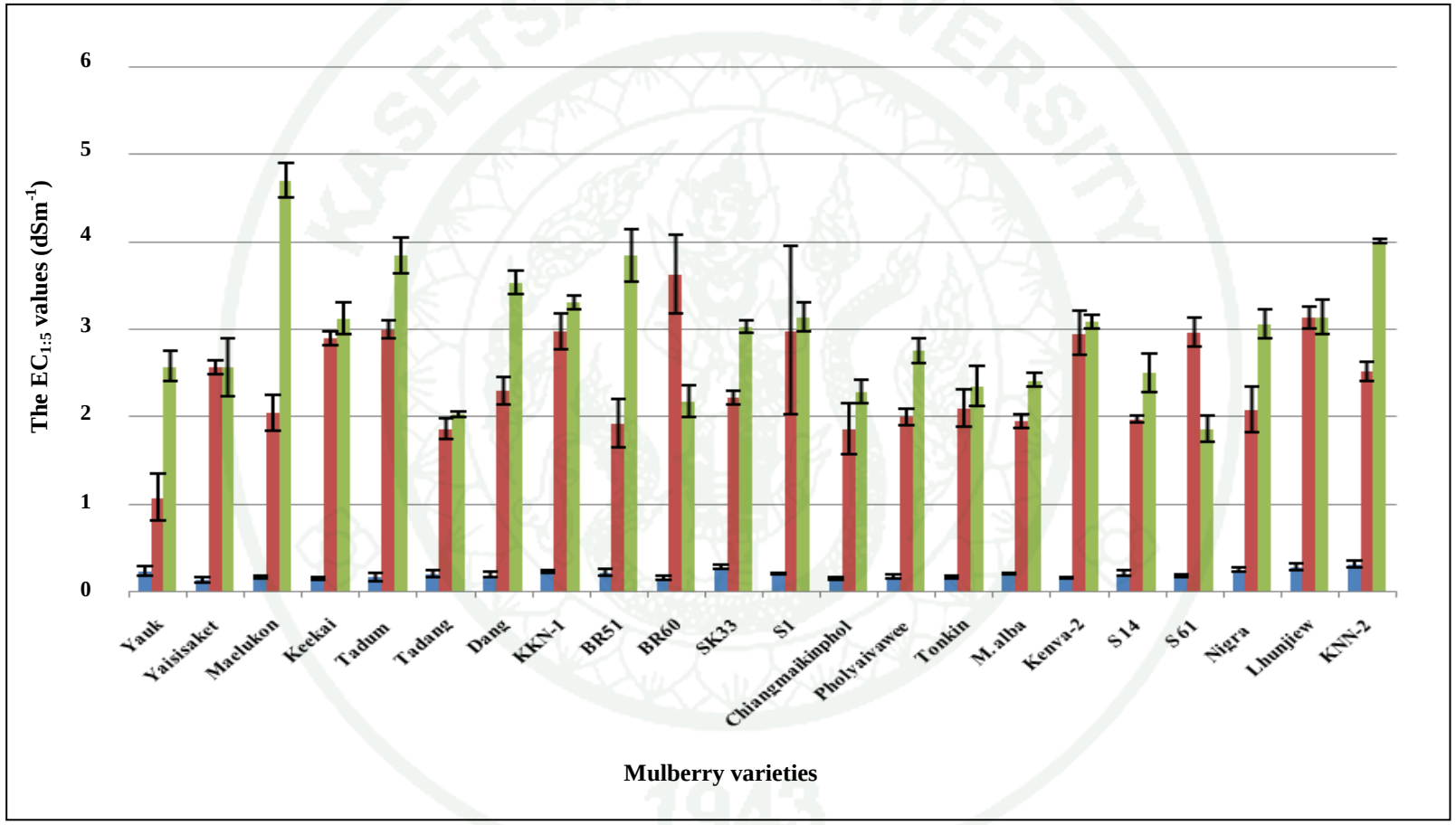

Figure 4 (Continued**)**

A

B

C

Figure 5 Growth characteristic of mulberry under salt stress (A) leaves yellow patch (B) leaves burning (C) leaves fall

The results showed that two salt tolerant varieties were identified, Som (female) and Plong (male). They were able to survive in soil $EC_{1:5}$ of 4.80 ± 0.1 and 4.07 ± 0.16 dSm^{-1}, respectively. The growth characteristics of these plants showed slightly wilting of leaves in third weeks. However, these varieties represented not significant different when compare with control plants (Figure 6A, B).

However a hybrid variety, BR51 (female), and an exotic variety, S61 (male), were classified as salt sensitive. All of treated plants died within one month with the soil $EC_{1:5}$ value of 2.18 ± 0.18 and 1.87 ± 0.15 dSm^{-1}, respectively. The growth characteristic of these plants appeared yellow patch and burn leaves within two weeks after treated with sodium chloride solution and and leaves fell within a week after (Figure 7A, B). The soil $EC_{1:5}$ of the control were about 11.8 ± 0.35 dSm^{-1}, 10.7 ± 0.37 dSm^{-1} included a salt tolerant and salt susceptible mulberry, respectively. Therefore, the difference of soil $EC_{1:5}$ in sensitive varieties were higher than tolerant varieties (Figure 8). When screening for salt tolerant mulberry were repeated on the 4 tolerant and susceptible varieties similar results were obtained in all seasons (Table 4).

In the present study, 44 mulberry genotypes were screened, from which two tolerant and two sensitive varieties were selected for further studies.

A

C1 C2 T1 T2 T3

B

C1 C2 T1 T2 T3

Figure 6 Growth characteristics of salt tolerant mulberry varieties (A) Som and (B) Plong were compared with controls. C1 and C2, control; T1, T2 and T3 treated with 200 mM sodium chloride for 30 days.

C

C1 C2 T1 T2 T3

D

C1 C2 T1 T2 T3

Figure 7 Growth characteristics of salt susceptible mulberry varieties (C) S61 and (D) BR51 were compared with controls. C1 and C2, control; T1, T2 and T3 treated with 200 mM sodium chloride for 30 days.

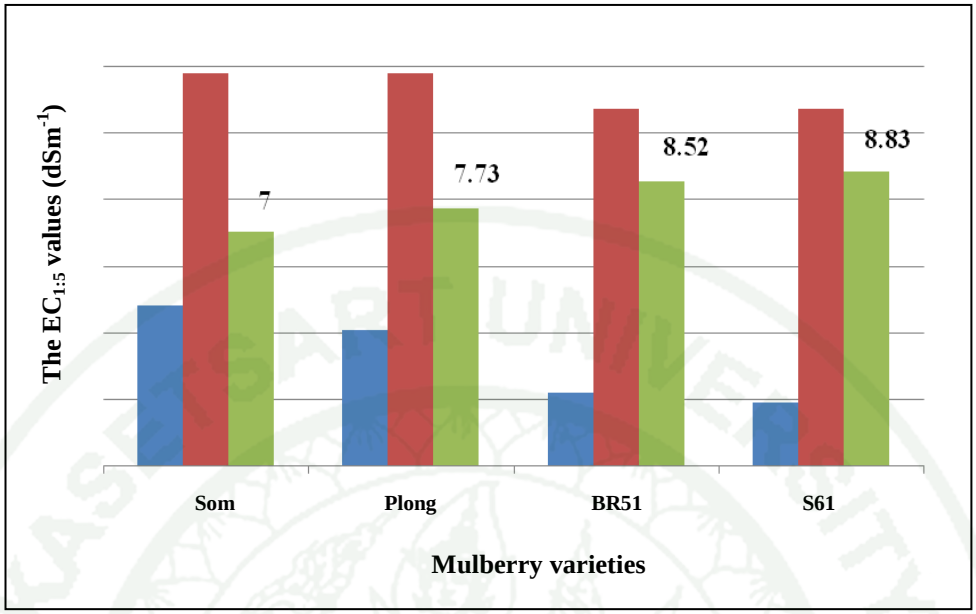

Figure 8 Soil $EC_{1:5}$ of salt tolerant and salt susceptible mulberry varieties were compared with soil $EC_{1:5}$ of controls for 30 days.
(; soil $EC_{1:5}$ for 30 days, ; soil $EC_{1:5}$ for control (a salt tolerant varieties; 11.8 dSm^{-1} , a salt sensitive varieties; 10.7 dSm^{-1} , ; differential of soil $EC_{1:5}$)

Table 3 Electrical conductivity values and growth characteristics of salt tolerant and susceptible mulberry varieties tested in rainy and winter seasons.

Mulberry Varieties	$EC_{1:5}$ (dSm^{-1})			Growth characteristics observation
	Rainy	Winter	Summer	
Som	4.80 ± 0.20	5.17 ± 0.15	4.79 ± 0.31	survive
Plong	4.07 ± 0.47	3.51 ± 0.54	4.01 ± 0.77	survive
BR51	2.18 ± 0.27	2.28 ± 0.11	2.22 ± 0.42	died
S61	1.87 ± 0.21	2.23 ± 0.13	2.77 ± 0.39	died

2. Differential protein expression of salt tolerant and susceptible mulberry varieties during salt stress

High-resolution 2D-PAGE is very useful for separating complex protein mixtures. Som and S61 were selected and used as salt tolerant and salt sensitive mulberry varieties in this experiment. Two months-old mulberry plant were treated with100 ml of 200 mM NaCl for 0 and 7 days. Proteins were extracted from leaves, separated by 2D-PAGE and stained with Coomassie Brilliant Blue. The 113 protein spots were detected in Som variety while 115 protein spots were detected in S61 varieties (Figure 9-10). The ratio of percentage volumes of protein spots were obtained from 2D-PAGE gels in control and NaCl treated mulberry using ImageMaster 2D Platinum 7.0 software. Of these, 13 proteins were differentially expressed under salt stress in one or other varieties (Figure 11-14).

In Som variety, of 12 proteins, the percentage total volume ratio of 6 proteins increased from 1.95-fold (spot 1,9) to 31.4-fold (spot 3), and 6 proteins were down-regulated by 1.92-fold (spot 8) to 8.61-fold (spot 2) under NaCl treatment compared with the control plants. In S61 variety, 9 proteins were differentially expressed under NaCl treatment. The percentage total volume ratio of 2 proteins increased by 1.60 - fold (spot 9) to 1.98-fold (spot 2), and those of 2 proteins decreased by 1.33-fold (spot 7) to 1.53-fold (spot 12), and 5 proteins were slightly down-regulated by less than 1.30-fold (spot 4-8 and 12) (Figure 15 a, b and Appendix Table 3). To determine the differences between the two cultivars in terms of their protein expression under salt stress, the distribution of up- or down-regulated proteins was compared in Som and S61. From 8 up-regulated protein spots, 1 of these was commonly found in both varieties and only one protein was specifically up-regulated in S61 and six of them were up-regulated only in Som. One protein in Som and two proteins in S61 were specifically down-regulated; however, 5 proteins were similarly down-regulated in both cultivars (Figure 16).

3. Identification of amino acid sequences

The total of 13 protein spots showing up or down-regulation were selected and subjected to identify by Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Table 3 shows the accession numbers and putative names of proteins that correspond to specific spots shown in (Figure 12-15) along with their experimental *pI* and molecular mass values. Protein spots stained with Coomassie Brilliant Blue of mulberry leaves were subjected to trypsin digestion and analyzed by nano LC-MS/MS. Of these, 8 protein spots were completely identified by nano LC-MS/MS alongside MASCOT database searching and/or Blast homology searching. However, 5 proteins could not be identified and these are shown as "Not hit" in Table 3.

In Som (tolerant mulberry variety), three up-regulated proteins were identified as 18 kDa winter accumulating protein A (spot 9), RuBisCO large subunit (spot 3), and RuBisCO small subunit (spot 12). These proteins are categorized as being involved in stress protein and photosynthesis. On the other hand, five down-regulated proteins were found, namely 33kDa Oxygen-evolving enhancer protein 1 (spot 4), Oxygen-evolving enhancer protein 2 (spot 6), Rieske Fe/S protein of cytochrome b6/f complex (spot 7) and the fragments of RuBisCO large subunit (spot 8) are mainly associated in photosynthesis and one protein that involved in defense-related mechanisms is 23kDa polypeptide of the oxygen evolving complex of photosystem II (spot 5).

In S61 (sensitive mulberry variety), two up-regulated proteins were identified as fragments of RuBisCO large subunit (spot 8) and 18 kDa winter accumulating protein A (spot 9). On the other hand, five down-regulated proteins were found, namely 33kDa Oxygen-evolving enhancer protein 1 (spot 4), 23kDa polypeptide of the oxygen evolving complex of photosystem II (spot 5), Oxygen-evolving enhancer protein 2 (spot 6) Rieske Fe/S protein of cytochrome b6/f complex (spot 7) and RuBisCO small subunit (spot 12).

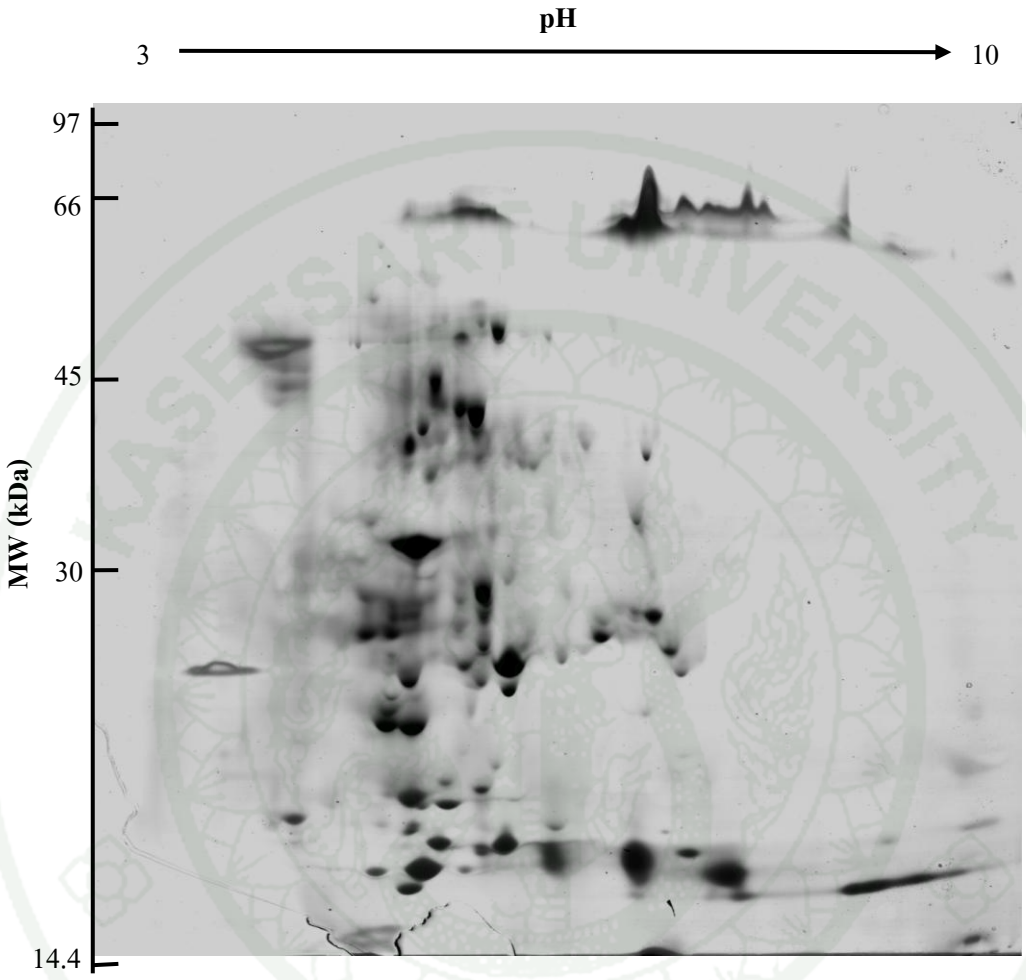

Figure 9 Protein expression patterns in the control leaves of Som mulberry variety, a salt tolerant mulberry variety. Proteins were extracted from leaves, separated by 2D-PAGE, and stained with silver nitrate. Proteins were applied in each experiment, and repeated three times.

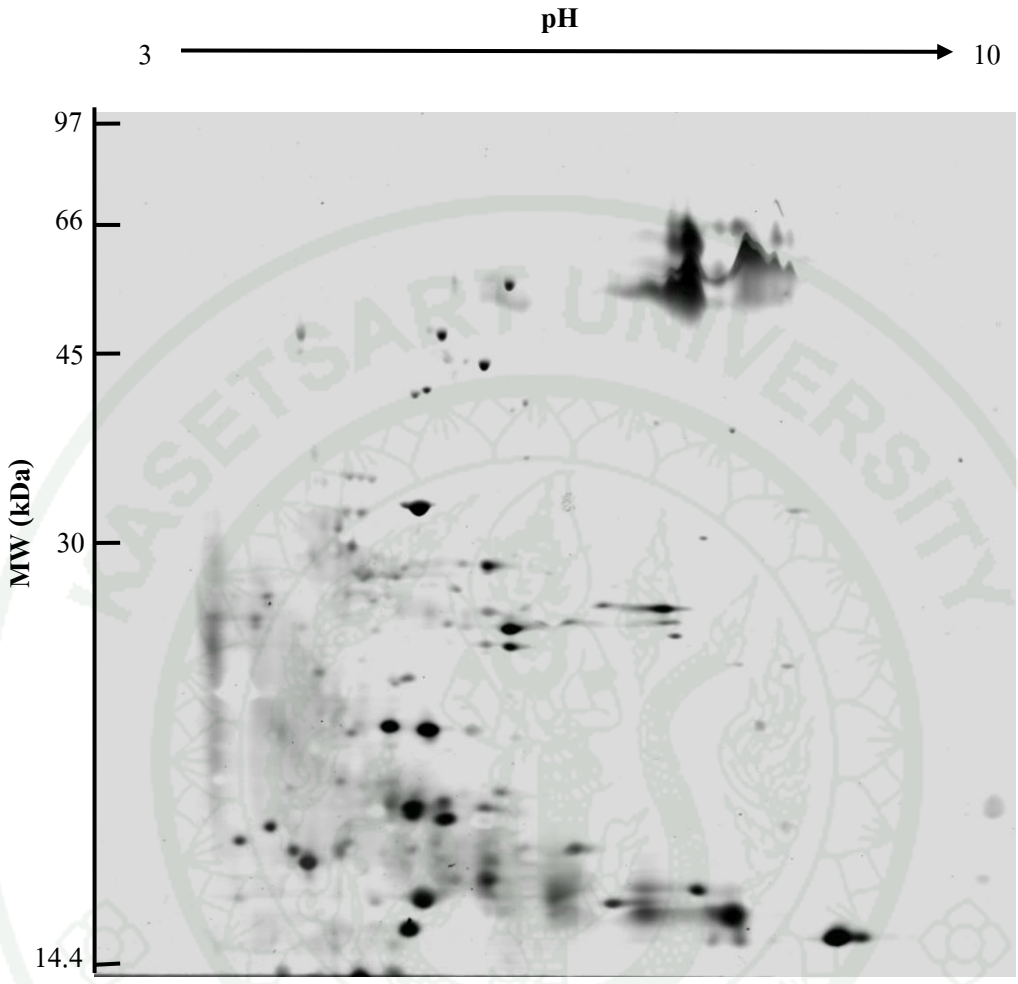

Figure 10 Protein expression patterns in the control leaves of S61, a salt sensitive mulberry variety. Proteins were extracted from leaves, separated by 2D-PAGE, and stained with silver nitrate. Proteins were applied in each experiment, and repeated three times.

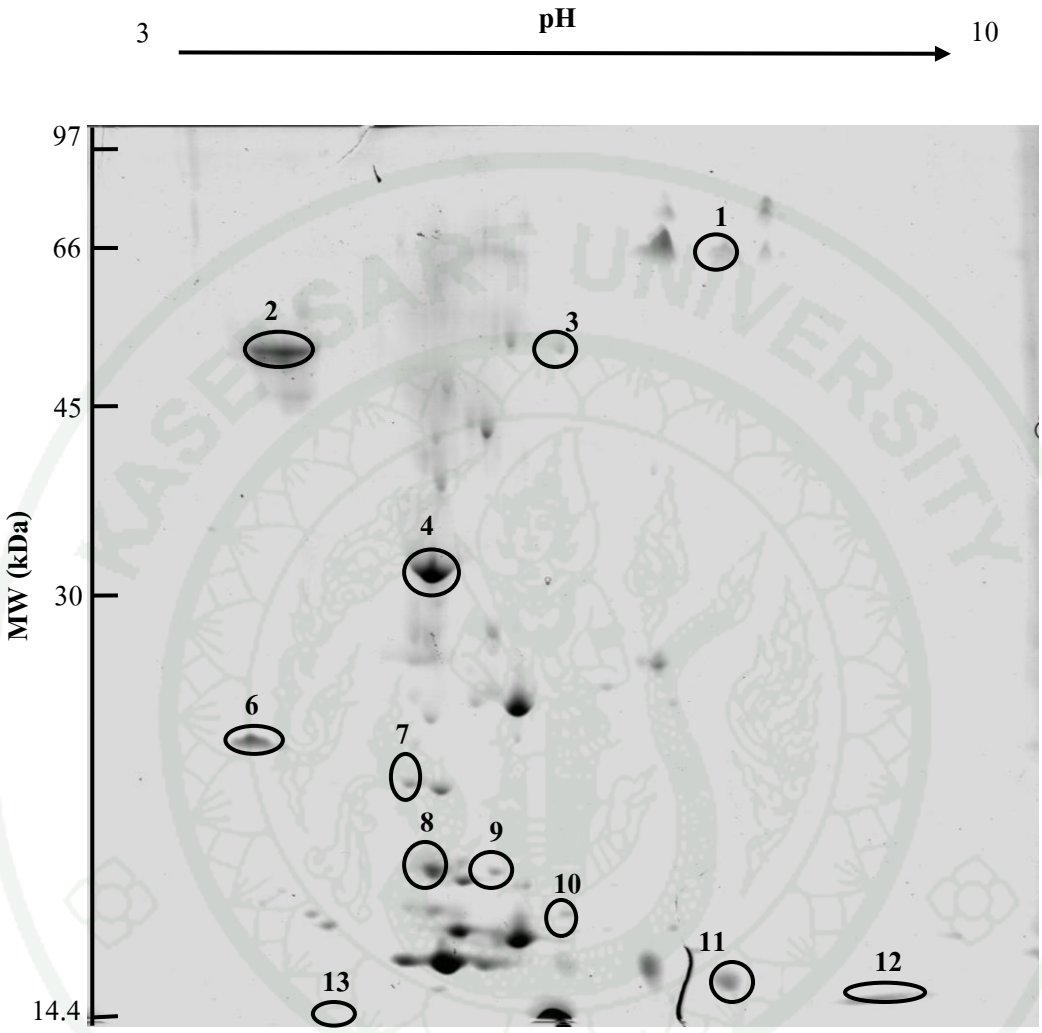

Figure 11 Protein expression patterns in the control leaves of Som, a salt tolerant mulberry variety. Proteins were extracted from the leaves, separated by 2D-PAGE , and stained by Coomassie brilliant blue. Circles mark the position of the differential expressed proteins after salt stress.

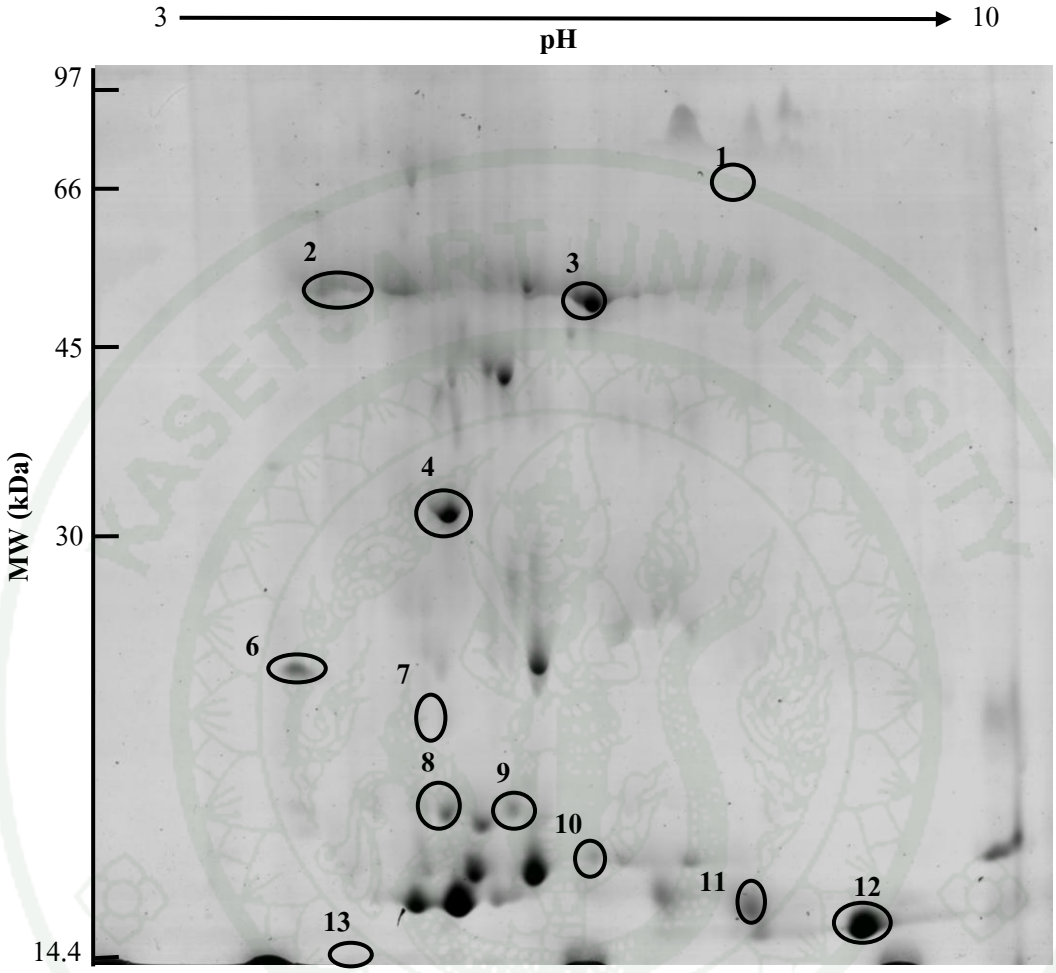

Figure 12 Protein expression patterns in the stressed leaves of Som, a salt tolerant mulberry variety. Proteins were extracted from the leaves after 1 weeks of treatment, separated by 2D-PAGE, and stained by Coomassie brilliant blue. Circles mark the position of the differential expressed proteins after salt stress.

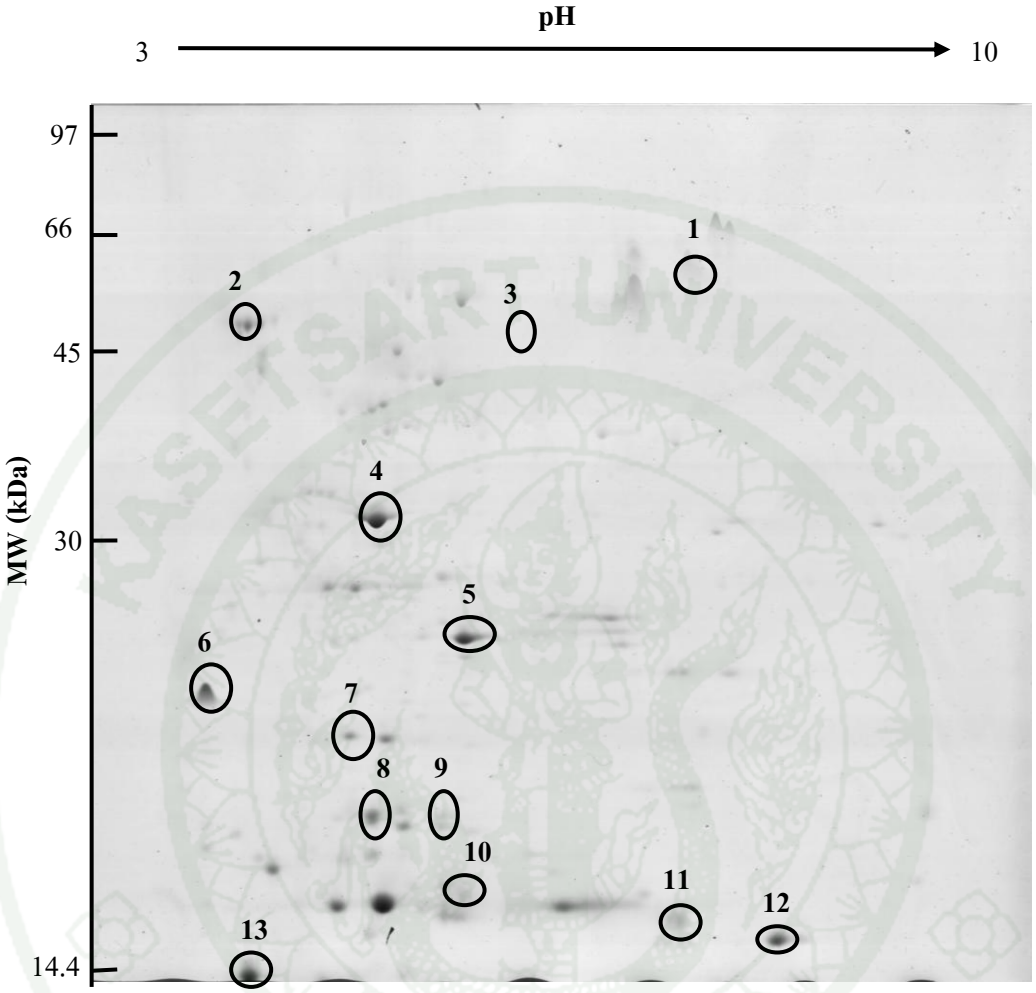

Figure 13 Protein expression patterns in the control leaves of S61, a salt sensitive mulberry variety. Proteins were extracted from the leaves, separated by 2D-PAGE , and stained by Coomassie brilliant blue. Circles mark the position of the differential expressed proteins after salt stress.

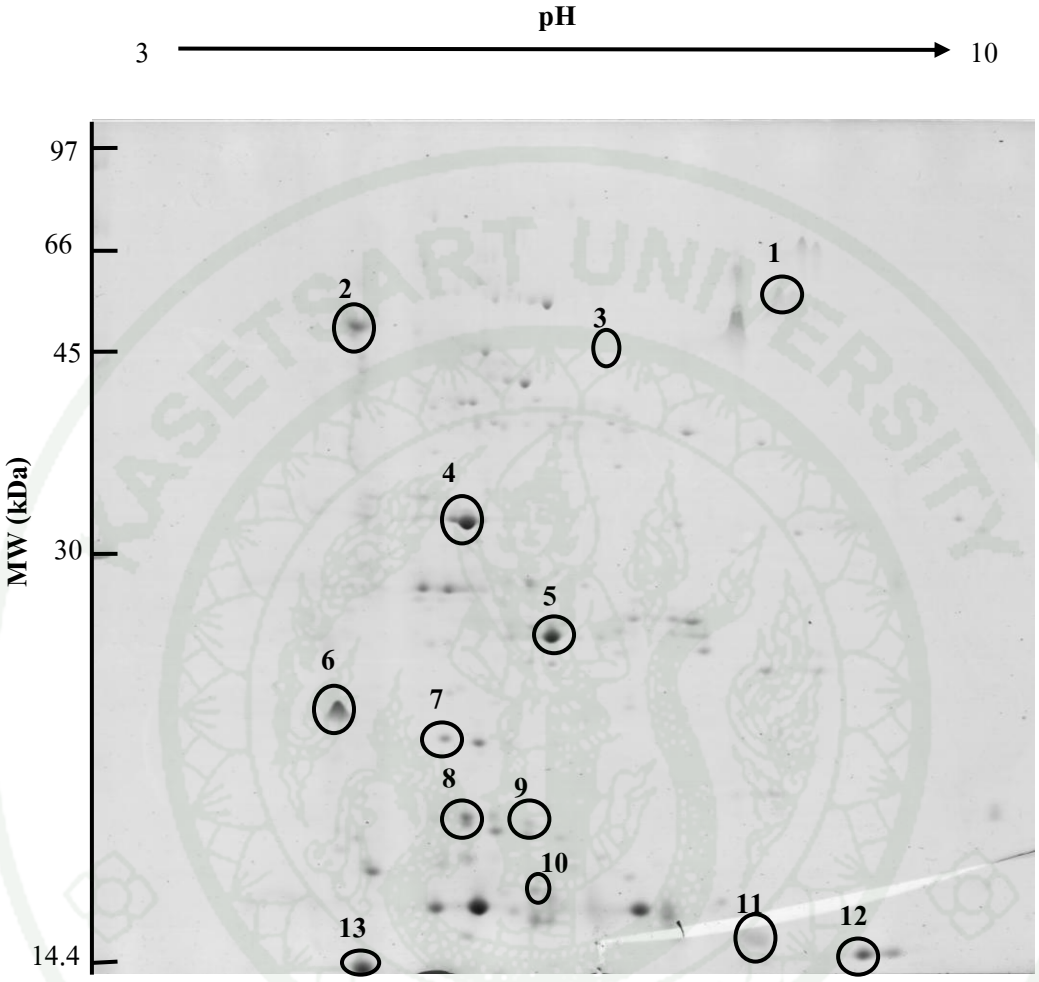

Figure 14 Protein expression patterns in the stressed leaves of S61, a salt sensitive mulberry variety. Proteins were extracted from the leaves after 1 weeks of treatment, separated by 2D-PAGE, and stained by Coomassie brilliant blue. Circles mark the position of the differential expressed proteins after salt stress.

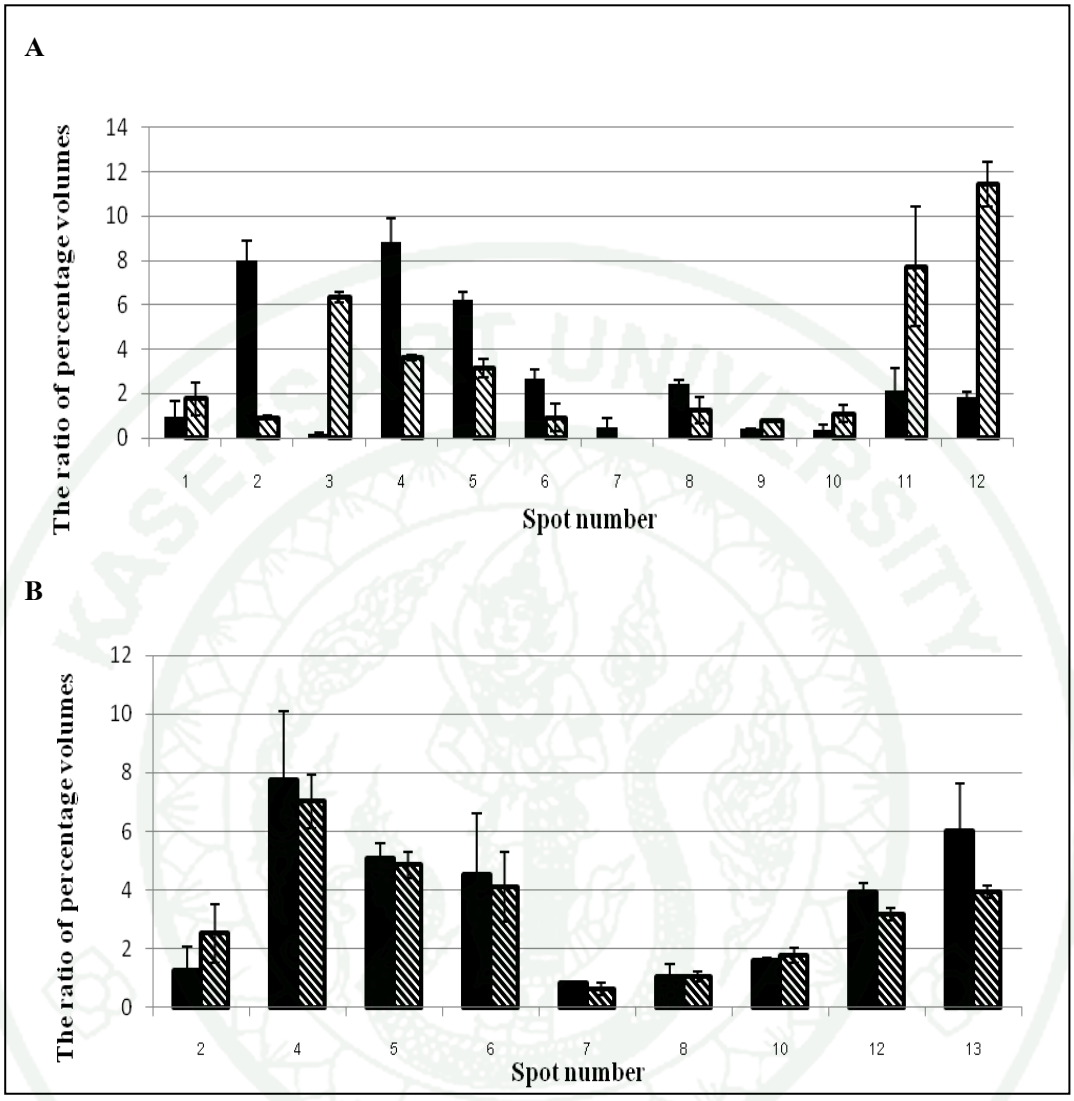

Figure 15 The relative levels of protein expression were analyzed in (A) Som and (B) S61 using image Master 5.0 2D Platinum software. Values were identified from three independent experiments.
(■ ; control, ▨ ; stress)

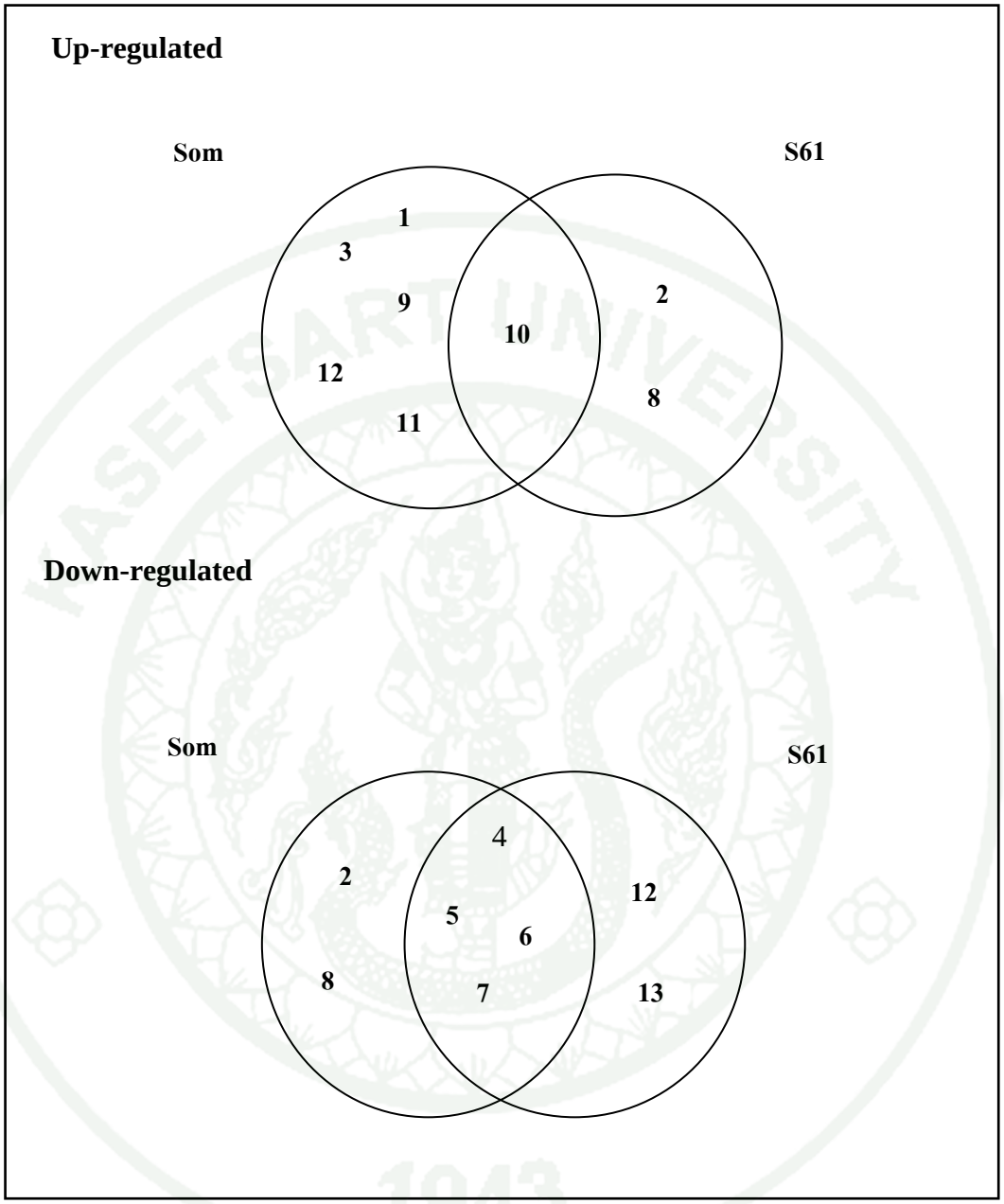

Figure 16 Venn diagram analysis showing up- or down-regulated proteins that overlapped between the two mulberry cultivars. Effects of salt stress on *Som* and *S61* were compared according to their up- and down- regulated protein spots at 200 mM NaCl treatment. Numbers correspond to the protein spots in the 2D-PAGE gels. Up- or down-regulation of protein spots was determined based on their relative intensity measured by using imageMaster 5.0 2D Platinum software, as shown in Figure 15A, B.

Table 5 List of protein spots which were differentially expressed in mulberry leaves under NaCl treatment.

Spot no.	Protein name	Plant species	Category	Accession no.	Matched peptide	Theoretical		Score
						M_r	pI	
1	Not hit	-	-	-	-	-	-	-
2	Not hit	-	-	-	-	-	-	-
3	RuBisCo large subunit	*Morus indica*	Photosynthesis	gi 114804273	8	53	6	302
4	33kDa Oxygen evolving enhancer protein 1	*Morus nigra*	Photosynthesis	gi 152143640	5	28.5	5.48	391
5	23kDa polypeptide of the Oxygen evolving complex of photosystem II	*Sonneratia alba*	Defense	gi 146454486	3	25.2	5.98	89
6	Oxygen-evolving enhancer protein 2	*Pisum sativum*	Photosynthesis	gi 131390	1	28	8.29	52
7	Rieske Fe/S protein of cytochrome b6/f complex	*Nicotiana tabacum*	Photosynthesis	gi 19999	2	24.1	7.59	90
8	RuBisCo large subunit	*Coreopsis grandiflo*ra	Photosynthesis	gi 289907	3	52.6	5.87	126
9	18 kD winter accumulating protein A	*Morus bombycis*	Stress related	gi 54311115	2	16.7	5.18	90
10	Not hit	-	-	-	-	-	-	-
11	Not hit	-	-	-	-	-	-	-
12	RuBisCo small subunit	*Fagus crenata*	Photosynthesis	gi 3914585	1	20.6	9.19	54
13	Not hit	-	-	-	-	-	-	-

Discussions

Salinity inhibits plant growth for two reasons: first, due to water deficit and second salt-specific or ion-excess effects, especially on glycophytes (Munns *et al*., 2006). The most commonly responds of glycophytes to salinity are loss of turgor, decreased photosynthesis, growth reduction, early senescence, tissue necrosis and even death of the plant (Cheeseman, 1988). Mulberry was classified as glycophyte with a moderate salinity tolerance by EC_e of 4-8 dSm^{-1} (Heidi, 2008).

This is the first report of screening salt tolerant and salt sensitive mulberry in Thailand. Two local varieties were salt tolerant whereas hybrid and exotic varieties were salt sensitive mulberries. In this study, the growth characteristics were focused on leaves due to salinity condition was more affecting in leaves than roots and easy to observe. Several researchers have shown that the leaves of many crop plants are more sensitive to salinity condition than root (Salem, 1989 and Perez-Alfocea *et al.*, 1993). Chartzoulakis *et al.* (2002) reported that the effect of salinity on leaf area was greater than plant height and dry weight. The decline in leaf growth is the earliest response of glycophytes exposed to salt stress (Munns *et al*., 2006).

The result of this work demonstrated a significant effect of NaCl on mulberry varieties. Different growth characteristics of mulberry plants were shown on second weeks after treating with NaCl solution and the soil $EC_{1:5}$ about 2-3 dSm^{-1}. After three weeks, the accumulation of Na^+ and Cl^- ions were increased in mulberry plants, these ions causes the physiological changes in mulberry leaves. The major reason for the detrimental effects of low to moderate salt concentrations is the negative osmotic potential caused by salts in the root zone (Jacoby, 1994). Similar results have been reported by Bernstein *et al.* (1974), these effects depending upon ion toxicities and nutritional deficiencies may also arise because of the predominance of a specific ion or competitions among cations or anions. The same effects were observed on mulberry leaves such as yellow patches and wilting. According to Vijayan *et al.* (2008) data, the first visible symptom of salt injury in mulberry is the appearance of yellow patches in young leaves under low to moderate salinity. The yellowing of leaf

may be due to degradation of chlorophyll by the increasing activity of chlorophyllase (Singh *et al.*, 2000). According to Munns (1993), this characteristic could be explained due to an imbalance among cations as a result of the complex interaction in the xylem transport system. In the last weeks, the soil $EC_{1:5}$ were increased in all varieties. The effect of salinity stress appeared on mulberry leaves such as a wilting, tissue necrosis, senescence and burning lesions. These growth characteristics result of water deficit and toxicity from salt ions were due to an increasing of soil $EC_{1:5}$ and ion accumulation in cell. The accumulation of high concentrations of Na^+ or Cl^- in the leaves generally results in the formation of burning like lesions (Zhu, 2002). According to Vijayan *et al*. (2008) report, under higher salinity condition a burnt like lesions appeared in the leaves. Early senescence of older leaves and retardation of growth under higher salinity as the salt promotes senescence of leaves by increasing the production of ABA and ethylene (Kefu *et al.*, 1991; Zhao *et al.*, 1992). Leaf injury in plants was attributed to toxic levels of Cl^- and Na^+ in leaves (Storey and Walker, 1999). However, some mulberry varieties did not show leaf injury like other varieties, especially, the salt tolerant varieties, Som and Plong. The leaves of treated plants showed no change during salt treatment, whereas, leaves of the salt sensitive varieties, BR51and S61, fell in all of treated plants and died eventually.

The different growth characteristics of these varieties may result from amount of ion accumulation in the plant cell, especially, in salt sensitive mulberry varieties showing that the soil $EC_{1:5}$ decreased much more than other varieties when compare with $EC_{1:5}$ value of the control. The $EC_{1:5}$ value of the control (treated soil without mulberry plant) was 11.8 ± 0.351 dSm^{-1}. The data indicated that mulberries had taken up and accumulated the potentially toxic Na^+ and Cl^-. Thus, a high uptake and accumulation of salt (Na^+ and/or Cl^-) in salt sensitive mulberry varieties may cause the plants dead. Similar results were reported by Wahome (1998), plants responded to increased salinity by the occurrence of leaves injury which was normally followed by leaf fall. Part of the shoot, the whole shoot or even the whole plant may die in extreme cases. Banuls and Primo-Millo (1995) showed a close relationship between the Cl^- content of the leaves and leaf fall associated with salt stress. In contrast, higher soil $EC_{1:5}$ in salt tolerant mulberry varieties indicated less uptake and accumulation of salt

(Na^+ and/or Cl^-). This may be a reason to explain why Som and Plong varieties more resistant to salinity than the others. According to reported by Greenway and Munns (1980) salt tolerance in glycophytes is associated with the ability to limit uptake and/or transport of saline ions, mainly Na^+ and Cl^- from the root zone to aerial parts. Similar results have been reported by Tattini (1994), the resistant mechanism of salt-tolerant olive varieties was probably related to saline ions exclusion by roots. In a field experiment by Haq *et al.* (2002), four Brassica species (relatively salt tolerant, *B. napus* and *B. carinata*; salt sensitive, *B. campestris* and *B. juncea*) showed a close association between their degree of salt tolerance and ability to exclude both Na^+ and Cl^- was found. Jones *et al.* (1984) suggested the partial exclusion of toxic ions as the primary selection criterion for salt tolerance in this crop. (Chaubey and Senadhira, 1994). Moreover, the exclusion of sodium ion from the cytoplasm or compartmentalization in the vacuoles is done by a salt-inducible enzyme Na^+/H^+ antiporter such as V-ATPase and VPPase (Apse *et al.*, 1999). According to reported by Chingkitti (2008) ATPase showed significant high activity in root of mulberry after salt stress. Wang *et al.* (2001) reported that the main strategy of salt tolerance in the halophyte *S. salsa* seems to be an up-regulation of V-ATPase activity, which is required to energize the tonoplast for ion uptake into the vacuole, while V-PPase plays only a minor role. Similar reported by Wang *et al.* (2001), the main strategy of salt tolerance in the *S. salsa* seems to be an up-regulation of V-ATPase activity, which is required to energize the tonoplast for ion uptake into the vacuole. The uptake and accumulation of ions in plants has attractions as an indicator of salinity tolerance since they are genetically regulated, though also affected by the environment.

This experiment clearly showed that there are significant of growth characteristics differences in salt tolerance and salt sensitive among mulberry varieties. Therefore, Som and Plong varieties could be identified as salt tolerant while S61 and BR51 could be identified as salt sensitive varieties. The salt tolerant mulberry varieties are local varieties but salt sensitive mulberry varieties are exotic and hybrid. The result indicated that local varieties have more tolerance capability to salt stress than exotic and hybrid varieties. The local varieties might had different physiological of roots, which adapted to tolerate under salt stress. The results correlated with

Vijayan *et al.* (2003; 2004) and Ahmad and Sharma (2008). Salt tolerant ability of local mulberry varieties was as a result of their adaptations to the environment. Local varieties could adaptation to environment better than others group. The result showed that most of the local mulberry varieties could be tolerant to saline soil for example Som, Plong, Kaewchonnabot and Maelukon etc. Their salt tolerant abilities, will be very useful for grafting, by using local mulberry varieties as the stock, in order to increase productivity of mulberry in Thailand. Estan *et al.* (2005) reported grafting provides an alternative way to enhance salt tolerance in plant and evidence is reported that the rootstock is able to reduce ionic stress.

Proteomic analysis of the leaves from salt sensitive (S61) and salt tolerant variety (Som) revealed that a group of proteins associated with photosynthesis was differentially expressed upon salt stress. RuBisCo are the most prevalent enzymes in the plant. They are about 30–50% of the total soluble protein content of the chloroplast (Chen and Harmon, 2006). Oxidative stress may lead to small-subunit degradation, subsequently leading to translational arrest of the large subunit (Razavizadeh *et al.*, 2009). Alternatively, oxidative stress could initially arrest large subunit translation, resulting in a rapid degradation of the unassembled small subunit. In this study, RuBisCO large subunit (spot 3) and RuBisCO small subunit were up-regulated by salt stress in only salt tolerant variety (Som) and were slightly down-regulated in salt sensitive variety (S61). RuBisCO is essential for CO_2 fixation in photosynthesis. Increased activity of RuBisCO subunits in maize and rice under salt stress has also been demonstrated (Zorb *et al.*, 2004; Kim *et al.*, 2005). This result was also consistent with Fan *et al.* (2009) and Veeranagamallaiah *et al.* (2008), who reported an increase in the abundance of RuBisCo in Populus cathayana Rehder and foxtail millet, respectively. These proteins have important roles in photosynthesis and down-regulation of these proteins shows that one of the target points of salt stress on mulberry plants is photosynthetic system which results in growth reduction.

A winter accumulating protein A (WAP) are up-regulated in both varieties (Som and Plong). WAP proteins are stress-induced proteins that is responsed in mulberry under cold stress. This protein has been reported that its amino acid

sequence, located and activity similar with small heat shock protein (smHSPs) (Ukaji *et al.*, 1999). HSP proteins are among the most well-known stress related proteins in plants which have been induced under several types of stress conditions such as heat, osmotic and salt stress. These proteins can act as chaperones which helps in correct folding of proteins and protects them from denaturing under stress conditions (Zhu *et al.*, 1995). Induction of these proteins in this study is one of the mechanisms which may confer salt tolerance to tolerant cultivar. Thus, it has been suggested that smHSPs are also important for desiccation tolerance (Scho *et al.* 1998). Accumulation of ER-localized smHSPs (WAP), which are thought to act as a molecular chaperone to stabilize proteins, might have possible roles in the adaptation to low temperature in winter, in water reduction as a result of cold acclimation (Ukaji *et al.*, 1999). Salt stress also reduces the ability of plants to absorb water which might cause up-regulated of WAP protein in this study, though, the function of this protein has not been reported about function of WAP in plant responses to salt stress. It can be assumed that the up-regulation of WAP protein under salt stress might be involved in increasing salt tolerance of mulberry. Although this protein has been induced in both varieties (Som and Plong) under salt stress, their up-regulation in salt tolerant varieties are higher than sensitive varieties. This suggested that this WAP protein may contribute to salt tolerance.

OEE1 and OEE2 are group of proteins associated with photosynthesis. The decline in growth is a general phenomenon in many plants when subjected to salinity stress and is often associated with a decrease in their photosynthetic capacity. In this study, it was found that these proteins were down-regulated in both varieties. These proteins are involved in light-induced oxidation of water in photosystem II of plants (Thornton *et al.*, 2004). Down-regulation of these proteins caused a decrease in rate of photosystem II (PSII). Decreased expression of OEE1 under salt stress has been also reported in potato and foxtail millet (Aghaei *et al.*, 2008; Veeragamallaiah *et al.*, 2008). For most glycophytes, salt accumulation will reduce photosynthetic efficiency, which subsequently inhibits the plant growth and development (Wang *et al.*, 2008). In contrast, increased level of expression of OEE2 under salt stress has been reported to occur in other plants such as rice, mangrove, potato and tobacco (Sugihara *et al.*,

2000; Abbasi and Komatsu, 2004; Aghaei *et al.*, 2008 and Razavizadeh *et al.*, 2009). They suggested that OEE2 could be involved in a mechanism for maintaining PSII activity in the presence of NaCl. However, the expression level of OEE2 was down-regulated in mulberry but the plant might use other mechanisms to survive under salt stress.

OEC and Rieske Fe/S protein of cytochrome b6/f complex are rate limiting step proteins in photosynthesis. The result showed that these proteins were significant down-regulated in salt tolerant varieties. An unfortunate consequence of salinity stress in plants is the excessive generation of reactive oxygen species (ROS) /intermediates such as superoxide, hydrogen peroxide and hydroxyl radical (Foyer *et al.*, 1994). The excess production of ROS during salinity stress results from impaired electron transport processes in chloroplast, especially in photosystem II (Vaidyanathan *et al.*, 2003). Reduction of these proteins in this study might reduce toxicity from ROS, which is normally very high toxic to plant cells during salt stress.

Salt stress resulted in different expression of five new proteins which has no information available in the NCBI database. Three of these (spots 1, 10 and 12) were up-regulated only in the tolerant variety and one protein (spot 13) was down-regulated only in the sensitive variety.

It is suggested that these new salt responsive proteins may play an important role in salt tolerance of mulberry. Especially, novel proteins were found only tolerant variety. These proteins might be useful for marker-assisted selection in salt tolerant mulberry in the future.

CONCLUSION AND RECOMMENDATION

Conclusion

In conclusions, an integrated physiological and proteomic approach has been used here to systematically investigate the salt stress responses of mulberry (*Morus* spp.).

1. My screening results showed that two local mulberry varieties, Som and Plong, were tolerant to salt soil (still survive) while the varieties BR51 (hybrid variety) and S61 (exotic variety) were sensitive to salt and died after treated with 200 mM NaCl.

2. The $EC_{1:5}$ values in soil extract of mulberry varieties after treated with 200 mM NaCl for 0, 15 and 30 days suggested that lowering NaCl influx into root might be the major mechanism of salt tolerance in Som and Plong varieties.

3. Approximately 100 protein spots were reproducibly detected on each 2D-PAGE. Of these, 13 proteins were differentially expressed in leaves of salt tolerant and salt sensitive mulberry varieties under sodium chloride treatment. These proteins could be functionally identified into three categories, most of them involved in photosynthesis such as RuBisCO, OEE1, *OEE*2, Rieske Fe/S protein of cytochrome b6/f complex, followed by defense related, OEC2 protein and stress related, WAP (smHSP) protein.

4. The combination of proteomic and physiological studies provide us a new knowledge of salt-tolerance mechanism in mulberry. My results suggested that mulberry variety (Som) may tolerate to severe salt stress by decreasing uptake salt ion from soil and maintaining RuBisCo activity under salt stress. In addition, some defense mechanism through the up-regulation of small heat shock protein or other three new proteins detected in tolerant variety can be considered as salt-responsive proteins in mulberry.

Recommendation

1. The proteins that could not be identified may involve in unknown mechanism to response to salt stress in mulberry plant. Therefore, these proteins should be re-analyzed.

2. Other metabolites that related salt stress such as metabolites, peptides, amino acid, DNJ, GABA were suggested to monitor in plant stressed with NaCl at various time points

LITERATURE CITED

Abbasi, F.M. and S, Komatsu. 2004. A proteomic approach to analyze salt responsive proteins in rice leaf sheath. **Proteomics** 4: 2072-2081.

Adams, P., J.C. Thomas, D.M. Vernon, H.J. Bohnert and R.G. Jensen. 1992. Distinct cellular and organismic responses to salt stress. **Plant Cell Physiol**. 33: 1215-1223.

Aghaei, Keyvan., A.A. Ehsanpour and S. Komatsu. 2008. Proteome analysis of potato under salt stress. **J. Proteome Res**. 7: 4858-4868.

Aharon, G. S., M. P. Apse, S.H. Duan, X.HX. Zhang and E. Blumwald. 2003. Characterization of a family of vacuolar Na^+/H^+ antiporters in *Arabidopsis thaliana*. **Plant Soil** 253:245-256.

Ahmad, P. and S. Sharma. 2010. Physio-biochemical attributes in two cultivars of mulberry (*Morus alba* L.) under $NaHCO_3$ stress. **IJPP.** 4: 79-86.

Ali, G., P. S. Srivastava and M. Iqbal. 1999. Proline accumulation, protein pattern and photosynthesis in regenerants grown under NaCl stress. **Biol. Plant**. 42: 89-95.

Apse, M.P., G.S. Aharon, W.A. Snedden and E. Blumwald. 1999. Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. **Science** 285: 1256-1258.

Aruga, H. 1994. **Principle of Sericulture**. A.A. Balkema/Rotterdam. 375 p.

Ashraf, M. 1994. Organic substances responsible for salt tolerance in *Eruca sativa*. **Biol. Plant.** 36: 255- 259.

Ashraf, M. 1999. Breeding for salinity tolerance proteins in plants. **Crit. Rev. Plant Sci**. 13:17- 42.

_____. 2004. Some important physiological selection criteria for salt tolerance in plants. **Flora** 199: 61-76.

Askari, H., J. Edqvist, M. Hajheidari, M. Kafi and G.H. Salekdeh. 2006. Effect of salinity levels on proteome of *Suaeda aegyptica* leaves. **Proteomics** 6: 2546-2554.

Ayala, F., M. Ashraf and W. O'Leary. 1997. Plasma membrane H^{+}-ATPase activity in salt-tolerant and salt-sensitive lines of spring wheat (*Triticum aestivum* L.). **Acta Bot. Neerl**. 46: 315–324.

Banuls, J., and E.P. Millo. 1995. Effect of salinity on some citrus scion-rootstock combinations. **Ann. Bot**. 76: 97-102.

Basavaiah D., and B. Rajan. 1989. Microsporogenesis in Hexaploid *Morus serrata* Roxb. **Cytologia** 54: 747-751.

Benavides, M.P., P.L. Marconi, S.M. Gallego, M.E. Comba and M.L. Tomaro. 2000. Relationship between antioxidant defence systems and salt tolerance in *Solanum tuberosum*. **Aust. J. Plant Physiol**. 27: 273-278

Bernstein, L., L.E. Francois and R.A. Clark.1974. Interactive effects of salinity and fertility on yields of grains and vegetables. **Agro. J**. 66: 412-421.

Binzel, M.L. 1995. NaCl-induced accumulation of tonoplast and plasma membrane H^{+}-ATPase message in tomato. **Physiol. Plant**. 94: 722–728.

Borsani1, O., V. Valpuesta and M.A. Botella. 2003. Developing salt tolerant plants in a new century: a molecular biology approach. **Plant Cell Tiss. Org. Cul**. 73: 101-115.

Bray, E.A., S.J. Bailey and E. Weretilnyk. 2000. Responses to abiotic stresses, pp 324-353. *In*: Gruissem W, Buchannan B, Jones R (eds). **Biochemistry and Molecular Biology of Plants**. American Society of Plant Physiologists, Rockville, MD.

Butt, M. S., A. Nazir, M. T. Sultan and K. Schroen. 2008. *Morus alba* L. nature's functional tonic. **Trends Food Sci. Technol**. 19: 505-512.

Cadenas, G.A., F.R. Tadeo, , E.P. Millo and M. Talon. 1998. Involvement of ABA and ethylene in the responses of citrus seedlings to salt shock. **Plant Physiol**. 103: 475-484.

Chartzoulakis, K., M. Loupassaki, M. Bertaki and I. Androulakis. 2002. Effects of NaCl salinity on growth, ion content and CO_2 assimilation rate of six olive cultivars. **Sci. Hort**. 96: 235-247.

Chaubey, C.N. and D. Senadhira. 1994. Conventional plant breeding for tolerance to problem soils, pp 37– 60. *In*: Yeo, A. R. and Flowers, T. J. (eds.). **Soil Mineral Stresses, Approaches to Crop Improvement.** -Springer. Berlin.

Cheeseman, J.M. 1988. Mechanisms of salinity tolerance in plants. **Plant Physiol**. 87: 57-550.

Chen, C.H. 2008. Review of a current role of mass spectrometry for proteome research. **Anal. Chim. Acta.** 624: 16-36.

Chen, S. X. and A.C. Harmon. 2006. Advance in plant proteomics. **Proteomics** 6: 5504-5516.

Cherian, S., M.P. Reddy and J.B. Pandya. 1999. Studies on salt tolerance in *Avicennia marina* (Forstk.) Vierh.: effect of NaCl salinity on growth, ion accumulation and enzyme activity. **Indian. J. Plant Physiol.** 4: 266-270.

Chingkitti, N. 2008. **Salt Stress Response of Mulberry (*Morus rotunbiloba*)**. M.S. thesis, Kasetsart University.

Chitteti, R.B. and Z. Peng. 2007. Proteome and phosphoproteome differential expression under salinity stress in rice (*Oryza sativa*) roots. **J. Proteome Res**. 6: 1718-1727.

Claes, B., R. Dekeyser, R. Villarroel, M.V.D. Bulcke, G. Bauw, M.V. Montagu, and A. Caplan. 1990. Characterization of a rice gene showing organ specific expression in response to salt-stress and drought. **Plant Cell** 2: 19-27.

Cornillon, P. and A. Palliox. 1997. Inflence of sodium chloride on the growth and mineral nutrition of pepper cultivars. **J. Plant Nutr**. 20: 1085-1094.

Cramer, G.R., A. Läuchli and E. Epstein. 1986. Effect of NaCl and $CaCl_2$ on ion activities in complex nutrient solutions and root growth of cotton. **Plant Physiol.** 81: 792-797.

Das, B.C. and S. Krishnaswami. 1965. Some observations on inter specific hybridization in mulberry. **Indian J. Seric**. 4: 1-8.

Dietz, K.J., N. Tavakoli, C. Kluge, T. Mimura, S.S. Sharma, G.C. Harris, A.N. Chardonnens and D. Golldack. 2001. Significance of the V-type ATPase for the adaptation to stressful growth conditions and its regulation on the molecular and biochemical level. **J. Exp. Bot**. 52: 1969-1980.

Dionisio, S.M.L and S. Tobita. 1998. Antioxidant responses of rice seedlings to salinity stress. **Plant Sci**. 135: 1-9.

Elstner, E.F. 1987. Metabolism of activated oxygen species, pp 234-264. *In*: Davies, D.D. (Ed.), **The Biochemistry of Plants. vol. II, Biochemistry of Metabolism**. Academic Press, San Diego, CA.

Estan, M., T. Maria, M.M. Rodriguez, P.A. Francisco, J.F Timothy and M.C. Bolarin. 2005. Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot. **J. Exp. Bot**. 56: 703-712.

Fan, Y., X.W. Xiao, S. Zhang , H. Korpelainen and L. Chunyang. 2009. Salt stress responses in *Populus cathayana* Rehder. **Plant Sci**. 176: 669-677.

Flowers, T.J. and A.R. Yeo. 1995. Breeding for salinity resistance in crop plants-where next. **Aust. J. Plant Physiol**. 22: 875-884.

Foyer, C.H., M. Lelandais and K.J. Kunert. 1994. Photooxidative stress in plants. **Physiol. Plant**. 92: 696–717.

Fridovich, I. 1986. Biological effects of the superoxide radical. **Arch. Biochem. Biophys**. 247: 1-11.

Fukuda, T., M. Sudo, M. Matuda, T. Hayashi, T. Kurose and Y.H.M. Florrin. 1959. Formation of silk protein during the growth of silkworm larvae *Bombyx mori* L. **Insect** 12: 90-112.

Golombek, S.D. and P. Lüdders. 1993. Effect of short-term salinity on leaf gas exchange of the fig (*Ficus carica* L.). **Plant Soil** 148: 21-27.

Greenway, H. and R. Munns. 1980. Mechanisms of salt tolerance in non-halophytes. **Ann. Rev. Plant Physiol**. 31: 149-190.

Haidi, K., S. Olsen, L. Rupp, G. Cardon and R. Heflebower. 2008. **Soil Salinity and Ornamental Plant Selection Horticulture**. Utah State University.

Halliwell, B. and J.M.C. Gutteridge. 1985. **Free Radicals in Biology and Medicine**. Clarendon Press, Oxford.

Harinasut, P., D. Poonsopa, K. Roengmongkol and R. Charoensataporn. 2003. Salinity effects on antioxidant enzymes in mulberry cultivar. **Sci. Asia**. 29 : 109-113.

Hasegawa, P.H., R.A. Bressan, J.-K. Zhu and H.J. Bohnert. 2000. Plant cellular and molecular responses to high salinity. **Ann. Rev. Plant Physiol. Plant Mol. Biol.** 51: 463-499.

Hautala, E.L., A. Wulff and J. Oksanen. 1992. Effects of deicing salt on visible symptoms, element concentrations and membrane damage in first-year needles of roadside Scots pine (*Pinus sylvestris*). **Ann. Bot. Fenn**. 29: 179-185.

Haq, T.U. J. Akhtar, A.U. Haq and M. Hussain. 2002. Effect of soil salinity on the concentration of Na^+, K^+ and Cl^- in the leaf sap of the four Brassica species. **Int. J. Agric. Biol**. 4: 385-388.

Hernandez, J., A. Jimenez, P. Mullineaux and F. Sevilla. 2000. Tolerance of pea plants (*Pisum sativum*) to long-term salt stress is associated with induction of antioxidant defences. **Plant Cell Environ**. 23: 853–862.

Horling, F., P. Lamkemeyer, J. Konnig, I. Finkemeir, A. Kandlbinder, M. Baier and K.J. Dietz. 2003. Divergent light-, ascorbate-, and oxidative stress-dependent regulation of expression of the peroxiredoxin gene family in *Arabidopsis*. **Plant Physiol**. 131: 317-325.

Imlay, J.A. and S. Linn. 1988. **DNA Damage and Oxygen Radical Toxicity**. Science 240: 1302-1309.

Irfan, M.Q., S. Qadirb and L. Zollaa. 2007. Proteomics-based dissection of stress-responsive pathways in plants. **J. Plant Physiol.** 164: 1239-1260.

Jacoby, B. 1994. Mechanisms involved in salt tolerance by plants, pp 97-123. *In* Pessarakli, M. (Ed.) **Handbook of Plant and Crop Stress**. Marcel Dekker, New York.

Jafari, M. 1998. Availability of potassium and its relations with salinity. **Iranian J. Nat. Resour.** 51 : 37-45.

Jelloulia, N., H.B. Jouiraa, H. Skourib, A. Ghorbela, A. Gourgourib and A. Mlikia. 2008. Proteomic analysis of Tunisian grapevine cultivar Razegui under salt stress. **J. Plant Physiol**. 165: 471-481.

Jesús, V., J. Novoa, A.M. Maldonadoa, S.E. Zomeñoa, L. Valledorb, M.A. Castillejoa, M. Curtoa, J. Valeroa, B. Sghaiera, G. Donosoa and I. Redondoa. 2009. Plant proteomics update (2007–2008): Second-generation proteomic techniques, an appropriate experimental design, and data analysis to fulfill MIAPE standards, increase plant proteome coverage and expand biological knowledge. **J. Proteome.** 72: 85-314.

Jones, W.R.G. 1981. Salt tolerance, pp. 271- 292. *In* Johnson, C.B.(ed.) : **Physiological Processes Limiting Plant Productivity**. Butterworths, London.

Jones, W.R.G., J. Gorham and E. Mcdonnell. 1984. Organic and inorganic solute contents as selection criteria for salt tolerance in the Triticeae, pp 189– 203. *In*: Staples, R. and Toennissen, G. H. (eds.): **Salinity Tolerance in Plants: Strategies for Crop Improvement**. Wiley, New York.

Jyothsnakumari, G., M.Thippeswamy, G. Veeranagamallaiah and C. Sudhakar. 2009. Differential expression of LEA proteins in two genotypes of mulberry under salinity. **Biol. Plant.** 53: 145-150.

Kashyap, S. and S. Sharma. 2006. In vitro selection of salt tolerant *Morus alba* and its field performance with bioinoculants. **Hort. Sci.** 33 : 77-86.

Katsube, T., N. Imawaka, Y. Kawano, Y. Yamazaki, K. Shiwaku and Y. Yamane. 2006. Antioxidant flavonol glycosides in mulberry (*Morus alba* L.) leaves isolated based on LDL antioxidant activity. **Food Chem.** 97: 25-31.

Kawamura, N., R. Dewaraja, Y. Ishii, N. Mendis and N. Mendis. 2006. Proteomics analysis of the psychiatric disorders and protein biomarkers for psychological stress. **International Congress Series**. 1287: 86-90.

Kefu, Z., R. Munns and R.W. King.1991. Abscisic acid levels in NaCl-treated barley, cotton, and saltbush. **Aust. J. Plant Physiol.** 18: 17-24.

Kennedy, B.F. and L.F.D. Fillippis. 1999. Physiological and oxidative response to NaCl of the salt tolerant *Grevillea ilicifolia* and the salt sensitive *Grevillea arenaria*. **J. Plant Physiol**. 155: 746=754.

Kim, D.W., R. Rakwal, G.K. Agrawal, Y.H. Jung, J. Shibato and N.S. Jwa. 2005. A hydroponic rice seedling culture model system for investigating proteome of salt stress in rice leaf. **Electrophoresis** 26: 4521-4539.

Kratsch, H., S. Olsen, L. Rupp, G. Cardon and R. Heflebower. 2008. **Soil Salinity and Ornamental Plant Selection Horticulture**. Utah State University.

Kumar, G.S.A., M. Reddy and C. Sudhakar. 2003. NaCl effects on proline metabolism in two high yielding genotypes of mulberry (*Morus alba* L.) with contrasting salt tolerance. **Plant Sci**. 165: 1245-1251

Kumar, G.S.A., G.J. Kumari and C. Sudhakar. 2008. Modulations in key enzymes of nitrogen metabolism in two high yielding genotypes of mulberry (*Morus alba* L.) with differential sensitivity to salt stress. **Environ. Exp. Bot**. 8: 1-36.

Kumari, G.j., S.G. Kumar, M. Thippeswamy, A. Annapurnadivi, S.T. Naik and C. Sudhakar. 2007. Effect of salinity on growth and proteomic changes in two cultivars of mulberry (*Morus alba* L.) with contrasting salt tolerance. **IJBT**. 6: 508-518.

Lal, S., V. Gulyani and P. Khurana. 2007. Overexpression of HVA1 gene from barley generates tolerance to salinity and water stress in transgenic mulberry (*Morus indica*). **Transgen Res.** 17: 651-663.

Land development department**. Saline soil**. Available Source: http://www.ldd.go.th/EFiles_project/ld-technique/Technique/k07.htm, June 9, 2010.

Lee, Joohyun and B. Cooper. 2006. Alternative workflows for plant proteomic Analysis. **Mol. BioSyst**. 2: 621-626.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin-Phenol reagent. **J. Biol. Chem.** 193: 265-275.

Machii, H. 1989. Varietal differences of nitrogen and amino acid contents in mulberry leaves. **Acta. Seric. Entomol.** 1: 51-61.

_____. 1990. On gamma-aminobutylic acid contained in mulberry leaves. **J. Seric. Sci. Jpn.** 59: 381-382.

_____, H and K. Katagiri**.** 1991. Varietal differences in nutritive values of mulberry leaves for rearing silkworms. **JARQ.** 25: 202-208

_____, H., A. Koyama and H. Yamanouchi. 1999. A list of genetic mulberry resources maintained at National Institute of Sericultural and Entomological Science. **Inst. Sericul. Entomol.** Sci. 26: 1-77.

Mahajan, S. and N. Tuteja. 2005. Cold, salinity and drought stresses: An overview. **Arch. Biochem. Biophys.** 444: 139-158.

Mansour, M. M. F. 2000. Nitrogen containing compounds and adaptation of plants to salinity stress. **Biol. Plant.** 43: 491-500.

Martino, C., S. Delfine, R. Pizzuto, F. Loreto and A. Fuggi. 2003. Free amino acids and glycine betaine in leaf osmoregulation of spinach responding to increasing salt stress. **New Phytol**. 158: 455-463.

Melander, W. and C. Horvath. 1977. Salt effects on hydrophobic interactions in precipitation and chromatography of proteins: An interpretation of the lyotropic series. **Arch. Biochem. Biophys.** 183: 200-215.

Moons, A., G. Bauw, E. Prinsen, M. Van Montagu and D. Straeten. 1995. Molecular and physiological responses to abscisic acid and salts in roots of salt-sensitive and salt-tolerant Indica rice varieties. **Plant Physiol**. 107: 177-186.

Murakeozy, E.P., Z. Nagy, C. Duhaze, A. Bouchereau and Z. Tuba. 2003. Seasonal changes in the levels of compatible osmolytes in three halophytic species of inland saline vegetation in Hungary**. J. Plant Physiol**. 160: 395-401.

Muuns, R. 2002. Comparative physiology of salt and water stress. **Plant Cell Environ**. 25: 239–250.

_____, R. James and A. Lauchli. 2006. Approaches to increase the salt tolerance of wheat and other cereals. **J. Exp. Bot**. 57: 1025-1043.

Muuns, R. and M. Tester. 2008. Mechanisms of salinity tolerance. **Ann. Rev. Plant Physiol.** 59: 651-681.

Murakeozy, E.P., Z. Nagy, C. Duhaze, A. Bouchereau and Z. Tuba. 2003. Seasonal changes in the levels of compatible osmolytes in three halophytic species of inland saline vegetation in Hungary. **J. Plant Physiol**. 160 : 395-401.

Navarro, R.A. 2000. Potassium transport in fungi and plants. **Biochem. Biophys. Acta**. 1469: 1-30.

Niidonme, T., K. Takahashi, Y. Goto, S. M. Goh, N. Tanaka and K. Kamei. 2007. Mulberry leaf extract prevents amyloid beta-peptide fibril formation and neurotoxicity. **Neuroreport** 18: 813-816.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. **J. Biol.Chem.** 250: 4007-4021.

Opiteck, G.J., S.M. Ramirez, J.W. Jorgenson and M.A. Moseley. 1998. Comprehensive two- dimensional high-performance liquid chromatography for the isolation of overexpressed proteins and proteome mapping. **Anal. Biochem**. 258: 349–61.

Parida, A.K. and A.B. Das. 2005. Salt tolerance and salinity effects on plants. **Ecotoxicol. Environ. Saf.** 60: 324-349.

Parihar, J.S., D. Singh and B.D. Baijal. 1990. Effect of salt stress on ATPase, Na^+ and K^+ uptake in berseem (*Trifolium alexandrinum* L.). ***Acta Bot.***18: 51–54.

Parker, R., T. Flowers, A.L. Moore and N.V.J. Harpham. 2006. An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. **J. Exp. Bot**. 57: 1109-1118.

Peltzer, D., E.Dreyer and A. Polle. 2002. Differential temperature dependencies of antioxidative enzymes in two contrasting species: *Fagus sylvatica* and *Coleus blumei*. **Plant Physiol. Biochem**. 40: 141-150.

Perez, A.F., M.T. Estan, M. Caro and M.C. Bolarin. 1993. Response of tomato cultivars to salinity. **Plant Soil.** 50: 203-211.

Pappin, D.J.C., P. Hojrup and A.J. Bleasby. 1993. Rapid identification of proteins by peptidemass fingerprinting. **Curr. Biol.** 3: 327-332.

Popova, L.P., Z.G. Stoinova and L.T. Maslenkova. 1995. Involvement of abscisic acid in photosynthetic process in *Hordeum vulgare* L. during salinity stress. **J. Plant Growth Regul**. 14: 211–218.

Price, A. and G. Hendry. 1991. Iron-catalyzed oxygen radical formation and its possible contribution to drought damage in nine native grasses and three cereals. **Plant Cell Environ**. 14: 477-484.

Qureshi, R.H. and E.G. Barrett. 1998. **Saline Agriculture for Irrigated Land**. Australian Centre for International Agricultural Research, Australia.

Raharjo, T.J., I. Widjaja, S. Roytrakul and R. Verpoorteb. 2004. Comparative proteomics of *cannabis sativa* plant tissues. **J. Biomol. Tech**. 15: 97-106.

Reddy, M.P., S. Sanish and E.R.R. Iyengar. 1992. Photosynthetic studies and compartmentation of ions in different tissues of *Salicornia brachiata* Roxb. under saline conditions. **Photosynthetica** 26: 173-179.

Rorat, T. 2006. Plant dehydrins-tissue location, structure and function. **Cell. Mol. Biol. Lett.** 11 : 536-556.

Razavizadeh, R., A.A. Ehsanpour, N. Ahsan, S. Komatsu. 2009. Proteome analysis of tobacco leaves under salt stress. **Peptides** 30: 1651-1659.

Salim,M. 1989. Effects of salinity and relative humidity on growth and ionic relations of plants. **New Phytol**. 113: 13-20.

Sanchez, M.D. 2000. **Mulberry: An Exceptional Forage Available Almost Worldwide**. World Animal Review.

Sauter, A. and W. Hartung. 2000. Radial transport of abscisic acid conjugates in maize roots: its implication for long distance stress signals. **J. Exp. Bot**. 51: 929-935.

Schoffl, F., R. Prandl and A. Reindl. 1998. Regulation of the heat-shock response. **Plant Physiol**. 117: 1135-1141.

Serrano, R., J.M. Mulet, G. Rios, J.A. Marquez, I.F. Larriona, M.P. Leube, I. Mendizabal, A. Pascual, M.R.R. Proft and C. Montesinos. 1999. A glimpse of the mechanism of ion homeostasis during salt stress. **J. Exp. Bot**. 50: 1023-1036.

Shi, H., B.H. Lee, S.J. Wu and J.K. Zhu. 2003. Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. **Nat. Biotechnol**. 21: 81-85.

Singh, N.K., C.A. Bracker, P.M. Hasegawa, A.K. Handa, S. Buckel, M.A. Hermodson, E. Pfankock, F.E..Regnier and R.A. Bressan. 1987. Characterization of osmotin. Athumatin-like protein associated with osmotic adaptation in plant cells. **Plant Physiol**. 85: 126-137.

Singh, S.K., H.C. Sharma, A.M. Goswami, S.P. Datta and S.P. Singh. 2000. *In vitro* growth and leaf composition of grapvine cultivars as affected by sodium chloride. **Biol. Plant**. 43: 283-286.

Sohn, H.Y., K.H. Son, C.S. Kwon, G.S. Kwon and S.S. Kang. 2004. Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: *Morus alba* L., *Morus mongolica Schneider, Broussnetia papyrifera* (L.) Vent, *Sophora flavescens Ait* and *Echinosophora koreensis Nakai*. **Phytomed.** 11: 666-672.

Srivastava, S., B. Fristensky and N.N.V. Kav. 2004. Constitutive expression of a PR10 protein enhances the germination of *Brassica napus* under saline conditions. **Plant Cell Physiol** . 45: 1320-4.

Storey, R. and R.R. Walker. 1999. Citrus and salinity. **Sci. Hort**. 78: 39-81.

Sugihara, K., N. Hanagata, Z. Dubinsky, S. Baba and I. Karube. 2000. Molecular characterization of cDNA encoding oxygenevolving enhancer protein 1 increased by salt treatment in the mangrove *Bruguiera gymnorrhiza*. **Plant Cell Physiol**. 41: 1279-1285.

Sudhakar, Chinta., A. Lakshmi and S. Giridarakumar. 2001. Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. **Plant Sci**. 161: 613-619.

Szabolcs, I. 1994. Soil salinization, pp 95-121. *In* Pessarakli, M. (Ed.), **Handbook of Plant Crop Stress**. Marcel Dekker, New York.

Taiz, L. and E. Zeiger. 2002. **Plant Physiology**. 3rd edition. –Sinauer Assoc., Sunderland.

Tattini, M., C. Ponzio, M.A. Coradeschi, R. Tafani and M.L. Traversi. 1994. Mechanisms of salt tolerance in olive plants. **Acta Hort.** 356: 181-184.

The Queen Sirikit Institute of Sericulture. 2009. **Production of silkworm**. Available Source: http://www.qthaisilk.com, May 5, 2010.

Thornton, L., H. Ohkawa, J.H. Roose, Y. Kashino, N. Keren and H.B. Pakrasi. 2004. Homolog of plant psbP and psbQ proteins are necessary for regulation of photosystem II activity in the *Cyanobacterium synechcysti*. **Plant Cell** 16: 2157-75.

Tutej, N. 2007. Mechanisms of high salinity tolerance in plants. **Meth. Enz**. 428: 419-438.

Ukaji, Norifumi.,C. Kuwabara, D. Takezawa, K. Arakawa, S.Yoshida, and S. Fujikawa. 1999. Accumulation of small heat-shock protein homologs in the endoplasmic reticulum of cortical parenchyma cells in mulberry in association with seasonal cold acclimation. **Plant Physiol**. 120: 481-489.

United State Salinity Laboratory Staff. 1954. **Diagnosis and Improvement of Saline and Alkali Soil**. Agriculture Handbook No. 60. New York.

Vaidyanathan, H., P. Sivakumar1, R. Chakrabarty, G. Thomas. 2003. Scavenging of reactive oxygen species in NaCl-stressed rice (*Oryza sativa* L.) differential response in salt-tolerant and sensitive varieties. **Plant Sci**. 165: 1411-1418.

Vijayan, K., S.P. Chakraborti and P.D. Ghosh. 2003. In vitro screening of mulberry (*Morus* spp.) for salinity tolerance. **Plant Cell Rep**. 22: 350-357.

_____, _____ and _____ . 2004. Screening of mulberry (*Morus* spp.) for salinity tolerance through *in vitro* seed germination. **IJBT**. 3: 47-51.

Vijayan, K., S.P. Chakraborti and P.D. Ghosh. 2004. Effect of salinity on leaf anatomy in mulberry. **Bulletin of Indian Academy of Sericulture 8: 16-21.**

_____, _____, S. Ercisli and P.D. Ghosh. 2008. NaCl-induced biochemical and anatomical changes in mulberry (*Morus* spp.). **Plant Growth Regul**. 56: 61-69.

_____. 2009. Approaches for enhancing salt tolerance in mulberry (*Morus* L). **Plant Omics J**. 2: 41-59.

Veeranagamallaiah, G., G. Jyothsnakumari, M. Thippeswamy, P. Chandra Obul Reddy, G.K. Surabhi, G. Sriranganayakulu, Y. Mahesh, B. Rajasekhar, Ch. Madhurarekha, C. Sudhakar. 2008. Proteomic analysis of salt stress responses in foxtail millet (*Setaria italica* L. cv. Prasad) seedlings. **Plant Sci.** 175: 631-641.

Wahome, P.K., Jesch, H.H. and I. Grittner. 2001. Mechanisms of salt stress tolerance in two rose rootstocks: *Rosa chinensis* `Major' and *R. rubiginosa*. **Sci. Hort.** 87: 207-216.

Wang, H. 1998. ICK1, a cyclin-dependent protein kinase inhibitor from *Arabidopsis thaliana* interacts with both Cdc2a and CycD3, and its expression is induced by abscisic acid. **Plant J**. 15: 501-510.

Wang, W.X., B. Vinocur, O. Shoseyov and A. Altman. 2001. Biotechnology of plant osmotic stress tolerance: physiological and molecular considerations. **Acta Hort**. 560: 285-292.

Wang, W.X., B. Vinocur and A. Altman. 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. **Planta** 218: 1-14.

Wang, X., P. Fan, H. Song, X. Chen, X. Li and Y. Li. 2009. Comparative proteomic analysis of differentially expressed proteins in shoots of *Salicornia europaea* under different salinity. **J. Proteome Res**. 8: 3331-3345.

Wilkins, M.R., J.C. Sanchez, A.A. Gooley and R.D. Appel. 1996. Progress with proteome project:why all proteins expressed by a genome should be identified and how to do it. **Biotechnol. Genet. Eng. Rev**. 13: 19-50.

Yang, C.G., J.S.Granite 1, J.E. Van Eyk and R.L. Winslow. 2006. MASCOT HTML and XML parser: an implementation of a novel object model for protein identification data. **Proteomics** 21: 5688-5693.

Yokoyama, T. 1962. **Synthesized Science of Sericulture**. Japan, pp. 39-46.

Yoojun, S. 2008. **Proteomic Analysis of Protein Expression in Mad Crap (*Scylla olivacia*) Hemocytes upon White Spot Syndrome Virus Infection**. M.S. thesis, Mahidol University

Yuvaniyama, A., and P. Pongwician. 2005. Sustainable use of problem soils in rainfed agriculture (FAO/TCP/THA/2906), pp 267- 307. *In* Proceedings of the Mid-Term Workshop on Integrated Management for Sustainable Use of Low Fertility and Salt-Affected Soils in Rainfed Agriculture**, Including Salt-Affected Soils in Coastal Areas.** FAO Project TCP/THA/2906. Khon Kaen, Thailand.

Zhao, K.F., A. Littlewood and P.J.C. Harris. 1992. Responses of *Gleditsia triacanthos* seedlings to salt stress. **Inter. Tree Crops J**. 7:149-153.

Zhou, K.Y., J. Zhou, Z. XuDong, S. Xiang and L. Zuo. 2009. Effects of salt stress on photosynthetic characteristics of mulberry seedlings. **Sci. Sil. Sin.** 45: 61-66.

Zhu, B., T.H. Chen and P. H. Li. 1995. Expression of three osmotinelike protein genes in response to osmotic stress and fungal infection in potato. **Plant Mol. Biol**. 28: 17-26.

Zhu, J.K., J. Shi, U. Singh, S.E. Wyatt, R.A. Bressan, P.M. Hasegawa and N.C. Capita. 1993. Enrichment of vitronectin and fibronectin like proteins in NaCl-adapted plant cells and evidence for their involvement in plasma membrane-cell wall adhesion. **Plant J**. 3: 637-646.

_____, P.M. Hasegawa and R.A. Bressan. 1997. Molecular aspects of osmotic stress. **Crit. Rev. Plant Sci**. 16: 253-277.

_____. 2002. Salt and drought stress signal transduction in plants. **Ann. Rev. Plant Physiol.** 53: 247-273.

_____. 2003. Regulation of ion homeostasis under salt stress. **Curr. Opin. Plant Biol**. 6: 441-456.

Zorb, C., S. Schmitt, A. Neeb, S. Karl, M. Linder and S. Schubert. 2004. The biochemical reaction of maize (*Zea mays* L.) to salt stress is characterized by amitigation of symptoms and not by a specific adaptation. **Plant Sci**. 167: 91-100.

APPENDIX

REAGENTS

1. Solution and buffer for SDS-PAGE

1.1 Polyacrylamide gel

- 12.5% Separating gel

Distilled water	1.92 ml
1.5 M Tris-HCl pH 8.8	1.5 ml
30% Acrylamide 0.8% bis-acylamide	2.52 ml
10% Ammonium persulfate	30 µl
10% SDS	60 µl
TEMED	5 µl
Total volume	10 ml

- 5% Stacking gel

Distilled water	1.86 ml
1.5 M Tris-HCl pH 6.5	0.72 ml
30% Acrylamide 0.8% bis-acylamide	0.04 ml
10% Ammonium persulfate	23 µl
10% SDS	30 µl
TEMED	1.7 µl
Total volume	3 ml

1.2 1.5 M Tris-HCl, pH 8.8

Tris base	18.15 g
Distilled water	80 ml

Adjust to pH 8.8 with concentrated HCl and add distilled water to 100 ml.

1.3 0.5 M Tris-HCl, pH 6.8

Tris base	6 g
Distilled water	80 ml

Adjust to pH 6.8 with concentrated HCl and add distilled water to 100 ml.

1.4 10X Running buffer

Tris base	30.2 g
Glycine	141.4 g
SDS	10 g
Distilled water to	1 l

1.5 10% w/v trichloroacetic acid solution

TCA	10 g
β-mercaptoethanol	0.07 ml
Acetone solution	100 ml

2. Solution for 2-DE

2.1 Protein lysis buffer

Tris base (1 M)	3 ml
Thiourea	15.22 g
Urea	42 g
CHAPS	4 g

Adjust to pH 8.5 with diluted HCl**,** make up to 100 ml, filtrate through 0.22 μm filter membrane and store at 4 °C.

2.2 Rehydration buffer

Urea	13.5	g
CHAPS	1	g
Bromophenol Blue	50	µl

Make up to 25 ml, filtrate through 0.22 µm filter membrane and store at -20 °C.

2.3 Equilibration buffer

Tris-HCl (1.5 M, pH 8.8)	50	ml
Urea	360.35	g
Glycerol	345	ml
SDS	20	g
Bromophenol Blue	400	µl

Make up to 1 l, filtrate through 0.45 µm filter membrane and store at -20 °C.

2.4 1.25X Coomassie brilliant blue G-250

$(NH_4)_2SO_4$	100	g
H_3PO_4	20	ml
Brilliant blue G-250	1	g
Milli Q water to	1	l

Stir for overnight.

Appendix Table 1 Soil $EC_{1:5}$ determined after NaCl treated for 0, 15 and 30 days.

Mulberry varieties	$EC_{1:5}$ (dSm^{-1}) / Time		
	0 day (mean ± SD)	15 days (mean ± SD)	30 day (mean ± SD)
Local varieties			
Yaiburirum	0.17 ± 0.01	1.08 ± 0.27	2.58 ± 0.17
Khunpai	0.17 ± 0.04	2.11 ± 0.16	2.93 ± 0.30
Phoe	0.12 ± 0.01	2.60 ± 0.13	2.60± 0.80
Mee	0.18 ± 0.01	2.59 ± 0.87	4.14 ± 0.30
Plong	0.16 ± 0.01	3.02 ± 0.19	4.07 ± 0.16
Jak	0.20 ± 0.08	2.05 ± 0.17	2.07 ± 0.15
Somyai	0.16 ± 0.02	2.61±0.24	2.13±0.10
Hangplalod	0.16 ± 0.02	2.60 ±0.13	3.84 ± 0.20
Kaewkrasang	0.12 ± 0.04	2.33 ± 0.76	3.35 ± 0.86
Baiphoe	0.16 ± 0.01	2.60 ± 0.15	2.97 ± 0.19
Chiangkam	0.12 ± 0.04	2.96 ± 0.12	2.96 ± 0.18
Baimon	0.22 ± 0.01	3.53 ± 0.18	2.22 ± 0.10
Kreu	0.17 ± 0.01	2.62 ± 0.12	3.70 ± 0.19
Soi	0.17 ± 0.01	2.25 ± 0.28	2.25 ± 0.70
Kam	0.14 ± 0.01	2.41 ± 0.14	2.41 ± 0.23
Som	0.15 ± 0.01	2.53 ± 0.16	4.70 ± 0.10
Kaewchonnabot	0.19 ± 0.01	2.25 ± 0.218	5.69 ± 0.47
Noi	0.13 ± 0.07	2.63 ± 0.88	2.63 ± 0.72
Kaew	0.17 ± 0.02	2.26 ± 0.87	3.91 ± 0.95
Sieda	0.20 ± 0.02	2.26 ± 0.10	4.40 ± 0.13
Paiubon	0.20 ± 0.02	2.61 ± 0.26	2.61 ± 0.19
Kaewsateuk	0.21 ± 0.01	1.75 ± 0.40	3.73 ± 0.60
Dang	0.19 ± 0.03	1.97 ± 0.39	2.51 ± 0.21
Yauk	0.23 ± 0.06	2.57 ± 0.70	2.57 ± 0.33
Yaisisaket	0.13 ± 0.03	2.05 ± 0.20	4.71 ± 0.19
Maelukon	0.17 ± 0.01	2.90 ± 0.80	3.13 ± 0.18

Appendix Table 1 (Continued)

Mulberry varieties	$EC_{1:5}$ (dSm^{-1})/Time		
	0 day (mean ± SD)	**15 days (mean ± SD)**	**30 days (mean ± SD)**
Keekai	0.15 ± 0.01	3.00 ± 0.10	3.85 ± 0.20
Tadum	0.16 ± 0.04	1.87 ± 0.11	2.03 ± 0.26
Tadang	0.20 ± 0.03	2.30 ± 0.15	3.54 ± 0.13
KKN-1	0.23 ± 0.01	2.52 ± 0.10	4.01 ± 0.21
Average	**0.17 ± 0.030**	**2.41 ± 0.52**	**3.25 ± 0.63**
Hybrid varieties			
BR51	0.21 ± 0.04.	3.63 ± 0.45	2.18 ± 0.18
BR60	0.16 ± 0.02.	2.96 ± 0.25	3.09 ± 0.82
S1	0.21 ± 0.08	2.22 ± 0.77	3.03 ± 0.72
Sisaket 33	0.29 ± 0.02	2.98 ± 0.20	3.31 ± 0.78
Average	**0.22 ± 0.05**	**2.68 ± 0.76**	**3.04 ± 0.68**
Fruit varieties			
Chiangmaikinphol	0.15 ± 0.01	1.87 ± 0.29	2.29 ± 0.13
Pholyaivawee	0.18 ± 0.02	2.30 ± 0.13	2.80 ± 0.21
Average	**0.17 ± 0.01**	**2.43 ± 0.79**	**2.71 ± 0.60**
Exotic varieties			
Tonkin	0.16 ± 0.01	2.00 ± 0.98	2.76 ± 0.14
M. alba	0.20 ± 0.01	2.10 ± 0.21	2.35 ± 0.22
Kenva	0.15 ± 0.01	1.95 ± 0.83	2.42 ± 0.80
S61	0.18 ± 0.02	2.97 ± 0.17	1.87 ± 0.15
M. Nigra	0.25 ± 0.02	2.09 ± 0.26	3.07 ± 0.16
Lhunjiew	0.29 ± 0.03	3.14 ± 0.12	3.15 ± 0.19
KNN-2	0.31 ± 0.03	2.99 ± 0.96	3.14 ±0.16
S14	0.21 ± 0.02	1.92 ± 0.27	3.85 ± 0.30
Average	**0.22 ± 0.06**	**2.34 ± 0.47**	**2.76 ± 0.64**

Appendix Table 2 Growth characteristics of mulberry varieties under salt stress For 30 days

Mulberry Varieties	Growth characteristics				
	control	control	treated	treated	treated
Yaiburirum	N	N	W	W	W
Khunpai	N	N	W	W	W
Phoe	N	N	W	D	W
Mee	N	N	D	W	W
Plong	N	N	S	S	S
Jak	N	N	W	D	W
Somyai	N	N	W	W	W
Hangplalod	N	N	D	W	W
Kaewkrasang	N	N	D	W	W
Baiphoe	N	N	W	W	D
Chiangkam	N	N	W	W	D
Baimon	N	N	W	W	D
Kreu	N	N	S	W	W
Soi	N	N	W	D	D
Kam	N	N	D	W	D
Som	N	N	S	S	S
Kaewchonnabot	N	N	W	D	W
Noi	N	N	D	S	W
Kaew	N	N	S	W	W
Sieda	N	N	W	W	D
Paiubon	N	N	D	W	D
Kaewsateuk	N	N	W	S	W
Yauk	N	N	W	W	D
Yaisisaket	N	N	W	W	W
Maelukon	N	N	W	W	W
Keekai	N	N	W	W	W

Appendix Table 2 (continued)

Varieties	Growth characteristics				
	control	**control**	**treated**	**treated**	**treated**
Tadum	N	N	D	W	W
Tadang	N	N	W	W	W
Dang	N	N	W	D	W
S14	N	N	W	W	W
BR51	N	N	D	D	D
BR60	N	N	W	W	W
S1	N	N	D	D	W
Sisaket 33	N	N	W	W	W
KKN-1	N	N	W	D	W
KNN-2	N	N	W	W	W
Chiangmaikinphol	N	N	W	D	W
Pholyaivawee	N	N	W	W	W
Tonkin	N	N	W	W	W
M. alba	N	N	S	W	W
Kenva	N	N	W	W	W
S61	N	N	D	D	D
M. nigra	N	N	W	W	W
Lhunjiew	N	N	W	S	W

Note: N; normal, S; survive, W; wilted , D; died

Appendix Table 3 Up- and Down- regulated proteins in mulberry leaves under NaCl treatment.

Spot no.	Amount of spot (% volume)				Up- or down regulation(folds)	Experimental	
	Som strain		S61 strain		Som/S61 strains	M*r* (kDa)	p*I*
	Control	stress	Control	stress			
1	0.924±0.729	1.770±0.753	-	-	Up: 1.95 /ND	71.5	7.5
2	8.030±0.871	0.933±0.056	1.268±0.796	2.511±0.999	Down: 8.16 / up: 1.98	57.2	3.8
3	0.200±0.034	6.34±0.234	-	-	Up: 31.4 / ND	56.9	6.1
4	8.836±1.050	3.615±0.143	7.753±2.333	7.012±0.913	Down: 2.44 / SD	34.8	5
5	6.249±0.333	3.165±0.415	5.090±0.485	4.846±0.440	Down: 1.97 / SD	26.4	5.8
6	2.663±0.434	0.921±0.602	4.533±2.061	4.102±1.174	Down: 2.89 / SD	24.4	3.6
7	0.465±0.434	0	0.825±0.055	0.621±0.215	Down 4.65 / Down: 1.33	22.3	4.9
8	2.407±0.200	1.255±0.573	1.609±0.400	1.778±0.160	Down: 1.92 / SD	18.3	5.1
9	0.413±0.023	0.808±0.042	0.365±0.056	0.586±0.264	Up: 1.95 / Up: 1.60	18.5	5.6
10	0.381±0.197	1.104±0.381	-	-	Up: 2.90 / ND	16.9	5.1
11	2.114±1.040	7.727±2.710	-	-	Up: 3.66 / ND	14.6	7.5
12	1.827±0.251	11.443±1.026	3.917±0.308	3.175±0.224	Up: 6.26 / SD	13.9	8.6
13	-	-	6.007±1.641	3.925±0.218	ND / Down: 1.53	13.5	3.8

Appendix Table 4 List of match peptides, which were identified with proteins available in NCBI database using MASCOT program.

Spot No.	Protein name	% Sequence coverage	Match peptide
3	Rubisco	17	DTDILAAFR
			TFQGPPHGIQVER
			AVYECLR
			FLFCAEAIYK
			EITLGFVDLLR
			VALEACVK
			DLAVE GNEIIR
			WSPELAAACEVWK
4	OEE1	40	RLTYDEIQSK
			GTGTANQCPTIEGGVDSFAFK
			LTYTLDEIEGPFEVANDGTVK
			DGIDYAAVTVQLPGGE RVPFLFTIK
			GGSTGYDNAVALPAGGRGDEEELTKENIK
5	OEC	11	EVEYPGQVLR
			VDYLLGK
			KEYYFLSVLTR
6	OEE2	5	SITDYGSPEEFLSK
7	Rieske Fe/S	11	DALGNDVIASEWLK
			VVFVPWVETDFR
8	Rubisco	16	KDTDILAAF R
			EITLGFVDLLR
			DLAVEGNEII
9	WAP1	16	AAVLDADNLFPK
			LVEGYLEANPSAYN
12	Rubisco small subunit	8	QVQCISFIAYKPPAK

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PUBLICATIONS

1) Chehasan Cheubong, Bantawan Marcheaw, Malee Na Nakorn, Sittiruk Roytrakul, and Amornrat Promboon. 2008. Screening of salt tolerant mulberry in soil. In The first Sericulture forum in Thailand. September 22-23, 2008. pp. 76. Department of Biochemistry, Faculty of Science, Kasetsart University, Thailand. (Poster Presentation)

2) Bantawan Marcheaw, Chehasan Cheubong, Malee Na Nakorn, Sittiruk Roytrakul, Ratree Wongpanya and Amornrat Promboon. 2009. Screening of salt tolerant mulberry in Thailand. In The 3rd conference of Botany in Thailand. March 25-27, 2009. pp. 70. Department of Botany, Faculty of Science, Mahidol Univesity, Thailand. (Poster Presentation)

3) Bantawan Marcheaw, Chehasan Cheubong, Malee Na Nakorn, Sittiruk Roytrakul, Ratree Wongpanya and Amornrat Promboon. 2009. Screening of salt tolerant mulberry, *Morus spp*., in Thailand. Proceedings of 35th Congress on Science and Technology of Thailand, October15-17, pp. 245. (Poster Presentation)

4) Chehasan Cheubong, Bantawan Marcheaw, Malee Na Nakorn, Sittiruk Roytrakul, Retree Wongpanya and Amornrat Promboon. 2010. Differential protein expression of salt tolerant and salt sensitive mulberry varieties under salt stress. In The 4th conference of Botany in Thailand. March 24-26, 2010. pp. 90. Lotus Pang Suan Khaw Hotel, Chiangmai, Thailand. (Poster Presentation).
