METABOLIC EXPRESSION PROFILE IN VARIOUS VARIETIES OF THAI RICE RESPONSE TO LOW TEMPERATURE

CHAPTER 1

INTRODUCTION

Rice (*O. sativa* L.) is the most economically important crop of Thailand. Among all varieties, the Indica rice is cultivated throughout the country, as well as received the extensive interest in improving grain quality and yield. However, Indica rice is indigenous to tropical climate (Yoshida *et al.*, 1996) and sensitive to low temperature (LT) that commonly occurs in the winter season on the upland of the northern part. Therefore, LT is the limiting factor for rice cultivation and productivity in this area. To develop LT-tolerant varieties of rice, it is important to understand the responses and protective mechanisms against LT in plants.

The responses to LT at metabolic level have been extensively studied in many plants. It was found that LT induced the changes of plant metabolome (Kaplan *et al.*, 2004). In addition, many studies indicated the correlation between the level of LT tolerance and the accumulation of some metabolites, suggesting the role of these metabolites in protecing plants against LT (Pillai and Akiyama, 2004).

Therefore, the comparative study on metabolic accumulation patterns between LT-tolerant and sensitive rice varieties in response to LT may contribute to the characterization of metabolites that provide the protective roles against LT to rice plants. In addition, the study on the expression of genes involved in biosyntheses of those metabolites may bring about more insights into plant responses to LT at molecular level. Differential responses to LT at metabolic and molecular levels may be used as marker for screening of LT-tolerant rice varieties, as well as they may facilitate the generation of transgenic rice with the ability to withstand LT stress.

CHAPTER 2

LITERATURE REVIEW

Rice is one of the most important crops cultivated worldwide. Rice plants originated in tropical and subtropical climates can be categorized into 3 major subspecies: Indica, Japonica and Javanica.

LT is the major limiting environmental factor affecting rice cultivation and productivity in temperate areas and at high altitude in the tropics (Bertin *et al.*, 1996). Many studies indicated the detrimental effects of LT on rice plants at any stage of growth and development, ranging from germination to maturity. During the early growth stages in rice, the occurrence of LT stress affects seed germination, leading to poor seedling establishment and subsequent decrease in vigor of vegetative growth (Zhang *et al.*, 2003). In addition, LT at the reproductive stage in rice can lead to the decrease in flowering and pollen production, especially in LT-sensitive varieties. It also causes male sterility that in turn contributes to massive yield loss.

Plant adaptation to LT stress is associated with biochemical and physiological changes. Under LT condition, plants respond by perceiving stress signal, relaying signal into cells by generating second messengers and initiating phosphorylation cascades. These signals can activate or inhibit transcription factors, which control the expression of genes (Sung *et al.*, 2003; Mahajan and Tuteja, 2005). Protein products derived from of gene transcription can be classified into 2 groups. The first group includes protein factors involved in regulation of signal tranduction or gene expression. The second group contains functional proteins, such as water channel proteins, molecular chaperones, late-embryogenesis abundant (LEA) proteins, reactive oxygen species (ROS)-detoxifying enzymes and key enzymes for metabolisms of protective metabolites, for example polyamines, compatible solutes and flavonoids (Maruyama *et al.*, 2004).

Polyamines are low molecular weight polycations that are found in both free bases and conjugated or bound forms. Three major polyamines (putrescine, spermidine, spermine) have been implicated in a wide range of biological processes including DNA replication, cell division, growth, development, leaf senescence, as well as environmental stress responses (Bouchereau *et al.*, 1999). Regarding to their polycationic nature at a physiological condition, polyamines can bind strongly to the negative charges in cellular components, such as nucleic acid, phospholipids, proteins and may help these substances to maintain their integrity and functions under LT

condition (Pillai and Akiyama, 2004). Polyamines can inhibit programmed cell death by directly functioning as ROS scavengers or inhibiting the activities of ROS generating enzymes, such as NADPH oxidase (Papadakis *et al.*, 2005).

Compatible solutes are the low molecular weight, highly soluble compounds. These solutes can be categorized into 3 types including quaternary ammonium compounds, amino acids and sugars or sugar alcohols (Ashraf and Foolad, 2007). The major functions of these compounds are to maintain turgor pressure of cells and protect plants against LT-induced dehydration. However, analysis of transgenic plants reveals other plausible protective roles of compatible solutes, such as stabilizing the quaternary structures of complex proteins or enzymes, maintaining membrane integrity and scavenging ROS (Chen and Murata, 2002). Moreover, proline is the source of carbon, nitrogen and energy during recovery from stress (Kishor *et al.*, 2005). Proline biosynthesis mediates higher $NADP^+$/NADPH ratio that leads the carbon flux to pentose phosphate pathway, providing the precursors for the biosynthesis of flavonoids and other phenylpropanoid compounds (Shetty, 2004).

Flavonoids are ubiquitous plant secondary metabolites with a vast array of biological functions. They have received the considerable attention for their antioxidative properties as components in human diets and ROS scavengers of plants (Pourcel *et al.*, 2006). Yamasaki *et al.* (1997) indicated that flavonoids can detoxify H_2O_2 by working in concert with peroxidase.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Plant materials and tissue culture systems

Seeds of various varieties of Thai rice were derived from Rice Research Center at Sanpatong, Chiangmai, Thailand. These varieties consist of SMGC02002, Namroo, SPTC80182, Supanburee 1 and KDML105. All plant cultures were performed in plant tissue culture room with the controlled temperature and light systems.

3.2 Reagents and laboratory apparatus

1. Standard chemicals, namely L-proline, sucrose, polyamines (putrescine, spermidine and spermine) (Sigma)
2. Chemicals used to analyze chlorophyll, proline, total soluble sugars, polyamines and flavonoids
3. Chemicals used to prepare NB medium
4. Laboratory apparatuses, including
   - Glass wares
   - Mortars and pestles
   - Spatulas
   - 15- and 50-ml centrifuge tubes
   - Micropipettes
   - Cylinders

3.3 Instruments

1. High pressure liquid chromatography (HPLC) system, composed of LC-10AD VP pump, SDP-10A VP UV-VIS detector, C-R7A plus chromatopac (SHIMADZU) and equipped with Inertsil ODS-3 column (4.6 mm × 250 mm, GL Sciences Inc.)
2. Spectrophotometer (BioMate 3, Thermo Electron Corp.)
3. Centrifuge (Mikro 22R, Hettich Zentrifugen)
4. Laminar air flow cabinet (HS123, ISSCO)

3.4 Methods

3.4.1 Plant materials, growth conditions and low-temperature treatment

Seeds of various varieties of Thai rice were dehusked and then surface-sterilized by soaking successively in 70% ethanol for 10 min, 5% clorox for 60 min and 30% clorox for 30 min. After extensive washing with sterilized water, seeds were germinated on NB medium and grown under conditions of 16 h photoperiod and 26°C/23°C day/night temperature.

Rice seedlings at the 2-leaf stage with uniformity in appearance were subjected to LT by growing at 26°C/10°C for 7 days while the control seedlings were maintained at 26°C/23°C. Samples were harvested at the end of night period of the 7^{th} day, immediately frozen in liquid nitrogen and kept at -80°C until the samples are used.

3.4.2 Analysis of relative growth rate

Rice plants after 0 and 7 days of treatment were collected and then dried at 70°C for 24 h. Dry weight (DW) was measured after allowing samples to cool down to room temperature in a desiccator. Relative growth rate (RGR) was calculated as the following formula (Noggle and Fitz, 1976).

$$RGR = \frac{\ln DW_7 - \ln DW_0}{7}$$

DW_0 and DW_7 are dry weight of sample at 0 and 7 days of low-temperature treatment, respectively. The denominator (7) represents total days of treatment.

3.4.3 Chlorophyll contents

Chlorophyll a and b were measured as described by Cha-um *et al.* (2005). Aerial parts of rice plants were ground in liquid nitrogen. Then, the fresh powder (50 mg) was extracted with 1.5 ml acetone and allowed to stand for 48 h at 4°C. After that, the sample was centrifuged at 2,000 g for 5 min and the absorbance of the extract was measured at 663 and 645 nm (A_{663} and A_{645}, respectively). The chlorophyll contents were calculated as the following formulas and represented as μg/g fresh weight (μg/g FW).

$$\text{Chlorophyll a} = 9.784A_{663} - 0.99A_{645}$$

$$\text{Chlorophyll b} = 21.42A_{645} - 4.65A_{663}$$

3.4.5 Metabolite analyses

1) Total soluble sugar contents

The extraction and analysis of total soluble sugars were performed as described in Shou *et al.* (2004). Fresh powder (0.1 g) of whole rice plants was extracted with 80% ethanol for 15 min at 80°C, shaken for 1 h at room temperature and allowed to stand overnight at 4°C. Then, the insoluble residuals were removed by centrifugation at 2,000 g for 10 min. After that, the extract, diH_2O and chloroform with the same volume (400 µl each) were mixed, vortexed vigorously and then centrifuged at 2,000 rpm for 10 min to removed chlorophyll.

The aqueous phase was diluted to 10- fold and analyzed for total soluble sugar contents using phenol-sulfuric acid method. 500 µl sample was mixed with 500 µl of 5% phenol, then 2.5 ml sulfuric acid was rapidly added to the mixture. After vortexing and allowing to stand for 10 min, the reaction mix was cooled down using tap water. The absorbance was measured at 490 nm. The concentration of the total soluble sugars was determined by comparing the absorbance with the standard graph of sucrose and represented as µg/g FW.

2) Proline content

Fresh powder (0.2 g) of whole rice plants was extracted with 3% sulfosalicylic acid by shaking for 1 h at room temperature. After allowing to stand overnight at 4°C, the sample was centrifuged at 2,000 g for 30 min. The supernatant was used to analyze for proline content using acid-ninhydrin method (Gilmour *et al.*, 2000). The mixture of equal volume (450 µl) of the extract, glacial acetic acid and acid-ninhydrin reagent was heated in the boiling water for 45 min. After rapid cooling, the reaction mixture was partitioned against the equal volume of toluene and shaken for 10 min at room temperature. The absorbance of organic phase was measures at 520 nm. The concentration of proline was determined by comparing the absorbance with the standard graph and represented as µg/g FW.

3) Polyamine contents

Free polyamines (putrescine, spermidine and spermine) were quantified by the protocol of Flores and Galston (1982). Fresh powder (0.1 g) of whole rice plants was extracted with 1 ml of 5% perchloric acid for 1 h in an ice bath. Then, the sample was centrifuged at 2,000 g for 25 min to remove the insoluble residuals. Next, 750 µl extract was mixed with 1 ml of 2 N NaOH and 10 µl benzoyl chloride. The mixture was vortexed for 10 s and incubated by shaking

at 37°C for 20 min. After that, 2 ml saturated NaCl was added to stop benzoylation reaction.

Benzoyl polyamines were extracted with 2 ml diethyl ether by shaking for 30 min at room temperature. Then, 1 ml of the ether phase was collected, evaporated to dryness, redissolved in 100 µl methanol and stored at -20°C until the samples are used.

The samples were then analyzed by HPLC system (SHIMADZU) equipped with Inersil ODS-3 column and eluted isocratically with 64% methanol at the flow rate of 1 ml/min. The seperated peaks were monitored by the absorbance at 254 nm and reported as peak area. The concentration of each polyamine was determined by comparing the peak area with the corresponding standard graph and represented as µmol/g FW.

4) Analysis of flavonoid levels

Fresh powder of whole rice plants was extracted overnight with 60% of methanol/HCl (99:1) at the ratio of 1 g sample/4 ml solvent. After that, chlorophyll was removed from the extract by adding chloroform, vortexing vigorously and centrifuging at 1,000 g for 10 min. The level of each flavonoid was determined as the absorbance at the specific wavelength as described in Harborne (1998).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Physiological responses

In this study, the effects of LT on 5 varieties of Thai rice were determined at physiological level, including the changes in relative growth rate and chlorophyll contents. The differences in physiological responses between LT-treated and control plants were used as the criteria for evaluating the LT tolerant ability of rice.

Seedlings of all rice varieties exhibited the growth inhibition after LT treatment (10°C in the dark period for 7 days). The levels of inhibition of SPTC80182, SMGC02002 and Numroo were lower than that of Supanburee 1 and KDML105 (figure 1).

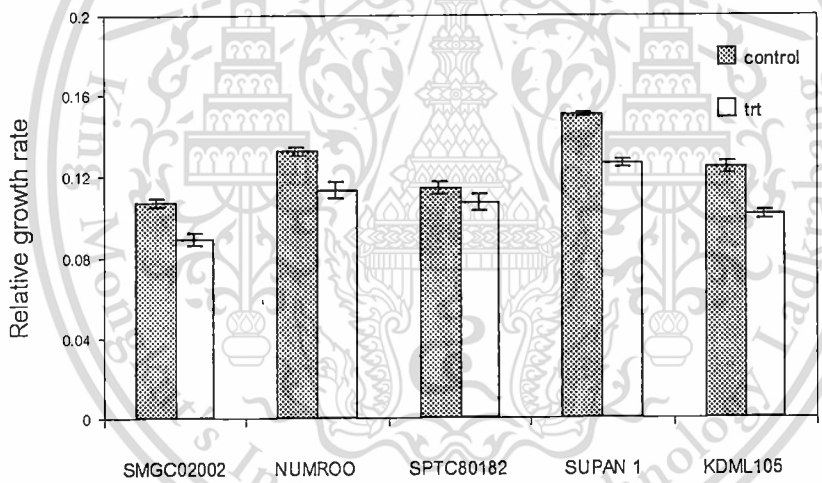

Figure 1 Relative growth rate of control (grey bar) and LT-treated (blank bar) rice seedlings All values are mean ± SE of results from 6 replications.

The similar trend was observed for the chlorophyll contents. Interestingly, LT-treated seedlings of SMGC02002 exhibited the higher levels of both chlorophylls than the control plant. However, this change was not significant (figure 2).

Therefore, the rice varieties used in this study could be separated in to two groups: LT-tolerant and LT-sensitive groups, based on these physiological responses. LT-tolerant varieties consist of SPTC80182, SMGC02002 and Numroo while LT-sensitive varieties include Supanburee 1 and KDML105.

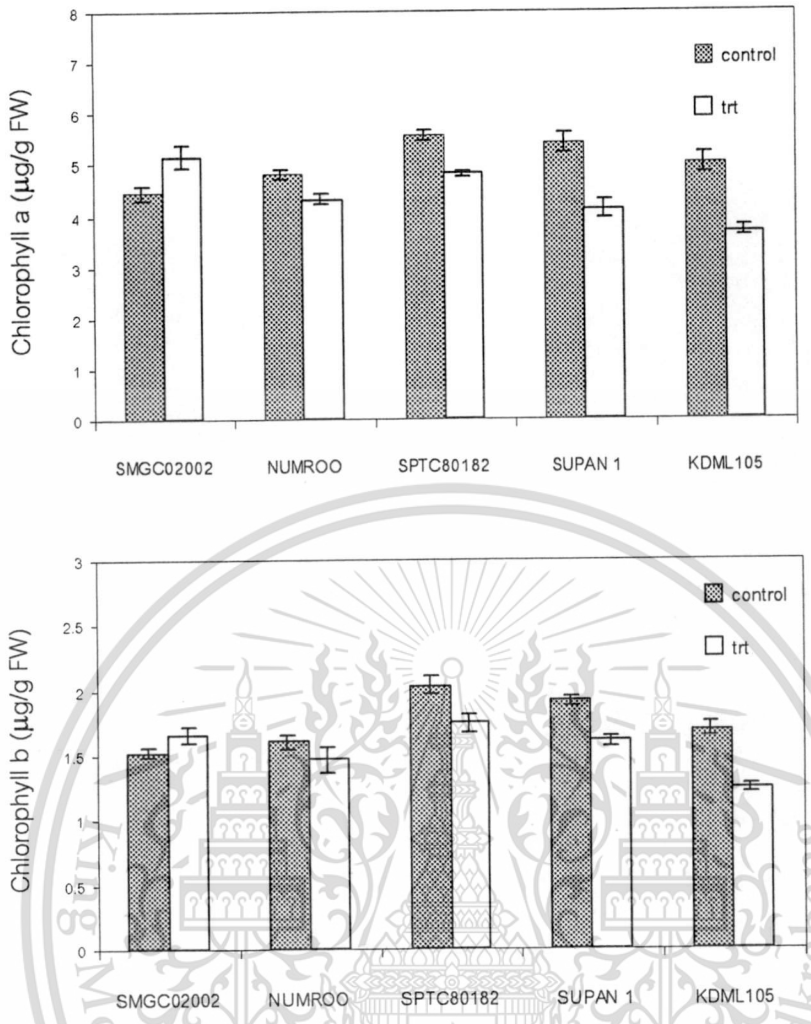

Figure 2 Chlorophyll contents of control (grey bar) and LT-treated (blank bar) rice seedlings All values are mean ± SE of results from 6 replications.

4.2 Metabolic responses

The changes in levels of some metabolite involved in stress protection were examined in both LT-tolerant and sensitive varieties. After LT treatment, the levels of total soluble sugars as well as proline in LT- sensitive group were increased with the greater extent than those in LT-tolerant group (figure 3 and 4).

LT also effected the levels of 3 major polyamines (putrescine, spermidine and spermine). It was found that the level of putrescine increased in almost all varieties in response to LT. The same trend was observed for spermidine level, except those in LT-tolerant SPTC80182. There was the lower level of spermine in all varieties, except Supanburee 1, when compared with there own controls (figure 5).

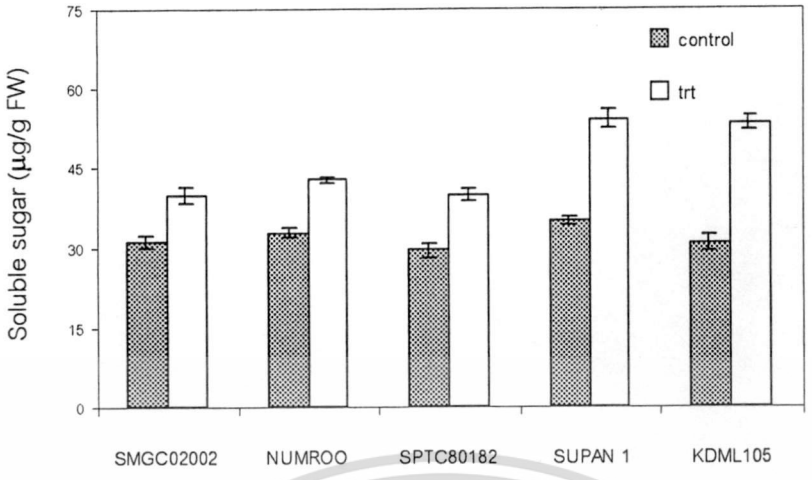

Figure 3 Soluble sugar levels of control (grey bar) and LT-treated (blank bar) rice seedlings

All values are mean ± SE of results from 6 replications.

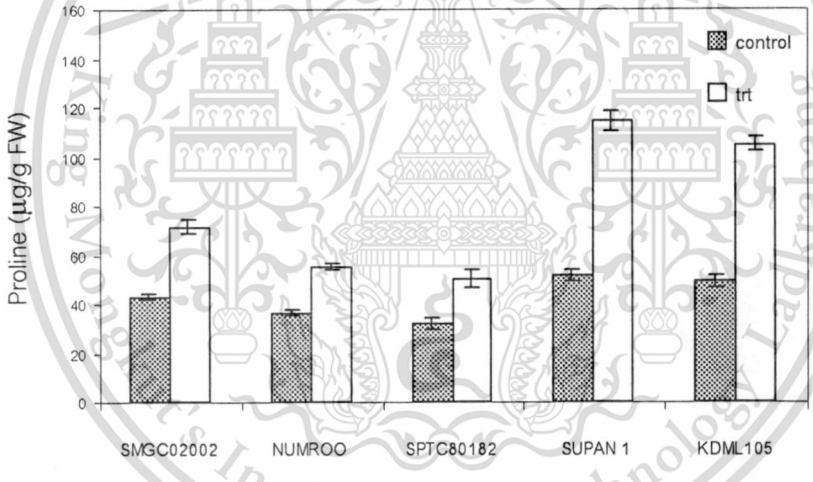

Figure 4 Proline levels of control (grey bar) and LT-treated (blank bar) rice seedlings

All values are mean ± SE of results from 6 replications.

The relative values of polyamines (level of polyamine in control plants / level of the same polyamine in LT-treated plants) were also compared. It was found that the most increased polyamine in LT-tolerant group was putrescine, while that in LT-sensitive group was spermidine.

The correlation between LT tolerance and changes in flavonoid levels was unclear. When compared with the control plants, the decrease in anthocyanin as well as the increase in flavonol and flavone levels were observed in LT-tolerant SMGC02002 and SPTC80182. However, the

opposite trend was present in Numroo, the LT-tolerant varieties, and all LT-sensitive varieties (figure 6).

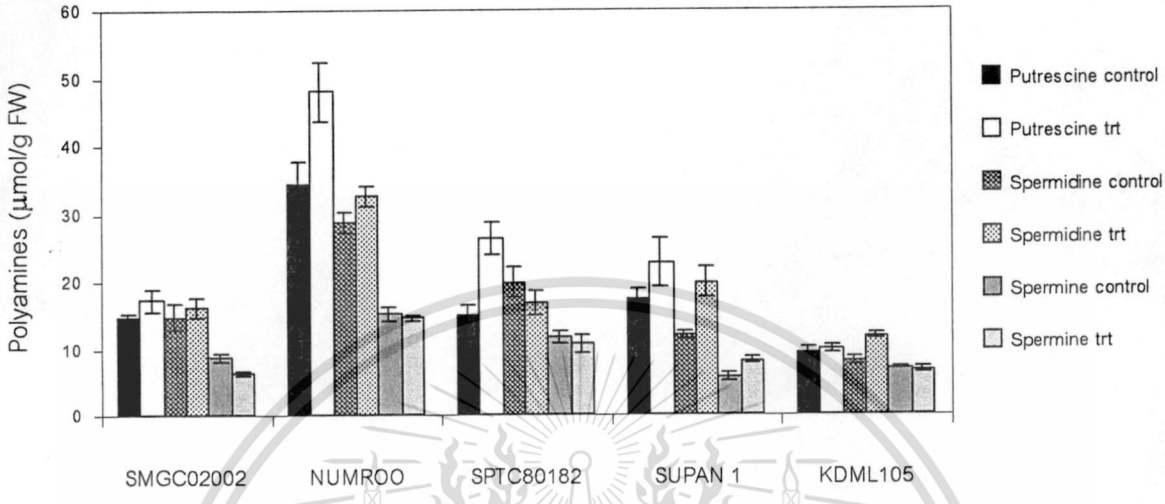

Figure 5 Polyamine levels of control and LT-treated rice seedlings
All values are mean ± SE of results from 6 replications.

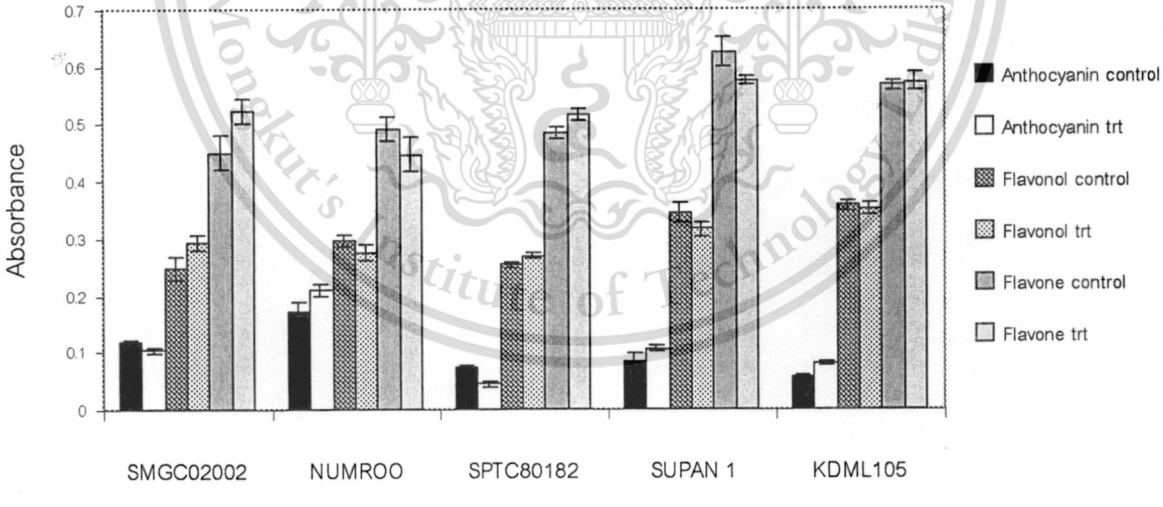

Figure 6 Anthocyanin levels of control and LT-treated rice seedlings
All values are mean ± SE of results from 6 replications.

CHAPTER 5

CONCLUSION

The differential responses of LT-tolerant and sensitive varieties were observed after LT treatment. These included the changes in relative growth rate, chlorophyll contents as well as levels of soluble sugar, proline and polyamines. The results of this study may be used as marker for screening of LT-tolerant rice varieties as well as facilitate the generation of transgenic rice with the ability to tolerate LT stress.

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