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DIFFERENTIAL EXPRESSION OF PROLINE AND FLAVONOIDS IN THAI RICE CULTIVARS UNDER SALINITY AND DROUGHT

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การทดลองแสดงให้เห็นความสัมพันธ์ในเชิงบวกบวกระหว่างปริมาณสารโพรลีนกับระดับการแปลรหัสของยีน *OsP5CS* และ *OsP5CR* และการสะสมสารเฟลโวนอยด์กับระดับการแปลรหัสของยีน *OsCHS* และ *OsDFR* ในสายพันธุ์ข้าว การเปลี่ยนแปลงปริมาณของสารเหล่านี้อาจเป็นการตอบสนองเพื่อเป็นกลไกลดความเป็นพิษเมื่อต้นกล้าพืชอยู่ภายใต้สภาวะเครียดทางกายภาพมีผลให้คงลักษณะทางด้านสัณฐานวิทยาและสรีรวิทยา

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ABSTRACT

Salinity (100 mM NaCl) and drought (100 mM mannitol) could induce stresses in seedlings of 10 Thai rice cultivars during incubating for 2 and 4 days under photoautotrophic culture condition, as indicated by the decrease level of plant growth characters, relative water content (RWC) and photosynthetic pigment contents and the increase level of lipid peroxidation. Multivariate parameters of rice seedlings cultivated under salt or drought stresses were subjected to Hierarchical cluster analysis in SPSS software and then classified into four groups, salt-tolerant (KDML105, SY, KD, KLD and TD49), salt-sensitive (PT1, KS, KK1, KK2 and BSR), drought-tolerant (KS, KK1, KK2 and BSR) and drought-sensitive (KDML105, PT1, SY, KD, KLD and TD49) cultivars. Proline and flavonoid metabolites from rice groups were measured. Stress-tolerant groups highly accumulated proline more than stress-sensitive groups when exposed to either salt or drought conditions. The decreases in flavanone were observed in stressed-seedlings cultivars, while flavones and flavonols in the stressed-tolerant cultivars were increased. Anthocyanin and tannin contents in stressed-tolerant cultivars were enriched more than those in stressed-sensitive cultivars. Moreover, the results showed that the positive correlation between proline content and the transcriptional levels of the *OsP5CS* and *OsP5CR* genes was observed and also the flavonoid accumulation was positively correlated with the transcriptional levels of the *OsCHS* and *OsDFR* genes in rice cultivars. The alteration contents of these compounds might be responsible for the detoxification defense mechanisms in rice seedlings exposed to abiotic stresses, leading to maintain morphological and physiological characters.

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CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

Rice (*Oryza sativa* L. ssp. *indica*) is a main staple crop around the world, feeding and providing the necessary daily calories for millions of people. The environmental stresses of salinity and drought reduce growth and agricultural productivity more than other factors (Bohnert *et al.,* 1995; Greenway and Munns, 1980). It is estimated that less than 10% of the world's arable lands may free from the major environmental stresses, i.e. drought and salinity (Ashraf, 1994). Plants have to evolve the complex internal systems responding to the environmental changes because of their immobility (Carimi *et al.,* 2003; Xu and Hanson, 2000). Rice, a major crop, is well known to be relatively sensitive to salt stress and water deficit. However, the differences within genetic gene pool present a distinctive tolerance among each rice cultivar. Salt and drought stresses result a number of plant detrimental processes such as a toxic action of Na^+ from salt, a modification in plant water status and a generation of oxidative stress affected a production of reactive oxygen species (ROSs) in plant cells (Zhu, 2002; Zhu, 2001). The effect of salt and drought stresses on rice in term of physiological and biochemical changes have been investigated. These stresses have been reported to diminish the rice growth and photosynthetic activity because theirs can damage DNA, protein, chlorophyll and membrane functions. To mitigate and repair damages initiated by osmotic and ROS, plants have developed the complex network of osmoprotectants and antioxidant systems (del Rio *et al.,* 2002; van Breusegem *et al.,* 2001; Mansour, 1998). Therefore, the common abiotic stress responses in plants are overproduction of different types of osmoprotectants and antioxidants in order to maintain cell for growth and survival (Schutzendubel and Polle, 2002; Zhang *et al.,* 2001; Mata and Lamattina, 2001; Hasegawa *et al.,* 2000; Martina *et al.,* 1999).

Under osmotic stress, plant cell requires the control of intracellular cytoplasm and accumulation of low molecular weight compounds or osmoprotectants such as proline and glycine betaines (Abdel-Nasser and Abdel-Aal, 2002; Bourot *et al.,* 2000). In response to drought or salinity stress, proline accumulation normally occurs in the cytosol of plant where it contributes substantially to the cytoplasmic osmotic adjustment (Ramajulu and Sudhakar, 2000). Proline accumulation has been reported to be a key role in protecting enzymes from denaturizing,

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stabilizing the protein synthesis, regulating the cytosolic acidity and acting as a reservoir of carbon and nitrogen sources (Nikolopoulos and Manetas, 1991; Venekamp *et al.,* 1989). Accumulation of proline under stress in many plant species has been correlated with stress tolerance, and its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants, such as mulberry (Kumar *et al.,* 2003), mustard (Mandan *et al.,* 1995) and rice (Hsu *et al.,* 2003). Despite the presence of a strong correlation between stress tolerance and high accumulation of proline in plants, this relationship may not be universal. Many reports also presented the negative relationship between proline and tolerance level. For example, the accumulation of proline in rice leaf grown under salt stress is deemed to be a symptom of salt injury rather than an indication of salt tolerance (Lutts *et al.,* 1999), and also similar with sorghum, proline accumulation in shoot and leaf is not a plant response associated with tolerance (de-Lacerda *et al.,* 2003).

Oxidative stress may be a significant factor in relation to salinity and drought induced injury (Mittler, 2002). Higher plants have ROS-scavenging systems consisting of several antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase and catalase and some low molecules of non-enzyme antioxidants, such as ascorbic acid, glutathione and flavonoids (Vaidyanathan *et al.,* 2003). These systems protect membranes from the deleterious effects of ROSs, such as superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen, which are produced at elevated rates when plants are exposed to abiotic stress conditions (Guo *et al.,* 2005). Flavonoids one of non-enzyme antioxidants are water-soluble pigments, found in various tissues of higher plants. They are secondary metabolites that serve as antioxidants to play an important role in the interactions between plant and environments and are being responsive to such factors as visible and UV-B radiation, drought and low temperature (Cortes *et al.*, 2006; Kong *et al.*, 2003). The flavonoid biosynthetic pathway in crop has been reported in responses to both biotic (diseases) and abiotic (UV-B, cold and drought) situations (Knight and Knight, 2001; Chalker-Scott, 1999; Kinoshita and Maekawa, 1986). The protective effects of flavonoids in biological systems are ascribed to their capacity to transfer electrons free radicals and chelate metal catalysts (Heim *et al.,* 2002; Ferrali *et al.,* 1997). However, the accumulation of various metabolites in flavonoid biosynthesis responded to salt and drought stress of *indica* rice, especially Thai rice cultivars has a few reports.

Therefore, using a wide range of rice cultivars may help towards a better understanding of the relationship between salt and drought tolerance and the accumulation of proline and flavonoid

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compounds. The comparative study on proline and flavonoid accumulation in various cultivars of Thai rice may provide the protective information of rice plants against salt and drought stresses. In addition, differential responses to salt and drought stresses at morphological, physiological and genes expression levels involved in biosynthetic pathway of proline and flavonoid metabolites may potentially be further utilized as biochemical indices or markers for salt- and drought-tolerant screening in rice breeding program.

Goal and objectives

The goal and objectives of this research are as follows:

1. To study the morphological and physiological characterizations responses to salt and drought stresses among Thai rice cultivars.

2. To study the comparative level of proline and flavonoid metabolites and their protective roles in rice crop against salt and drought stresses.

3. To determine the influences of salt and drought stresses on the expression of genes involved in the proline and flavonoid biosynthesis of Thai rice cultivars.

1.3 Scope of the study

In this study, the effect of salt and drought stresses on morphological, physiological and metabolic levels in various Thai rice cultivars grown under photoautotrophic growth condition was determined. The morphological and physiological responses such as growth ability, relative water contents, photosynthetic pigments and lipid peroxidation levels among Thai rice varieties were compared either with or without stressed conditions. The contents of proline osmoprotective agent and flavonoids antioxidant metabolites in rice seedlings were measured. Moreover, the expression of genes encoding enzymes related to those metabolites biosynthesis in rice seedlings grown under salt- and drought stresses were detected.

1.4 Expected results

The expected results from this research are as follows:

1. The basic knowledge of morphological and physiological characterization responses to salt and drought stresses among Thai rice cultivars should be known.

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2. The proline and flavonoid accumulation may play important roles in rice crop against salt and drought stresses. These metabolites expressed in salt- and drought-stressed seedlings may potentially be further utilized as indices for the salt and drought tolerant screening of Thai rice cultivars.

3. The candidate genes relating to salt- and drought-responsive metabolites, proline and flavonoids may be further applied for rice breeding and genetic engineering.

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CHAPTER 2

LITERATURE REVIEW

2.1 Rice

Rice is belonging to Graminae or Poaceae family, which is composing of *Oryza sativa* and *Oryza glaberrima*. These plants are native to tropical and subtropical Southern and Southeastern Asia and in Africa. *O. sativa* is cultivated worldwide, whereas *O. glaberrima* is grown in a few countries in West Africa. In *O. sativa*, there are three subspecies, including *indica* (long grain), *japonica* (round grain) and *javanica* (medium grain). *Indica* rice generally cultivates in the tropical climate belt, from Thailand, India, Pakistan and Brazil. *Japonica* type is mostly grown in temperate countries, Japan, Korea and Northern China. In addition, *javanica* type only found in Indonesia (Oka, 1991). Rice crop is a monocarpic annual plant, growing to 1–1.8 m tall, occasionally more depending on the variety and soil fertility. It has long, slender leaves 50–100 cm long and 2–2.5 cm broad. The small wind-pollinated flowers are produced in a branched arching to pendulous inflorescence 30–50 cm long. Rice seed is a grain (caryopsis) 5–12 mm long and 2–3 mm thick (Crawford and Shen, 1998; Smith and Bruce, 1998).

Rice is one of the most important crops in the world and is the staple food for about three billion people (Toenniesen, 1996). Production and consumption are concentrated in Asia where more than 90% of all rice produced and consumed (David, 1996). Rice is the world's largest crop following maize (corn) and wheat for 700, 692 and 626 million metric tons in 2005, respectively (Heong *et al.*, 2005). Rice cultivation is well-suited to countries and regions with low labour costs and high rainfall, as it is very labour-intensive to cultivate and requires plenty of water for irrigation, much like the licorice crops found in Eastern Europe. Rice can be grown practically anywhere, even on steep hillsides. Although its species are native to South Asia and certain parts of Africa, centuries of trade and exportation have made it commonplace in many cultures (Londo *et al.*, 2006; Huang *et al.*, 2005; Jahn *et al.*, 2004). Rice is the significant commodity for Thai's economy. It is the country's staple food, by-productions of rice is also importance for human and animal consumption. Thailand is one of the world rice producers among others in Southeast Asia such as China, India, Indonesia and Vietnam (Table 2.1). World production of rice has risen steadily from about 200 million tons of paddy rice in 1960 to 600 million tons in 2004 (Heong *et*

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al., 2005). Milled rice is about 68% of paddy rice by weight. In the year 2004, the top three producers are China (31% of world production), India (20%), and Indonesia (9%). World trade figures are very different, as only about 5–6% of rice produced is traded internationally. The largest three exporting countries are Thailand (26% of world exports), Vietnam (15%), and the United States (11%), while the largest three importers are Indonesia (14%), Bangladesh (4%), and Brazil (3%).

The taxonomic definition of rice is as follows (Tassongchant, 1987).

Division	Spermatophyta
Class	Angiospermae
Subclass	Monocotyledonae
Order	Graminales
Family	Graminae
Genus	*Oryza*
Species	*sativa*
Subspecies	*indica*
	japonica
	javanica

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Table 2.1 Top paddy rice producers in 2005

Countries	Product (million metric ton)
China	185
India	129
Indonesia	54
Bangladesh	40
Vietnam	36
Thailand	27
Myanmar	25
Pakistan	18
Philippines	15
Brazil	13
Japan	11
World Total	700

Source: Londo *et al.* (2006)

2.2 Environmental stresses of higher plants

Environmental stresses trigger a wide variety of plant responses, ranging from altered physiology, cellular metabolism and gene expression. Several abiotic factors affect the plant growth and productivity. Among the environmental stresses, salinity and drought stresses are the main abiotic factors that limit agricultural crop production (Reddy *et al.,* 2004; Chaves *et al.,* 2003; Rontein *et al.,* 2002). Drought and salinity affect more than 10 percent of arable land and are rapidly increasing on a global scale. These stresses are declining yields for most major crop plants by more than 50 percent (Burg *et al.,* 1996). Numerous physiological changes that occur under these conditions have been documented. Therefore, the understanding on plants response to external environment is a greater importance and also a fundamental topic for stress tolerant improvement. Salt and drought stresses have largely remained a mystery until recently and the

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responses of plants to both stresses are highly complex, involving deleterious or adaptive changes (Xiong and Zhu, 2001; Hasegawa *et al.,* 2000; Amtmann and Sanders, 1999; Epstein *et al.,* 1980).

2.2.1 Salinity and drought stresses

Saline soil is a major abiotic stress in plant agriculture world-wide. Current estimates indicate that 10-35 percent of the world's agricultural land is now affected by this problem (Parida and Daş, 2005). Cultivable lands of Thailand are also affected by this problem. There are 3.5 million ha of saline soil area in the Northeast region of Thailand (Department of Environmental Quality Promotion, 2008b). The increasing of saline soil area usually occurs from the unsustainable irrigation which causes water logging and salinity development in rainfed. Salinity exerts complex effects on the plant as a result of ionic and osmotic stresses (Kawasaki *et al.,* 2001; Hoshida *et al.,* 2000).

Drought conditions currently exist in many parts of Southeast Asia, particularly in Indochina, Thailand, Vietnam, Myanmar, Cambodia, and Laos. The drought has stressed rice, coffee, sugar cane, and other crops in this region. The largest crop losses have been reported in Thailand; moreover the drought has also damaged crops in southern China, Cambodia, Vietnam, and Laos. Approximately 76% of the total 9.2 million ha of rice growing areas in Thailand are under rainfed conditions. The three rainfed rice ecosystems, upland, lowland and deep water. Rainfed lowland occupies 6.8 million ha, covering 75% of the total rice growing areas under drought condition. The majority of the rainfed lowland areas are found in the Northeast (4.8 million ha) and North (1.4 million ha) regions of Thailand (Department of Environmental Quality Promotion, 2008a). These areas are a little improvement in yield from plant breeding (Jongdee *et al.,* 2006; Fukai *et al.,* 1999). Moreover, Thai people always faced with water shortage about 13,000-24,000 villages or 6-10 million people, which mostly located in the Lower Northeast. Water source was lacked periodically, and became severity. Plant experience drought stresses either when the water supply to roots becomes difficult or when the transpiration rate becomes very high. In plants and other photoautotroph, water plays the additional role of providing the energy necessary to drive photosynthesis. Water molecules are oxidized, in a process termed autolysis, to yield the electrons that are used to drive the energy yielding photosystem II reaction center (Salisbury and Ross, 1992). One of the major consequences of drought stress is the loss of protoplasmic water (Hartung *et al.,* 1998). Additionally, the concentration of photoplasmic constituents and the loss of water from the cell lead to the formation of glassy state. In this state, liquid is left in the cell that it has a very high viscosity, increasing the chances of molecular

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interactions that cause protein denaturation and membrane fusion (Mundree *et al.*, 2002; Hoekstra *et al.*, 2001).

2.2.1.1 Ionic stress

Tolerance mechanisms can be categorized as those that function to minimize ion disequilibrium or osmotic stress. The chemical potential of the saline solution initially establishes a water potential imbalance between the apoplast and symplast that leads to turgor decrease, which if severe enough can cause growth reduction. Cellular dehydration begins when the water potential difference is greater than it can be compensated for by turgor loss (Sander, 2000; Bohnert *et al.*, 1995). The cellular response to turgor reduction is osmotic adjustment. The cytosolic and organellar machinery of plants is equivalently Na^+ and Cl^- sensitive, thus osmotic adjustment is achieved in these compartments by accumulation of compatible osmolytes and osmoprotectants. High concentrations of Na^+ in the soil or increased Na^+ accumulation in the plant system may be recognized by extracellular and intracellular sensors that the effective counteraction mechanisms will be initiated. Na^+ and Cl^- are motivating osmolytes accumulation for osmotic adjustment and are compartmentalized into the vacuole to minimize cytotoxicity (Blumwald *et al.*, 2000). Movement of ions into the vacuole might occur directly from the apoplast into the vacuole through membrane or a cytological process (Hasegawa *et al.*, 2000). Then compartmentalization could be achieved with minimal or no exposure of the cytosol to toxic ions. The bulk of Na^+ and Cl^- movement from apoplast to the vacuole likely is mediated through ion transport system located in the plasma membrane and tonoplast. Coordinate regulation of these ion transport system is required in order to control net influx across the plasma membrane and vacuolar compartmentation. The SOS signal pathway is a regulator of key transport systems required for ion homeostasis (Yokoi *et al.*, 2002; Hasegawa *et al.*, 2000; Zhu, 2000) (Fig. 2.).

Ion transporters are considered to play an important role in salt tolerance. In principle, three mechanisms exist to prevent excess Na^+ accumulation in the symplast of plant cells (Bartels and Sunkar, 2005; Zhu, 2003; Apse and Blumwald, 2002):

- Restricting the Na^+ permeation and entry into plants by Na^+ transporters.
- Compartmentalizing the Na^+ in the vacuole.
- Extruding Na^+: cytosolic Na^+ can be transported back to the external medium or the apoplast *via* plasma membrane Na^+/H^+ antiporter activity.

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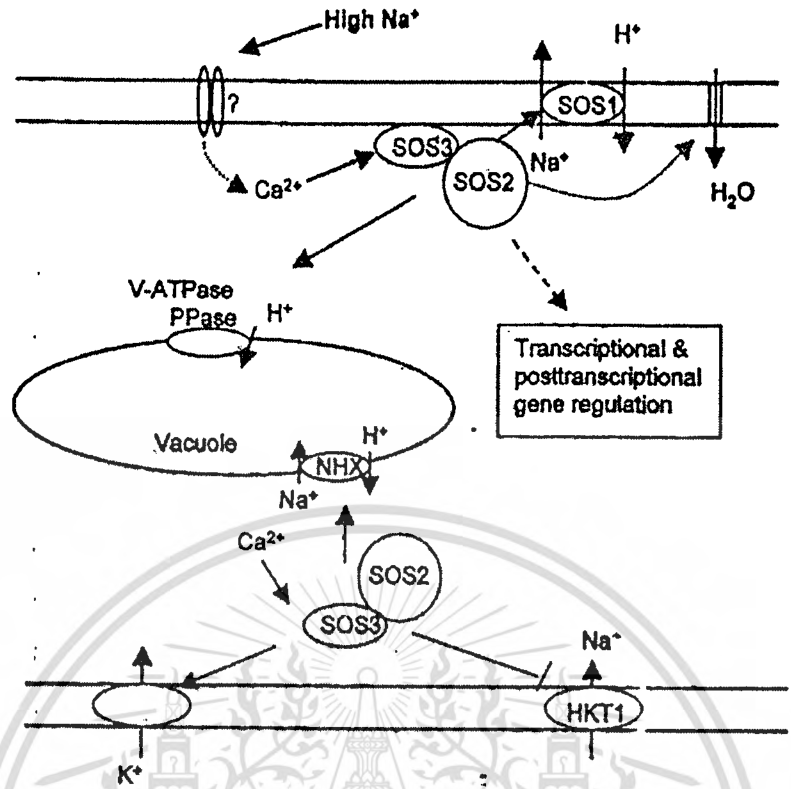

Fig. 2.1 Salt stress-induced, Ca^{2+}-dependent signaling that mediates Na^{+} homeostasis and salt tolerance. SOS1, plasma membrane Na^{+}/H^{+} antiporter; SOS2, serine/threonine kinase; SOS3, Ca^{2+} binding protein; PPase, vacuolar H^{+}-ATPases and pyrophophatases; NHX, vacuolar Na^{+}/H^{+} exchanger

Source: Zhu (2002)

Molecular interaction and complementation analysis indicate that *SOS3* is required for activation of *SOS2* that regulates *SOS1* transcription. The *SOS3* gene encodes a Ca^{2+} sensor protein similar to the calcineurin B subunit from yeast, a Ca^{2+} dependent protein phosphatase, and neuronal calcium sensors from animals (Liu and Zhu, 1998; Shi *et al.*, 2000). *SOS2* encodes a serine/threonine protein kinase with an N-terminal kinase domain and a unique C-terminal domain with FISL motif that is sufficient for interaction with *SOS3* (Liu *et al.*, 2000). The *SOS1* gene encodes a plasma membrane Na^{+}/H^{+} antiporter, which is further evidence for the interaction of the three *SOS* genes. *SOS3* forms a complex is necessary for the phosphorylation and subsequent activation of *SOS1* (Qiu *et al.*, 2002). The SOS pathway also regulates the vacuolar Na^{+}/H^{+} exchange activity and contributes to Na^{+} compartmentalization. The SOS pathway coordinately regulates plasma membrane and tonoplast Na^{+}/H^{+} antiporter activity which leads to Na^{+} homeostasis and thus salt tolerance (Bartels and Sunkar, 2005; Qiu *et al.*, 2004).

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2.2.1.2 Osmotic stress

Salt and drought tolerance requires that compatible solutes accumulate in the cytosol and organelles where these function in osmotic adjustment and osmoprotection. Compatible solute accumulation as a response to osmotic stress is a ubiquitous process in organism. However, the solute accumulations vary in the organism and even between plant species. Compatible solutes are non-toxic molecules. These molecules may have a primary role of turgor maintains but they may also be involved in stabilizing proteins and cell structures (Burg *et al.,* 1996; Yancey *et al.,* 1982). Initially, it was thought that compatible solutes have their main role in osmotic adjustment, but there is increasing discussion of other roles. The accumulation of these solutes *per se* may not only be important for osmotic stress tolerance but also for plant adaptation (Chen and Murata, 2002; Serraj and Sinclair, 2002; Hasegawa *et al.,* 2000). A major category of organic osmotic solutes consists of simple sugar, sugar alcohols and complex sugar (Bohnert and Jensen, 1996; Rhodes and Hanson, 1993) and other quaternary amino acid derivatives such as proline, glycine betaine and β-alanine betaine (Nuccio *et al.,* 1998) (Fig. 2.2). Many organic osmolytes are presumed to be osmoprotectants, as their levels of accumulation are insufficient to facilitate osmotic adjustment. Furthermore, many types of the osmoprotectants enhance stress tolerance of plants when expressed as transgene products (Table 2.2).

$$CH_3 - N^+(CH_3)_2 - CH_2 - COO^-$$

Glycinebetaine

Proline

$$CH_3 - N^+(CH_3)_2 - CH_2 - CH_2 - COO^-$$

β-alanine betaine

$$CH_2OH - OHCH - OHCH - HCOH - HCOH - CH_2OH$$

D-Mannitol

$$(CH_3)_2S^+ - CH_2 - CH_2 - COO^-$$

3-Dimethylsulphoniopropionate

Fig. 2.2 Structures of representative osmoprotectants accumulated by stress-tolerant plants

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Source: Rathinasabapathi (2000)

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2.2.1.3 Oxidative stress

A secondary response of salinity and drought stresses in plants are the oxidative stress that stress-induced production of reactive oxygen species (ROS) including superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\cdot}$) and singlet oxygen ($O_2^{\cdot}$) (Table 2.3). ROS are a product of altered chloroplast and mitochondrial metabolism during stress. These species cause oxidative damage to different cellular components including membrane lipids, protein and nucleic acids (Glenn *et al.,* 1999; Haliwell and Gutteridge, 1986). The alleviation of this oxidative damage could provide enhanced plant resistance to salt and drought stresses. Plants use low molecular mass antioxidants such as ascorbic acid, reduced glutathione, carotenoid, tocopherol, flavanones and anthocyanins and employ a diverse array of enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and monodehydroascorbate reductase (MDAR) to scavenge ROS (Apse and Blumwald, 2002). ROS are predominantly generated in the chloroplast by direct transfer of excitation energy from chlorophyll to produce singlet oxygen, or by univalent oxygen reduction at photosystem I and to some extent in mitochondria (Allen, 1995; Foyer *et al.,* 1994). Chloroplasts are the first targets in plant cells since this is the major site of ROS production. Stress-enhanced photorespiration and NADPH activity also contributes to the increased H_2O_2 accumulation. The toxicity of H_2O_2 is not due to its reactivity *per se*, but requires the presence of a metal reductant to from the highly reactive hydroxyl radical, which potentially reacts with all biological molecules (Apel and Hirt, 2004; Mittler, 2002; Haliwell and Gutteridge, 1986). Engineering the synthesis of antioxidants has been a relatively successful approach to obtain stress tolerant plants (Table 2.2).

2.3 Stressed-induced responses of photosynthesis

Higher plant species can be divided into three major photosynthetic types, namely C_3, C_4 and crassulacean acid metabolism (CAM) plants. C_4 and CAM plants possess anatomical, physiological and biochemical features that allow them to adapt to arid and semi-arid areas (Ku *et al.,* 1996). However, more than 90% of the plant species including many important crops are C_3 plants, and most of them do not survive in arid and semi-arid regions. Nowadays, arid and semi-arid regions is increasing, thus C_3 plants are subjected to abiotic stress and are studied the responsive activity. Therefore, reducing the adverse effect or conferring tolerance to abiotic stress in C_3 crop plants plays an important role in overcoming the barriers of crop productivity. When

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plants are subjected to abiotic stress, water content in leaves decrease, abscisic acid is induced and effected rapid stomatal closure. However, such stomatal closure restricts CO_2 entry into leaves and decreases the availability of CO_2 to the photosynthetic apparatus (Chaves, 1991). These conditions lead to an overreduction of the photosynthetic electron transport chain (Osmond and Grace 1995), which induces oxidative stress (Borsani *et al.,* 2001). In addition, salt and drought stresses generally promote the senescence of leaves. Salt- and drought-induced decrease in photosynthesis pigment concentrations by damage chloroplasts and thylakoid membrane and reduction of photosynthetic efficiency has been reported (Demiral and Türkan, 2006; Yamane *et al.,* 2003).

Stomatal limitation was generally accepted to be the main determinant of reduced photosynthesis under abiotic stress (Cornic, 2000). This has been attributed to decreases in both photosynthesis rate and internal CO_2 concentration, which finally inhibits total photosynthetic metabolism. Several nonstomatal effects are also attributed for stomatal closure during stress. These include photophosphorylation, ribulose-1,5-bisphosphate (RuBP) regeneration, ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) activity and ATP synthesis (Lawlor, 2002; Tezara *et al.,* 1999). It is well known that leaf water status always interacts with stomatal conductance and a good correlation between leaf water potential and stomatal conductance always exists, even under abiotic stress. The closure of stomata under drought has also been implicated due to changes in plant nutritional status. Decrease in relative water content (RWC) has been known to induce stomatal closure and parallel decrease photosynthesis rate (Cornic, 1994). Thus, stomatal movements are very dynamic, involving complex regulation by several environmental factors, and stomatal conductance should be taken as an integrative parameter to assess photosynthetic responses under stress. The limitation of photosynthesis under stress through metabolic impairment is a more complex phenomenon than stomatal limitation. Changes in the cellular carbon metabolism are likely to occur early in the dehydration processes. Drought and salinity generally reduces the biochemical capacity for carbon assimilation and utilization. The rate of photosynthesis in higher plants depends on the activity of Rubisco as well as synthesis of RuBP (Parry *et al.,* 2002; Tezara *et al.,* 1999).

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Table 2.2 Stress-responsive genes contributing to salinity and drought tolerance in transgenic plants

Function of transformed gene	Plant	Remarks	Reference
Synthesis of ion transporters			
Na^+/H^+ antiport (*OsNHX1*)	Rice	Improved salt tolerance	Fukuda *et al.* (1999)
	Perennial ryegrass	Improved salt tolerance	Wu *et al.* (2005a)
Na^+/H^+ antiport (*nhaA*)	Rice	Improved salt and drought tolerance	Wu *et al,* (2005b)
Na^+/H^+ antiport (*SOD2*)	Rice	Improved salt tolerance	Zhao *et al.* (2006)
Na^+/H^+ antiport (*AtNHX1*)	*Arabidopsis*	Improved salt tolerance	Apse *et al.* (1999)
Synthesis of protective proteins			
Late embryogenesis protein (*Hva1*)	Rice	Improved salt and drought tolerance	Rohila *et al.* (2002)
	Rice	Improved drought tolerance	Babu *et al.* (2004)
	Chinese cabbage	Improved salt and drought tolerance	Park *et al.* (2005)
Heat shock protein (*rHsp90*)	Rice	Improved salt drought high pH and high temperature tolerance	Liu *et al.* (2006a)

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Table 2.2 (Continued) Stress-responsive genes contributing to salinity and drought tolerance in transgenic plants

Function of transformed gene	Plant	Remarks	Reference
Synthesis of compatible solutes			
S-adenosylmethionine decarboxylase (*SAMDC*)	Rice	Improved salt tolerance	Roy and Wu (2002)
Arginine decarboxylase (*AtADC2*)	*Arabidopsis*	Improved salt tolerance	Urano *et al.* (2004)
Δ^1Pyrroline-5-carboxylate synthetase (*p5cs*)	Rice	Improved salt and drought tolcrance	Su and Wu (2004)
	Rice	Improved salt and drought tolerance	Zhu *et al.* (1998)
	Potato	Improved salt tolerance	Hmida-Sayari *et al.* (2005)
	Tobacco	Improved salt tolerance	Kavi Kishor *et al.* (1995)
Choline oxidase (*COX*)	Rice	Improved salt tolerance	Su *et al.* (2006)
Choline oxidase (*codA*)	*Arabidopsis*	Improved salt and cold tolerance	Hayashi *et al.* (1997)
Choline dehydrogenase (*betA*)	Cabbage	Improved salt tolerance	Bhattacharya *et al.* (2004)
Trehalose-6-phosphate synthase/phosphatase (*TPSP*)	Rice	Improved salt drought and low temperature tolerance	Garg *et al.* (2002)

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Table 2.2 (Continued) Stress-responsive genes contributing to salinity and drought tolerance in transgenic plants

Function of transformed gene	Plant	Remarks	Reference
Trehalose-6-phosphate synthase (*TPS1*)	Tomato	Improved salt and drought tolerance	Cortina and Culianez-Macia (2005)
Betaine aldehyde dehydrogenase (*BADH*)	Rice	Improved salt high and low temperature tolerance	Kishitani *et al.* (2000)
Mannitol-1-phosphate dehydrogenase (*Mt1D*) and glucitol-6-phosphate dehydrogenase (*GutD*)	Loblolly pine	Improved salt tolerance	Tang *et al.* (2005)
Synthesis of antioxidants			
Anthocyanidin synthase (*ANS*)	Rice	Increased flavonoids content	Reddy *et al.* (2007)
Ascorbate peroxidase (*APX*)	Tobacco	Improved salt and drought tolerance	Badawi *et al.* (2004)
Dehydroascorbate reductase (*DHAR1*)	*Arabidopsis*	Improved salt tolerance	Ushimaru *et al.* (2006)
Mn superoxide dismutase (*MnSOD*)	Rice	Improved drought tolerance	Wang *et al.* (2005)
	Rice	Improved salt tolerance	Tanaka *et al.* (1999)
	Alfalfa	Improved drought tolerance	McKersie *et al.* (1996)
	Alfalfa	Improved salt tolerance	Wang *et al.* (2004)

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Table 2.2 (Continued) Stress-responsive genes contributing to salinity and drought tolerance in transgenic plants

Function of transformed gene	Plant	Remarks	Reference
Fe superoxide dismutase (*FeSOD*)	Tobacco	Improved salt and oxidative tolerance	van Camp *et al.* (1996)
Vitamin B-6 biosynthetic genes (*PDX1* and *PDX2*)	Tobacco	Improved salt tolerance	Herrero and Daub (2007)
Glyoxalase (*MG*)	Tobacco	Improved salt tolerance	Yadav *et al.* (2005)

Table 2.3 Chemical and biological effects of ROS

ROS	Description
superoxide radicals ($O_2^{\cdot-}$)	One-electron reduction state of O_2, formed in many autoxidation reactions and by the electron transport chain. Rather unreactive but can release Fe^{2+} from iron-sulphur proteins and ferritin. Undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis and is a precursor for metal-catalyzed •OH formation.
hydrogen peroxide (H_2O_2)	Two-electron reduction state, formed by dismutation of $•O_2$- or by direct reduction of O_2. Lipid soluble and thus able to diffuse across membranes.
hydroxyl radicals ($OH^{\cdot}$)	Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive, will attack most cellular components.
peroxy radicals ($ROO^{\cdot}$)	Oxygen centred organic radicals. Lipid forms participate in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.

Source: Rice-Evans and Gopinathan (1995)

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The quantity of Rubisco in leaves is controlled by the rate of synthesis and degradation of the enzyme, even in stressful environments. Decreased synthesis of Rubisco under stress was evidenced by a rapid decrease in the abundance of Rubisco small subunit (rbcS) in tomato (Vu *et al.,* 1999). Parry *et al.* (2002) suggested that Rubisco activity is regulated to match the capacity of the leaf to regenerate RuBP. Loss of Rubisco activity has been reported in several plants under abiotic stress (Chaitanya *et al.,* 2003; Parry *et al.,* 2002). The rate of photosynthesis also depends on the synthesis of RuBP. The reactions of RuBP to 3-PGA always decreased with reducing RWC, which suggest that regeneration of RuBP was inhibited under drought. RuBP contents and synthesis are presumed to be under the control of the Clavin cycle or the supply of ATP and NADPH to the Clavin cycle (Fig. 2.3). ATP synthesis was known to limit photosynthesis rate at low RWC because of the inhibition of photophosphorylation in water stressed sunflower leaves (Lawlor, 2002). Under conditions of environmental stress, reduction in chloroplast volume might also lead to desiccation within the chloroplast, which in turn leads to conformational changes in Rubisco. Abiotic stress conditions are also known to acidify the chloroplast stoma, resulting in inhibited Rubisco activity (Meyer and Genty, 1999).

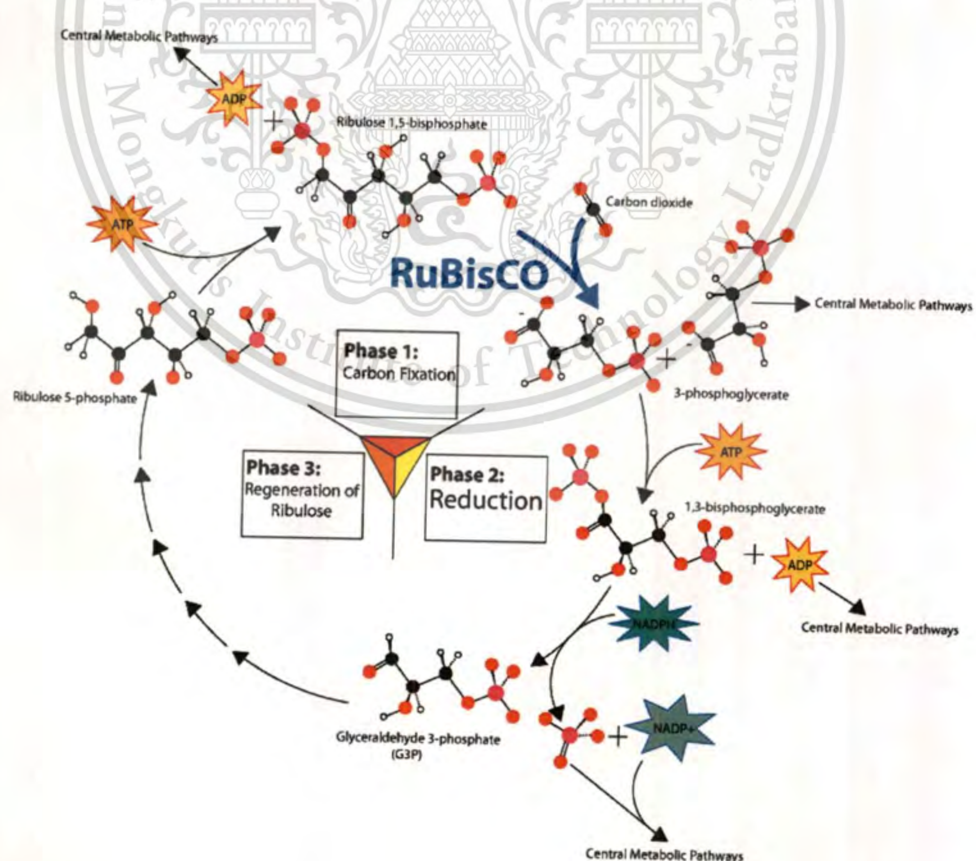

Fig. 2.3 The Clavin cycle

Source: Bassham (2003)

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2.4 The biosynthesis of proline in high plants

Proline is one of the well-known osmoprotectants and its accumulation is widely observed in various organisms under abiotic stress, such salt, cold and drought (Ueda *et al.,* 2001; Yoshiba *et al.,* 1997). Proline accumulation is regulated by multiple factors, such as its synthesis, catabolism, utilization for protein synthesis and transport from other tissues. Additionally, proline is also utilized for protein synthesis, and large part of hydroxyproline, a derivative of proline through hydroxylation, is found in structural proteins, such as collagen in animals or hydroxyproline rich protein in plants (Hall and Cannon, 2002; Kavi Kishor *et al.,* 1995). Proline and hydroxyproline in structural proteins are clearly distinguished from free proline, which serves to regulate osmotic adjustment. In *Arabidopsis*, proline can account for up to 20% of the free amino acid pool after salt stress (Verbruggen *et al.,* 1993). There are two alternative routes in proline biosynthesis in higher plants: the L-ornithine and the L-glutamate pathways. It is also known that, as in plants, both ornithine and glutamate are precursors of proline biosynthesis in microorganisms and mammals. The plant glutamate pathway differs from that in bacteria and human. In bacteria and human, the conversion of glutamate to glutamate-5-semialdehyde is catalyzed by two enzymes *via* two consecutive reactions, whereas, in higher plants the conversion is catalyzed by a bi-functional enzyme in a single reaction (Hu *et al.,* 1992).

Pathway of proline biosynthesis *via* glutamate

Proline biosynthetic pathway starts with the phosphorylation of glutamate, which gets converted to γ-glutamyl phosphate and then to glutamic-γ-semialdehyde (GSA) by the enzymes γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase respectively. GSA gets converted to pyrroline 5-carboxylate (P5C) by spontaneous cyclization. On the other hand, glutamate is directly catalysed to GSA by pyrroline 5-carboxylate synthetase (P5CS) in plants and other eukaryotes (Fig. 2.4) (Hare *et al.,* 1998; Hu *et al.,* 1992). P5C is then reduced to proline by P5C reductase (P5CR) in both prokaryotes and eukaryotes.

Pathway of proline biosynthesis *via* arginine/ornithine

Proline is synthesized not only from glutamate but also from arginine/ornithine (Adams and Frank, 1980). Arginine gets converted to ornithine by the enzyme arginase. In bacteria, ornithine is degraded to α-keto-δ-aminovalerate by the enzyme ornithine α-aminotransferase (α-OAT), which then spontaneously gets cyclized to pyrroline 5-carboxylate (P5C). P5C is finally catalysed to proline by P5C reductase (Fig. 2.4). In plants, GSA is derived directly from ornithine by the enzyme ornithine δ-aminotransferase (δ-OAT) (Peng *et al.,* 1996).

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Proline is a compatible osmolyte which is not charged at neutral pH. It is highly soluble in water. Moreover, at high concentrations, it has little or no perturbing effect on macromolecule solvent interactions (Chadalavada *et al.,* 1994). Accumulation of compatible solutes results in an increase in cellular osmolarity that can drive influx of water or reduce the efflux. This provides the turgor that is necessary for cell expansion. Under osmotic or dehydration stress conditions, membrane integrity must be maintained to prevent protein denaturation. Moreover, proline contributes to stabilizing sub-cellular structures (e.g. membranes and proteins), heavy metal chelation (Mehta and Gaur, 1999) and buffering cellular redox potential under stress conditions (Kavi Kishor *et al.,* 2005). Besides, the emerging evidence implicates the antioxidative activity of proline. For example, Mehta and Gaur (1999) demonstrated a reduced heavy metal (Cu, Cr, Ni, Zn) dependent lipid peroxidation and K^+ efflux from *Chlorella vulgaris* pretreated with exogenous proline. Further, the transgenic green microalga *Chlamydomonas reinhardtii* expressing the mothbean Δ^1-pyrroline-5-carboxylate synthetase (P5CS) is shown to contain 80% higher free proline levels than the wild type cells and exhibit substantially enhanced tolerance to toxic Cd concentrations (Siripornadulsil *et al.,* 2002). Free proline levels correlated well with malondialdehyde levels or lipid peroxidation in heavy metal treated algae. Free proline is suggested to act as an antioxidant in Cd stressed cells. Proline dependent scavenging of ROS such as hydroxyl radicals (Smirnoff and Cumbes, 1989) and singlet oxygen (Alia *et al.,* 2001) has also been reported.

Transgenic approach to improve plant stress tolerance *via* over-producing proline has been widely reported. For example, engineered tobacco plants over-producing proline significantly reduced the level of free radicals and improved tolerance to 200 mM NaCl (Hong *et al.,* 2000). In *Arabidopsis*, plants engineered with an antisense proline dehydrogenase cDNA resulted in an increase in accumulation of proline and a constitutive tolerance to freezing temperatures as well as salinity (up to 600 mM NaCl) (Nanjo *et al.,* 2003). Also, introduction of mothbean *P5CS* gene *via Agrobacterium* into carrot resulted in enhanced salt tolerance. Transgenic cell lines of carrot exhibited six-time increased degree of tolerance to 250 mM NaCl (Han and Hwang, 2003). Recently, *OsP5CS* is introduced into rice and the gene in transgenics plants are found to be salt inducible and is also essential for salt and cold tolerance (Hur *et al.,* 2004).

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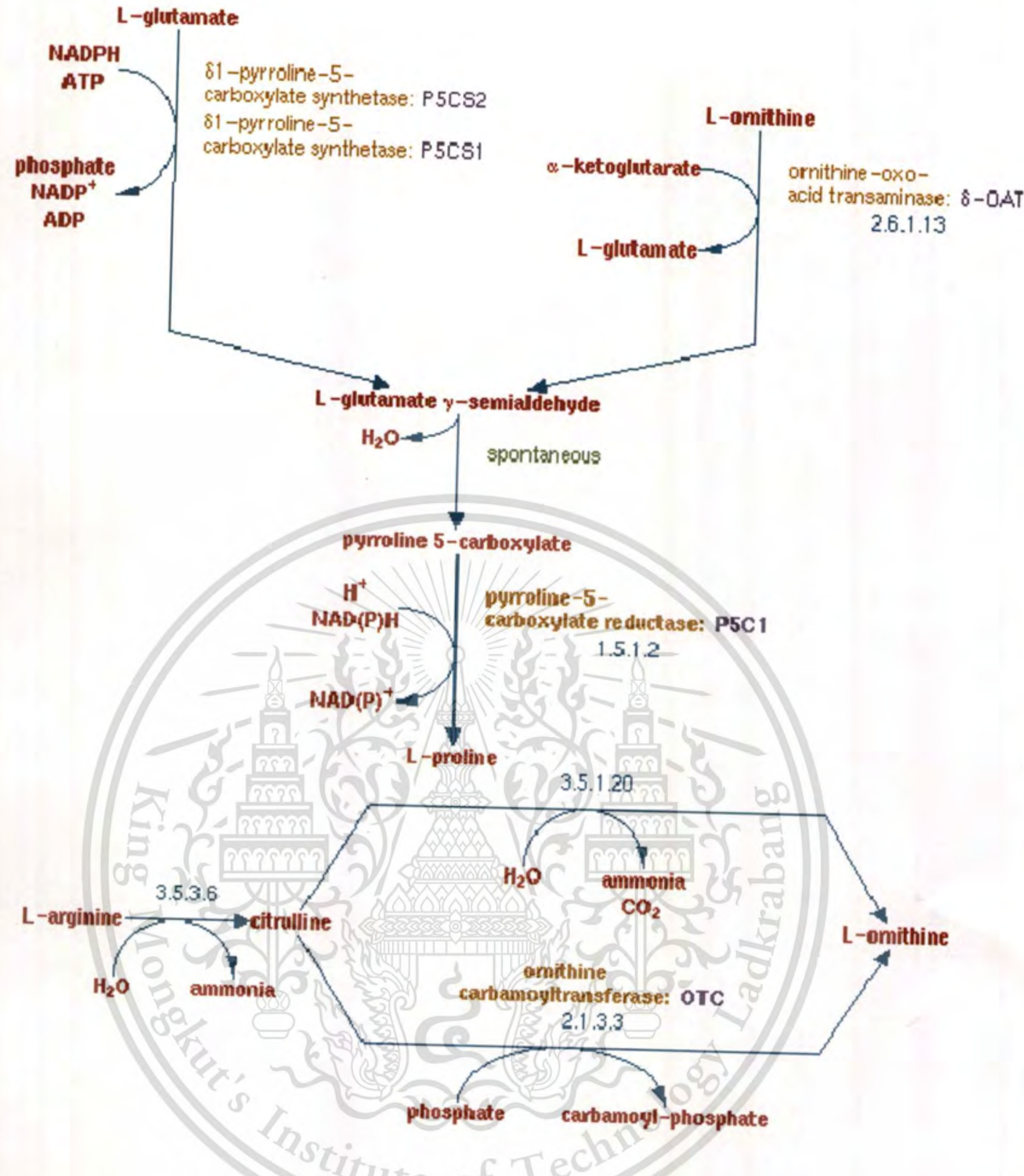

Fig. 2.4 Proline biosynthesis pathways in plants

Source: Hu *et al.* (1992)

Exogenous application of proline can play an important role in enhancing plant stress tolerance. This role can be in the form of either osmoprotection or cryoprotection (Santarius, 1992; Songstad *et al.,* 1990). For example in rice, exogenous application of 30 mM proline counteracted the adverse effects of salinity on early seedling growth, though higher concentrations of proline resulted in reduced growth (Roy *et al.,* 1993). Exogenous application of proline to salt- or drought stressed halophyte (*Allenrolfea occidentalis*) affected an increase of their growth and a halt in increased ethylene production (Chrominski *et al.,* 1989). Growth of

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tobacco suspension cells under salt stress is promoted by exogenous application of 10 mM proline, which is proposed to be due to proline action as a protectant of enzymes and membranes (Okuma *et al.,* 2000). In soybean cell cultures maintained under salt stress, exogenous application of proline increases the activities of superoxide dismutase and peroxidase, which normally contribute to increased salt tolerance (Hua and Guo, 2002). Although exogenous application of proline to plants exposed to abiotic stresses generally provides a stress preventing or recovering effect, high concentrations of proline may be harmful to plants, including inhibitory effects on growth or deleterious effects on cellular metabolisms (Ehsanpour and Fatahian, 2003; Nanjo *et al.,* 2003). For example in mung bean (*Vigna radiata*), it is determined that the addition of 20-33 mM proline to cell cultures mitigated the adverse effects of NaCl stress, concentrations of 50 mM or higher are inhibitory to the growth of both salt-stressed and non-stressed cultures (Kumar and Sharma, 1989). In rice, exogenous application of 30 mM proline is the effective concentration in improving germination and seedling growth under salt stress, whereas higher concentrations (40 or 50 mM) resulted in seedling growth reduction (Roy *et al.,* 1993). Therefore, it is essential to determine optimal concentrations of proline that provide beneficial effects in different plant species.

2.5 The biosynthesis of flavonoids in high plants

Flavonoids have been proposed to exert beneficial effects in a multitude of disease states, including cancer, cardiovascular disease, and neurodegenerative disorders. Many of the biological actions of flavonoids have been attributed to their antioxidant properties, either through their reducing capacities *per se* or through their possible influences on intracellular redox status (Rice-Evans *et al.,* 1996).

Flavonoids are a broad class of low molecular weight, secondary plant phenolics characterized by the flavan nucleus. Flavonoids are ubiquitous plant secondary products that are best know as the characteristic red, blue and purple anthocyanin pigments of plant tissues (Winkel-Shirley, 2001; Dooner, 1983). Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings and are classified according to substitutions. Flavonoids are characterized by the presence of two benzene rings (ring A and B) that are linked by a 3-carbon bridge (to from chalcones) or by a pyrane or pyrone ring (ring C). The basic flavonoid structure contains the flavan nucleus, which consists of 15 carbon atoms derived from a C_6-C_3-C_6 skeleton (Fig. 2.5). On the basis of the position of and the modifications to the A, B and C rings, the more

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than 4000 flavonoids discovered to date can be classified in its several classes, including six major subgroups that found in most higher plants: the chalcones, flavones, flavonols, flavandiols, anthocyanins and condensed tannins (Fig. 2.6) (Taylor and Grotewold, 2005; Mol *et al.,* 1998).

Fig. 2.5 Basic flavonoid structure including the numbering system

Source: Cook and Samman (1996)

Structural diversity among the flavonoids is brought about by a variety of modifications, including specific hydroxylation, glycosylation, acylation, prenylation, sulfation and methylation (Mol *et al.,* 1998; Dixon and Palva, 1995). The first key step in the biosynthesis of all flavonoids is condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA catalyzed by chalcone synthase (CHS); the chalcone is stereospecifically cyclized by the action of chalcone isomerase (CHI). Hydroxylation of flavanone at the 3-position by flavanone 3-hydroxylase (F3H) leads to the formation of dihydroflavonol, which is catalyzed by dihydroflavonol 4-reductase (DFR) to leucoanthocyanidin, and hydroxylation of the 3'- and 5'-positions of the B-ring is catalyzed by flavonoid 3'-hydroxlase (F3'H) and flavonoid 3',5'-hydroxlase (F3',5'H), respectively. Hydroxyl groups in the B-ring are known to affect the absorption spectrum of anthocyanin, and especially, F3',5'H is important for formation of blue colored delphinidin. On the other side of the reaction, flavone synthase II (FS2) is a key enzyme for flavone biosynthesis; it catalyzes introduction of the double bond between C-2 and C-3 of flavanone to produce flavone. Almost all anthocyanidin and anthocyanin undergoes several modifications, such as glycosylation and acylation, by leucoanthocyanidin dioxygenase (LDOX), O-methyltransferase (OMT), rhamnosyl transferase (RT), and UDPGflavonoid glucosyl transferase (UFGT) (Fig. 2.7).

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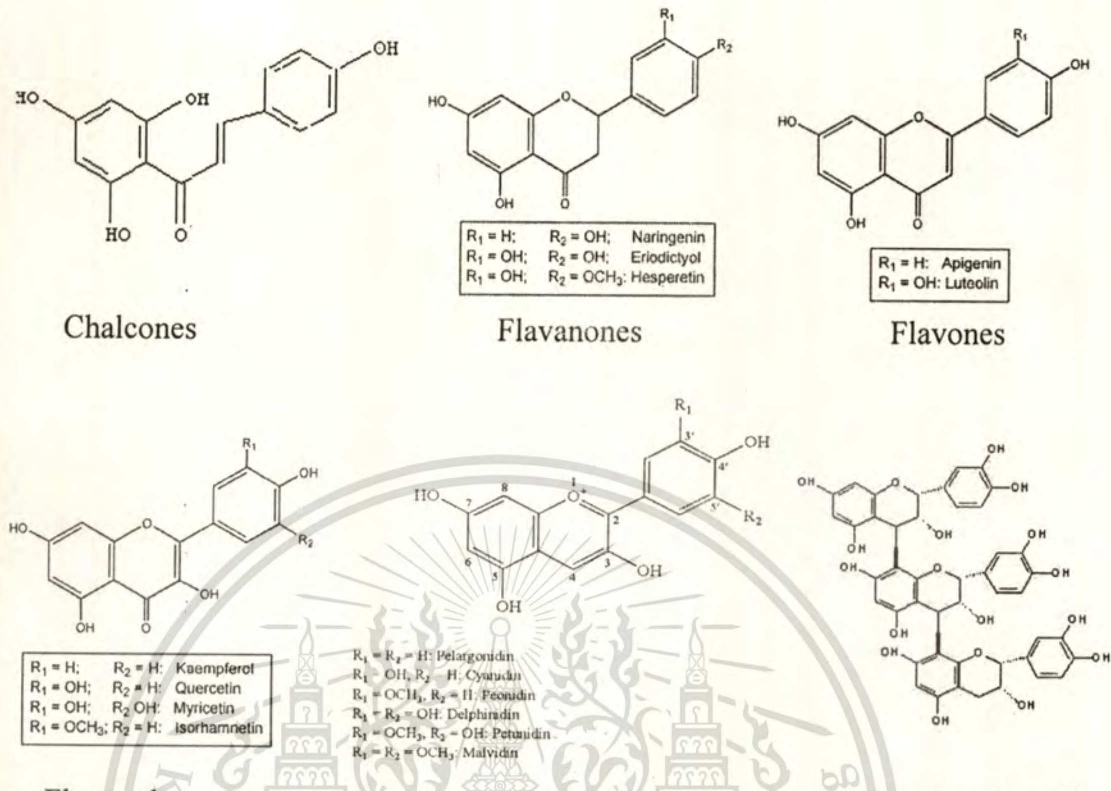

Fig. 2.6 Structure of the main classes of flavonoids, in which R1 and R2 indicate the sites of possible substitutions. OGly indicate a glycosidic linkage.

Source: Taylor and Grotewold (2005)

Flavonoids brightly colored pigments play a major role in insect mediated pollination in many plant species and, in addition, are implicated in several important plant-associated functions such as modulation of hormone response, UV-B protection and photoperception of foliage (Shirley, 2002; Dixon and Palva, 1995; Holton and Cornish, 1995;). Recently, flavonoids play important role in plant response and human health by the detoxification of ROS that stress-induced production of oxidative stress (Reddy *et al.,* 2007; Treutter, 2005).

Flavonoids that serve as antioxidants are play an important role in interactions between the plant and the environmental being responsive to such factors as visible and UV-B radiation and low temperature (Kong *et al.,* 2003; Chalker-Scott, 1999). In addition, flavonoids also play a role in rice protection against salt and drought stresses through their antioxidative ability (Reddy *et al.,* 2007; Ithal and Reddy, 2004; Reddy *et al.,* 2004).

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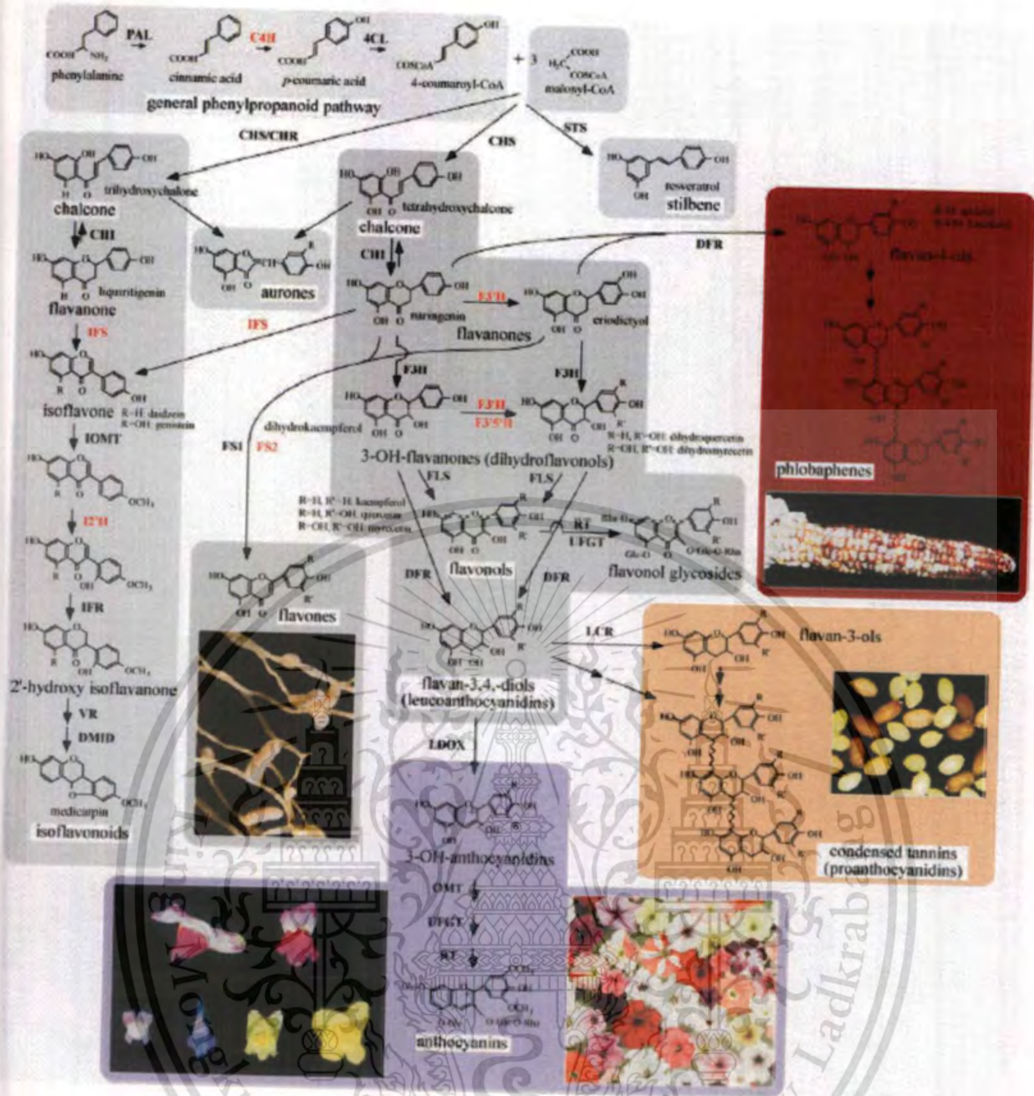

Fig. 2.7 Schematic of the major branch pathways of flavonoid biosynthesis, starting with general phenylpropanoid metabolism. Enzyme names are abbreviated as follows: cinnamate-4 hydroxylase (C4H), chalcone isomerase (CHI), chalcone reductase (CHR), chalcone synthase (CHS), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase (DMID), flavanone 3-hydroxylase (F3H), flavone synthase (FSI and FSII), flavonoid 3' hydroxylase (F3'H) or flavonoid 3'5' hydroxylase (F3'5'H), isoflavone O-methyltransferase (IOMT), isoflavone reductase (IFR), isoflavone 2'-hydroxylase (I2'H), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR), O-methyltransferase (OMT), Phe ammonia-lyase (PAL), rhamnosyl transferase (RT), stilbene synthase (STS), UDPG flavonoid glucosyl transferase (UFGT), and vestitone reductase (VR).

Source: Winkel-Shirley (2001)

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Antioxidant activity of flavonoids has already been shown about 40 years ago. Their antioxidant activity depends on the reactivity of hydroxyl substituents in oxygen atom abstraction reactions. The B-ring hydroxyl configuration is the most significant determinant of scavenging of ROS (Burda and Oleszek, 2001; Sekher *et al.,* 2001). Hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical. The reactions are the reactions with peroxyl radicals, hydroxyl radicals and superoxide.

$$ROO^{\cdot} + FlOH \longrightarrow ROOH + FlO^{\cdot}$$

$$HO^{\cdot} + FlOH \longrightarrow H_2O + FlO^{\cdot}$$

Fl = flavonoids

Moreover, the dioxygenases of the flavonoids pathway show high sequence similarity with each other and catalyze a variety of two-electron oxidations including hydroxylations, desaturations and oxidative ring closures (Pietta, 2000; Madhuri and Reddy, 1999; Schofield and Zhang, 1999).

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CHAPTER 3

RESEARCH METHODOLOGY

3.1 Plant materials and tissue culture systems

Thai rice seeds including KDML105, Pathumthani1 (PT1), Klum Sakol (KS), Klum Khonkaen1 (KK1), Klum Khonkaen2 (KK2), Black sticky (BSR), Sang Yod (SY), Kulanbdang (KLD), Dang (D) and TD49 were derived from Pathumthani, Phitsanulok and Sakolnakorn Rice Research Center, Rice Research Institute, Department of Agriculture and Cooperative, Thailand. All plant cultures were performed in plant tissue culture room with the controlled air temperature and light intensity and photoperiod.

3.2 Reagents and laboratory apparatus

1. Standard chemicals, namely (L-proline)
2. Chemicals used to analyze photosynthetic pigments, lipid peroxidation, proline and flavonoids
3. Chemicals used to prepare NB medium
4. Chemicals used to RNA extraction and RT-PCR analysis
5. Laboratory apparatuses, including
    - Glass wares
    - Mortars and pestles
    - Spatulas
    - 15- and 50-mL centrifuge tubes
    - Micropipettes
    - Cylinders
    - Plastic chambers

3.3 Instruments

1. Autoclave (Hirayama, HV-50, Japan)
2. Spectrophotometer (BioMate 3, Thermo Electron Corp.)
3. Centrifuge (Mikro 22R, Hettich Zentrifugen)
4. Laminar air flow cabinet (HS123, ISSCO)

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3.4 Methods

3.4.1 Plant material and stress treatment

Seeds of ten rice cultivars (*Oryza sativa* L. ssp. *indica*), KDML105, Pathumthani1 (PT1), Klum Sakol (KS), Klum Khonkaen1 (KK1), Klum Khonkaen2 (KK2), Black sticky (BSR), Sang Yod (SY), Kulanbdang (KLD), Dang (D) and TD49 were dehusked by hand, sterilized once in 70% ethanol for 2-3 min, once in 5% commercial bleach (5.25% sodium hypochlorite) for 40 min, 30% commercial bleach for 30 min and then rinsed 4-5 times with sterile distilled-water. Surface-sterilized seeds were placed on sterile tissue paper and then germinated on NB medium (Li *et al.,* 1993) containing 3% (w/v) sucrose and 0.8% (w/v) agar. Seedlings were culture ***in vitro*** under conditions of 25±2°C air temperature and 60±5 μmol $m^{-2}s^{-1}$ photosynthetic photon flux (PPF) with 16 hd^{-1} photoperiod for 7 days. After 7 days, seedlings were placed on NB-liquid medium under photoautotrophic growth condition (CO_2 as a carbon source) using vermiculite as a supporting material. Air-exchanges of culture vessel were adjusted to 2.32 μmol CO_2 h^{-1} by punching a hole on plastic cap (ϕ 1 cm) and covering with micro porous filter (0.2 μm of pore size). Fourteen-days-old rice seedlings were treated with culture medium containing 100 mM NaCl (salt stress) or 100 mM mannitol (drought stress) for 0, 2 and 4 days. Rice seedlings were harvested, frozen in liquid nitrogen and stored at -80°C prior to analysis.

3.4.2 Morphological and physiological determinations

Shoot length, root length, fresh weight (FW) and dry weight (DW) from both control and stressed seedlings were determined as growth. DW were measured in a sample incubated in hot-air oven at 70°C for 24 h. Relative water content (RWC) was calculated using the formula following Sumithra *et al.* (2006) method:

$$\% RWC = (FW-DW) \times 100 / FW$$

3.4.3 Photosynthetic pigment contents

Chlorophyll A (Ch_A), chlorophyll B (Ch_B) and total carotenoid (Car) concentrations were analyzed following the methods of Shabala *et al.* (1998) and Lichtenthaler (1987). Leaf tissue (0.05 g) was ground in liquid nitrogen. Pigments were extracted with acetone at 4°C for 48 h. The pigments were measured using an UV-visible spectrophotometer (Thermo Electron Model Bio

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Mate 3, Massachusetts, USA)) at wavelengths 662, 644 and 470 nm. The Ch_A, Ch_B and Car (μgg^{-1} FW) concentrations were calculated according to the following equations:

$$\text{Chlorophyll A } (Ch_A) = 9.784\, D_{662} - 0.99 D_{644}$$

$$\text{Chlorophyll B } (Ch_B) = 21.42\, D_{644} - 4.65 D_{662}$$

$$\text{Total carotenoid (Car)} = (1000\, D_{479} - 1.9 Ch_A - 63.14\, Ch_B)/214$$

When D_i is the optical density at wavelength i.

3.4.4 Lipid peroxidation

The levels of lipid peroxidation were determined in terms of malondiadehyde (MDA) content according to the method of Hodges *et al.* (1999). MDA of whole rice seedlings were extracted with 0.1% (w/v) trichloroacetic acid by shaking for 1 h at room temperature. Then, the sample was centrifuged at 12,000 rpm for 30 min to remove the insoluble residues. Five-hundred microliter aliquot of supernatant was mixed with either (i) –TBA solution comprised of 20.0% trichloroacetic acid and 0.01% butylated hydroxytoluene, or (ii) +TBA solution containing the above substances plus 0.65% TBA. After that, the mixtures were heated at 95°C for 25 min and then cooled on ice. The absorbance of each sample was measured at 490, 532 and 600 nm. MDA equivalents were calculated as the following formulas and represented as $\mu molL^{-1}$.

$$A = [(A_{532+TBA}) - (A_{600+TBA}) - (A_{532-TBA} - A_{600-TBA})]$$

$$B = [(A_{440+TBA} - A_{600+TBA})\, 0.0571]$$

$$\text{MDA equivalents } (\mu molL^{-1}) = [(A-B)/157{,}000] \times 10^6$$

3.4.5 Proline content

Free proline content was determined according to Gilmour *et al.* (2000). Two-hundred milligram samples from each group were homogenized in 1 mL 3% (w/v) sulphosalicyclic acid and shaking at 250 rpm for 1 h at room temperature and then stored at 4°C over night. The homogenate sample was centrifuged at 12,000 rpm at 4°C for 30 min. The supernatant was added 450 µL acid ninhydrin and 450 µL glacial acetic acid. The mixture was heated at 100°C 45 min in water bath. Reaction was stopped by using an ice bath. The proline was extracted with toluene

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and was measured using UV-visible spectrophotometer at wavelength 519 nm. Proline concentrations were determined using calibration curve and expresses as mg proline g^{-1} FW.

3.4.6 Analysis of flavonoid levels

Flavonoid content was determined according to Harborne (1998). Seedling (0.5 g) was ground in liquid nitrogen and extracted with 4 mL methanol 1 % (w/v) HCl for 2 h at room temperature by stirring, added 1 mL chloroform. The homogenate was centrifuged at 4,000 rpm at 4°C for 15 min. The level of each flavonoid was determined as the absorbance at the specific wavelength (Table 3.1).

3.4.7 Experimental design and statistic analysis

The changes of morphological (FW, DW, SL and RL) and physiological (RWC, photosynthetic pigments and MDA content) characters in all stressed cultivars were analyzed using Hierarchical cluster analysis in SPSS software (SPSS for Windows version 15, SPSS Inc., Chicago, USA) for classified into tolerant and sensitive groups of stressed-seedlings cultivars.

All experiments were designed in completely randomized design (CRD) with 5 replicates (n=5). Statistical analysis for each experiment was performed with Duncan's multiple range test (DMRT) using SPSS software (SPSS for Windows version 15, SPSS Inc., Chicago, USA).

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Table 3.1 Wavelengths used in measuring levels of flavonoids

Flavonoids	Wavelengths (nm)
Flavanone	
Naringenin	330
Flavones	
Luteolin	350
Apigenin	336
Flavonols	
Myricetin	378
Quercetin	374
Kaempferol	368
Tannin	
Gallotannin	550
Anthocyanins	
Delphinidin	546
Cyanidin	535
Pelargonidin	520

Source: Harborne (1998)

3.4.8 Gene expression analysis

Total RNA extraction was performed according to the protocol modified by Sompornpailin (2005). Whole rice plants were ground in liquid nitrogen. Then, total RNA was extracted with the mixture of equal volume of phenol and extraction buffer (10 M LiCl, 10% SDS, 1 M Tris-HCl pH9.0 and 0.25 M EDTA pH 8.0). After vortexing for 5 min, the samples were centrifuged at 6,000 rpm for 30 min. Total RNA was precipitated from the aqueous phase by adding LiCl to the final concentration of 2 M. After keeping at 4°C for 4-16 h, the samples were centrifuged to separate total RNA. The RNA pellets were washed with 70% ethanol and dissolved with RNA free water. The concentration of RNA was determined by measuring the absorbance at 260 nm.

The expression of *OsP5CS*, *OsP5CR*, *OsCHS* and *OsDFR* was analyzed by RT-PCR. The primer pair for amplifying *OsP5CS* was designed from the conserved nucleotide sequences of *OsP5CS* (GenBank accession No. D49714), *SofP5CS* (GenBank accession No. EU005373) from

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Saccharum officinarum, *TaeP5CS* (GenBank accession No. AY888045) from wheat and *SarP5CS* (GenBank accession No. EU113257) from *Saccharum arundinaceum.* The primer pair for amplifying *OsP5CR* was designed from the conserved nucleotide sequences of *TaeP5CR* (GenBank accession No. AY880317) from wheat, *ZmaP5CR* (GenBank accession No. DQ026301) from maize, *HvuP5CR* (GenBank accession No. AY177684) from barley and putative *OsP5CR* (GenBank accession No. NM001051928) from *japonica* rice. The amplification of *OsCHS* (GenBank accession No. X89859) and *OsDFR* (GenBank accession No. Y07956) was performed using gene specific primers. The *Actin* primers were used in the control reaction. The sequences of primers used in this experiment were listed in Table 3.2.

Total RNA (3 μg) samples from stress or control plants were reverse transcribed using iScript™cDNA Synthesis Kit (Bio-Rad, CA, USA). PCR was subsequently performed in 50 μL of a reaction mix containing 2.0 mM KCl, 200 pmol dNTP, 50 pmol of each gene-specific primer pair, 1μL first-strand cDNA and 1 U of *Taq* DNA polymerase (Fermentas Life Sciences). The expression of genes was analyzed by RT-PCR. The primers used in this experiment were listed in table 3.2. A Mastercycle EPgradient (Eppendrof) was used with an initial denaturation step of 95°C for 5 min. Then, the reaction was followed by 3 step-cycles:

Denaturation	94°C	45 s
Annealing	60°C	2 min for *OsP5CS*
	55°C	2 min for *OsP5CR*
	65°C	2 min for *OsCHS*
	60°C	2 min for *OsDFR*
Extension	72°C	2 min

This step was performed for 30 cycles (*OsP5CS* and *OsP5CR*) and 40 cycles (*OsCHS and OsDFR*), followed by a final extension step of 72°C for 7 min. The RT-PCR products were separated on a 1.0 % agarose gel containing ethidium bromide and visualized under UV light. The intensity of each amplification product was quantified by scanning densitometry using a gel document (SynGene).

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Table 3.2 Primer sequences for gene amplification in this experiment

Genes	Primer sequences		% G + C content
OsP5CS	Forward	5'-AT GGA TCC ATG GCC AGC GTC GAC CCG-3'	65
OsP5CS	Reverse	5'-CG GGA TCC TCA TTG CAA AGG AAG GCT CTT ATG-3'	52
OsP5CR	Forward	5'- GTT GGA CAA GCA GCA TCA GTG ATG-3'	50
OsP5CR	Reverse	5'-GCA GCA ACA ACG GCA TTT ATC AGC-3'	50
OsCHS	Forward	5'-AT GGA TCC ATG GCA GCG GCG GTG ACG GTG-3'	69
OsCHS	Reverse	5'-AT GGA TCC TCA GGC GGC GGC GCC GGC-3'	77
OsDFR	Forward	5'-AT GGA TCC ATG GGC GAG GCG GTG AAG-3'	62
OsDFR	Reverse	5'-CG GGA TCC TCA TTT GAC CAA CGC TTC TGT-3'	52
Actin	Forward	5' GTG ACA ATG GAA CTG GAA TGG TNA AGG 3'	44-48
	Reverse	5' CAC CAT CAC CAG AAT CGA GCA CAA TAC 3'	48

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CHAPTER 4

RESULTS

4.1 Effect of salt or drought stresses on morphological and physiological of Thai rice

Abiotic stresses like drought and salinity are the most limiting factors for crop production world-wide especially for developing countries, where the largest populations with enormous demand on food supplies. Salt and drought stresses can cause the limiting capacity of a plant to absorb water and nutrients thus these affect plant survival, biomass, plant height and plant morphology (Parida and Das, 2005). These stresses can be caused hyperionic and hyperosmotic effects on plants leading to membrane disorganization, increase in reactive oxygen species (ROS) and metabolic toxicity (Jaleel *et al.*, 2007a). Stresses altered a wide array of metabolic processes culminating in stunted growth, reduced enzyme activities and biochemical constituents (Jaleel *et al.*, 2007b).

Seeds of ten rice cultivars were germinated and grown in 100 mM concentrations of NaCl or mannitol and analyzed for growth parameters on the 2nd and 4th day after stresses. Based on fresh weight (FW), dry weight (DW), shoot length (SL), root length (RL) and the relative water content (RWC) of the seedlings all of rice cultivars showed the reduction of morphological and physiological parameters under stress conditions when compared to the control (non-stresses). Cultivars which are considered as salt-tolerant, KDML105, SY, D, KLD and TD49, showed slightly decreased FW (2.94-8.38%), DW (0.79-5.48%), SL (1.01-5.27%), and RL (4.41-7.14%) values at 100 mM salinity level compared to the non salt-treated plants. The salt sensitive cultivars, KS, KK1, KK2, BSR and PT1, on the other hand, showed higher decreased than the salt tolerant cultivars in this growth parameter (Table 4.1 and 4.3). Under drought condition, control seedlings maintained higher FW, DW, SL, RL and RWC than those of stressed-seedlings. Maximum decrease in mean FW, DW, SL, RL and RWC was observed in drought sensitive cultivars (KDML105, SY, D, KLD, TD49 and PT1) whereas there was slightly difference in FW (2.01-9.20%), DW (1.85-7.16%), SL (1.17-8.92%), and RL (2.63-9.33%) between control and drought treated seedlings in drought-tolerant (KS, KK1, KK2 and BSR) (Table 4.2 and 4.4).

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Under both stresses, RWC of stressed-seedlings were none significantly differ with control seedlings in stressed-tolerant cultivars. However, stressed-tolerant cultivars showed reduction of RWC less than stressed-sensitive cultivars (Table 4.5 and 4.6). Salt- and drought-tolerant cultivars were slightly decreased 0.33-0.82% and 0.04-0.43%, respectively whereas salt- and drought-sensitive cultivars were reduced 1.05-2.64% and 1.55-2.47%, respectively. Parallel to RWC, a considerable decrease in water potential and an effective decrease of plant growth were observed under both stress treatments (Table 4.1-4.6). The relationship between morphological and physiological characteristics of control and stress imposed seedlings, the rice cultivars were categorized into 4 groups for further experiments using Hierarchical cluster analysis in SPSS software. First, the salt-tolerant group consists of KDML105, SY, D, KLD and TD49. The second group was salt-sensitive cultivars, namely, KS, KK1, KK2, BSR and PT1. The third group was drought-tolerant, KS, KK1, KK2 and BSR. The last group was drought-sensitive, KDML105, SY, D, KLD, TD49 and PT1 (Fig. 4.1 and 4.2).

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Table 4.1 Changes in the fresh weight (FW) and dry weight (DW) of rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl for 2 or 4 days

Cultivars	Days	Treatments	FW (mg)	DW (mg)
KS	0	0	175.44 a	27.66 a
	2	0	194.90 bc (100 %)	30.28 c (100 %)
		100 mM NaCl	171.72 a (88.11 %)	28.30 b (93.46 %)
	4	0	218.82 c (100 %)	32.68 e (100 %)
		100 mM NaCl	190.16 b (86.90 %)	30.32 cd (92.77 %)
KK1	0	0	135.34 a	19.90 a
	2	0	225.92 c (100 %)	31.50 c (100 %)
		100 mM NaCl	184.22 b (81.54 %)	28.40 b (90.16 %)
	4	0	256.38 d (100 %)	37.58 d (100 %)
		100 mM NaCl	210.26 b (82.01 %)	32.70 c (87.01 %)
KK2	0	0	105.64 a	13.84 a
	2	0	188.20 c (100 %)	30.04 d (100 %)
		100 mM NaCl	159.74 b (84.88 %)	27.22 b (90.61 %)
	4	0	192.92 d (100 %)	30.40 d (100 %)
		100 mM NaCl	162.78 b (84.38 %)	27.56 c (90.66 %)
BSR	0	0	151.76 a	23.10 a
	2	0	193.66 b (100 %)	26.00 c (100 %)
		100 mM NaCl	158.08 a (81.63 %)	23.64 ab (90.92 %)
	4	0	200.62 b (100 %)	27.92 d (100 %)
		100 mM NaCl	153.50 a (76.51 %)	22.92 a (88.15 %)
KDML105	0	0	194.38 a	25.24 a
	2	0	211.72 c (100 %)	29.24 bc (100 %)
		100 mM NaCl	205.50 b (97.06 %)	28.52 b (97.54 %)
	4	0	236.16 d (100 %)	32.98 d (100 %)
		100 mM NaCl	229.66 d (97.25 %)	32.72 d (99.21 %)
SY	0	0	119.06 a	18.36 a
	2	0	143.82 b (100 %)	21.14 c (100 %)
		100 mM NaCl	139.28 b (96.84 %)	20.66 bc (97.26 %)
	4	0	176.26 d (100 %)	25.86 e (100 %)
		100 mM NaCl	166.68 c (94.56 %)	25.20 d (97.45 %)

Means within the same cultivar followed by the same letters are not significantly different by DMRT at $P \leq 0.05$.

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Table 4.1 (Continued) Changes in the fresh weight (FW) and dry weight (DW) of rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl for 2 or 4 days

Cultivars	Days	Treatments	FW (mg)	DW (mg)
D	0	0	112.30 ab	18.60 a
	2	0	125.10 c (100 %)	19.12 a (100 %)
		100 mM NaCl	115.60 b (92.41 %)	18.18 a (95.08 %)
	4	0	170.54 e (100 %)	21.76 b (100 %)
		100 mM NaCl	160.26 d (93.97 %)	21.10 b (96.97 %)
KLD	0	0	116.98 a	21.88 a
	2	0	125.74 bc (100 %)	23.84 c (100 %)
		100 mM NaCl	117.26 b (93.26 %)	22.55 b (94.59 %)
	4	0	129.66 c (100 %)	26.64 d (100 %)
		100 mM NaCl	121.02 b (93.34 %)	25.72 d (96.55 %)
TD49	0	0	109.24 a	16.78 a
	2	0	170.28 c (100 %)	21.52 c (100 %)
		100 mM NaCl	156.94 b (92.16 %)	20.34 bc (94.52 %)
	4	0	183.96 d (100 %)	26.74 d (100 %)
		100 mM NaCl	168.54 c (91.62 %)	25.68 d (96.04 %)
PT1	0	0	162.16 bc	22.28 b
	2	0	203.24 d (100 %)	25.14 b (100 %)
		100 mM NaCl	158.56 ab (78.02 %)	22.14 ab (88.06 %)
	4	0	217.36 e (100 %)	30.16 d (100 %)
		100 mM NaCl	165.90 c (76.33 %)	26.78 c (88.79 %)

Means within the same cultivar followed by the same letters are not significantly different by DMRT at $P \leq 0.05$.

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Table 4.2 Changes in the fresh weight (FW) and dry weight (DW) of rice seedlings cultured under photoautotrophic system with or without 100 mM mannitol for 2 or 4 days

Cultivars	Days	Treatments	FW (mg)	DW (mg)
KS	0	0	175.44 a	27.66 a
	2	0	194.90 cd (100 %)	30.28 bc (100 %)
		100 mM mannitol	190.98 bc (97.99 %)	29.72 b (98.15 %)
	4	0	218.82 f (100 %)	32.68 e (100 %)
		100 mM mannitol	206.16 e (94.21 %)	31.86 de (97.49 %)
KK1	0	0	135.34 a	19.90 a
	2	0	225.92 c (100 %)	31.50 c (100 %)
		100 mM mannitol	208.08 b (92.10 %)	29.62 b (94.03 %)
	4	0	256.38 e (100 %)	37.58 d (100 %)
		100 mM mannitol	235.58 d (91.89 %)	36.14 d (96.17 %)
KK2	0	0	105.64 a	13.84 a
	2	0	188.20 c (100 %)	30.04 c (100 %)
		100 mM mannitol	181.06 b (96.21 %)	29.66 bc (98.74 %)
	4	0	192.92 d (100 %)	30.40 c (100 %)
		100 mM mannitol	186.10 c (96.46 %)	29.76 bc (97.89 %)
BSR	0	0	151.76 a	23.10 a
	2	0	193.66 c (100 %)	26.00 c (100 %)
		100 mM mannitol	175.04 b (91.66 %)	24.16 b (92.92 %)
	4	0	200.62 c (100 %)	27.92 d (100 %)
		100 mM mannitol	182.16 b (90.80 %)	25.92 c (92.84 %)
KDML105	0	0	194.38 ab	25.24 a
	2	0	211.72 c (100 %)	29.24 c (100 %)
		100 mM mannitol	174.78 a (82.55 %)	26.56 b (90.83 %)
	4	0	236.16 d (100 %)	32.98 d (100 %)
		100 mM mannitol	191.84 ab (81.23 %)	29.80 c (90.36 %)
SY	0	0	119.06 a	18.36 a
	2	0	143.82 b (100 %)	21.14 c (100 %)
		100 mM mannitol	112.98 a (78.56 %)	18.98 b (89.78 %)
	4	0	176.26 c (100 %)	25.86 e (100 %)
		100 mM mannitol	138.18 b (78.40 %)	23.18 d (89.64 %)

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Table 4.2 (Continued) Changes in the fresh weight (FW) and dry weight (DW) of rice seedlings cultured under photoautotrophic system with or without 100 mM mannitol for 2 or 4 days

Cultivars	Days	Treatments	FW (mg)	DW (mg)
D	0	0	112.30 ab	18.60 b
	2	0	125.10 c (100 %)	19.12 b (100 %)
		100 mM mannitol	95.92 a (76.67 %)	15.92 a (83.26 %)
	4	0	170.54 e (100 %)	21.76 c (100 %)
		100 mM mannitol	129.30 d (75.82 %)	18.96 b (87.13 %)
KLD	0	0	116.98 b	21.88 b
	2	0	125.74 c (100 %)	23.84 c (100 %)
		100 mM mannitol	100.94 a (80.28 %)	20.46 a (85.82 %)
	4	0	129.66 c (100 %)	26.64 d (100 %)
		100 mM mannitol	106.82 ab (82.38 %)	24.02 c (90.17 %)
TD49	0	0	109.24 a	16.78 a
	2	0	170.28 c (100 %)	21.52 c (100 %)
		100 mM mannitol	133.96 b (78.67 %)	18.88 b (87.73 %)
	4	0	183.96 d (100 %)	26.74 d (100 %)
		100 mM mannitol	132.94 b (72.27 %)	21.84 c (81.68 %)
PT1	0	0	162.16 a	22.28 a
	2	0	203.24 d (100 %)	25.14 b (100 %)
		100 mM mannitol	162.14 a (79.78 %)	22.30 a (88.70 %)
	4	0	217.36 e (100 %)	30.16 d (100 %)
		100 mM mannitol	176.46 ab (81.18 %)	26.84 c (88.79 %)

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Table 4.3 Changes in the shoot length (SL) and root length (RL) of rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl for 2 or 4 days

Cultivars	Days	Treatments	SL (cm)	RL (cm)
KS	0	0	24.7 b	3.7 ab
	2	0	24.8 b (100 %)	3.8 b (100 %)
		100 mM NaCl	22.2 a (89.52 %)	3.4 a (89.47 %)
	4	0	25.6 c (100 %)	4.2 c (100 %)
		100 mM NaCl	22.8 a (89.06 %)	3.8 b (90.48 %)
KK1	0	0	22.5 a	5.2 b
	2	0	26.4 c (100 %)	5.3 b (100 %)
		100 mM NaCl	23.6 b (89.39 %)	4.7 a (88.68 %)
	4	0	29.5 d (100 %)	6.2 c (100 %)
		100 mM NaCl	26.8 c (90.85 %)	5.3 b (85.48 %)
KK2	0	0	19.0 a	4.3 a
	2	0	28.5 d (100 %)	6.0 d (100 %)
		100 mM NaCl	25.6 b (89.82 %)	5.2 b (86.67 %)
	4	0	29.3 e (100 %)	6.7 e (100 %)
		100 mM NaCl	26.4 c (90.10 %)	5.8 c (86.57 %)
BSR	0	0	21.1 ab	3.8 a
	2	0	24.0 c (100 %)	4.8 c (100 %)
		100 mM NaCl	21.4 b (89.17 %)	4.2 b (87.50 %)
	4	0	25.5 d (100 %)	4.9 c (100 %)
		100 mM NaCl	20.8 a (86.67 %)	4.2 b (85.71 %)
KDML105	0	0	26.2 a	6.4 a
	2	0	28.6 b (100 %)	6.8 b (100 %)
		100 mM NaCl	28.2 b (98.60 %)	6.5 a (95.59 %)
	4	0	29.8 c (100 %)	7.1 c (100 %)
		100 mM NaCl	29.5 c (98.99 %)	6.8 b (95.77 %)
SY	0	0	21.4 a	5.2 a
	2	0	25.6 c (100 %)	7.1 c (100 %)
		100 mM NaCl	25.1 b (98.05 %)	6.8 b (95.77 %)
	4	0	26.5 d (100 %)	7.2 c (100 %)
		100 mM NaCl	25.6 c (96.60 %)	6.8 b (94.44 %)

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Table 4.3 (Continued) Changes in the shoot length (SL) and root length (RL) of rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl for 2 or 4 days

Cultivars	Days	Treatments	SL (cm)	RL (cm)
D	0	0	24.8 a	5.4 a
	2	0	25.4 b (100 %)	5.9 b (100 %)
		100 mM NaCl	24.4 a (96.06 %)	5.5 a (93.22 %)
	4	0	28.7 d (100 %)	7.3 d (100 %)
		100 mM NaCl	27.6 c (96.17 %)	6.8 c (93.15 %)
KLD	0	0	22.6 a	3.9 a
	2	0	24.4 c (100 %)	4.4 b (100 %)
		100 mM NaCl	23.4 b (95.90 %)	4.1 a (93.18 %)
	4	0	24.8 c (100 %)	4.6 c (100 %)
		100 mM NaCl	23.7 b (95.56 %)	4.3 ab (93.48 %)
TD49	0	0	22.3 a	4.9 a
	2	0	26.8 c (100 %)	7.0 c (100 %)
		100 mM NaCl	25.4 b (94.78 %)	6.5 b (92.86 %)
	4	0	28.4 d (100 %)	7.1 c (100 %)
		100 mM NaCl	26.9 c (94.73 %)	6.6 b (92.96 %)
PT1	0	0	25.1 b	5.7 c
	2	0	25.5 bc (100 %)	6.2 d (100 %)
		100 mM NaCl	22.4 a (87.84 %)	4.7 a (75.81 %)
	4	0	25.9 c (100 %)	6.2 d (100 %)
		100 mM NaCl	22.6 a (87.26 %)	5.1 b (82.26 %)

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Table 4.4 Changes in the shoot length (SL) and root length (RL) of rice seedlings cultured under photoautotrophic system with or without 100 mM mannitol for 2 or 4 days

Cultivars	Days	Treatments	SL (cm)	RL (cm)
KS	0	0	24.7 b	3.7 a
	2	0	24.8 b (100 %)	3.8 a (100 %)
		100 mM mannitol	24.3 ab (97.98 %)	3.7 a (97.37 %)
	4	0	25.6 c (100 %)	4.2 b (100 %)
		100 mM mannitol	25.3 c (98.83 %)	4.1 b (97.62 %)
KK1	0	0	22.5 a	5.2 a
	2	0	26.4 c (100 %)	5.3 a (100 %)
		100 mM mannitol	25.3 b (95.83 %)	5.0 a (94.34 %)
	4	0	29.5 e (100 %)	6.2 c (100 %)
		100 mM mannitol	28.2 d (95.59 %)	5.9 b (95.16 %)
KK2	0	0	19.0 a	4.3 a
	2	0	28.5 c (100 %)	6.0 c (100 %)
		100 mM mannitol	27.6 b (96.84 %)	5.8 b (96.67 %)
	4	0	29.3 d (100 %)	6.7 d (100 %)
		100 mM mannitol	28.2 c (96.25 %)	6.4 c (95.52 %)
BSR	0	0	21.1 a	3.8 a
	2	0	24.0 d (100 %)	4.8 c (100 %)
		100 mM mannitol	22.1 b (92.08 %)	4.4 b (91.67 %)
	4	0	25.5 e (100 %)	4.9 c (100 %)
		100 mM mannitol	23.7 c (92.94 %)	4.5 b (91.84 %)
KDML105	0	0	26.2 a	6.4 b
	2	0	28.6 c (100 %)	6.8 c (100 %)
		100 mM mannitol	26.1 a (91.26 %)	5.4 a (87.10 %)
	4	0	29.8 d (100 %)	7.1 c (100 %)
		100 mM mannitol	27.1 b (90.94 %)	6.2 b (87.32 %)
SY	0	0	21.4 a	5.2 a
	2	0	25.6 d (100 %)	7.1 c (100 %)
		100 mM mannitol	23.1 b (90.23 %)	6.0 b (84.51 %)
	4	0	26.5 e (100 %)	7.2 c (100 %)
		100 mM mannitol	24.1 c (90.94 %)	6.0 b (83.33 %)

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Table 4.4 (Continued) Changes in the shoot length (SL) and root length (RL) of rice seedlings cultured under photoautotrophic system with or without 100 mM mannitol for 2 or 4 days

Cultivars	Days	Treatments	SL (cm)	RL (cm)
D	0	0	24.8 b	5.4 ab
	2	0	25.4 c (100 %)	5.9 b (100 %)
		100 mM mannitol	22.6 a (88.98 %)	5.1 a (86.44 %)
	4	0	28.7 d (100 %)	7.3 d (100 %)
		100 mM mannitol	24.9 b (86.76 %)	6.2 c (84.93 %)
KLD	0	0	22.6 b	3.9 a
	2	0	24.4 c (100 %)	4.4 b (100 %)
		100 mM mannitol	21.8 a (89.34 %)	3.8 a (86.38 %)
	4	0	24.8 c (100 %)	4.6 c (100 %)
		100 mM mannitol	22.2 b (89.52 %)	4.0 a (86.96 %)
TD49	0	0	22.3 a	4.9 a
	2	0	26.8 c (100 %)	7.0 c (100 %)
		100 mM mannitol	24.0 b (89.55 %)	6.2 b (88.57 %)
	4	0	28.4 d (100 %)	7.1 c (100 %)
		100 mM mannitol	24.8 b (87.32 %)	6.2 b (87.32 %)
PT1	0	0	25.1 b	5.7 b
	2	0	25.5 bc (100 %)	6.2 c (100 %)
		100 mM mannitol	22.1 a (86.67 %)	4.6 a (74.19 %)
	4	0	25.9 c (100 %)	6.2 c (100 %)
		100 mM mannitol	22.3 a (86.10 %)	4.9 a (79.03 %)

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Table 4.5 Percentage of relative water content (%RWC) in the seedlings of rice cultivars exposed 2 and 4 days to 100 mM NaCl. Each value is the mean of at least five independent experiments

Cultivars	Days	Treatments	% RWC	± SD	% RWC_t/RWC_c
KS	0	0	84.2339	4.7667	
	2	0	84.4638	5.6453	
		100 mM NaCl	83.5197	5.3652	98.8822
	4	0	85.0654	4.3233	
		100 mM NaCl	84.0555	5.5455	98.8129
KK1	0	0	85.2963	4.6566	
	2	0	86.0570	6.5364	
		100 mM NaCl	84.5836	5.7657	98.2879
	4	0	85.3421	5.7516	
		100 mM NaCl	84.4478	6.5453	98.9522
KK2	0	0	86.8989	4.7576	
	2	0	84.0383	4.6455	
		100 mM NaCl	82.9598	4.7647	98.7167
	4	0	84.2422	5.7536	
		100 mM NaCl	83.0692	4.7667	98.6076
BSR	0	0	84.7786	3.7656	
	2	0	86.5744	5.4253	
		100 mM NaCl	85.0455	6.0937	98.2340
	4	0	86.0831	6.3283	
		100 mM NaCl	85.0684	5.4534	98.8212
KDML105	0	0	87.0151	5.7342	
	2	0	86.1893	6.4324	
		100 mM NaCl	86.1217	4.6246	99.9215
	4	0	86.0349	4.6756	
		100 mM NaCl	85.7529	5.7656	99.6722

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Table 4.5 (Continued) Percentage of relative water content (%RWC) in the seedlings of rice cultivars exposed 2 and 4 days to 100 mM NaCl. Each value is the mean of at least five independent experiments

Cultivars	Days	Treatments	% RWC	± SD	% RWC_t/RWC_c
SY	0	0	84.5792	4.2331	
	2	0	85.3011	4.6356	
		100 mM NaCl	85.1666	5.6764	99.8423
	4	0	85.3285	5.7566	
		100 mM NaCl	84.8812	4.2767	99.4758
D	0	0	83.4372	5.7753	
	2	0	84.7162	5.6445	
		100 mM NaCl	84.2734	5.7596	99.4772
	4	0	87.2405	6.4214	
		100 mM NaCl	86.8339	4.6256	99.5339
KLD	0	0	81.2959	4.6453	
	2	0	81.0402	4.6516	
		100 mM NaCl	80.7692	4.5656	99.6656
	4	0	79.4540	5.7555	
		100 mM NaCl	78.7473	5.7672	99.1106
TD49	0	0	84.6393	4.6565	
	2	0	87.3620	5.4232	
		100 mM NaCl	87.0396	5.5353	99.6310
	4	0	85.4642	5.7567	
		100 mM NaCl	84.7633	5.7656	99.1798
PT1	0	0	86.2605	5.4345	
	2	0	87.6304	6.5435	
		100 mM NaCl	86.0368	6.5364	98.1815
	4	0	86.1244	6.3423	
		100 mM NaCl	83.8577	6.2112	97.3682

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Table 4.6 Percentage of relative water content (%RWC) in the seedlings of rice cultivars exposed 2 and 4 days to 100 mM mannitol. Each value is the mean of at least five independent experiments

Cultivars	Days	Treatments	% RWC	± SD	% RWC_t/RWC_c
KS	0	0	84.2339	4.7667	
	2	0	84.4638	5.4343	
		100 mM mannitol	84.4382	5.1323	99.9696
	4	0	85.0654	4.8787	
		100 mM mannitol	84.5460	5.7676	99.3894
KK1	0	0	85.2963	4.6566	
	2	0	86.0570	5.4232	
		100 mM mannitol	85.7651	4.9765	99.6608
	4	0	85.3421	5.3422	
		100 mM mannitol	84.6591	4.8787	99.1998
KK2	0	0	86.8989	4.7576	
	2	0	84.0383	4.6565	
		100 mM mannitol	83.6187	5.8787	99.5007
	4	0	84.2422	4.6564	
		100 mM mannitol	84.0086	5.4544	99.7227
BSR	0	0	84.7786	3.7656	
	2	0	86.5744	5.4323	
		100 mM mannitol	86.1974	4.9867	99.5646
	4	0	86.0831	5.3242	
		100 mM mannitol	85.7708	4.8776	99.6371
KDML105	0	0	87.0151	5.7342	
	2	0	86.1893	4.6554	
		100 mM mannitol	84.8038	4.6576	98.3924
	4	0	86.0349	5.3243	
		100 mM mannitol	84.4662	4.7655	98.1767

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Table 4.6 (Continued) Percentage of relative water content (%RWC) in the seedlings of rice cultivars exposed 2 and 4 days to 100 mM mannitol. Each value is the mean of at least five independent experiments

Cultivars	Days	Treatments	% RWC	± SD	% RWC_t/RWC_c
SY	0	0	84.5792	4.2331	
	2	0	85.3011	5.7665	
		100 mM mannitol	83.2006	6.1322	97.5375
	4	0	85.3285	6.2122	
		100 mM mannitol	83.2248	4.8676	97.5346
D	0	0	83.4372	5.7753	
	2	0	84.7162	6.2332	
		100 mM mannitol	83.4028	5.4384	98.4497
	4	0	87.2405	5.4342	
		100 mM mannitol	85.3364	4.9866	97.8174
KLD	0	0	81.2959	4.6453	
	2	0	81.0402	5.4733	
		100 mM mannitol	79.7305	4.6532	98.3839
	4	0	79.4540	5.7877	
		100 mM mannitol	77.5136	5.3434	97.5579
TD49	0	0	84.6393	4.6565	
	2	0	87.3620	4.6575	
		100 mM mannitol	85.9062	5.6223	98.3337
	4	0	85.4642	5.4343	
		100 mM mannitol	83.5715	5.8765	97.7854
PT1	0	0	86.2605	5.4345	
	2	0	87.6304	5.4343	
		100 mM mannitol	86.2465	4.6543	98.4207
	4	0	86.1244	4.7676	
		100 mM mannitol	84.7898	4.7867	98.4503

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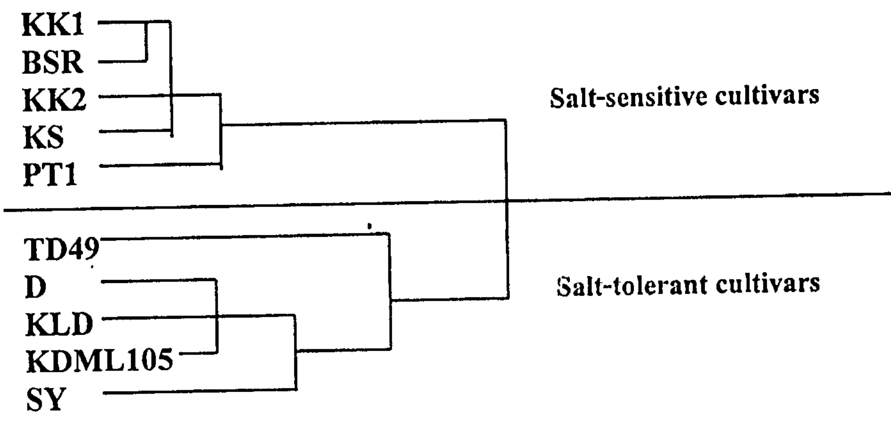

Fig. 4.1 Characterization of cluster in the fresh weight, dry weight, RWC, photosynthetic pigments and MDA content of rice seedlings cultured under photoautotrophic system with 100 mM NaCl for 2 and 4 days by Hierarchical cluster analysis in SPSS software

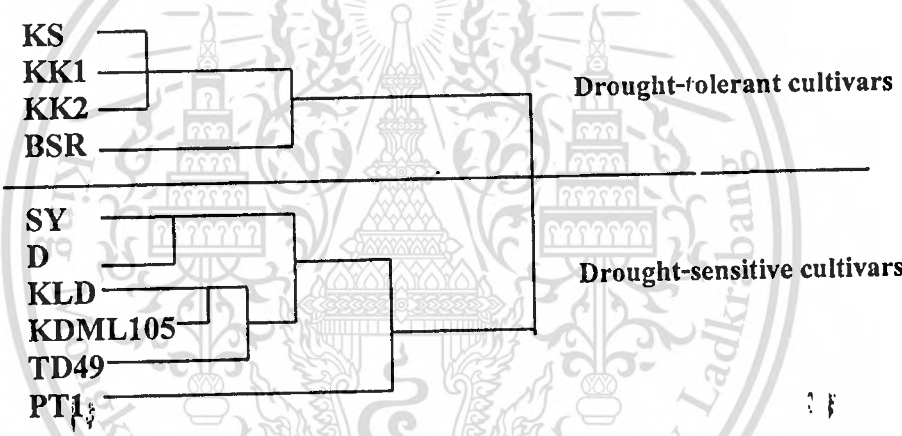

Fig. 4.2 Characterization of cluster in the fresh weight, dry weight, RWC, photosynthetic pigments and MDA content of rice seedlings cultured under photoautotrophic system with 100 mM mannitol for 2 and 4 days by Hierarchical cluster analysis in SPSS software

4.2 Effect of salt or drought stresses on photosynthetic pigment contents of Thai rice

Photosynthetic system is responsible for growth and development of plants. However, photosynthetic efficiency of a particular plant is influenced by surrounding environmental conditions (Wise, 1995). The capacity of plants to utilize the light absorbed by them declines significantly when they are exposed to environmental stresses such as drought, salt, extreme temperature and extreme light intensity (Dubey, 1997; He *et al.*, 1995). Adverse environmental

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conditions, such as high temperature, salinity and water stress that strongly limit photosynthetic carbon metabolism can intensify reduction in photosynthetic capacity of the plant. Therefore, the measurement of photosynthetic pigments is a useful tool for the effect of stress on photosynthetic system (Uchida *et al.*, 2002; Huner *et al.*, 1993).

The results for photosynthetic pigment contents of the tested cultivars are shown in Tables 4.7 and 4.8. Salt or drought stresses significantly affected the reduction of photosynthetic pigment contents (Ch_A, Ch_B and Car) after 2 and 4 days. Under salt or drought stresses, a gradual reduction in Ch_A, Ch_B and Car contents were observed in all five salt-tolerant and all four drought-tolerant cultivars. The difference in the reduction of photosynthetic pigment contents between relatively tolerant and sensitive cultivars was much more apparent under both stress conditions. The extent of reduction in Ch_A, Ch_B and Car contents after 4 days of salt stress was lower in tolerant cultivars KDML105 (4.92%, 6.83%, 2.94%, respectively), SY (5.08%, 7.24%, 3.12%, respectively), D (10.69%, 9.41%, 7.74%, respectively), KLD (10.17%, 10.28%, 9.11%, respectively) and TD49 (13.36%, 11.45%, 10.74%, respectively) and more pronounced in relatively sensitive KS (30.29%, 45.74%, 36.43%, respectively), KK1 (41.70%, 39.77%, 30.34%, respectively), KK2 (49.75%, 52.08%, 32.07%, respectively), BSR (45.48%, 42.23%, 36.21%, respectively) and PT1 (45.35%, 40.92%, 30.79%, respectively) (Table 4.7).

In the meanwhile, the decline in photosynthetic pigment contents observed in plants subjected to drought stress is often associated with a decrease in their Ch_A, Ch_B and Car. The Ch_A, Ch_B and Car of stressed-seedlings at 100 mM mannitol were decreased as compared to the control. The degradation range of photosynthetic pigment contents for drought-sensitive cultivars was more than drought-tolerant cultivars under stress condition after 4 days varies from 33.42-50.60% in KDML105, 30.55-33.54% in SY, 45.16-56.58% in D, 38.02-43.15% in KLD, 36.01-48.26% in TD49 and 31.81-46.29% in PT1.

In this experiment, high levels of Ch_A, Ch_B and Car contents in 2 and 4 days stressed-tolerant cultivars indicated that rice seedlings were able to maintain photosynthetic pigment contents of photosynthesis system at 100 mM NaCl and mannitol levels and demonstrate a relatively high degree of stresses tolerance (Table 4.8).

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Table 4.7 Chlorophyll A (Ch_A), chlorophyll B (Ch_B) and total carotenoids (Car) contents of rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl for 2 and 4 days

Cultivars	Days	Treatments	Ch_A ($\mu g\ g^{-1}$ FW)	Ch_B ($\mu g\ g^{-1}$ FW)	Car ($\mu g\ g^{-1}$ FW)
KS	0	0	625.2734 c	139.1106 ab	224.7138 c
	2	0	669.7510 d	259.4222 e	240.7840 d
		100 mM NaCl	455.4320 a	170.5546 c	138.6252 a
	4	0	667.1758 d	244.8974 d	247.2120 d
		100 mM NaCl	465.0270 b	135.3130 a	157.1556 b
KK1	0	0	709.5024 c	182.2280 c	239.4528 b
	2	0	820.6324 d	206.3092 d	262.7294 c
		100 mM NaCl	559.4028 b	128.0170 a	182.2596 a
	4	0	863.4980 e	220.9838 e	279.3096 d
		100 mM NaCl	503.4224 a	133.0798 b	194.5130 a
KK2	0	0	600.1994 c	159.9678 c	222.8542 b
	2	0	709.5686 e	191.8444 d	235.5954 c
		100 mM NaCl	437.2592 b	109.0712 b	164.8464 a
	4	0	700.0370 d	190.3670 d	238.6912 c
		100 mM NaCl	351.7646 a	91.20860 a	162.1376 a
BSR	0	0	563.9330 c	141.1206 c	220.0542 c
	2	0	749.7696 d	177.3706 d	280.2384 e
		100 mM NaCl	465.8852 b	109.9642 b	193.6688 b
	4	0	748.5526 d	174.7934 d	275.5606 d
		100 mM NaCl	415.5962 a	100.9752 ab	175.8068 a
KDML105	0	0	718.4462 b	210.0492 b	244.0156 a
	2	0	760.1462 c	215.1082 c	306.0994 b
		100 mM NaCl	697.7272 a	199.1934 a	247.7494 a
	4	0	894.9786 e	228.3330 d	331.2902 d
		100 mM NaCl	850.9218 d	212.7402 c	321.5572 c

Means within the same cultivar followed by the same letters are not significantly different by DMRT at $P \leq 0.05$.

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Table 4.7 (Continued) Chlorophyll A (Ch_A), chlorophyll B (Ch_B) and total carotenoids (Car) contents of rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl for 2 and 4 days

Cultivars	Days	Treatments	Ch_A ($\mu g\ g^{-1}$ FW)	Ch_B ($\mu g\ g^{-1}$ FW)	Car ($\mu g\ g^{-1}$ FW)
SY	0	0	679.6498 b	162.5624 ab	252.9558 b
	2	0	745.9176 d	183.2752 d	264.8016 c
		100 mM NaCl	676.1018 ab	167.9354 b	243.1164 a
	4	0	708.7030 c	170.1876 c	260.5540 c
		100 mM NaCl	672.7138 ab	157.8578 a	252.4052 b
D	0	0	489.6124 a	132.3358 a	185.7714 a
	2	0	603.5204 d	149.4508 b	219.7672 c
		100 mM NaCl	532.0228 b	135.6654 a	201.1128 b
	4	0	659.1314 e	169.0666 d	245.6150 e
		100 mM NaCl	587.9934 c	153.1694 c	226.9982 d
KLD	0	0	520.2032 a	138.0492 a	212.6292 a
	2	0	777.2126 d	185.2258 c	278.8484 c
		100 mM NaCl	684.5724 b	163.5742 b	247.7494 b
	4	0	824.4870 e	210.1534 d	308.6820 e
		100 mM NaCl	740.5756 c	188.5418 c	283.5396 d
TD49	0	0	771.5564 c	214.7120 c	261.9252 b
	2	0	801.5888 d	228.4816 d	288.4116 c
		100 mM NaCl	707.5520 b	202.4570 b	250.2394 a
	4	0	805.1278 e	222.9448 d	295.6598 d
		100 mM NaCl	697.5836 a	197.4390 a	263.8796 b
PT1	0	0	428.1036 c	102.8202 b	173.7494 a
	2	0	542.0904 d	130.5306 c	242.0906 c
		100 mM NaCl	350.4554 b	89.43840 a	170.2722 a
	4	0	574.6916 e	145.9008 d	245.4318 c
		100 mM NaCl	325.5338 a	86.20620 a	169.8818 a

Means within the same cultivar followed by the same letters are not significantly different by DMRT at $P \leq 0.05$.

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Table 4.8 Chlorophyll A (Ch_A), chlorophyll B (Ch_B) and total carotenoids (Car) contents of rice seedlings cultured under photoautotrophic system with or without 100 mM mannitol for 2 and 4 days

Cultivars	Days	Treatments	Ch_A ($\mu g\ g^{-1}$ FW)	Ch_B ($\mu g\ g^{-1}$ FW)	Car ($\mu g\ g^{-1}$ FW)
KS	0	0	625.2734 c	139.1106 a	224.7138 b
	2	0	669.7510 d	259.4222 d	240.7840 c
		100 mM mannitol	604.8250 a	228.9592 b	220.4818 a
	4	0	667.1758 d	244.8974 c	247.2120 d
		100 mM mannitol	613.3324 b	223.5134 b	222.4972 b
KK1	0	0	709.5024 a	182.2280 a	239.4528 b
	2	0	820.6324 d	206.3092 c	262.7294 d
		100 mM mannitol	732.4550 b	182.3160 a	230.3846 a
	4	0	863.4980 e	220.9838 d	279.3096 e
		100 mM mannitol	771.8120 c	197.0676 b	244.6914 c
KK2	0	0	600.1994 a	159.9678 a	222.8542 b
	2	0	709.5686 de	191.8444 d	235.5954 c
		100 mM mannitol	635.9680 c	170.3954 c	214.2598 a
	4	0	700.0370 d	190.3670 d	238.6912 cd
		100 mM mannitol	629.3882 b	169.3312 b	214.6752 a
BSR	0	0	563.9330 a	141.1206 a	220.0542 ab
	2	0	749.7696 d	177.3706 e	280.2384 e
		100 mM mannitol	659.7198 c	156.3910 b	241.1996 c
	4	0	748.5526 d	174.7934 d	275.5606 d
		100 mM mannitol	629.3882 b	169.3312 c	214.6752 a
KDML105	0	0	718.4462 b	210.0492 b	244.0156 c
	2	0	760.1462 c	215.1082 c	306.0994 d
		100 mM mannitol	464.5664 a	110.6670 a	212.4156 a
	4	0	894.9786 d	228.3330 d	331.2902 e
		100 mM mannitol	469.5406 a	112.7886 a	220.3720 b

Means within the same cultivar followed by the same letters are not significantly different by DMRT at $P \leq 0.05$.

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Table 4.8 (Continued) Chlorophyll A (Ch_A), chlorophyll B (Ch_B) and total carotenoids (Car) contents of rice seedlings cultured under photoautotrophic system with or without 100 mM mannitol for 2 and 4 days

Cultivars	Days	Treatments	Ch_A ($\mu g\ g^{-1}$ FW)	Ch_B ($\mu g\ g^{-1}$ FW)	Car ($\mu g\ g^{-1}$ FW)
SY	0	0	679.6498 b	162.5624 c	252.9558 c
	2	0	745.9176 d	183.2752 e	264.8016 de
		100 mM mannitol	471.5372 a	117.122[illegible] b	188.9086 ab
	4	0	708.7030 c	170.1876 d	260.5540 d
		100 mM mannitol	471.0312 a	110.2790 a	180.9576 a
D	0	0	489.6124 c	132.3358 c	185.7714 c
	2	0	603.5204 d	149.4508 d	219.7672 d
		100 mM mannitol	408.1404 b	100.8086 b	150.6546 b
	4	0	659.1314 e	169.0666 e	245.6150 e
		100 mM mannitol	300.8454 a	73.3974 a	137.1278 a
KLD	0	0	520.2032 c	138.0492 c	212.6292 c
	2	0	777.2126 d	185.2258 d	278.8484 d
		100 mM mannitol	320.8636 a	77.7616 a	152.8524 a
	4	0	824.4870 e	210.1534 e	308.6820 e
		100 mM mannitol	468.6782 b	120.2008 b	191.1176 b
TD49	0	0	771.5564 c	214.7120 c	261.9252 c
	2	0	801.5888 d	228.4816 e	288.4116 d
		100 mM mannitol	552.1960 b	140.7218 a	149.9100 a
	4	0	805.1278 de	222.9448 d	295.6598 e
		100 mM mannitol	500.6176 a	142.6456 ab	152.9582 ab
PT1	0	0	428.1036 c	102.8202 b	173.7494 ab
	2	0	542.0904 d	130.5306 c	242.0906 c
		100 mM mannitol	362.5496 b	80.07460 a	170.5830 a
	4	0	574.6916 e	145.9008 d	245.4318 c
		100 mM mannitol	348.6312 a	78.34900 a	167.3630 a

Means within the same cultivar followed by the same letters are not significantly different by DMRT at $P \leq 0.05$.

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4.3 Effect of salt or drought stresses on lipid peroxidation of Thai rice

As a secondary consequence, salinity and drought also effect the generation of oxidative stress in plant tissues and the increasing level of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) which then in return damage membranes, photosynthetic pigments, proteins, DNA and lipids (Fadzilla *et al.*, 1997; Hernandez *et al.*, 1995). Steady-state concentrations of ROS that are constantly produced in chloroplasts, mitochondria, peroxisomes and plasma membranes, are suppressed to low levels by the small antioxidant molecules and by the enzymatic and non-enzymatic activities that scavenge ROSs (del Rio *et al.*, 2002; Salin, 1991). Malondialdehyde (MDA) amount is a secondary end product of polyunsaturated fatty acid oxidation, which is widely applied to measure the extent of lipid peroxidation as indicator of oxidative stress (Lin and Kao, 2000). Determining the MDA content and hence, the extent of membrane lipid peroxidation, has often been used as a tool to assess the degree of plant sensitivity to oxidative damage (Blokhina *et al.*, 2003).

In salt or drought stressed seedlings of the ten rice cultivars, the level of lipid peroxides was measured in terms of MDA content (Fig 4.3 and 4.4). A salt treatment level of 100 mM led to 26–88% increase in MDA level of salt-sensitive cultivars (KS, KK1, KK2, BSR and PT1) whereas salt-tolerant cultivars led to 8–25% (KDML105, SY, D, KLD, and TD49) enhancement in the lipid peroxide level in stressed-seedlings of 2 and 4 days grown seedlings compared to control (Fig 4.3).

Under drought stress, drought-tolerant cultivars had a slight increase of MDA level at 2 days and a great increase at 4 days. MDA level in six drought-sensitive cultivars, KDML105, SY, D, KLD TD49 and PT1, accumulated 1.56, 1.65, 1.67, 1.54, 1.71 and 1.95-folds more than that in control seedlings, respectively. These increase level of MDA in drought-sensitive cultivars was more than the four drought-tolerant cultivars, KS, KK1, KK2 and BSR at 4 days following drought treatment. Four drought-tolerant cultivars accumulated 1.08, 1.19, 1.17 and 1.41-folds more than that in control seedlings, respectively (Fig. 4.4). The results indicated that stressed-tolerant were less damaged under both stresses than stressed-sensitive.

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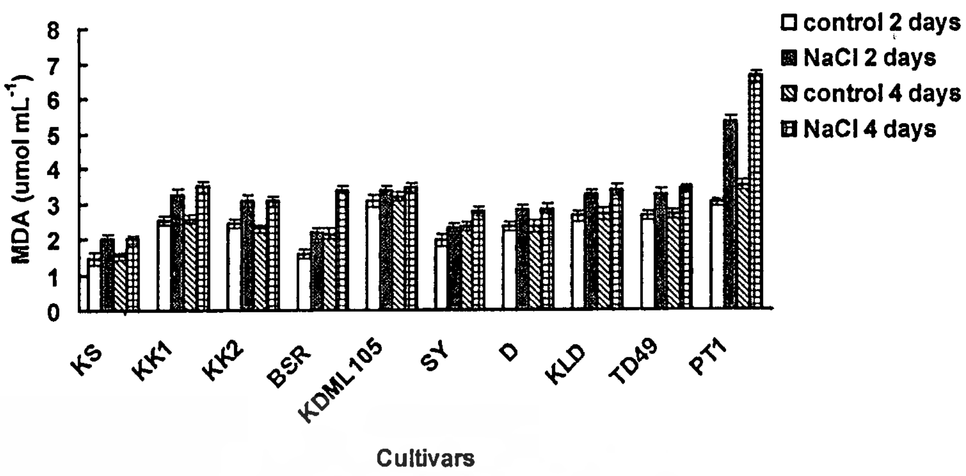

Fig. 4.3 MDA content of Thai rice cultivars cultured under photoautotrophic system with or without 100 mM NaCl (salt stress) for 2 and 4 days. Error bars represent by ±SE (n=5).

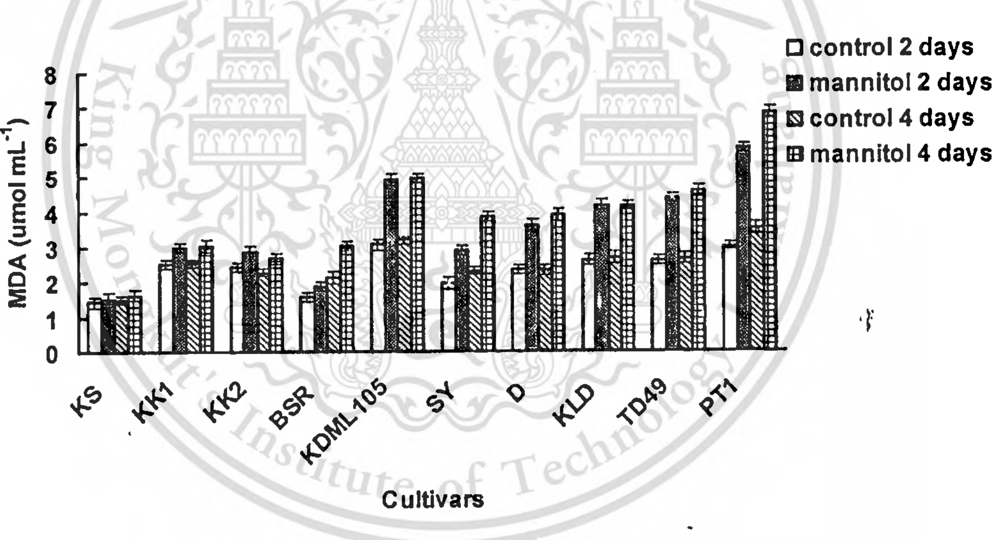

Fig. 4.4 MDA content of Thai rice cultivars cultured under photoautotrophic system with or without 100 mM mannitol (drought stress) for 2 and 4 days. Error bars represent by ±SE (n=5).

4.4 Effect of salt or drought stresses on proline content of Thai rice

The plant's defense against abiotic stress requires osmotic adjustment and this can be done through synthesis of intracellular solutes (Serrano *et al.,* 1999). It is well known, that one of the most common responses to water deficit and saline environments is the accumulation of proline,

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which acts as a compatible solute, an osmoprotectant, and a protective agent for cytosolic enzymes and cellular organelles (Fendina and Popova, 1996; Bohnert *et al.*, 1995; Rajesakaran, 1988). Proline has also been considered as carbon and nitrogen sources for rapid recovery from stress, a stabilizer for membranes and some macromolecules and also a free radical scavenger (Jain *et al.*, 2001).

A significantly higher level of free proline content was observed in stressed-seedlings grown under salt stress at 2 and 4 days. The salt-tolerant seedlings (KDML105, SY, D, KLD and TD49) grown at 100 mM NaCl had about 1.45-1.78-folds higher proline content than that at the control whereas the salt-sensitive seedlings (KS, KK1, KK2, BS and PT1) grown in salt stress had about 1.05-1.21-folds higher proline content than that at the control (Fig. 4.5).

Moreover, the changes in proline content in response to drought at 2 and 4 days were investigated (Fig. 4.6). As compared to control, the proline contents of seedlings increased dramatically in drought-tolerant cultivar (1.75-folds in KS, 1.49-folds in KK1, 1.56-folds in KK2 and 1.42-folds in BS) after 4 days of drought induction. Whereas, the proline contents of seedlings increased slightly in drought-sensitive cultivars (1.17-folds in KDML105, 1.32-folds in SY, 1.16-folds in D, 1.31-folds in KLD, 1.01-folds in TD49 and 1.14-folds in PT1) after 4 days of drought induction as compared to control (Fig. 4.6).

In this study, proline accumulation could be applied indicator for screening of salt- and drought-tolerant cultivars, which had stabilization of photosynthetic pigments and accumulation of proline higher than salt- and drought-sensitive cultivars.

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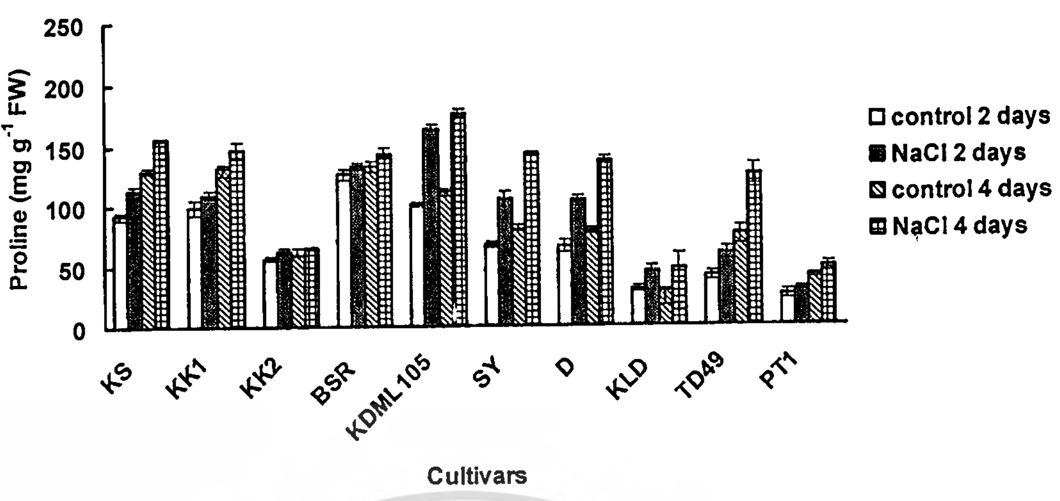

Fig. 4.5 Proline content of Thai rice cultivars cultured under photoautotrophic system with or without 100 mM NaCl (salt stress) for 2 and 4 days. Error bars represent by ±SE (n=5).

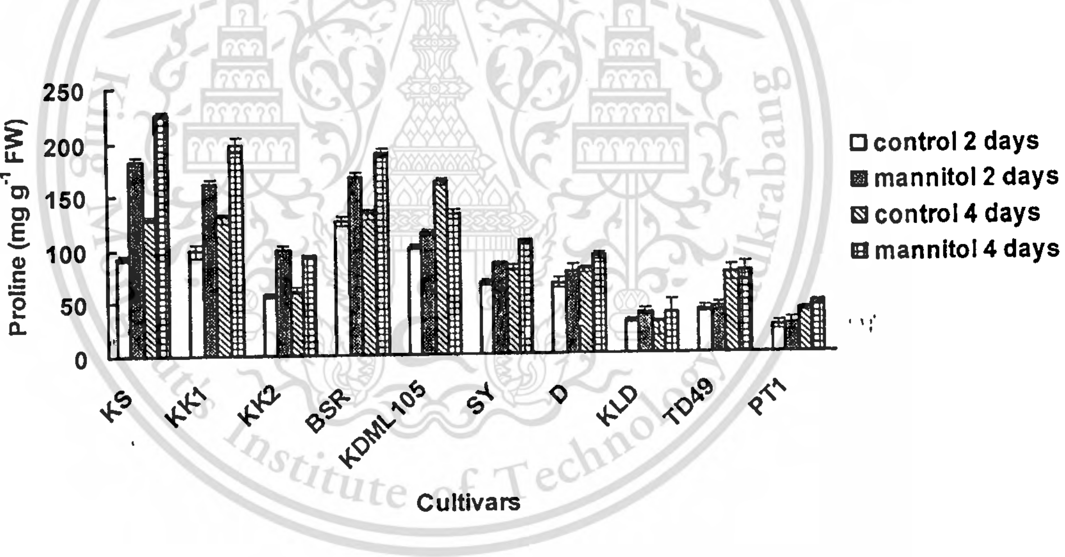

Fig. 4.6 Proline content of Thai rice cultivars cultured under photoautotrophic system with or without 100 mM mannitol (drought stress) for 2 and 4 days. Error bars represent by ±SE (n=5).

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4.5 Effect of salt or drought stresses on flavonoid levels of Thai rice

Flavonoids were extracted in acidified methanol and measured quantitative value by spectrophotometry. Flavonoids are polyphenolic family of antioxidant phytochemicals and are powerful hydrogen-donating antioxidants and scavengers of reactive oxygen species (Tsau and Deng, 2004). Flavonoids are classified the families of flavones, flavonols and anthocyanins. To prevent oxidative damages from abiotic stress, plants have evolved a number of strategies, including the accumulation of antioxidative agents (Tsau and Deng, 2004). It has been reported that flavonoids serve as the effective antioxidants *in vitro* as well as *in vivo* (Heim *et al.,* 2002; Bors *et al.*, 1990) and their contents are changed in response to abiotic stress (Treutter, 2006; Graham, 1998; Balakumar *et al.,* 1993). Therefore, the involvement of flavonoids and abiotic stress tolerance of Thai rice was studied by measuring major flavonoids, i.e. flavanone, flavonols, flavones anthocyanins and tannin.

The flavonoids content in different cultivars of Thai rice considered in this study were shown in Fig. 4.7-4.16. Effect of salt or drought on flavoniod accumulation in different rice cultivars was analyzed. Fourteen-day-old seedlings were treated separately with 100 mM NaCl and 100 mM mannitol and seedling samples were harvested 2 and 4 days after treatment.

After exposed to salt or drought stress, the declination levels of flavanone (naringenin) was observed in all cultivars (Fig. 4.7). Salt-tolerant cultivars (KDML105, SY, D, KLD and TD49) accumulated flavanone about 87-89% and 87-90% of the control under salt stress at 2 and 4 days, respectively. Whereas salt-sensitive cultivars (KS, KK1, KK2, BSR and PT1) accumulated flavanone more than salt-tolerant cultivars about 96-99% and 95-99% of the control under salt stress at 2 and 4 days, respectively (Fig 4.7A). In drought stress, drought-tolerant cultivars (KS, KK1, KK2 and BSR) also exhibited the similar responsive pattern of salt stress. Flavanone contents in drought-tolerant cultivars were decreased more than that in drought-sensitive cultivars (KDML105, SY, D, KLD, TD49 and PT1). Flavanone accumulations of drought-tolerant cultivars were 86-88% whereas drought-sensitive cultivars were 95-99% when compared to control after 2 and 4 days stressed (Fig. 4.7B).

Salt and drought stress caused the striking changes of flavonol levels. Among all salt-and drought-tolerant cultivars showed the remarkable increase in all detected flavonols (myricetin, quercetin and kaempferol). These seedlings exposed with each stress by 2 and 4 days contain flavonols about 102-116% and 103-150% of control, respectively. However, the content of

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flavonols in salt- and drought-sensitive cultivars were about 74-98% and 70-98% compared to the control after 2 and 4 days stressed (Fig. 4.8, 4.9 and 4.10).

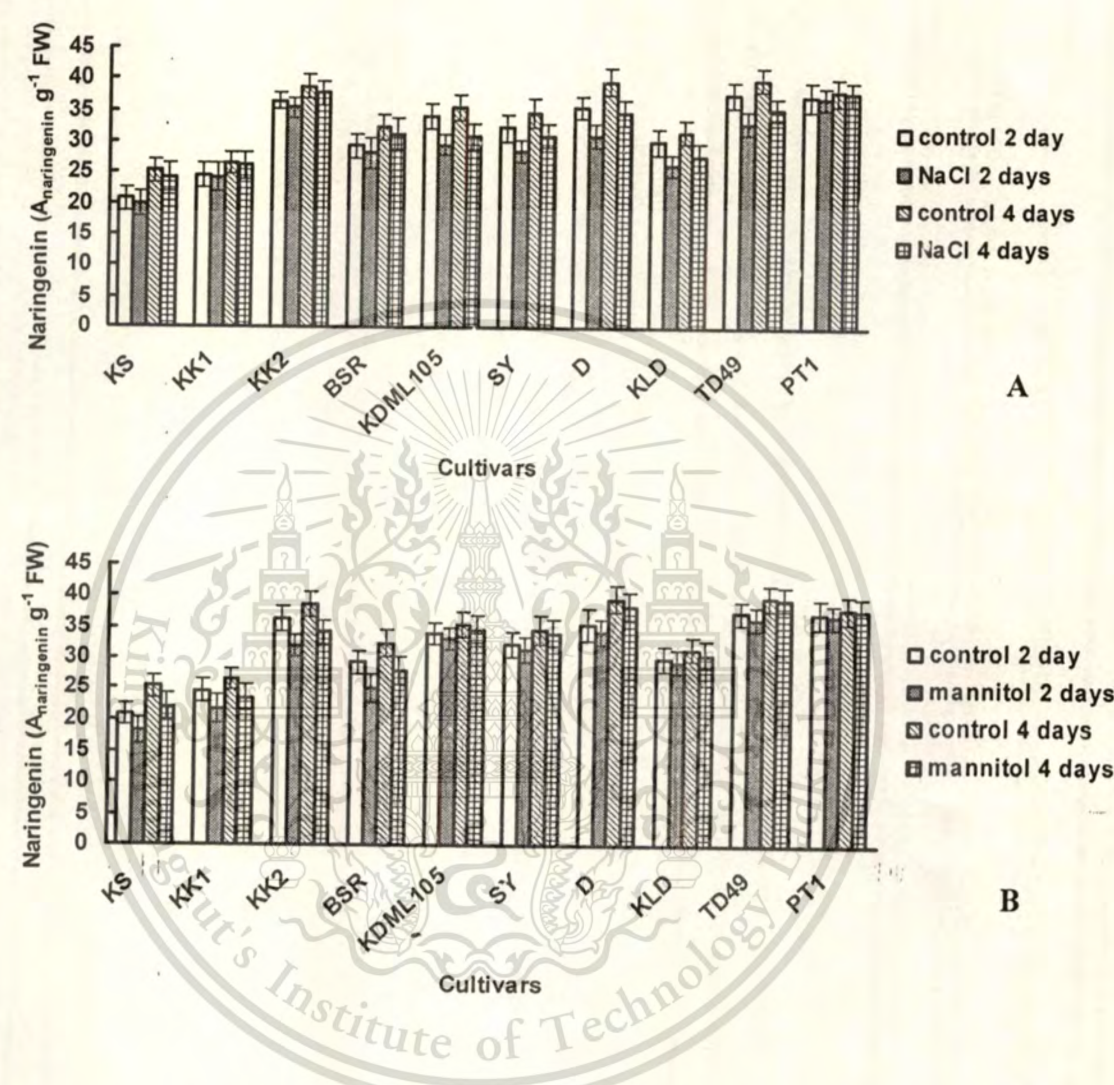

Fig. 4.7 Quantification of flavanone (naringenin) in Thai rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl (A) and with or without 100 mM mannitol (B) for 2 and 4 days. The absorbance values (A_{330}) of the acidified methanolic extracts of stressed-seedlings and control rice seedlings are shown as the amount of total flavanone. Error bars indicate the S.E. of the five independent replicates.

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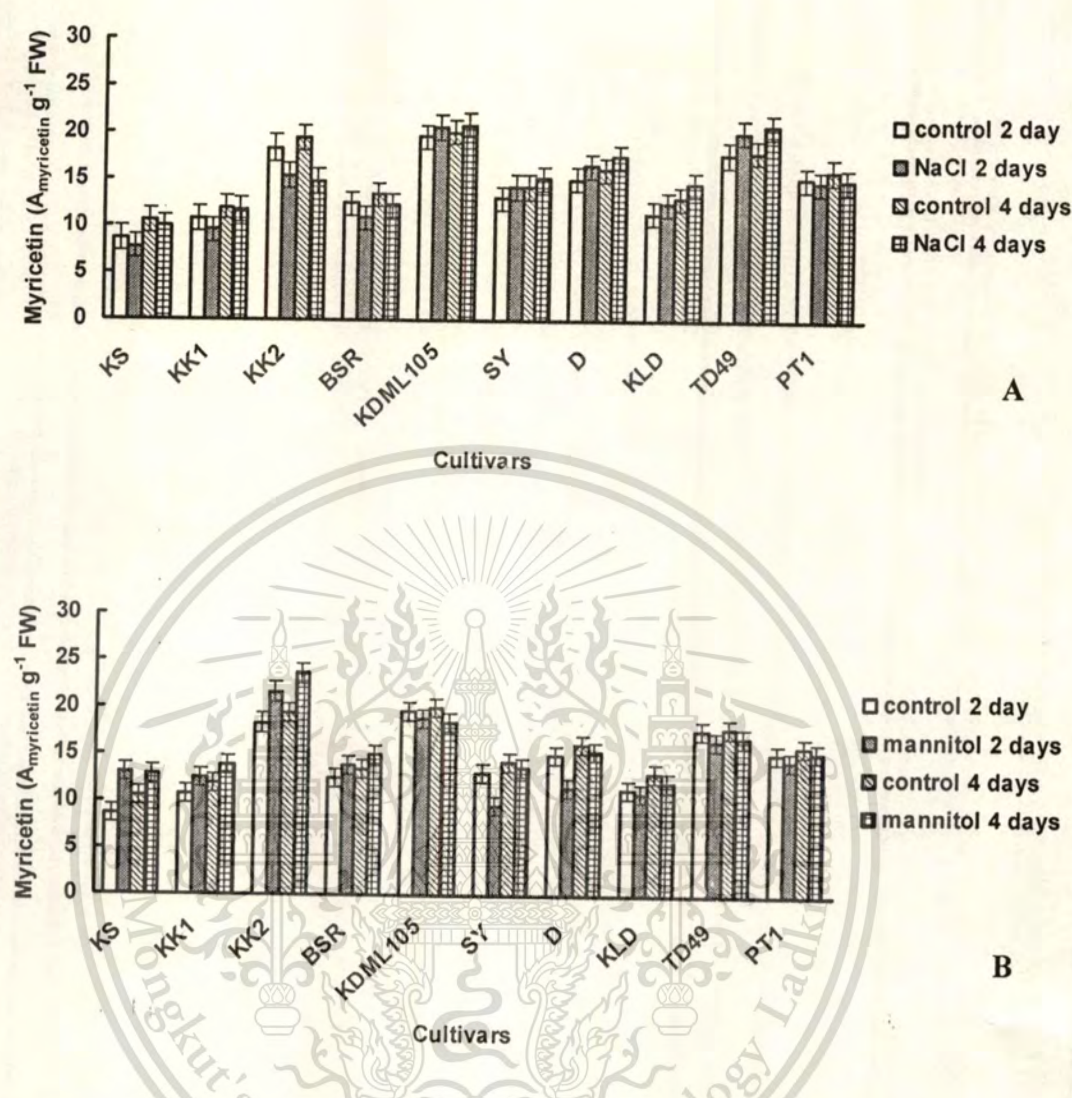

Fig. 4.8 Quantification of myricetin in Thai rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl (A) and with or without 100 mM mannitol (B) for 2 and 4 days. The absorbance values (A_{378}) of the acidified methanolic extracts of stressed-seedlings and control rice seedlings are shown as the amount of myricetin. Error bars indicate the S.E. of the five independent replicates.

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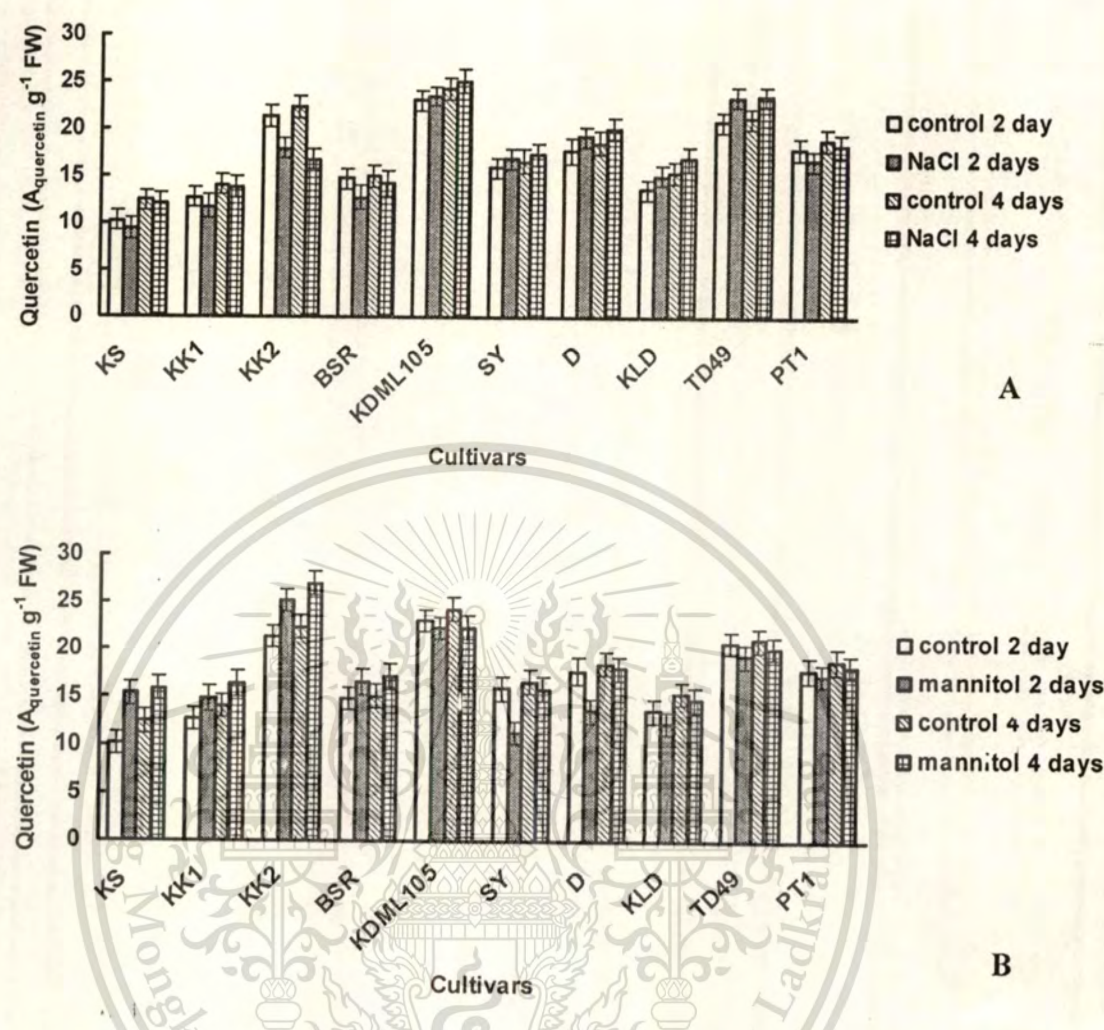

Fig. 4.9 Quantification of quercetin in Thai rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl (A) and with or without 100 mM mannitol (B) for 2 and 4 days. The absorbance values (A_{374}) of the acidified methanolic extracts of stressed-seedlings and control rice seedlings are shown as the amount of quercetin. Error bars indicate the S.E. of the five independent replicates.

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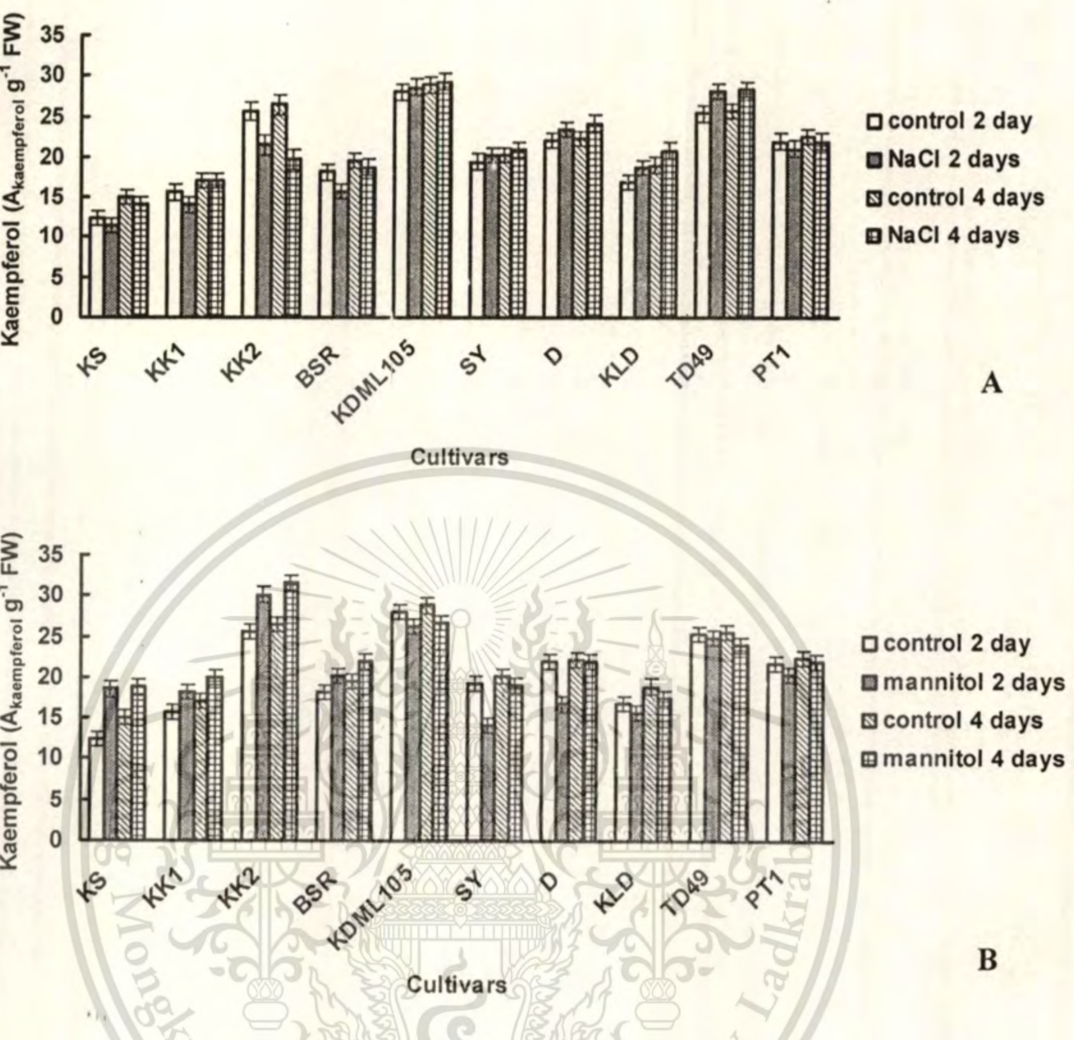

Fig. 4.10 Quantification of kaempferol in Thai rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl (A) and with or without 100 mM mannitol (B) for 2 and 4 days. The absorbance values (A_{368}) of the acidified methanolic extracts of stressed-seedlings and control rice seedlings are shown as the amount of kaempferol. Error bars indicate the S.E. of the five independent replicates.

The changes in the amount of flavones (luteotin and apigenin) followed the similar pattern to those of flavonols. Paralleling with the increase in flavonol levels, salt- and drought-tolerant cultivars exhibited 101-106% and 107-149% of control in all flavones response to 2 days of salt and drought stress, respectively. On the other hand at 4 days, the levels of flavones in salt-tolerant cultivars were about 73-96% of control whereas drought-tolerant cultivars were about 106-120% of control. However, the content of flavones in salt- and drought-sensitive cultivars

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was about 71-97% and 72-98% compared to the control, respectively after 2 and 4 days stressed (Fig. 4.11 and 4.12).

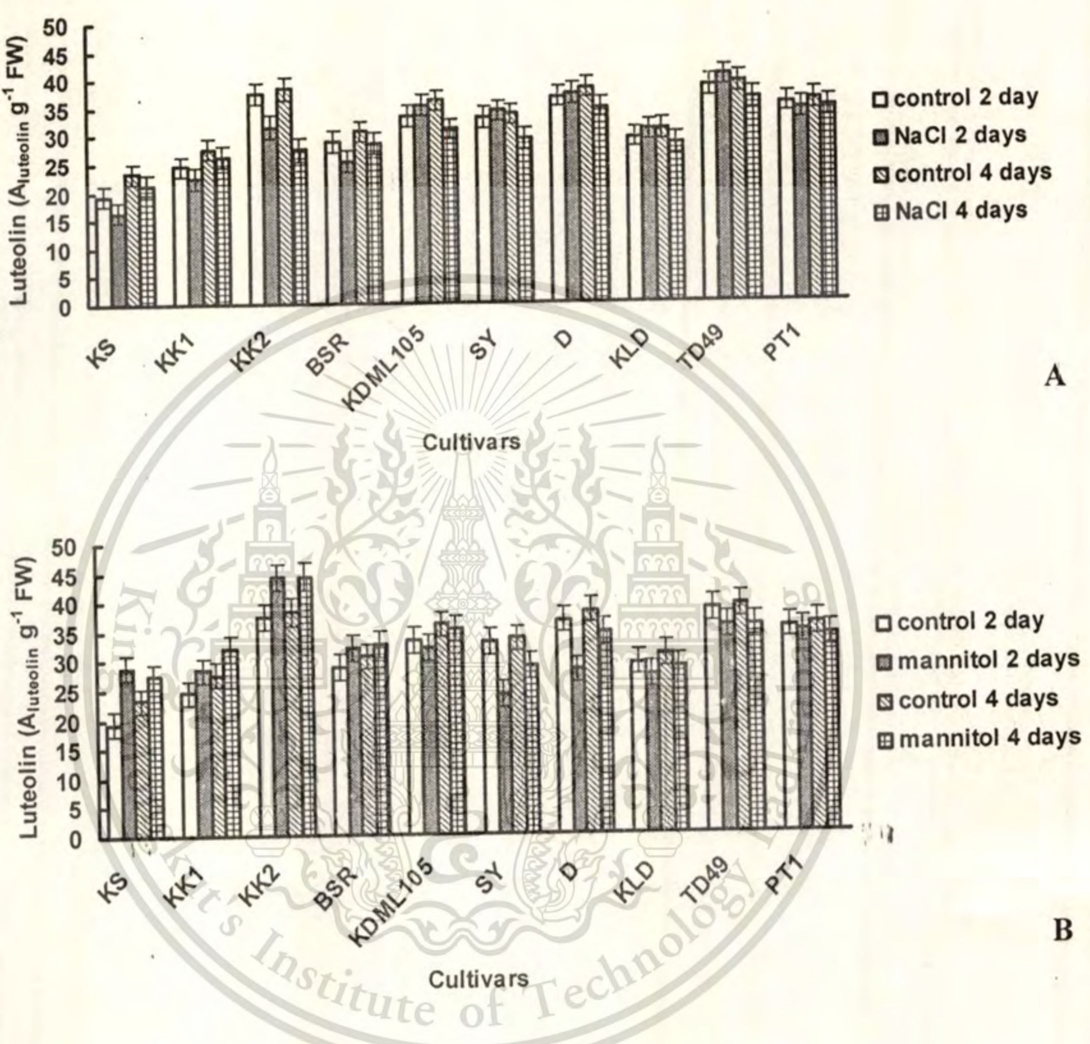

Fig. 4.11 Quantification of luteolin in Thai rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl (A) and with or without 100 mM mannitol (B) for 2 and 4 days. The absorbance values (A_{350}) of the acidified methanolic extracts of stressed-seedlings and control rice seedlings are shown as the amount of luteotin. Error bars indicate the S.E. of the five independent replicates.

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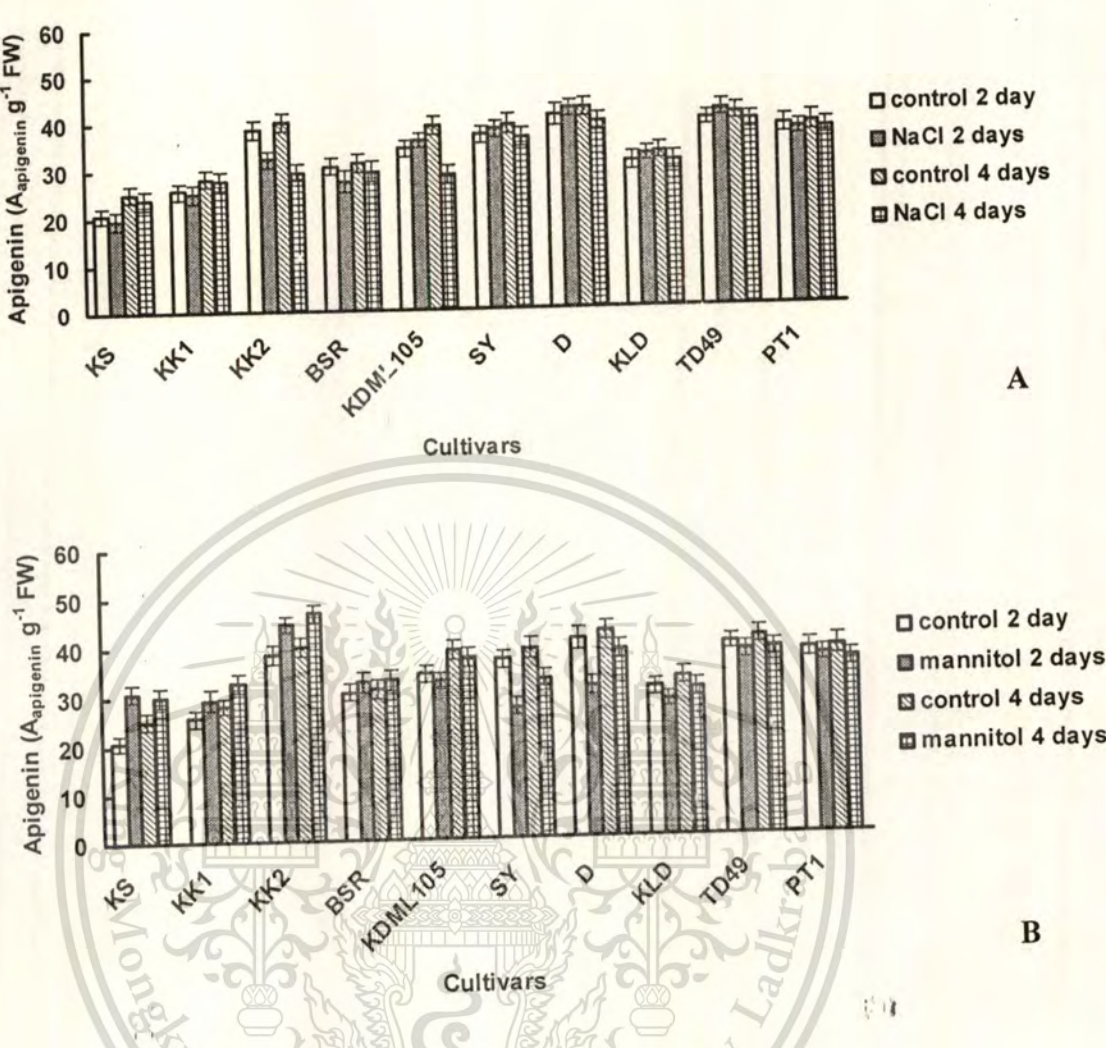

Fig. 4.12 Quantification of apigenin in Thai rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl (A) and with or without 100 mM mannitol (B) for 2 and 4 days. The absorbance values (A_{336}) of the acidified methanolic extracts of stressed-seedlings and control rice seedlings are shown as the amount of apigenin. Error bars indicate the S.E. of the five independent replicates.

After exposed to 2 and 4 days of salt or drought stress, the accumulations in the level of tannins (gallotannin) followed the similar pattern to those of anthocyanins (delphinidin, cyanidin and pelargonodin). Both stresses induced the accumulations of tannins and anthocyanins in all cultivars. Salt-tolerant cultivars were presented the increase of tannins and anthocyanins (about 137-217% and 140-388% of control, respectively) more than salt-sensitive cultivars (about 106-118% and 107-124% of control, respectively). By contrast, the decrease trend was found in PT1

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whose tannins and anthocyanin contents were about 95-96% and 87-90% less than those in the controls (Fig. 4.13A, 4.14A, 4.15A and 4.16A).

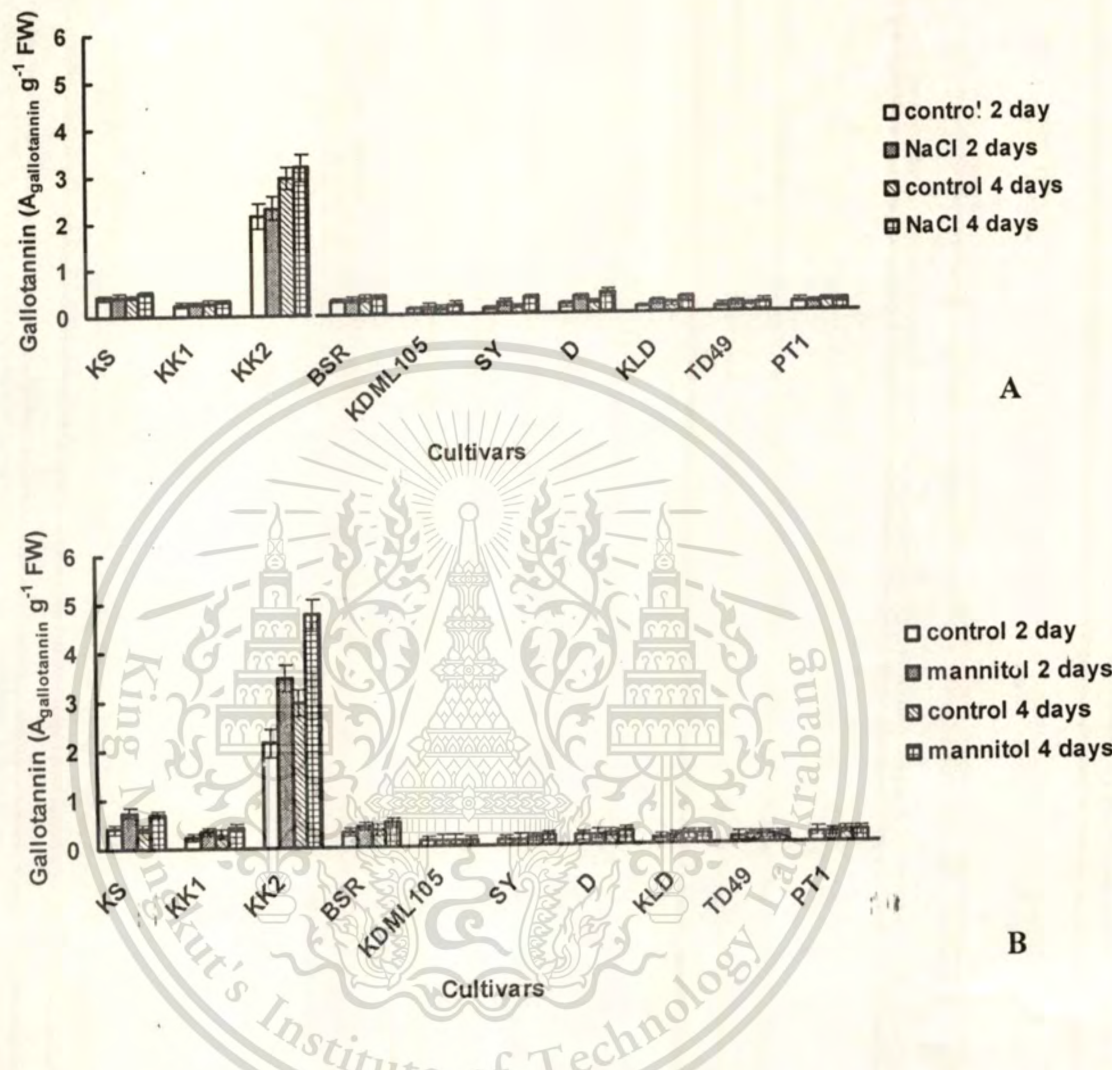

Fig. 4.13 Quantification of tannin (gallotannin) in Thai rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl (A) and with or without 100 mM mannitol (B) for 2 and 4 days. The absorbance values (A_{550}) of the acidified methanolic extracts of stressed-seedlings and control rice seedlings are shown as the amount of tannin (gallotannin). Error bars indicate the S.E. of the five independent replicates.

Under drought stress, drought-tolerant cultivars were dramatically increased in tannins and anthocyanins contents (135-182% and 119-197% of control, respectively) whereas drought-sensitive cultivars were slightly increased in tannins and anthocyanins contents (106-110% and

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104-113% of control, respectively). In contrast, PT1 were presented the decline of tannins and anthocyanins contents (94-95% and 87-98% of control, respectively) when subjected to 2 and 4 days of drought stress. The results indicated that flavonol, tannins and anthocyanins may be playing a protective role to Thai rice against salt or drought stresses in stressed-tolerant cultivars (Fig. 4.13B, 4.14B, 4.15B and 4.16B).

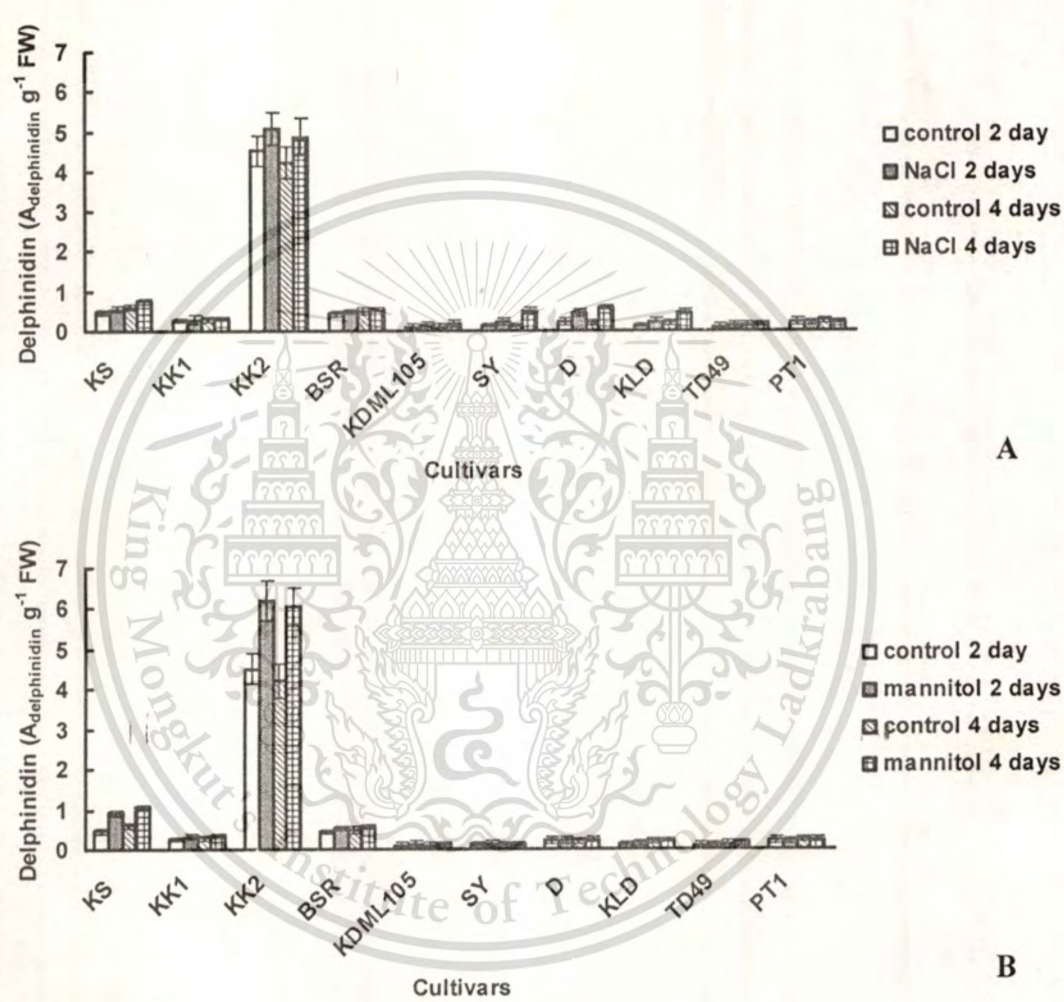

Fig. 4.14 Quantification of delphinidin in Thai rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl (A) and with or without 100 mM mannitol (B) for 2 and 4 days. The absorbance values (A_{546}) of the acidified methanolic extracts of stressed-seedlings and control rice seedlings are shown as the amount of delphinidin. Error bars indicate the S.E. of the five independent replicates.

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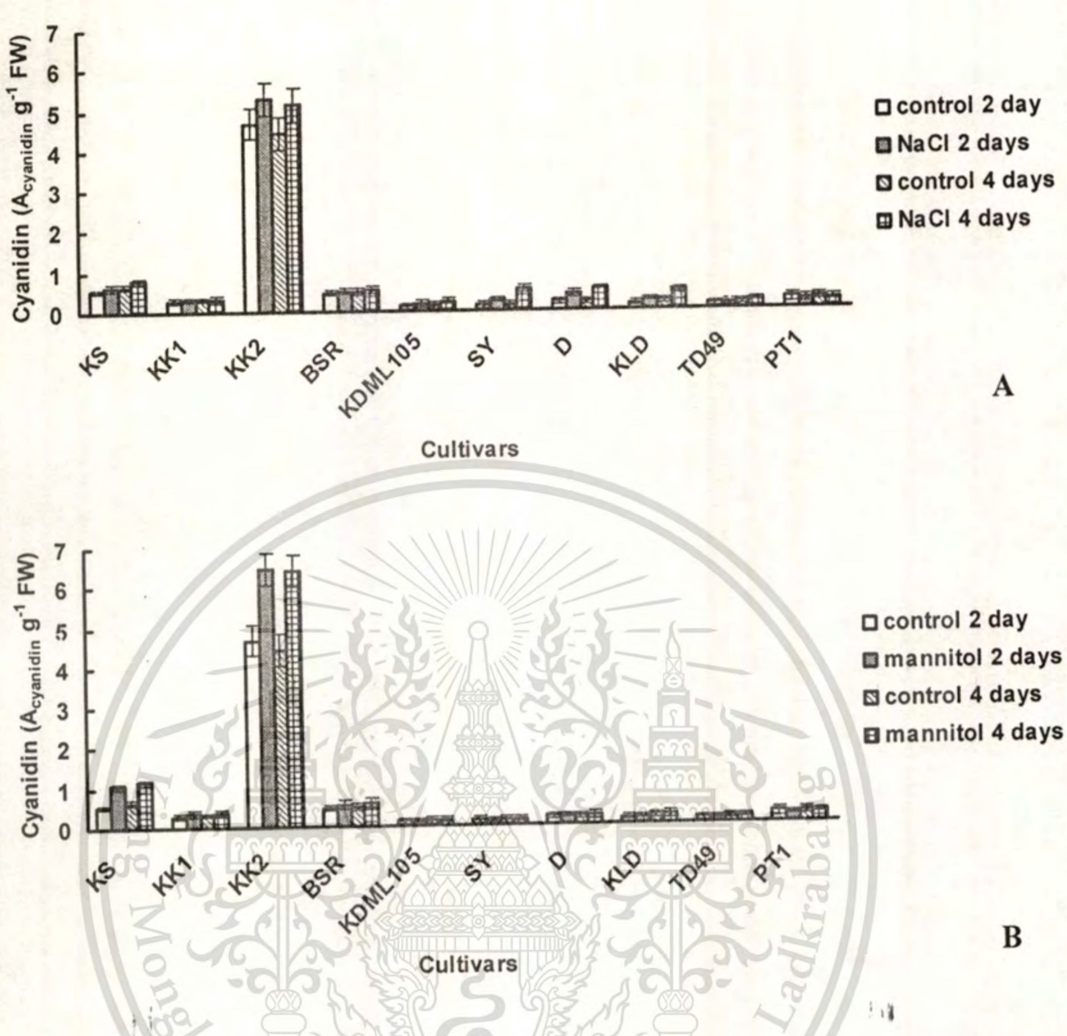

Fig. 4.15 Quantification of cyanidin in Thai rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl (A) and with or without 100 mM mannitol (B) for 2 and 4 days. The absorbance values (A_{535}) of the acidified methanolic extracts of stressed-seedlings and control rice seedlings are shown as the amount of cyanidin. Error bars indicate the S.E. of the five independent replicates.

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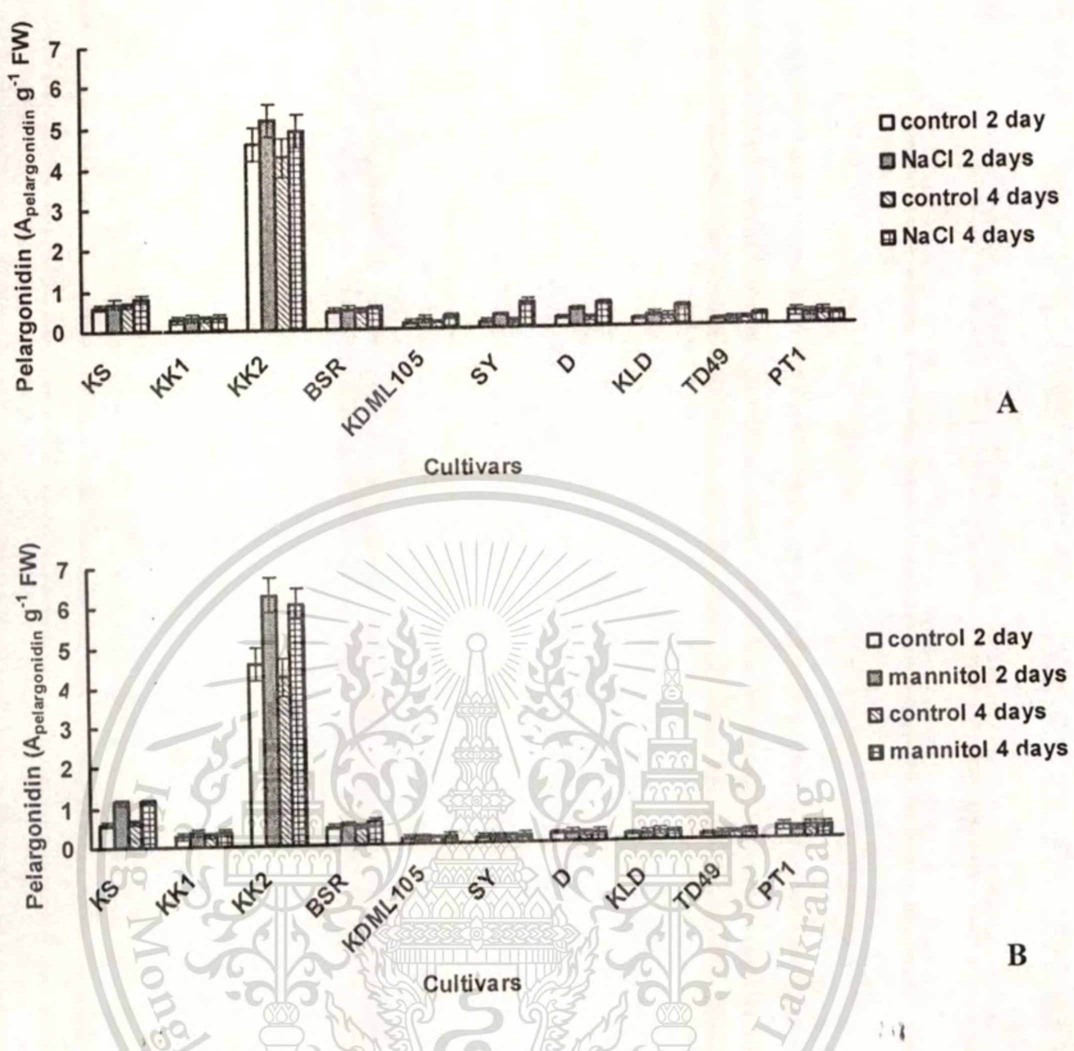

Fig. 4.16 Quantification of pelargonidin in Thai rice seedlings cultured under photoautotrophic system with or without 100 mM NaCl (A) and with or without 100 mM mannitol (B) for 2 and 4 days. The absorbance values (A_{520}) of the acidified methanolic extracts of stressed-seedlings and control rice seedlings are shown as the amount of pelargonidin. Error bars indicate the S.E. of the five independent replicates.

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4.6 Effects of salt or drought stresses on the expression of proline and flavonoids biosynthetic genes

Proline synthesis in plants is maintained mainly by the supply of the amino acids glutamate or ornithine or both. Each has been confirmed as a proline precursor (Rhodes *et al.,* 1999). Higher plant glutamate is derived from the activity of ferredoxin-dependent glutamate synthase (Fd-GOGAT). The capacity of this enzyme to supply glutamate under stress conditions is an important metabolic function during proline accumulation (Berteli *et al.,* 1995). The flavonoid biosynthetic pathway is one of the most intensively investigated pathways for elucidating many fundamental and applied biological and genetic processes. CHS and DFR are the different biosynthetic enzymes encoded produced flavonoid substances. CHS is the first enzyme in flavonoid biosynthesis pathway. DFR in the pathway is a key enzyme in the synthesis of anthocyanins (Turnbull *et al.,* 2004; Turnbull *et al.,* 2000).

In order to determine whether induction of proline and flavonoid accumulations by salt or drought stresses in Thai rice seedlings correlated with transcriptional levels. These levels were assessed by reverse transcriptase-polymerase chain reaction (RT-PCR). The expression of two key genes in proline metabolism, *P5CS* and *P5CR*, and two key genes in flavonoid metabolism, *CHS* and *DFR*, was monitored in the Thai rice seedlings of the experimental plants by RT-PCR at 2 days after salt or drought stresses.

The results from RT-PCR indicated that salt and drought treatments had effects on the expression of *OsP5CR* and *OsP5CS* in all cultivars. The significant accumulation of *OsP5CR* and *OsP5CS* in response to salt or drought stresses were observed in salt-tolerant (KDML105 and SY) and drought-tolerant (KS and KK2) cultivars, respectively. The increase in *OsP5CR* and *OsP5CS* mRNA levels was detected 10.28-28.71% and 22.83-69.07% in salt-tolerant cultivars, respectively whereas salt-sensitive cultivars were increased 1.41-2.53% and 3.59-5.74%, respectively under salt stress. In the same way, The *OsP5CR* and *OsP5CS* expression of drought-tolerant cultivars (28.43-69.81% and 26.06-38.64%, respectively) were higher than drought-sensitive cultivars (0.19-2.44% and 2.66-13.40%, respectively) (Fig. 4.17 and 4.19).

The expression of the *OsCHS* and *OsDFR* genes in rice stressed-seedlings was examined by RT-PCR. The expression of *OsCHS* and *OsDFR* was detected higher in salt- and drought-tolerant cultivars than salt- and drought-sensitive cultivars. *OsCHS* expression was detected in salt- and drought-tolerant cultivars increased 5.79-9.81% and 7.98-10.22%, respectively whereas

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salt- and drought-sensitive cultivars had the increase 0.35-3.14% and 0.71-3.57%, respectively. Like the expression of *OsDFR* gene in salt- and drought-tolerant cultivars were increased 79.29-98.48% and 58.65-81.42%, respectively whereas salt- and drought-sensitive cultivars had the increase 1.99-3.80%and 1.67-3.46%, respectively but PT1 were decreased 1.79 and 0.59% when exposed to salt and drought stresses, respectively (Fig. 4.18 and 4.20).

The results indicated that *OsP5CR* and *OsP5CS* gene expression may be playing proline accumulation in stressed-tolerant Thai rice cultivars. In addition, *OsCHS* and *OsDFR* gene of flavonoid biosynthesis pathway may be playing flavonoid accumulation especially, flavonol, tannins and anthocyanins accumulation when exposed to both stresses in Thai rice cultivars.

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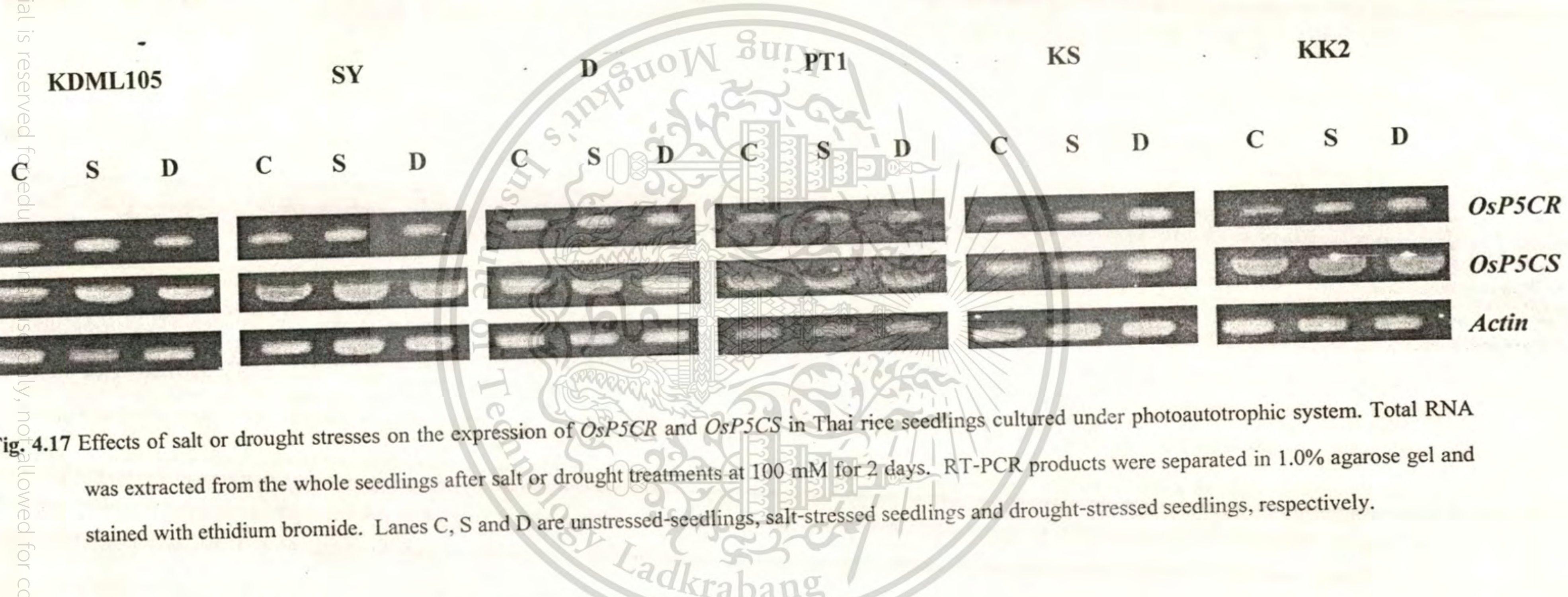

Fig. 4.17 Effects of salt or drought stresses on the expression of *OsP5CR* and *OsP5CS* in Thai rice seedlings cultured under photoautotrophic system. Total RNA was extracted from the whole seedlings after salt or drought treatments at 100 mM for 2 days. RT-PCR products were separated in 1.0% agarose gel and stained with ethidium bromide. Lanes C, S and D are unstressed-seedlings, salt-stressed seedlings and drought-stressed seedlings, respectively.

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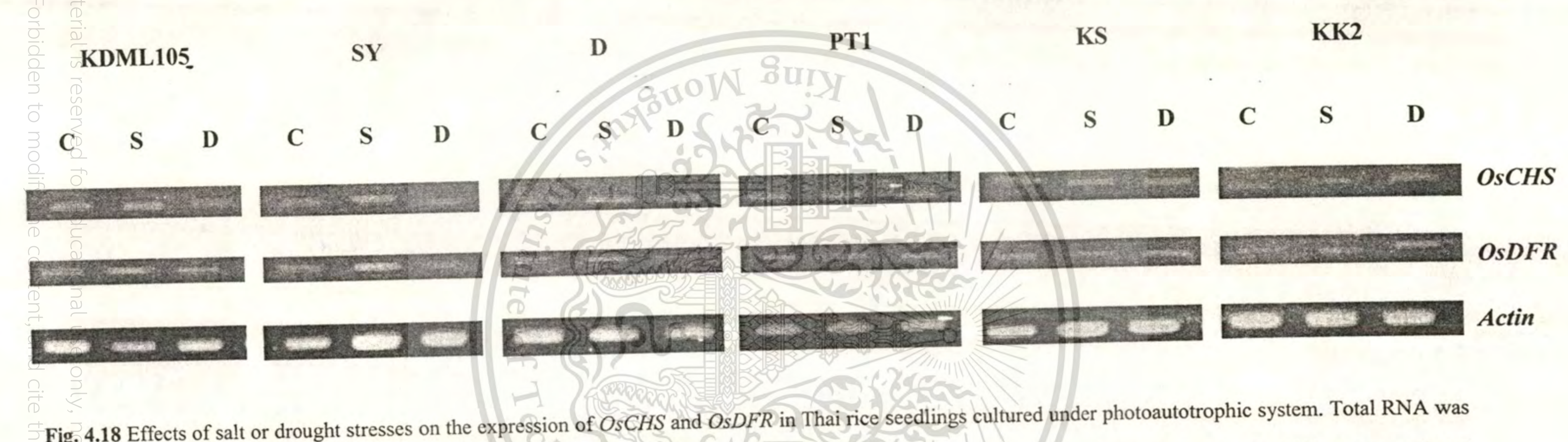

Fig. 4.18 Effects of salt or drought stresses on the expression of *OsCHS* and *OsDFR* in Thai rice seedlings cultured under photoautotrophic system. Total RNA was extracted from the whole seedlings after salt or drought treatments at 100 mM for 2 days. RT-PCR products were separated in 1.0% agarose gel and stained with ethidium bromide. Lanes C, S and D are unstressed-seedlings, salt-stressed seedlings and drought-stressed seedlings, respectively.

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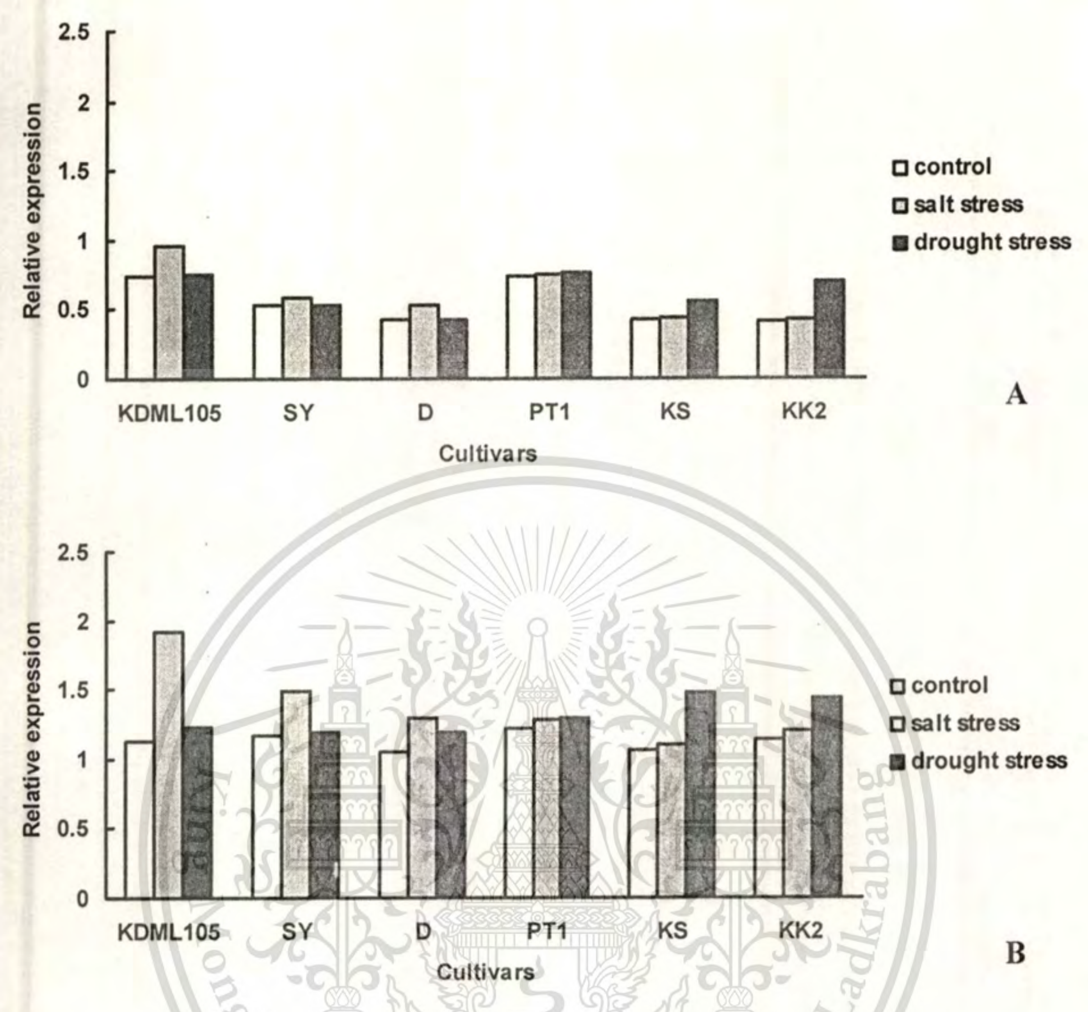

Fig. 4.19 Relative expression of proline biosynthesis genes of Thai rice seedlings cı ltured under photoautotrophic system in response to salt or drought stresses at 100 mM for 2 days. Relative expression of *OsP5CR* (A) and *OsP5CS* (B) represents the ratio of transcript level of each gene to that of *Ac:in* from the same sample.

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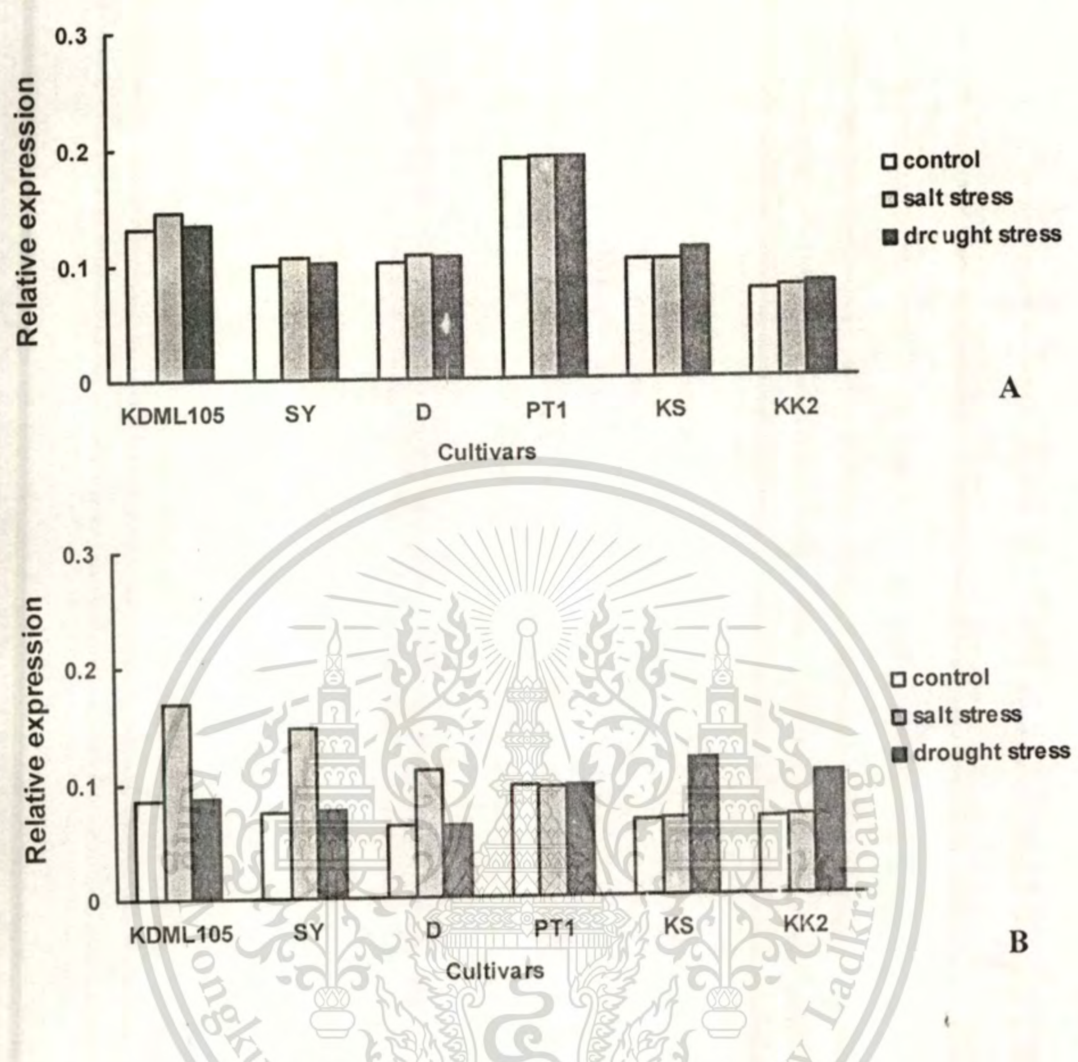

Fig. 4.20 Relative expression of flavonoid biosynthesis genes of Thai rice seedlings cultured under photoautotrophic system in response to salt or drought stresses at 100 mM for 2 days. Relative expression of *OsCHS* (A) and *OsDFR* (B) represents the ratio of transcript level of each gene to that of *Actin* from the same sample.

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CHAPTER 5

DISCUSSSION

5.1 Effect of salt or drought stresses on morphology and physiology of Thai rice

A morphological response to abiotic stress is an adjustment of plant growth such as reduction in seedling height, basal diameter, and total biomass (Munns, 2002). Under saline and drought conditions, seedlings are confronted with modification of ion balance, water status, mineral nutrition thus these seedlings had several physiological responses, including stomata behaviors and photosynthetic efficiency and a consequence of growth reduction (Lei *et al.,* 2006). In this study, stressed-tolerance cultivars were determined based on morphological and physiological characteristics, along with the small changes in FW, DW, SL, RL and RWC when compared to stressed-sensitive cultivars.

In crop plants, it has been demonstrated that the resistance to salt and drought is a quantitative trait and that RWC is a relevant tool for screening salt and drought tolerances (Teulat *et al.,* 2003; Liu and van Staden, 2001). In fact, RWC gives an idea at a specific time point of the level of water deficit, and uses as primary information about the response to abiotic stress. In this experiment, the loss of seedling turgor indicated by a decrease in relative water content (RWC) and the growth reduction were considered by the decreases in both FW and DW.

These results showed that stressed-sensitive cultivars have decrease in RWC, FW and DW more than stressed-tolerant cultivars after 2 and 4 days of both NaCl and mannitol stresses (Table 4.1, 4.2, 4.5 and 4.6). The water relations suggested that the stressed-tolerant cultivars in response to the externally imposed stress at 100 mM NaCl or mannitol have better water maintenance in plant seedling than those sensitive cultivars. The salt tolerant cultivars may achieve osmotic adjustment by lowering the osmotic potential leading to slightly decrease FW, DW, SL and RL when exposed to stress environments (Table 4.1-4.4).

Moreover osmotic stress, the NaCl stress also induced the accumulation of Na^+ and Cl^- ions which have been suggested to be one of the primary causes of growth inhibition in plants and threshold levels of these potentially toxic ions have been analyzed for various crop species (Tattini *et al.,* 1995). Elevated Na^+ and Cl^- content in environment affects many indispensable nutrients uptake through competitive interactions and by affecting the ion selectivity of membranes (Stepien and Klobus, 2006). Enzymes, organelles inside plant cell may be damaged

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by those toxic ions resulting in retard growth and development. For example, the reduction of cyclin-dependent kinase activity results in cell division reduction as well as inhibition of growth under water deficit condition (Liu and van Staden, 2001). Plant growth in response to a stress condition is largely under the control of plant hormones. Hormones, in particular ABA along with cytokinins and ethylene, have been implicated in the root–shoot signaling. An insufficient amount of ABA accumulation in the shoot would result in ethylene mediated growth inhibition, whereas, higher accumulation of ABA in the root would prevent the ethylene mediated growth inhibition (Sharp, 2002; Wilkinson and Davies, 2002). Therefore, stressed-sensitive cultivars were inhibited in SL and RL more than stressed-tolerant cultivars which may occurred from insufficient accumulation of ABA content under stress condition.

5.2 Effect of salt or drought stresses on photosynthetic pigment contents of Thai rice

During the dry season in tropical environments, high irradiance, high temperature, and water deficit cause inhibition of photosynthetic system since its sensibility. The salt stress condition leads to osmotic stress as well as drought stress in crop plants (Lefevre *et al.*, 2001). Under salt and drought conditions, restricted CO_2 availability due to stomatal closure may lead to increased susceptibility to photodamage. This evident also affect photosynthetic system by causing the depletion of chlorophyll (Hernádez and Almansa, 2002; Powles, 1984). Abiotic stress in leaves results in the destruction of photosynthetic pigments, which some extent can be attributed to the impairment of the primary photosynthetic machinery (Zgallai *et al.*, 2005; Kaiser, 1987). Inhibition of photosynthesis associated with drought stress limits a photosynthetic carbon metabolism and effects on plant growth and physiology (Hassan, 2006; Griffiths and Parry 2002; Lawlor, 2002). The important role of chlorophyll pigment, which transfer electrons for change electronic energy into chemical energy in photosystem II, in plant seedlings is absorbing light in photosynthetic system (Capellades *et al.*, 1991). Thus, the level of photosynthetic pigments has been analyzed in certain crop plants so as to understand the adaptation level. Photosynthetic pigments degradation in the stressed plants is one of the effective parameters which can be used to identify stressed-tolerance and stressed-sensitivity (Bahaji *et al.*, 2002).

Chloroplast membranes are in particular sensitive to oxidation stress damage caused by the generation of excessive amount of ROS in these membranes (Bowler *et al.*, 1992). Ion

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accumulation in leaves adversely affects chlorophyll content (Meloni *et al.*, 2003). In this experiment, the degradation of photosynthetic pigments was occurred in stressed-sensitive cultivars more than stressed-tolerant cultivars after 2 and 4 days of both stresses (Table 4.7 and 4.8). The observed decrease in chlorophyll and total carotenoid contents in the cucumber and rice plants grown under saline conditions may be attributed to both an increased degradation and inhibited synthesis of those pigments (Cha-um *et al.*, 2004; Mitsuya *et al.*, 2003; Garcia-Sanchez *et al.*, 2002; Sultana *et al.*, 1999). Stomatal closure is known to be an effective mechanism for economical water utilization under salt stress and limitation of the harmful salt ions uptake (Hasegawa *et al.*, 2000). Moreover, the reduced CO_2 assimilation rate at high salinity could be attributed, in part, to increased Na^+ concentration resulting in the reduced content of K^+ ions which are indispensable in maintaining the steady-state photosynthetic rate and contribute to better regulation of stomata closure (Cornic, 1994).

The photosynthetic systems in light and dark reaction are damaged when exposed to abiotic stress, leading to growth inhibition and productivity loss (Ali *et al.*, 2004; Mitsuya *et al.*, 2003). In this study, the chlorophyll stabilization in stressed-tolerant cultivars was comparatively tolerant owing to the better in photosynthetic parameters (Ch_A, Ch_B and total carotenoid) than stressed-sensitive cultivars. These data of stressed-seedlings directly affected in plant growth characters, which shown by the measurements of morphological change (FW, DW, SL and RL) (Table 4.1-4.4, 4.7 and 4.8).

5.3 Effect of salt or drought stresses on lipid peroxidation of Thai rice

A common effect of salt and drought stresses, similarly as of other environmental stresses, is to cause oxidative damage by generation of reactive oxygen species (ROS) which can damage cell constituents (Smirnoff, 1998). ROS are responsible for stress-dependent peroxidation of membrane lipids (Upadhyaya and Panda, 2004; Ratnayaka *et al.*, 2003; Chen *et al.*, 2000, Sreenivasulu *et al.*, 1999) protein degradation (Jiang and Zhang 2001) and nucleic acid damages (Hagar *et al.*, 1996). The MDA content is an indicator of membrane lipid peroxidation which could reflect the degree of damage at adverse condition. It is already known that free radical-induced peroxidation of lipid membrane is a reflection of stress-induced damage at the cellular level (Jain *et al.*, 2001).

Lipid peroxidation is often used as an indicator of increased oxidative damage (Jagtap and Bhargava, 1995). The results showed that MDA content in seedlings grown at both stresses were

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augmented by oxidative stress, suggesting an enhancement in lipid peroxidation of cell membrane. All Thai rice cultivars in this study exhibited an increase in MDA levels in response to both stresses (Fig. 4.3 and 4.4), indicating that they suffered from oxidative stress. The lower level of lipid peroxidation in stressed-tolerant cultivars suggests that, these cultivars are better protected themselves from oxidative damage under each stress than stressed-sensitive cultivars, especially KDML105 and KS which salt- and drought-tolerant cultivars, respectively were increased approximately 9 and 8% but PT1 which sensitive cultivar in both stresses was increased approximately 82 and 94% under salt and drought stress (Fig. 4.3 and 4.4). This result is in agreement with the previous experiments which performed experiment on stressed sesame, rice and wheat genotypes under salt and drought stresses (Khan and Panda, 2008; Fazeli *et al.*, 2007; Mandhania *et al.*, 2006).

5.4 Effect of salt or drought stresses on proline content of Thai rice

In order to counteract abiotic stress, several physiological and biochemical processes are induced or accelerated, such as compatible solutes accumulation and activation of several detoxification enzymes (Grene, 2002). The amino acid proline is one of the most widespread accumulated osmoprotectants. Free proline accumulation under stress conditions is the importance for plant adaptation during abiotic stresses. In stress conditions, proline may act as an osmotic adjustment mediator (Molinari *et al.*, 2004), a subcellular structure stabilizer (Schobert and Tschesche, 1978), a free radical scavenger (Matysik *et al.*, 2002) and a redox potential buffer (Hare *et al.*, 1998). Morover, proline is an important component of cell wall proteins (Nanjo *et al.*, 1999).

The levels of free proline content also appear to be related to resistant or sensitive characteristics. In these experiments, the amount of proline increased immediately in all stress-treated cultivars, the increase in stressed-tolerant cultivars was more than that in stressed-sensitive cultivars under both stress conditions (Fig. 4.5 and 4.6). To verify the role of proline as part of the antioxidant system during salt or drought stresses, MDA level were evaluated because it indicates lipid peroxidation, which is mediated by high levels of ROS. MDA contents obtained in this work are in agreement with the results of previous studies (Zhang *et al.*, 2006; Ghoulam *et al.*, 2002; Fu and Huang, 2001). In addition, the oxidative stress mediated is evaluated by measuring the photosynthetic pigment degradation. When plant is contacting with stresses, electron transport system was deteriorated and ROS is generated at lethal levels. This evidence led to membrane

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damage and chlorophyll loss (Claudia *et dl.*, 2008; Parida *et al.*, 2007; Liu *et al.*, 2006b; Rahnama and Ebrahimzadeh, 2004). In addition, oxygenase activity of Rubisco may increase during dehydration and antioxidant may protect Complex I (NADH: ubiquinone oxidoreductase to O_2), whereas the osmoprotectants (proline) may protect Complex II (succinate: ubiquinone oxidoreductase to O_2) because Complex I is disrupted by Na^+ as a results of oxidative stress and Complex II is disrupted by Na^+ toxicity under salt stress (Hamilton and Heckathorn, 2001). Photosynthetic pigment contents in leaf of stressed-tolerant plants are substantially higher compared to stressed-sensitive plants during the salt and drought stress periods. The protective effects of proline to the damage sustain the hypothesis of its role in ROS scavenging and osmoprotectant.

5.5 Effect of salt or drought stresses on flavonoid levels of Thai rice

Flavonoids are a functionally diverse group of secondary products with roles in pigmentation, plant-microbe interaction and reproduction. Flavonoids have also been linked to plant defense system against various stresses such as pathogens, wounding and UV light damage (Winkel-Shirley, 2001). In this experiment, the flavonoid pathway was induced during salinity or drought stresses in all Thai rice cultivars especially tannins and anthocyanin compounds (Fig. 4.13-4.16). The results demonstrate that the flavonoid accumulations of salt-tolerant and drought-tolerant cultivars to salinity or drought stresses are higher than other cultivars under specific treatment. Microarray analysis of gene expression in *Arabidopsis* where a full-length cDNA encoding anthocyanin synthase is shown to be up regulated by drought, cold or salt stress (Seki *et al.*, 2002). The exposure of rice seedlings to dehydration, high salt and ABA significantly increase in accumulation of anthocyanins in leaves (Ithal and Reddy, 2004). Anthocyanin antioxidant nature is defined mainly by the presence of a B-ring catechol group capable of readily donating hydrogen (electron) to stabilize a radical species (Rice-Evans, 2001). Flavonoids are unlikely to express beneficial action *in vivo* through out completing antioxidants such as ascorbate, which are accumulated at higher concentrations. Accumulating evidence suggests that the cellular effects of flavonoids may be mediated by their interaction with specific proteins central to intracellular signaling cascades (Schrieter *et al.*, 2002). Anthocyanins can also make complexes with other molecules (copigmentation). However, such complex molecules can protect partner compounds against oxidative damages, for example anthocyanins prevent ascorbic acid (AsA) against metal induced oxidation by forming a stable AsA-metal-anthocyanin co-ordinate

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complex. Above complex not only protects AsA from H_2O_2 and $OH^{\cdot}$, but also protects anthocyanins from oxidative damage (Sarma *et al.*, 1997).

The results of anthocyanin ROS scavenging have been reported in the protection of chlorophyll from $^1O_2$ ROS (Agati *et al.*, 2007). The data supported in the relation between the increase of anthocyanin content and the stabilization of photosynthetic pigments in stressed-tolerant cultivars more than stressed-sensitive cultivars (Table 4.7 and 4.8; Fig. 4.14-4.16). In addition, formation of the anthocyanin-DNA complex prior to expose to $OH^{\cdot}$ protected both anthocyanin and DNA from oxidative damage. Anthocyanin-DNA copigmentation may be a possible defense mechanism against the oxidative damage of DNA and may have *in vivo* physiological functions attributable to the antioxidant ability of anthocyanins (Kong *et al.*, 2003; Mas *et al.*, 2000; Sarma and Sharma, 1999). In this experiment, tannins content was increased in stressed-tolerant cultivars more than stressed-sensitive cultivars under both stresses. Tannin is beyond the branch to anthocyanin in flavonoid biosynthesis and has antioxidant activity in oxidative stress as well as anthocyanins (Winkel-Shirley, 2001; Yamasaki *et al.*, 1997). The relation between tannins and abiotic stress tolerance has been a few studies. Thus, these results demonstrate that tannins and anthocyanin products may play an important *in vivo* role in plant protection from oxidative stresses of salinity and drought.

5.6 Effects of salt or drought stresses on the expression of proline and flavonoids biosynthetic genes

Proline is a dominant organic molecule that accumulates in many organisms which exposured to environmental stresses, e.g., drought, high salinity, elevated temperatures, freezing, UV radiation, and heavy metals (Kuznetsov and Shevyakova, 1997; Kavi Kishor *et al.*, 1995; Saradhi *et al.*, 1995). The accumulation of proline in response to decreasing osmotic potential or water deficit has been observed in bacteria, algae and plants. This accumulation is a primary response against stresses (Voetberg and Sharp, 1991) and correlated with *P5CS* gene transcription in many plants such as tomato (Dombrowski *et al.*, 2008), potato (Schafleitner *et al.*, 2007), alfalfa (Ginzberg *et al.*, 1998), Arabidopsis (Yoshiba *et al.*, 1995) and rice (Igarashi *et al.*, 1997). *P5CR* gene encoding enzyme at secondary step of proline biosynthesis had a few reports. Plant P5CR cDNA were isolated from soybean, pea and *A. thaliana* (Delauney and Verma, 1993; Verbruggen *et al.*, 1993) The previous experiments reported that P5CS has a key role in the

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biosynthesis of proline under abiotic stress (Dombrowski *et al.,* 2008; Hong *et al.,* 2000; Ginzberg *et al.,* 1998; Yoshiba *et al.,* 1995). The relationship between the gene expressions of *P5CS* and *P5CR* and the accumulation of proline under osmotic stress has been characterized previously in *A. thaliana*. These studies have been revealed that the induction of the *OsP5CS* and *OsP5CR* gene precedes the accumulation of proline. Proline biosynthesis was regulated at the levels of both enzyme activity and gene expression of two enzymes, P5CS and P5CR in biosynthetic pathway.

RT-PCR of gene involved in proline biosynthesis showed significant differences in the level of expression between the control and the treatments (Fig. 4.17 and 4.18). In *A. thaliana* and rice, the gene encoding for *P5CS* is induced by high salt and dehydration, and the simultaneous accumulation of proline is observed as a result of both of the previous treatments (Yoshiba *et al.,* 1995). However, unlike in *A. thaliana* the accumulation and gene expression of proline in rice is observed only as a result of high salt treatment (Igarashi *et al.,* 1997) while this experiment presented the increase of gene expression in *OsP5CS* and *OsP5CR* under salt or drought stress. The finding that the salt or drought stress induced the *P5CS* and *P5CR* gene, that encodes for the key enzyme P5CS and P5CR, which catalyses the two steps of proline biosynthesis from glutamate in plants (Tripathi *et al.,* 2007 Sawahel and Hassan, 2002; Roosens *et al.,* 1999). These stresses inducible expression of the *OsP5CS* and *OsP5CR* genes resulted in an increased proline accumulation and related to the observed proline content in the seedlings of the Thai rice cultivars, which the higher expression of the *OsP5CS* and *OsP5CR* genes and the accumulation of proline content in stressed-tolerant cultivars than stressed-sensitive cultivars (Fig. 4.5, 4.6, 4.17 and 4.19). Other phenomena rather than transcriptional or post-transcriptional regulation of the *OsP5CS* and *OsP5CR* gene leading to differential proline accumulation in the seedlings of the tolerant and sensitive cultivars appear to play a role in the response to abiotic stress conditions (Roosens *et al.,* 2002; Gregorio *et al.,* 2002; Babu *et al.,* 2001; Hong *e al.,* 2000).

Flavonoids accumulation and transcript levels of the flavonoid biosynthetic genes are measured because phenotypically indistinguishable tissue might show differences at transcription level under a given stress (Pourcei *et al.,* 2006). Among the given treatments in this experiment, salinity and drought were found to be significantly more effective in activating the flavonoid pathway in Thai rice seedlings. The two genes of the flavonoid pathway, *OsCHS* and *OsDFR* encoding chalcone synthase and dihydroflavonol reductase, respectively, catalyze steps of the flavonoid pathway and relate to the production of anthocyanins. *OsCHS* and *OsDFR* genes are

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found to be responsive to both stress treatments showing enhanced transcripts in about 2 days after treatment (Fig. 4.18 and 4.20). The observed differences in the pattern of stress-induced accumulation of transcript of the *OsCHS* and *OsDFR* are somewhat similar to the reported differences in gene expression patterns of the maize orthologs under light and cold treatments of seedlings (Chiristie *et al.,* 1994; Taylor and Briggs, 1990). The observed expression pattern of the *OsCHS* and *OsDFR* transcript is in agreement with the results of microarray analysis of gene expression in *Arabidopsis*, which a full-length cDNA encoding CHS and DFR enzyme, is shown to be up-regulated by drought, cold or high salt stress (Seki *et al.,* 2002). The results have shown that the flavonoid biosynthesis genes were stress responsive. Further, the results suggest a possible role of the salinity and drought in stress responsive expression of *OsCHS* and *OsDFR* genes in Thai rice.

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CHAPTER 6

CONCLUSIONS

In summary, this study has shown that proline and flavonoid accumulations are salt or drought stresses response. Characterization of FW, DW, RWC and photosynthetic pigments containing of Thai rice seedlings, including the MDA level showed that salt-tolerant or drought-tolerant Thai rice cultivars have more better character than sensitive cultivars. These Thai rice cultivars produced significantly more proline and flavonoids, especially tannins and anthocyanin compounds, accumulation patterns. Proline may serve as osmoprotectant and antioxidant, which may also play as important role in protecting organelles, enzymes and protein in plant cell from salt, drought and oxidative stresses. Moreover, production of flavonoids, especially tannins and anthocyanin required to protect Thai rice seedlings from induced oxidative stress in both stresses. The evident correlations between the transcript up-regulation of gene with proline and flavonoid accumulation from both biosynthesis pathways under salt or drought stresses were observed. These results had parallel with the changes in *OsP5CS* and *OsP5CR* gene expression and proline content in proline biosynthesis. The *OsCHS* and *OsDFR* gene expressions in flavonoid biosynthesis change in parallel with tannin and anthocyanin accumulations. It is possible that the function and/or stabilization of *OsP5CS* and *OsP5CR* genes transcript were the necessary roles for proline biosynthesis as well as *OsCHS* and *OsDFR* were mainly functioned in flavonoid biosynthesis under abiotic stresses. Therefore, damage prevention of stressed rice seedlings required the synergic work between proline and flavonoids from salt and drought stresses including oxidative stress which induced by both stresses in Thai rice seedling cultivars.

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