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

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หัวข้อวิทยานิพนธ์	รูปแบบการแสดงออกทางเมแทบอลิซึมของข้าวไทยสายพันธุ์ต่างๆตอบสนองต่ออุณหภูมิต่ำ
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บทคัดย่อ

ในงานวิจัยนี้ ได้ตรวจสอบผลกระทบของอุณหภูมิต่ำต่อการเปลี่ยนแปลงทางสรีรวิทยา การสะสมสารเมแทบอไลท์ รวมถึงระดับการแสดงออกของยีนในข้าวไทยจำนวน 5 สายพันธุ์ ได้แก่ สุพรรณบุรี1 ขาวดอกมะลิ105 SPTC80182 SMGC02002 และน้ำรู

ต้นกล้าข้าว 3 สายพันธุ์ ได้แก่ SPTC80182 SMGC02002 และน้ำรู ที่ได้รับอุณหภูมิต่ำ แสดงการลดลงของอัตราการเจริญเติบโตจำเพาะและปริมาณคลอโรฟิลล์น้อยกว่าต้นกล้าข้าวสายพันธุ์สุพรรณบุรี1และขาวดอกมะลิ105 เมื่อเปรียบเทียบกับชุดควบคุม นอกจากนี้ ยังพบว่าสารมาโลนแอลดีไฮด์ในต้นกล้าข้าวสายพันธุ์สุพรรณบุรี1และขาวดอกมะลิ105 มีปริมาณสูงขึ้น สารชนิดนี้เป็นผลิตภัณฑ์ที่ได้จากปฏิกิริยาระหว่างอนุมูลอิสระกับไขมันที่เยื่อหุ้มเซลล์ แสดงให้เห็นถึงการสะสมของอนุมูลอิสระ ซึ่งทำลายเยื่อหุ้มเซลล์ ดังนั้น สามารถจัดจำแนกสายพันธุ์ข้าวได้ 2 กลุ่ม ได้แก่ สายพันธุ์ที่ทนต่ออุณหภูมิต่ำ (SPTC80182 SMGC02002 และน้ำรู) และสายพันธุ์ที่ไม่ทนต่ออุณหภูมิต่ำ (สุพรรณบุรี1และขาวดอกมะลิ105)

ในการศึกษาเปรียบเทียบการเปลี่ยนแปลงปริมาณของสารเมแทบอไลท์ที่เกี่ยวข้องกับการตอบสนองต่อสภาวะเครียดและความสามารถในการต้านอนุมูลอิสระ พบว่าอุณหภูมิต่ำชักนำให้เกิดการเพิ่มขึ้นของสารที่ทำหน้าที่รักษาระดับน้ำของเซลล์ ได้แก่ น้ำตาลและโพรลีน อย่างเด่นชัดในข้าวสายพันธุ์ไม่ทนต่ออุณหภูมิต่ำ ข้าวทั้ง 2 กลุ่มมีการสะสมสารกลุ่มพอลิเอมีนในรูปแบบที่แตกต่างกันเมื่อได้รับอุณหภูมิต่ำ โดยพบว่า อุณหภูมิต่ำส่งผลให้เกิดการเพิ่มของพูเทรสซีนมากที่สุดในข้าวสายพันธุ์ทนต่ออุณหภูมิต่ำ ขณะที่ข้าวสายพันธุ์ไม่ทนต่ออุณหภูมิต่ำมีปริมาณสเปอร์มิดีนเพิ่มขึ้นมากที่สุด การศึกษากิจกรรมการต้านอนุมูลอิสระของสารสกัดจากต้นข้าว แสดงให้เห็นว่า ข้าวสายพันธุ์ทนต่ออุณหภูมิต่ำมีความสามารถในการต้านอนุมูลอิสระเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อได้รับอุณหภูมิต่ำ อย่างไรก็ตาม จากการทดลองพบว่า ไม่สามารถระบุความสัมพันธ์ของความสามารถในการทนต่ออุณหภูมิต่ำและรูปแบบการเปลี่ยนแปลงของสารเฟลโวนอยด์ได้อย่าง

ชัดเจน ข้าวสายพันธุ์ทนต่ออุณหภูมิต่ำ ได้แก่ SPTC80182 และ SMGC02002 มีปริมาณของสารแอนโทไซยานินลดลง และมีปริมาณของเฟลโวนอล รวมถึงเฟลโวนเพิ่มขึ้นเมื่อได้รับอุณหภูมิต่ำ ขณะที่ข้าวสายพันธุ์ไม่ทนอุณหภูมิต่ำทั้งหมดและน้ำรูซึ่งเป็นข้าวสายพันธุ์ที่ทนต่ออุณหภูมิต่ำมีการเปลี่ยนแปลงของสารกลุ่มนี้ในรูปแบบที่ตรงกันข้าม โดยอุณหภูมิต่ำชักนำให้เกิดการเพิ่มของสารแอนโทไซยานิน แต่พบว่าเฟลโวนอลและเฟลโวนมีปริมาณลดลง

ในการศึกษาการแสดงออกของยีนจากชีวสังเคราะห์พอลิเอมีนซึ่งให้เอนไซม์ spermidine synthase (ยีน *SPDS*) และ S-adenosyl methionine decarboxylase (ยีน *OsSAMDC* และ *SAMDC*) โดยเทคนิค RT-PCR พบว่า ข้าวสายพันธุ์ไม่ทนอุณหภูมิต่ำมีการเปลี่ยนแปลงของระดับการแสดงออกโดยรวมของยีนที่ทำการศึกษาสอดคล้องกับระดับสเปอร์มิดีน แต่ไม่พบความสัมพันธ์นี้ในข้าวสายพันธุ์ทนอุณหภูมิต่ำ

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ABSTRACT

In this research, the effects of low temperature (LT) on 5 varieties of Thai rice, namely Supanburee1, KDML105, SPTC80182, SMGC02002 and Numroo, were determined on physiological changes, metabolite accumulation and gene expression.

Comparing with the controls, LT-treated seedlings of 3 rice varieties (SPTC80182, SMGC02002 and Numroo) exhibited the fewer extents of reduced relative growth rates and depleted chlorophyll contents than seedlings of Supanburee1 and KDML105 varieties. Moreover, the significant increase in the contents of malonaldehyde, a product of lipid peroxidation, was observed in seedlings of Supanburee1 and KDML105. The presence of malonaldehyde content implied the accumulation of reactive oxygen species that caused damages to membrane lipids. Thus, these rice varieties could be separated into LT-tolerant group (SPTC80182, SMGC02002 and Numroo) and LT-sensitive group (Supanburee1 and KDML105).

The changes in contents of some stress-related metabolites as well as antioxidative ability of plant extracts from both rice groups were investigated. It was found that LT significantly induced the accumulation of detected compatible solutes, i.e. soluble sugars and proline, in LT-sensitive group. These rice groups exhibited differential responses in polyamine accumulation when exposed to LT. While the most increased polyamine in LT-tolerant varieties was putrescine, that in LT-sensitive varieties was spermidine. The study of free radical-scavenging activities demonstrated that the significant increase in antioxidative ability in LT-tolerant varieties, but not in LT-sensitive rice plants. However, the correlation between LT tolerance and changes in flavonoid contents was unclear. In the presence of LT, the decrease in anthocyanin and the increase in flavonol and flavone levels were observed in only 2 LT-tolerant varieties, namely SPTC80182 and SMGC02002. By contrast, the opposite responsive pattern was presented in all sensitive varieties as well as Numroo, the other LT-tolerant rice.

The expression of genes from polyamine biosynthetic pathway encoding spermidine synthase (*SPDS*) and *S*-adenosyl methionine decarboxylase (*OsSAMDC* and *SAMDC*) was analyzed using RT-PCR. The results showed that correlation between spermidine contents and the overall transcript levels of the detected genes was observed in LT-sensitive rice but not in LT-tolerant rice.

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CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

Rice (*Oryza 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 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 protecting plants against LT (Pillai and Akiyama, 2004; Lee *et al.*, 1995; Saruyama and Tanida, 1995).

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 of rice plants against LT. In addition, the study on the expression of genes involved in biosynthesis of those metabolites may bring more insights into plant responses to LT at molecular level. Differential responses to LT at physiological, 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.

1.2 Goal and objectives

The goal and objectives of this research are as follows:

1. To study the physiological responses between LT-tolerant and sensitive Thai rice varieties
2. To characterize the metabolites that may provide the protective roles of rice plants against LT
3. To determine the influences of LT on the expression of genes involved in the biosynthesis of some metabolites in rice plants

1.3 Scope of the study

In this study, the influences of LT on 5 varieties of Thai rice, namely Supanburee1, KDML105, SMGC02002, Numroo and SPTC80182, were determined at both physiological and metabolic levels. The physiological responses including alterations in chlorophyll contents and relative growth rate, as well as the extent of malonaldehyde were detected. The changes in levels of some metabolites involved in stress protection in plants, including proline, sugars, polyamines, flavonoids, together with the free radical-scavenging activity of rice plant extracts were studied. The differences in metabolic expression profiles between LT-tolerant and sensitive varieties were used to characterize the metabolites that might provide the protective roles against LT to rice plants. Furthermore, LT-induced changes in expression of polyamine biosynthetic genes were analyzed.

1.4 Expected results

The expected results from this research are as follows:

1. The understanding in differential response between LT-tolerant and sensitive rice varieties

2. The metabolites that play important roles in protecting rice plants against LT could be characterized based on the differences in metabolic expression profiles between LT-tolerant and sensitive rice varieties when exposed to LT. These metabolites may be used as markers for the screening of rice varieties with the ability to tolerate LT.

3. This research could provide more insights into the responses to LT and the protective mechanisms that are essential for generating LT-tolerant rice by breeding and genetic engineering.

4. This research may bring about the potential solution to reduce the adverse effects of LT on rice cultivation in the northern part of Thailand and other temperate areas in the world.

CHAPTER 2

LITERATURE REVIEW

2.1 Influences of LT on rice plants

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). In South and Southeast Asia, it was estimated that about 7 million hectares cannot be planted with rice varieties because of LT stress (Andaya and Mackill, 2003).

2.1.1 Growth and development of rice under LT stress

Many studies indicated the detrimental effects of LT on rice plants at any stage of growth and development, ranging from germination to maturity. In general, indica rice is susceptible to temperature below 20°C. However, the critical temperature and the level of susceptibility to LT are variable depending on the varieties, duration of exposure to LT and growth stages. The indica rice subspecies are indigenous to tropical environment, thus they always show more severe symptoms upon LT treatment than Japonica subspecies (Mackill and Lei, 1997). The critical temperature and symptoms of LT injuries in rice plants at different growth stages are summarized in table 2.1.

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.*, 2005). LT treatment at 10°C for 5 days could slow down and lower germination of both indica and japonica rice seeds (Bertin *et al.*, 1996). During seedling stage, the effects of LT on rice plants include retarded growth, leaf wilting and discoloration (table 2.1). According to the study of Yoshida *et al.* (1996), the chlorosis of developing leaves was induced by the exposure of rice seedlings to LT in the dark. This symptom was closely related to the disturbance of chloroplast development and was more severe in indica rice than in Japonica rice.

Table 2.1 Critical temperature and symptoms of LT injuries in rice plants

Growth stage	Critical temperature (°C)	Symptoms of LT injuries
Germination	10	Poor, delayed germination
Seedlings	13	Retarded seedling growth, seedling rot, leaf discoloration, withering after transplanting
Vegetative	15	Inhibited rooting, growth and tillering, delayed panicle formation
Reproductive	17	Inhibited panicle development, spikelet sterility, disturbed meiosis and pollen formation, delayed heading
Heading	17	Inhibited pollination
Maturity	14	Poor grain filling and quality

Source: Lee (2001)

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. Within the reproductive stage, the early microspore phase when microspores are released from tetrads is considered to be the most susceptible to LT (Yamagushi *et al.*, 2004). The morphological alterations in rice microsporangia can be induced by 4-day-exposure of rice plants at early microspore phase to LT in the dark. The ultrastructural changes include microspore with poor pollen wall formation, abnormal vacuolation, unusual starch accumulation in the plastids of endothecium cell layer and anther wall abnormalities (Mamun *et al.*, 2006).

2.1.2 Development of LT-tolerant rice varieties

Due to the adverse influences of LT on rice growth, development as well as productivity, there are many efforts to generate rice germplasms that can produce high and stable yields in regions where LT is the limiting factor for rice cultivation. To this purpose, many techniques are developed and they include screening from nature, breeding and genetic engineering.

Screening

The successful screening for LT-tolerant rice varieties from nature depends on techniques in evaluation of LT sensitivity. The most widely used technique consists of direct exposure to LT following by visual damage assessment. Sthapit and Witcombe (1998) had purposed the screening method by determining the germination rate of rice and greening ability of plumules under LT condition.

Bertin *et al.* (1996) developed the screening techniques based on physiological responses to LT of various rice varieties. They concluded that the electrolyte leakage and changes in chlorophyll fluorescence were the good indicators of LT tolerance during the vegetative growth. Another assessment for LT tolerance is the measurement of radical growth as suggested by Saltveit (2000).

The reliable and standard rating system for evaluating the susceptibility to LT is one of major factors contributed to the successful screening. To this purpose, the International Rice Research Institute (IRRI) has developed the Standard Evaluation System (SES) based on phenotypic expression in response to the stress of interest including LT (IRRI, 2002).

Although the effective screening methods have been extensively developed, the screening process is still highly complex and sometimes impractical under field condition because the responses to LT can vary according to the environment that can fluctuate throughout the growing season and over years (Lee, 2001).

Breeding

Tolerance to LT is the multigenic trait. So, the improvement of tolerance by conventional breeding is considered to be difficult without the availability of effective genetic markers (Vinocur and Altmon, 2005). With the development of diverse DNA markers and the construction of high-density linkage maps for rice plants, the quantitative trait loci (QTL) analysis has become a powerful tool for the genetic dissection of multigenic traits. By this approach, several QTLs conferring LT tolerance at germination, vegetative and reproductive stages have been identified.

The QTLs associated with the ability to germinate under LT condition were identified by Fujino *et al.* (2004). The most effective QTL was designed as qLTG3-1 and mapped on chromosome 3.

The QTL responsible for LT-induced necrosis and wilting tolerance of rice seedlings was mapped on the short arm of chromosome 12 and designated as qCTS12 (Andaya and Mackill, 2003). The fine mapping of qCTS12 indicated that the most likely candidate genes underlying this QTL were *OsGSTZ1* and *OsGSTZ2*, which encodes enzyme members of zeta-class glutathione *S*-transferase, i.e. glutathione *S*-transferase and maleyactoacetate isomerase, respectively. (Andaya and Tai, 2006). These enzymes are implicated to contribute to tolerance against various stresses via the detoxification of ROS. Interestingly, overexpression of *OsGSTZ1* in transgenic rice results in higher germination rate and growth of seedlings at LT (Takesawa *et al.* 2002).

Saito *et al.* (2001) identified 2 QTLs conferring LT tolerance at reproductive stage of rice plants. These QTLs, designated as Ctb-1 and Ctb-2, were responsible for the proper length of anthers and mapped on the long arm of chromosome 4.

Genetic engineering

Gene transferring is another way to generate LT tolerant plants. This approach relies on the knowledge of plant responses and protective mechanisms against LT.

Temperate plants, such as arabidopsis, tobacco and potato, can increase the ability to tolerate LT by the acclimation process (Xin and Browse, 2000). On the contrary, most tropical and subtropical plant species, including rice, lack this ability and are typically injured by LT (Nogueira *et al.*, 2003). However, the study of gene expression and proteomic analyses indicated that rice shares the same LT-responsive genes and proteins with those of the temperate plants (Rabbani *et al.*, 2003; Bae *et al.*, 2003; Yan *et al.*, 2005; Rensink *et al.*, 2005). This evidence suggests that plants with and without the adaptive ability have the same responsive and protective pathways. These testimonies are very useful because they provide more candidate genes for genetic engineering.

The examples of transgenic rice engineered to enhance LT tolerance are summarized in table 2.2. The engineering strategies developed for rice rely on the expression of genes that are involved in signal transduction and regulatory pathway, or genes encoding enzymes that are responsible for detoxifying mechanisms or synthesis of protective metabolites.

Table 2.2 Application of genetic engineering to improve low temperature tolerance in rice

Functions of transformed genes	Sources of genes	Type of expression	Criteria for evaluating effects of stress	Results of genetic engineering	References
Signal transduction					
Calcium-dependent protein kinase (*OsCDPK7*)	Rice	Overexpression	Chlorophyll fluorescence	Rice seedlings enhanced LT and salt tolerance.	Saijo *et al.* (2000)
Mitogen-activated protein kinase (*OsMAPK5*)	Rice	Overexpression	Seedling survival after stress	Rice seedlings enhanced tolerance to LT, drought and salt stress.	Xiang and Yang (2003)
		Suppression	Seedling survival after stress	Rice seedlings showed reduction in LT, drought and salt stress.	
Transcription factors					
CBF1/DREB1b	Arabidopsis	Overexpression	Ion leakage, chlorophyll fluorescence and survival rate	LT tolerance of seedlings was not significantly different from wild type but the expression of genes encoding dehydrin was increased.	Lee *et al.* (2004)
CBF3/DREB1A	Arabidopsis	Overexpression	Chlorophyll fluorescence	Rice seedlings elevated LT tolerance in less extent than drought and salt tolerance.	Oh *et al.* (2005)

Table 2.2 (Continued)

Functions of transformed genes	Sources of genes	Type of expression	Criteria for evaluating effects of stress	Results of genetic engineering	References
Ammonium assimilation					
Chloroplast glutamine synthase (*GS2*)	Rice	Overexpression	Chlorophyll fluorescence	Rice seedlings enhanced tolerance to LT in less extent than salt stress.	Hoshida *et al.* (2000)
Detoxifying enzyme					
Zeta-class glutathione *S*-Transferase (*GST*)	Rice	Overexpression	Germination rate and growth rate	Transgenic rice contained higher GST and glutathione peroxidase activity and enhanced LT tolerance.	Takesawa *et al.* (2002)
Synthesis of compatible solutes					
Choline oxidase (*codA*)	*Arthrobacter globiformis*	Constitutive expression	Chlorophyll fluorescence	Transgenic rice accumulated glycine betaine and enhanced tolerance to LT and salt stress.	Sakamoto *et al.* (1998)

Table 2.2 (Continued)

Functions of transformed genes	Sources of genes	Type of expression	Criteria for evaluating effects of stress	Results of genetic engineering	References
Bifunctional fusion enzyme (*TPSP*) encoding enzymes for trehalose biosynthesis	*E. coli*	Constitutive expression	Chlorophyll fluorescence and growth inhibition	Transgenic rice accumulated more trehalose in leaves and seeds and enhanced LT, drought and salt tolerance.	Jang *et al.* (2003)
Synthesis of membrane lipids					
Oleate-selective glycerol-3-phosphate *O*-acyl transferase (*GPAT*)	Arabidopsis	Overexpression	Net photosynthesis rate	Transgenic rice contained higher level of unsaturated fatty acids and enhanced LT tolerance.	Yokoi *et al.* (1998)

2.2 Reactive oxygen species

Reactive oxygen species (ROS) are the partially reduced forms of atmospheric oxygen (O_2) that are known as the unavoidable by-products of aerobic metabolisms. ROS can be separated into 2 types according to their chemical properties: free radicals and non-radical molecules. Free-radical ROS, such as superoxide radical (O_2^-), hydroxyl radical ($OH^•$) and phenoxyl radical, contain unpaired electron in the outer orbital, making them easily reduce other molecules. Non-radical ROS contain more stable chemical structures than those of free-radical ROS but they are also highly reactive. The examples of non-radical ROS include singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and ozone (O_3) (Apel and Hirt, 2004).

2.2.1 The generation of ROS

There are 3 major sources of ROS production in plants: electron transport systems in chloroplasts and mitochondria, peroxidases and oxidases in peroxisomes, plasma membrane, cell wall and apoplast, as well as photoreceptors such as chlorophylls (Edreva, 2005). The sources of ROS, localization and types of primarily produced ROS are summarized in table 2.3.

Table 2.3 ROS generation in plants

Sources of ROS	Localization	Primary ROS
Electron transport systems		
Photosynthesis	Chloroplasts	O_2^-
Respiration	Mitochondria	O_2^-
Peroxidases and oxidases		
Glycolate oxidases	Peroxisomes	H_2O_2
Fatty acid β-oxidation	Peroxisomes	H_2O_2
Xanthine oxidase	Peroxisomes	O_2^-
Oxalate oxidase	Apoplast	H_2O_2
Amine oxidases	Apoplast	H_2O_2
NADPH oxidases	Plasma membrane	O_2^-
Peroxidases	Cell wall	H_2O_2, O_2^-
Photoreceptors		
Excited chlorophylls	Chloroplasts	$^1O_2$

Source: Mittler (2002)

Generation of ROS via electron transport systems

The electron transport systems in chloroplasts and mitochondria operate in the oxygen-rich environment. Therefore, the leakage of electrons from overloaded electron transport systems will inevitably yield ROS.

In chloroplasts, the main source of ROS production is considered to be photosystem I (PSI) (Dat *et al.*, 2000). Under normal electron transportation, the electrons that are generated by photooxidation of H_2O are passed through the ferridoxin, the component of PSI that located on the stromal side of thylakoid membrane. Ferridoxin is then used the electrons to reduce $NADP^+$, yielding NADPH. In case of excess excitation (light) energy, a part of electron flow is diverted from photosynthetic components to reduce oxygen, resulting in the formation of superoxide radical by the process called Mehler reaction. However, the study of Zhang *et al.* (2003) has established the probability of superoxide generation through the leakage of electrons from Q_B, the components of PSII.

Superoxide radicals once produced may follow the different pathways. On the lumen side of thylakoid membrane that is acidified under light condition, superoxide radicals may be protonated to form per hydroxyl radicals ($HO_2^{\bullet}$), which initiates lipid peroxidation. Superoxide radicals on the stromal side of thylakoid membrane may be dismutated enzymatically or spontaneously to form hydrogen peroxides (Alscher *et al.*, 2002). At Fe-S centers of ferridoxin where Fe^{2+} ions are available, hydrogen peroxides can react with superoxide radicals to generate hydroxyl radicals that are much more dangerous. This mechanism is called Haber-Weiss cycle (Edreva, 2005) (figure 2.1). Therefore, it is important to scavenge hydrogen peroxides before entering Haber-Weiss cycle (see the following sections).

In animal cells, mitochondria are the major sites for ROS formation and primary targets of ROS-mediated damages. By contrast, little is known about ROS turnover in plant mitochondria because they are not traditionally regarded as a major source of ROS in plants. However, The main sites of ROS generation in plant mitochondria are considered to be complex I (NADH : ubiquinone oxidoreductase) and complex III (cytochrome C oxidase) of respiratory electron transport system (Møller, 2002).

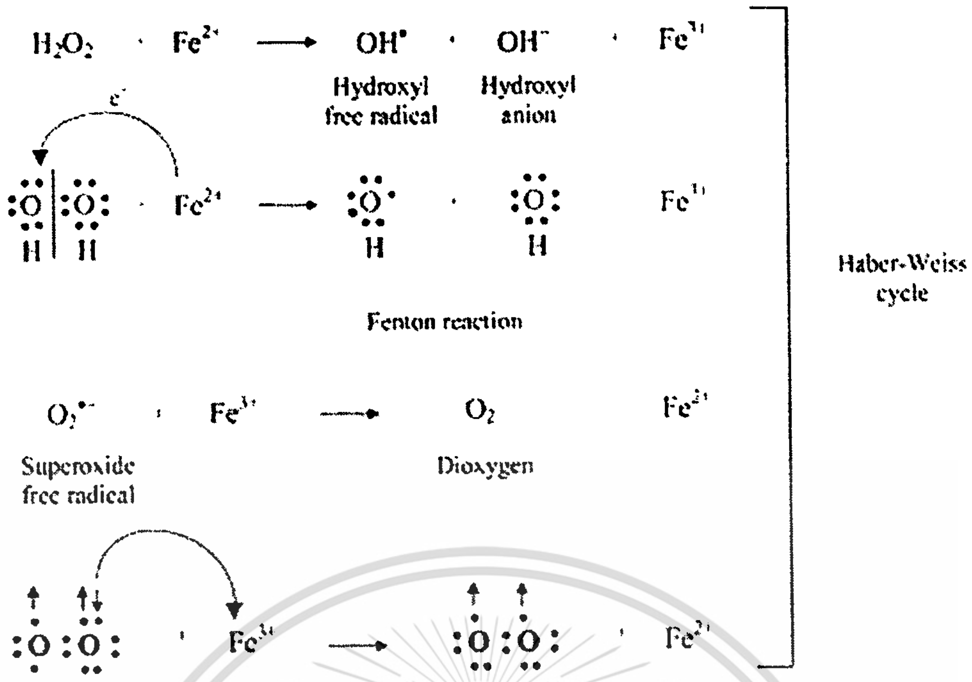


Figure 2.1 The generation of hydroxyl radicals through Haber-Weiss cycle. The reaction between hydrogen peroxide and Fe^{2+} is called Fenton reaction.

Source: Edreva (2005)

Generation of ROS via peroxidases and oxidases

Peroxidases and oxidases are the other sources for ROS generation in plants. In peroxisomes, hydrogen peroxide is produced through the action of glycolate oxidase, which is a part of photorespiration. The generation of hydrogen peroxide can also occur during lipid catabolism as a by-product of fatty acid oxidation. Moreover, superoxide radicals are produced in matrix of peroxisomes via the action of xanthine oxidase (Corpas *et al.*, 2001).

Plasma membrane NADPH oxidases have been the subject of intense investigation. They are thought to play a key role in ROS signaling for root development and plant-pathogen interaction. Structurally, NADPH oxidases contain multimeric flavocytochromes that form electron transport chains capable of reducing oxygen molecules to superoxide radicals. This process leads to the accumulation of apoplastic ROS, which is so-called oxidative burst (Torres and Dangl, 2005).

Amine oxidases are also the candidate of ROS producers. These enzymes can be separated into 2 types: copper-containing amine oxidases and flavin-containing polyamine oxidases, and responsible for catalyzing the deamination of polyamines and generate hydrogen peroxide as the by-product (Cona *et al.*, 2006).

Generation of ROS via photoreceptors

Excited chlorophylls mediate another mechanism for ROS generation in chloroplasts. Normally, the absorption of light energy leads the chlorophyll molecule to change its state from a ground state to a highly energetic state. The excited pigment can undergo charge separation, a process by which mediates the transfer of an electron to another photosynthetic molecule (Hopkins and Huner, 2004). If the electron is transfer to oxygen, it can result in the production of superoxide radical as previously described.

On the other hand, the excited chlorophyll may lose a part of its energy and then reach the triplet excited state at which it can transfer the remaining excited energy to the ground state oxygen, leading to the generation of singlet oxygen (Edreva, 2005).

2.2.2 Damaging effects of ROS

ROS are reactive, highly toxic molecules that lead to oxidative damages of lipids, proteins and nucleic acids, leading to the impairment of normal cellular structures and functions. ROS can also cause a chain reaction of cellular oxidation that results in lethality (Rodriguez and Redman, 2005). A range of environmental stresses, including LT, can cause oxidative stress via the perturbation of normal metabolisms that leads to the over accumulation of ROS (Kaplan *et al.*, 2004).

There are reports about the induction of ROS generation by LT stress. The study of Fadzillah *et al.* (1996) indicated the increase of hydrogen peroxide level in rice shoot cultures upon LT treatment for 8 days. The similar trend was also observed in seedlings of winter wheat (Okuda *et al.*, 1991) under LT stress.

Photosynthesis is the major target of ROS during LT stress. Normal photosynthetic mechanism relies on the balance between the light energy absorbed by photosystems and the reduction of CO_2 through Calvin cycle. Under LT condition, CO_2 fixation is inhibited, leading to the insufficient CO_2 for the absorbed excitation energy (Allen and Ort, 2001). This in turn contributes to the over-production of ROS. The damaging effects of ROS on chloroplastic components are summarized in table 2.4.

The polyunsatured fatty acids of cellular lipids are susceptible to ROS-induced oxidative damages, leading to the formation of cytotoxic aldehydes, alkenals and hydroxyalkeneals such as malonaldehydes (MDA) and 4-hydroxy-2-nonenal (HNE) (Taylor *et al.*, 2004).

Table 2.4 Damaging effects of ROS on chloroplastic components

Types of ROS	Damaging effects
$OH^{\bullet}$	Damages to all components
$^1O_2$	Membrane lipid peroxidation
	Chlorophyll destruction
H_2O_2	Calvin cycle enzyme inactivation
	Cross linking of D_1 and D_2 proteins
	Damages to oxygen-evolving complex at PSII
O_2^-	Membrane lipid peroxidation
	Chlorophyll destruction
	Denaturation of D1 by tyrosine modification

Source: Edreva (2005)

The example for lipid peroxidation mechanism through auto-oxidation is shown in figure 2.2. First-chain initiation involves the attack of $OH^{\bullet}$ on the methylene ($-CH_2-$) of arachinodic acid to abstract a hydrogen atom. This abstraction of an H from $-CH_2-$ group leaves behind an unpaired electron on the carbon. The presence of a double bond in the fatty acid weakens the C-H bonds on the carbon atom adjacent to the double bond and makes the H removal easier. The carbon radical tends to be stabilized by a molecular rearrangement to form a conjugated diene. These can undergo various reactions, the most common event is to combine with O_2, giving rise to the first peroxyl radical ($CHOO^{\bullet}$). This radical can abstract H atom from another adjacent polyunsaturated fatty acid, leading to the propagation of the chain reaction. The peroxyl radical can combine with the removed hydrogen to yield a lipid hydroperoxides which are then degraded nonenzymatically, generating alkoxyl radicals. In plants, these alkoxyl radicals are oxygenated to form HNE (Taylor *et al.*, 2004).

The damaging effects of HNE involve the inhibition of cellular metabolism through the modification of lipoic acid-containing enzymes. In isolated mitochondria of pea leaves, HNE can inhibit glycine-dependent respiration by the modification of H-protein in glycine decarboxylase. In addition, this effect can be induced upon LT treatment (Taylor *et al.*, 2002). Millar and Leaver (2000) studied the effects of HNE on O_2 consumption by potato tuber mitochondria. It was found that HNE could inhibit the activities of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase.

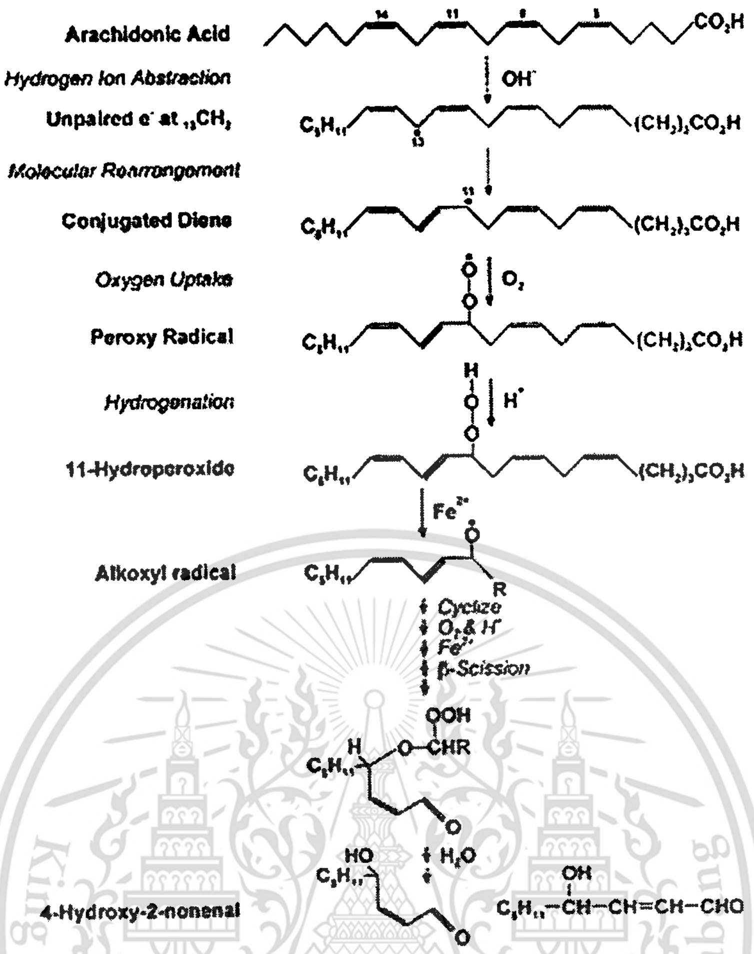


Figure 2.2 The mechanism for the production of HNE from arachidonic acid through auto-oxidative lipid peroxidation

Source: Taylor *et al.* (2004)

The oxidative damages of mitochondrial components can also be occurred by the direct action of ROS. In animal and human cells, it is well documented that ROS can cause mutation on mitochondrial DNA (mtDNA), contributing to aging as well as a number of diseases (Møller, 2002). Such damages are also likely to occur in plant mtDNA, but this area has received little attention to date. In addition, ROS can directly inhibit the activities of mitochondrial aconitase by the modification of Fe-S cluster (Verniquet *et al.*, 1991).

2.2.3 Protective mechanisms against oxidative damages of ROS

LT, as well as other stresses, can induce the over-accumulation of ROS. Plants adopt various mechanisms that can scavenge enhanced ROS to protect themselves against oxidative damages. These are collectively called antioxidative mechanisms and involved both enzymatic and non-enzymatic systems that work in concert to control ROS levels when plants are exposed to stresses.

Enzymatic systems

The major enzymatic systems of ROS-scavenging mechanisms in plants include superoxide dismutation, water-water cycle, ascorbate-glutathione cycle, glutathione peroxidase cycle and the detoxification of hydrogen peroxide by catalase (Holmberg and Bülow, 1998; Asada, 1999). The pathways of these systems are shown in figure 2.3.

Superoxide dismutases (SODs) are essential components of ROS-scavenging mechanisms and are found in almost all cellular compartments. The SOD isozymes can be divided into 3 classed according to their metal cofactors: CuZnSOD, MnSOD and FeSOD. In general, plants contain CuZnSOD and/or FeSOD in chloroplasts. CuZnSOD also localizes in cytosol while MnSOD is found in mitochondria. All SODs catalyze the dismutation of two superoxide radicals into O_2 and hydrogen peroxide, which is subsequently removed by other mechanisms.

The water-water cycle is located in thylakoid membranes of chloroplasts and plays a role in hydrogen peroxide scavenging as well as dissipation of excess excitation energy from photosynthetic electron transport system. In this cycle, thylakoid-bound ascorbate peroxidase (tAPX) catalyzes the reduction of hydrogen peroxide by ascorbate (AsA), yielding H_2O and monodehydroascorbate (MDA). Then, MDA accepts the electron from ferridoxin (fd), yielding AsA of another round of the cycle.

The ascorbate-glutathione cycle is the major ROS-scavenging mechanism in chloroplasts and other compartments, including mitochondria, cytosol, peroxisomes and apoplast. This process involves several enzymes, namely ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). These enzymes act in a highly coordinated manner to detoxify hydrogen peroxide. The turnover of AsA and reduced glutathione (GSH) requires NADPH as the electron donor.

The glutathione peroxidase (GPX) cycle and catalase (CAT) are essential for the scavenging of ROS in peroxisomes. In GPX cycle, GSH reduces hydrogen peroxide by the action of GPX. This cycle requires NADPH as the electron donor to convert oxidised glutathione (GSSH) into GSH for the other rounds of detoxification. Hydrogen peroxide can also be scavenged by CAT.

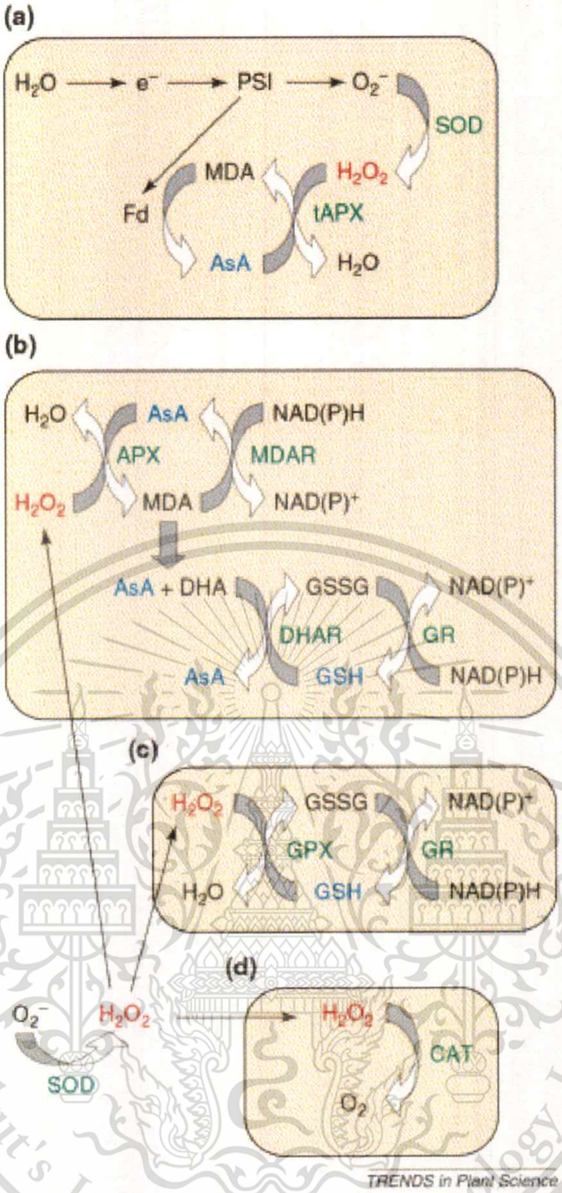


Figure 2.3 Pathways of enzymatically antioxidative mechanisms in plants

(a) The water–water cycle. (b) The ascorbate–glutathione cycle. (c) The glutathione peroxidase cycle. (d) Catalase

Abbreviations: Fd: ferridoxin, AsA: ascorbate, MDA: monodehydroascorbate, DHA: Dehydroascorbate, GSH: reduced glutathione, GSSG: oxidized glutathione, SOD: superoxide dismutase, tAPX: thylakoid-bound ascorbate peroxidase, MDAR: MDA reductase, DHAR: DHA reductase, GR: glutathione reductase, GSSG: oxidized glutathione, CAT: catalase

Source: Mittler (2002)

Non-enzymatic systems

Non-enzymatic systems for ROS scavenging in plants comprise of various substances, such as tocopherols, ascorbate, carotenoids, phenolic compounds including flavonoids (Tsau and Deng, 2004). These metabolites can directly detoxify ROS by chemical bonding.

2.2.4 Importance of antioxidative mechanisms on LT tolerance in plants

There are numerous studies indicating that antioxidative mechanisms are important for protecting plants for LT stress. Kuk *et al.* (2003) examined the effects of LT on the activity of various antioxidative enzymes in rice. During the recovery from LT stress, rice seedlings exhibited the higher activity of CAT and APX in leaves, suggesting the importance of these enzymes in conferring LT tolerance to rice plants.

The study of Sato *et al.* (2001) also supports the importance of APX on LT tolerance in rice. The prior exposure to heat-shock temperature enhanced activity of APX, but not SOD, in rice seedling. These plants were subsequently treated with LT. It was found that the enhanced activity of APX was related to less injury from LT.

Maeda *et al.* (2006) examined the roles of tocopherols in arabidopsis under various stress conditions by using mutants that were deficient in tocopherol biosynthesis and accumulation. These mutants exhibited the markedly different phenotypes from wild type, i.e. reduced growth and seed production and limited the export of photoassimilates under LT condition. This study, therefore, suggests the roles of tocopherol in LT adaptation in arabidopsis.

LT treatment could induce the accumulation of soluble phenolic compounds in tomato and watermelon. This induction was related to the higher activity of phenylalanine ammonia-lyase, the key enzyme for phenolic biosynthesis, as well as the lower activity of degrading enzymes such as polyphenol oxidase (Rivero *et al.*, 2001).

Antioxidative mechanisms are also crucial for cold acclimation. It was found that the activity of enzymes for ascorbate-glutathione cycle, including APX, MDAR, DHAR and GR, as well as the levels of ascorbate and glutathione were induced in cold-acclimated Scot pine seedlings. In addition, there was the correlation between levels of induction and the freezing tolerance (Tao *et al.*, 1998).

The relationship between levels of LT tolerance and the capacity of antioxidative systems have been extensively detected in many plants. Santis *et al.* (1999) studied the germination of maize seeds at low temperature and indicated that the high germination rate correlated with low level of lipid peroxidation in isolated mitochondria. This suggests that LT tolerant varieties contain more efficient antioxidative mechanisms than LT sensitive varieties.

Saruyama and Tanida (1995) indicated that both LT-tolerant and sensitive rice varieties exhibited the lower activities of CAT and APX during LT treatment, suggesting the LT- labile nature of these enzymes. During recovery from LT, however, LT-tolerant varieties could regain the activity of CAT and APX with the greater extent as well as showed higher growth than LT-sensitive varieties. The authors concluded that the levels of LT tolerance in rice plants involved the ability to recover the activities of CAT and APX.

Guo *et al.* (2006) studied the responses of antioxidative mechanisms to LT in various varieties of rice plants. During LT stress, LT-tolerant rice exhibited lower levels of electrolyte leakage, hydrogen peroxide and lipid peroxidation than LT-sensitive varieties. Such responses were related with the higher activities of SOD, CAT and APX, as well as contents of ascorbate and glutathione in LT-tolerant rice. These findings also suggested the correlation between LT tolerance and the capacity of antioxidative systems under LT condition.

2.3 Protective metabolites against LT stress in plants

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 gene transcription can be classified into 2 groups. The first group includes protein factors involved in regulation of other signal cascades or gene expression. The second group contains functional proteins, such as water channel proteins, molecular chaperones, late-embryogenesis abundant (LEA) proteins and key enzymes for metabolisms of protective metabolites, for example polyamines, compatible solutes and flavonoids (Maruyama *et al.*, 2004).

2.3.1 Polyamines

Polyamines are low molecular weight polycations that are found in bacteria, animals and plants. There are 3 major polyamines in plants: putrescine, spermidine and spermine. All these compounds are present in both free bases and conjugated or bound forms (Takahashi *et al.*, 2003).

The biosynthesis of polyamines is shown in figure 2.4. The first step in biosynthesis involves the decarboxylation of ornithine or arginine to generate putrescine. The single enzyme, ornithine decarboxylase (ODC), catalyzes the ornithine pathway. By the contrary, the conversion of arginine to putrescine requires the successive action of 3 enzymes, including arginine decarboxylase (ADC), agmatine iminohydrolase (AIH) and N-carbamoylputrescine amido hydrolase (CPA). Many plants, such as rice and cucumber, generate putrescine from both pathways (Trung-Nghia *et al.*, 2003; Shen *et al.*, 2000). However, there are no detectable ODC activity in arabidopsis, suggesting that putrescine is produced exclusively through arginine pathway (Hanfrey *et al.*, 2001).

Spermidine and spermine are formed by the addition of aminopropyl groups from decarboxylated S-adenosyl methionine (dcSAM) to putrescine and spermidine, respectively. The generation of dcSAM from S-adenosyl methionine (SAM) is the committing step and involes the action of S-adenosyl methionine decarboxylase (SAMDC). SAM also functions as the precursor for ethylene biosynthesis (Alcázar *et al.*, 2006).

Polyamines have been implicated in a wide range of biological processes including DNA replication, cell division, growth, development, as well as environmental responses. 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).

Polyamine can protect plants from oxidative damages. Papadakis *et al.* (2005) indicated that the application of polyamines to tobacco protoplasts could inhibit the activity of NADPH oxidase. This effect correlated with the number of amino group within polyamine molecules (spermine > spermidine > putrescine) and leads to the decrease in level of superoxide redical.

Shen *et al.* (2000) investigated the accumulation of polyamines in 2 cucumber varieties. They found that LT-tolerant variety increase the level of spermidine during LT treatment and the recovery periods. On the other hand, there were no significant changes of polyamine levels in LT-sensitive variety. The application of spermidine to the sensitive variety could alleviate the damaging effects of LT and inhibit the activity of NADPH oxidase.

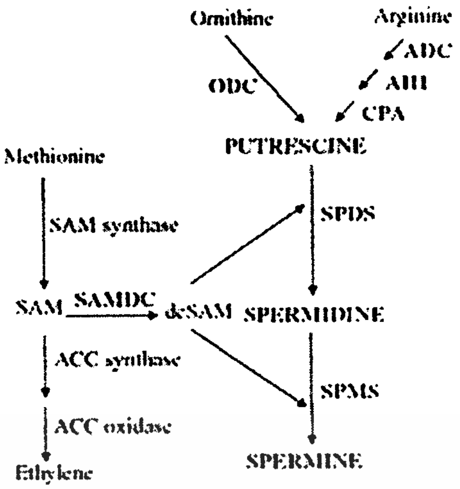


Figure 2.4 Polyamine biosynthesis in plants.

Abbreviations: ACC: 1-amino-cyclopropane-1-carboxylic-acid, ADC: arginine decarboxylase, AIH: agmatine iminohydrolase, CPA: N-carbamoylputrescine amidohydrolase, DAO: diamine oxidase, dcSAM: decarboxylated *S*-adenosyl methionine, GABA: γ-aminobutyric acid, ODC: ornithine decarboxylase, PAO: polyamine oxidase, SAM: *S*-adenosylmethionine, SAMDC: *S*-adenosylmethionine decarboxylase, SPDS: spermidine synthase, SPMS: spermine synthase

Source: Alcázar *et al.* (2006)

The regulation of polyamine biosynthesis is essential for the structural changes of light harvesting complex II (LHCII), the pigment-protein complex of PSII, under LT condition. Sfakianaki *et al.* (2006) found that *Phaseolus vulgaris* LHCII respond to LT by increasing their oligomeric forms and protein contents. These changes were related to the higher level of LHCII-associated spermine. They also suggest that spermine might provide the protective role to LHCII by reducing oxidative damages from LT.

The study of Lee (1997) demonstrated that putrescine played a role in controlling the growth of rice roots *in vitro*. At normal growth temperature, the level of putrescine as well as the activities of ADC and ODC increased with the progressive growth while the levels of spermidine, spermine and the activity of SAMDC decreased. Roots that were exposed to LT exhibited the growth inhibition and lost their re-growth ability during the recovery from LT. This inhibition can be alleviated by the exogenous putrescine. By contrast, the application of α-difluoromethyl arginine (DFMA), an ADC inhibitor, as well as exogenous spermidine and spermine exacerbated the inhibition.

Lee *et al.* (1995) also indicated the role of putrescine in protecting plants against LT stress. The exposure of rice seedling to LT induced the accumulation of putrescine in both shoots and roots. The increase in putrescine level was correlated with the ability to tolerate LT of rice plants. Moreover, these changes occurred coincidently with the increase in abscisic acid (ABA) level. The further investigations demonstrated that ABA regulated the polyamine biosynthesis by modulating the expression of *ADC*, *spermidine synthase* (*SPDS*) and *spermine synthase* (*SPMS*) genes in rice and arabidopsis under LT condition (Alcázar *et al.*, 2006; Imai *et al.*, 2004; Akiyama and Jin, 2006).

2.3.2 Compatible solutes

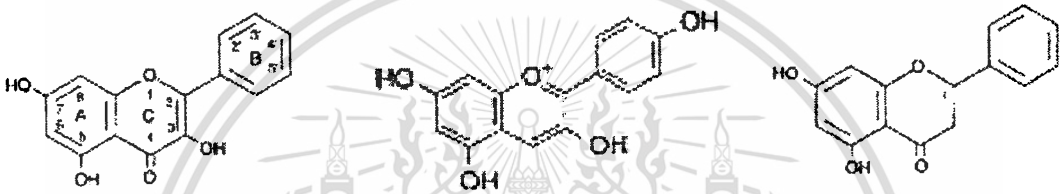
Compatible solutes are the low molecular weight, highly soluble compounds. These solutes can be categorized into 3 types including quaternary ammonium compounds, amino acids and sugars or sugar alcohols (Ashraf and Foolad, 2007). The major functions of these compounds are to maintain turgor pressure 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).

2.3.3 Flavonoids

Flavonoids are well-known plant secondary metabolites. The basic structure of flavonoids comprises two aromatic structures (A- and B-rings) linked by a heterocyclic C-ring (figure 2.5). Flavanoids are classified into 9 major subgroups. Six subgroups, including chalcones, aurones, isoflavones, flavonols and flavandiols are colorless flavonoids while the other 3 subgroups: anthocyanins, condensed tannins and phlobaphenes serve as pigments for plants (Shirley, 1996).

The biosynthesis of flavonoids starts with the combination of malonyl CoA and coumaroyl CoA through the action of chalcone synthase (CHS). This committing step yields the basic skeletons, which in turn follow several branched pathways for the synthesis of each subgroup (figure 2.6). The further modification by hydroxylation, methylation, glycosylation or acylation results in the diversity of these compounds (Mol *et al.*, 1998). Some flavonoids are restricted to certain plants species. For example, isoflavonoids are found in legumes and a small number of nonlegume plants. Similarly, sorghum, maize and gloxinia are among the few species that synthesize phlobaphenes (Winkel-Shirley, 2001).



Flavonol **Anthocyanin** **Flavone**

Figure 2.5 Structures of anthocyanins, flavonols and flavones

The basic structure consists of 3 rings: A, B and C. A- and C-ring fuse together while B-ring attaches its $1'$ position to the 2 position of C-ring.

Source: Rice-Evans *et al.* (1997)

Flavonoids exhibit a vast array of biological functions in plants. They are best known as pigments that are essential for plant reproduction by recruiting pollinators and seed dispensers. They also serve as photoreceptors for plant growth and development, control the polar transportation of auxin and act as signal during nodulation. Recently, flavonoids have gained lots of interest as they play important roles in human health by preventing degenerative diseases associated with oxidative stress (Treutter, 2005).

Flavonoids also play a role in plant protection against LT stress through their antioxidative ability. The accumulation of anthocyanins and other phenylpropanoids is increased response to LT in many plants, such as bilberry, winter oilseed rape and peach (Taulavuori *et al.*, 2004; Solecka and Kacperska, 1995; Leng and Qi, 2003).

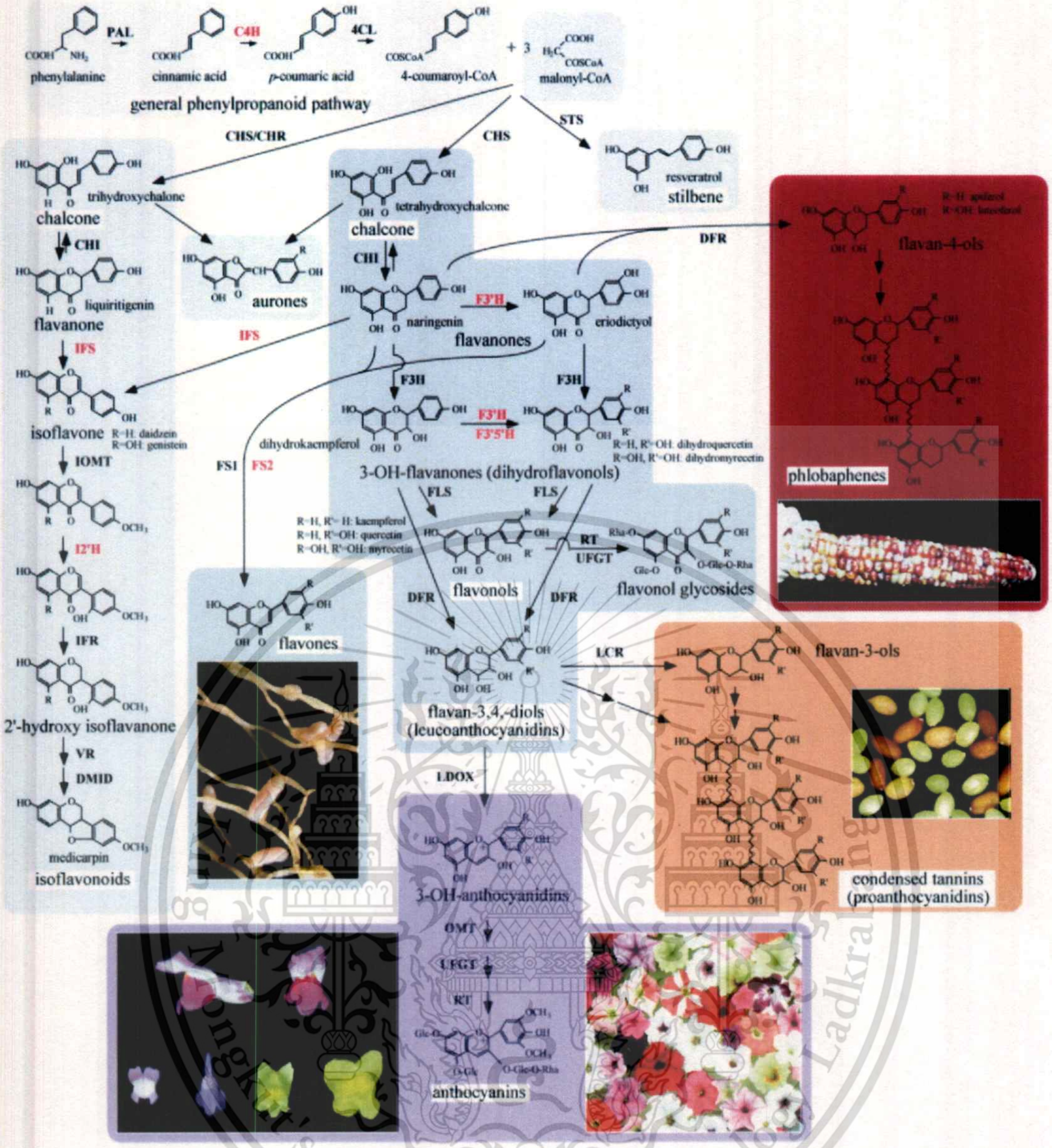


Figure 2.6 Flavonoid biosynthesis

Abbreviations: C4H: cinnamate-4-hydroxylase, CHI: chalcone isomerase, CHR: chalcone reductase, CHS: chalcone synthase, 4CL: 4-coumaroyl:CoA-ligase, DFR: dihydroflavonol 4-reductase, F3H: flavanone 3-hydroxylase, FS: flavone synthase, F3'H: flavonoid 3' hydroxylase, F3'5'H: flavonoid 3'5' hydroxylase, IOMT: isoflavone *O*-methyltransferase, IFR: isoflavone reductase, IFS: isoflavone synthase, LDOX: leucoanthocyanidin dioxygenase, LCR: leucoanthocyanidin reductase, OMT: *O*-methyltransferase (OMT), PAL: phenylalanine ammonia-lyase, RT: rhamnosyl transferase, UFGT: UDPG-flavonoid glucosyl transferase

Souce: Winkel-Shirley, 2001

The antioxidative mechanism of flavonoids has been investigated. Flavonoids can directly scavenge superoxide radicals and singlet oxygen in the *in vitro* experiment (Bors *et al.*, 1990). However, this activity is limited in plant cells because flavonoids are localized mainly in vacuoles that do not accumulate such radicals. Therefore, the major target for flavonoids seems to be hydrogen peroxide since it can diffuse into vaculoes.

The study of Yamasaki *et al.* (1997) suggests the mechanism of flavonoids in hydrogen peroxide detoxification. In this mechanism, peroxidase catalyzes the reaction between hydrogen peroxide and flavonoids, leading to the generation of H_2O and flavonoid phenoxyl radicals. These radicals were in turn reduced by ascorbate to produce flavonoids for other rounds of the cycle.

The phenoxyl radicals can also undergo the polymerization in browning process, resulting in the formation of semiquinones and quinines. These compounds can also act as antioxidants in plants (Pourcel *et al.*, 2006).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Plant materials and tissue culture systems

Seeds of 5 Thai rice varieties, namely Supanburee1, KDML105, SMGC02002, Numroo and SPTC80182 were derived from Rice Research Center at Sanpatong, Chiangmai, Thailand. 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, flavonoids and DPPH free radical-scavenging activity
3. Chemicals used to prepare NB medium
4. Chemical used for RNA extraction and RT-PCR analysis
5. Laboratory apparatuses, including
    - Glass wares
    - Mortars and pestles
    - Spatulas
    - 15- and 50-ml centrifuge tubes
    - Micropipettes

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 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 25±2°C day/night temperature.

Rice seedlings at the 2-leaf stage with uniformity in appearance were subjected to LT by growing at 25°C/10°C for 7 days while the control seedlings were maintained at the same temperature. Samples were harvested at the end of night period of the 7th 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 was calculated as the following formula (Noggle and Friz, 1976).

$$\text{relative growth rate} = \frac{\ln DW_7 - \ln DW_0}{7}$$

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 Reiss (1994). 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 MDA contents

The extraction and analysis of MDA were performed by using the protocol of Hodges *et al.* (1999). Fresh powder (1.5 g) of whole rice seedlings was extracted with 0.1% (w/v) trichloro acetic acid by shaking for 1 h at room temperature. Then, the sample was centrifuged at 2,000 g for 30 min to remove the insoluble residues. Next, 500 µl 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 nmol/ml-gFW.

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

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

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

3.4.6 Metabolite analyses

1) Total soluble sugar contents

The extraction and analysis of total soluble sugars were performed as described in Shou *et al.* (2003). 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$_2$O and chloroform with the same volume (400 µl each) were mixed, vortexed vigorously and then centrifuged at 2,000 g 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) and Asotra *et al.* (1987). 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 supernatant was divided into 2 parts: The first part was analyzed for flavonoid levels and the second part was determined for DPPH free radical (DPPH$^{\bullet}$)-scavenging activity.

The level of each flavonoid was determined as the absorbance at the specific wavelength (table 3.1).

5) DPPH$^{\bullet}$-scavenging activity

DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical that is deep violet in methanol. In the presence of antioxidant compounds, DPPH$^{\bullet}$ is reduced, leading to the change in color to light yellow. The discoloration of DPPH$^{\bullet}$ is associated with the degree of reduction and it can be monitored by measuring the absorbance at 517 nm (Miliauskas *et al.*, 2003).

DPPH$^{\bullet}$-scavenging activity was performed using the method of Brand-Williams *et al.* (1995) with little modification. 150 µl of 0.8 mM DPPH$^{\bullet}$ in 95% methanol was mixed with 0-150 µl of extract with one appropriate concentration and 95 % methanol was added to adjust the final volume to 1,500 µl, resulting that each reaction mixture contained the different concentration of sample. After vortexing, the mixture was kept in the dark at room temperature for 30 min and then the absorbance was measured at 517 nm.

The ability of extract in scavenging DPPH$^{\bullet}$ was determined as the amount of seedlings needed to decrease the initial DPPH$^{\bullet}$ concentration by 50% (EC$_{50}$), which was obtained from a graph of sample concentrations plotted against the percentages of remaining DPPH$^{\bullet}$.

6) Experimental design and statistic analysis

All experiments were designed in completely randomized design (CRD) with 6 replicates (n=6). Statistical analysis for each experiment was performed with ANOVA and Duncan's multiple range test (DMRT) using SPSS software version 11.

Table 3.1 Wavelengths used in measuring levels of flavonoids

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

Source: Harborne, 1998

3.4.7 Gene expression analysis

Total RNA extraction was performed according to the protocol of Sambrook and Russell (2001). Whole rice plants were ground in liquid nitrogen until they turned to fine powder. Then, the samples were extracted with the mixture of equal volume of phenol and RNA extracion 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 g 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 *OsSPDS*, *OsSAMDC1* and *OsSAMDC2* was analyzed by RT-PCR. The amplification of *OsSAMDC1* (GenBank accession No. Y07766) and *OsSAMDC2* (AJ251899) was performed using gene specific primers (Pillai and Akiyama, 2004). The primer pair for amplifying OsSPDS was designed from the conserved nucleotide sequences of *OsSPDS1* (AJ251296), *OsSPDS2* (AJ251297), putative *OsSPDS* from Indica rice (CT833007), *ZmSPDS* from *Zea mays* (AY730048), *AtSPDS3* form *Arabidopsis thaliana* (AY040013), *CaSPDS* from

Coffea arabica (AB015599) and *CsSPDS* from *Cucumis sativus* (Ay646352). 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 treatment 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). 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-cycle:

Denaturation	94°C 45 s
Annealing	55°C 2 min for *OsSAMDC1*
	50°C 2 min for *OsSAMDC2*
	56°C 2 min for *OsSPDS*
Extension	72°C 3 min

This step was performed for 23 cycles (*OsSAMDC1* and *OsSAMDC2*) or 30 cycles (*OsSPDS*), followed by a final extension step of 72 °C for 10 min. The RT-PCR products were separated on a 1.5 % 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).

Table 3.2 Primer sequences for amplification of *OsSPDS, OsSAMDC1* and *OsSAMDC2*

Genes		**Primer sequences**
OsSPDS	Forward	5' ATG TGG CC(T/A) GGA GA(G/A) GC 3'
	Reverse	5' GAA TCN AC(A/T) AT(G/A) ACN GCA TC 3'
OsSAMDC1	Forward	5' ATG GTG AAC CCT GCT CCT 3'
	Reverse	5' GCA GAA GGC AAA GGA AAT 3'
OsSAMDC2	Forward	5' ACG GAG AAA GCT GGT AAA 3'
	Reverse	5' TTA AAT GAC TTG TAT TAT ATT CC 3'
Actin	Forward	5' GTG ACA ATG GAA CTG GAA TGG TNA AGG 3'
	Reverse	5' CAC CAT CAC CAG AAT CGA GCA CAA TAC 3'

CHAPTER 4

RESULTS

4.1 Effects of LT on physiological characteristics of Thai rice

The ability to tolerate LT of rice seedlings is a crucial factor determining survival and productivity. Exposure to LT results in retarded seedling growth, desiccation, as well as leaf discoloration (Lee, 2001). A number of techniques have been used to develop LT-tolerant rice varieties. Among them, screening based on the physiological responses to LT is one of promising approaches (IRRI, 2002). The LT tolerance of Thai rice varieties was determined by measuring the relative growth rate and chlorophyll contents of rice after exposed to LT (10°C) in the dark period.

After 7 days of LT treatment, all varieties showed the sign of LT injuries, including growth retardation, chlorosis of newly emerged leaves, wilting and necrosis (Figure 4.1). Among all varieties, LT- treated Supanburee1 exhibited the most severe symptoms (Figure 4.1A) by significant reduction of relative growth rate (19% comparing with its control) (Figure 4.2). The comparable level of injuries (Figure 4.1B) and the decrease in relative growth rate were found for LT-treated KDML105 (20% decrease when compared to the control). The LT-induced injuries in SPTC80182, Numroo and SMGC02002 were less obvious (Figure 4.1C, D and E). The decline in relative growth rates of these varieties was less pronounced (6, 9 and 10% lower than those of the controls, respectively). Interestingly, LT-treated seedlings of SMGC02002 were more vigor and greener than the control (Figure 4.1E).

Paralleling with the changes in relative growth rates, the chlorophyll contents of Supanburee1 and KDML105 seedlings were diminished upon LT exposure (Figure 4.3). Compared to the controls, the levels of chlorophyll a and b in LT-exposed seedlings of Supanburee1 were about 80 and 73%, respectively. The chlorophyll contents of Numroo were least affected by LT, accounting for 8% chlorophyll a and 9% chlorophyll b lower than those of the control. However, there was the exception for SMGC02002 whose LT-treated seedlings contained slightly higher levels of chlorophylls than the untreated plants.

Based on these results, the rice varieties were categorized into 2 groups for further experiments. First, the "LT-tolerant" group consists of SPTC80182, Numroo and SMGC02002. The second group is composed of "LT-sensitive" varieties, namely, Supanburee1 and KDML105.

This categorization is in agreement with the data derived from field study from Rice Research Center at Sanpatong, Chiangmai, Thailand (Personal contact).

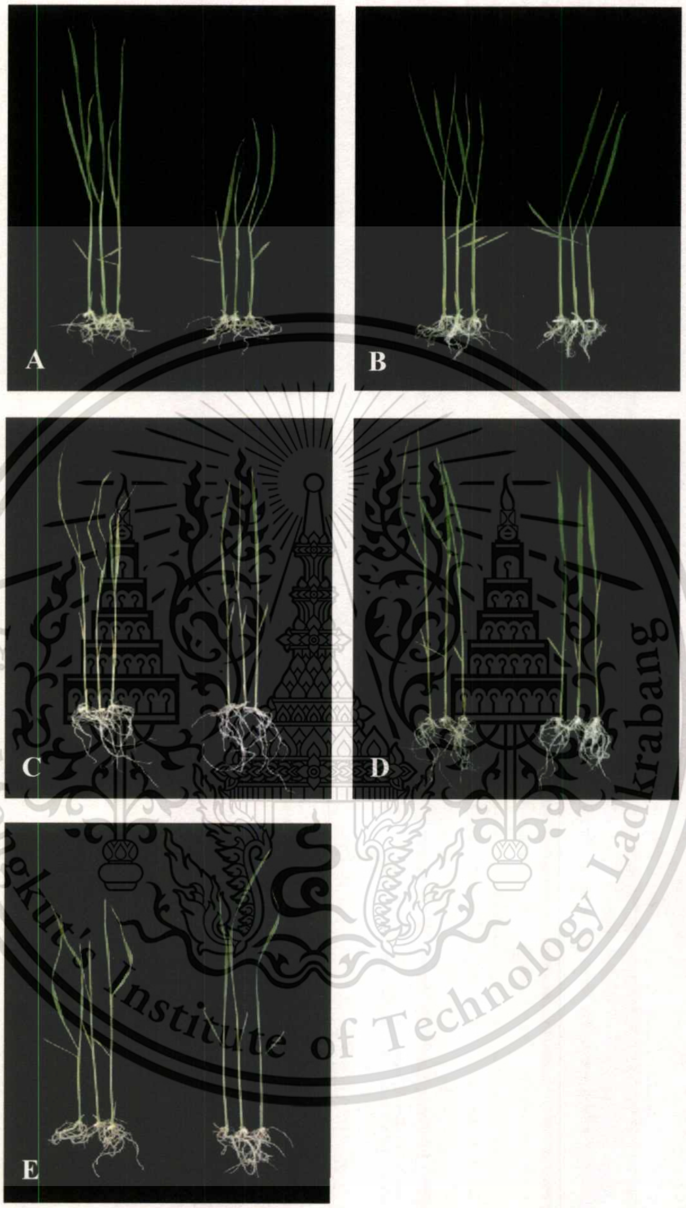


Figure 4.1 LT-induced physiological changes in rice seedlings

Ten-day-old seedlings of 5 Thai rice varieties, namely, Supanburee1 (A), KDML105 (B), SMGC02002 (C), Numroo (D) and SPTC80182 (E), were subjected to 10°C in the dark period for 7 days. The control and LT-treated seedlings were shown in the left and the right of each picture, respectively.

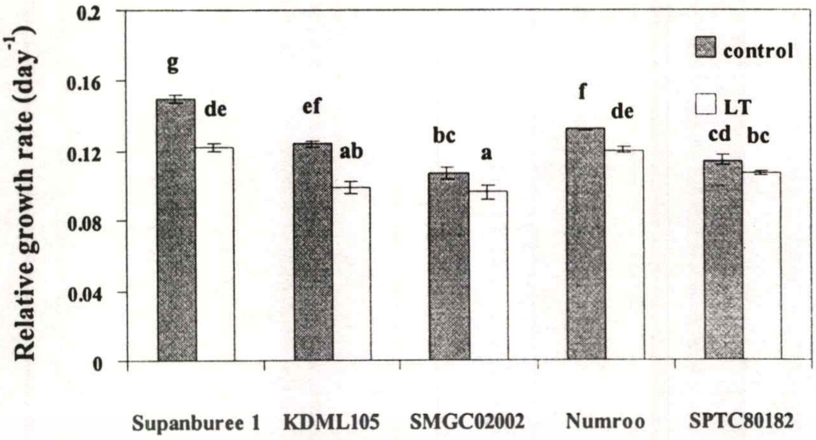


Figure 4.2 Effects of LT on relative growth rate of rice seedlings in comparison to the controls

Dry weights of seedlings were recorded at the end of dark period on day 0 and day 7 of the treatment at 10°C and they were used to calculate relative growth rates. The data represent means and SD of 6 replicates. Different letters above the bars indicate statistically significant difference ($P \leq 0.05$).

4.2 Effects of LT on lipid peroxidation

LT causes the disruption of cellular functions, leading to the overproduction of ROS (Mittler, 2002). This consequent oxidative stress can damage cell constituents, such as membrane lipids. Peroxidation of lipids brings about the production of MDA, which has been proven to be a reliable marker for oxidative damages (Taylor *et al.*, 2004). Therefore, the levels of MDA were measured to test the extent of LT-induced oxidative stress.

After 7 days of LT treatment, lipid peroxidation was higher in both LT-sensitive and tolerant varieties (Figure 4.4). In comparison with the controls, LT-treated Supanburee1 and KDML105 displayed the increase in MDA levels, which rose for 48 and 41%, respectively. The contents of MDA in seedlings of LT-tolerant group were less induced by LT, showing 11-25% increase in response to LT.

These results suggested that LT-tolerant group faced the less severe oxidative stress. This might be attributable to the more effective mechanisms that could prevent the seedlings of these varieties from the excessive accumulation of ROS.

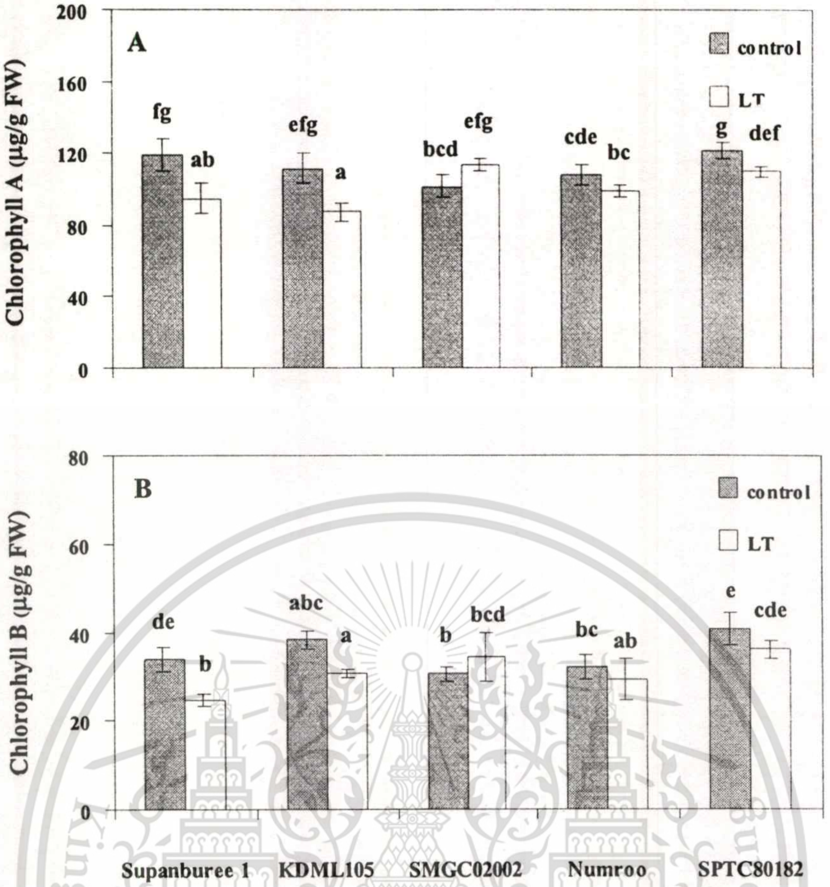


Figure 4.3 Chlorophyll contents of the control and LT-treated rice seedlings

The extents of chlorophyll a (A) and chlorophyll b (B) were measured from the aerial parts of seedlings after LT treatment at 10°C in the dark period for 7 days. The data represent means and SD of 6 replicates. Different letters above the bars indicate statistically significant difference ($P \leq 0.05$) for each experiment.

4.3 LT induced changed in levels of stress-related metabolites

LT stress causes dramatic changes to plant metabolome due to the changes in enzyme activities as well as the reconfiguration of metabolome (Kaplan *et al.*, 2004). In the latter evidence, some metabolites are accumulated in order to protect plants against LT stress. In this study, the responsive patterns of some stress-related metabolites, i.e. soluble sugars, proline, polyamines and flavonoids, were examined in order to characterize the metabolites that play a protective role to Thai rice against LT.

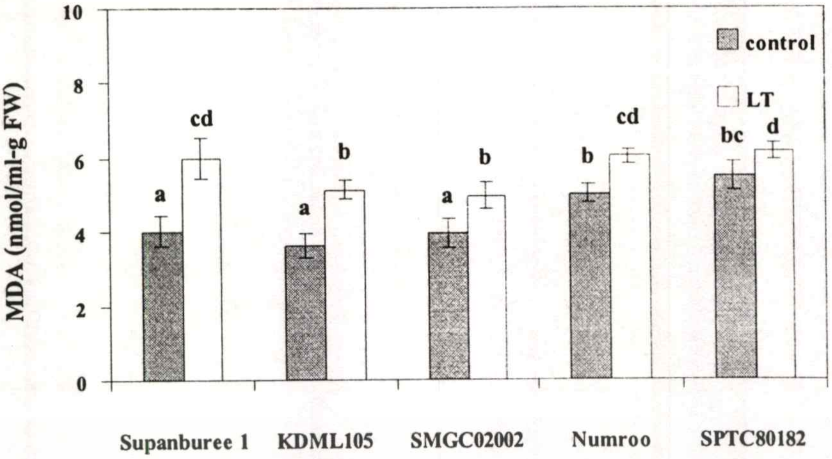


Figure 4.4 Lipid peroxidation of control and LT-treated rice seedlings

The extents of lipid peroxidation were assayed by determining the amount of MDA from the whole seedlings after LT treatment at 10°C in the dark period for 7 days. The data represent means and SD of 6 replicates. Different letters above the bars indicate statistically significant difference ($P \leq 0.05$).

4.3.1 Effects of LT on the contents of total soluble sugars and proline

It is well documented that the contents of compatible solutes in plant cells increase during LT stress. These compounds may protect plants from LT-induced dehydration (Ashraf and Fooland, 2007). Therefore, the levels of soluble sugars and proline, the well-known compatible solutes, were detected to evaluate whether they involve LT tolerance in Thai rice.

LT treatment provoked clearly distinct responses in sugar accumulation between LT-tolerant and LT-sensitive groups (Figure 4.5). Relative to the controls, sugar contents were substantially increased about 55 and 74% in LT-treated Supanburee1 and KDML105, respectively, while they were found to accumulate only about 30% in seedlings of SPTC80182, Numroo and SMGC02002.

Paralleling with the response in soluble sugar levels, LT also induced the more pronounced increase in proline content in LT-sensitive varieties (Figure 4.6). Proline accumulated up to 2 fold in LT-treated Supanburee1 and KDML105. On the contrary, seedlings of SPTC80182, Numroo and SMGC02002 exhibited only 1.5-1.7 fold increase in proline content when exposed to LT.

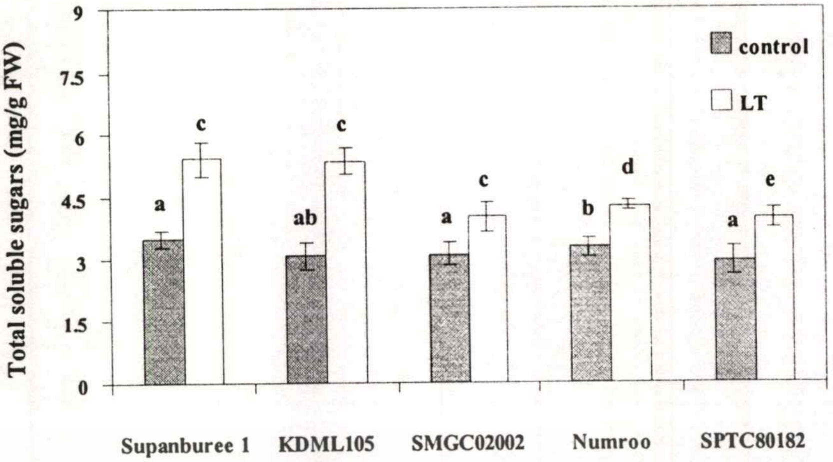


Figure 4.5 Effects of LT on the contents of total soluble sugars in comparison with the control rice seedlings

The extents of soluble sugars were measured from the whole seedlings after treatment at 10°C in the dark period for 7 days. The data represent means and SD of 6 replicates. Different letters above the bars indicate statistically significant difference ($P \leq 0.05$).

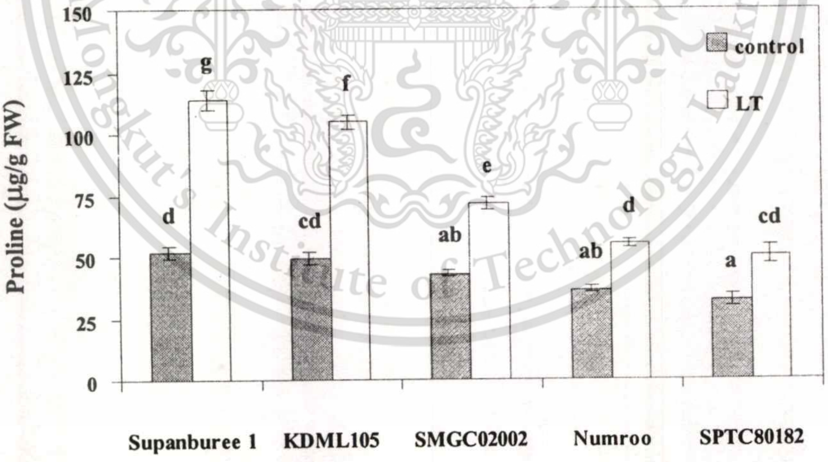


Figure 4.6 Proline contents of control and LT-treated rice seedlings

The contents of proline were assayed from the whole seedlings after LT treatment at 10°C in the dark period for 7 days. The data represent means and SD of 6 replicates. Different letters above the bars indicate statistically significant difference ($P \leq 0.05$).

4.3.2 Effects of LT on the contents of polyamines

There is strong experimental evidence supporting that polyamines are involved in protecting cell integrity and cellular components from LT-induced damages (Pillai and Akiyama, 2004). In this experiment, the contents of 3 major polyamines were measured to determine the involvement of polyamines and the ability to tolerate LT of Thai rice.

The induction of putrescine by LT was observed (Figure 4.7A). Among all varieties, Numroo contained the striking level of putrescine. Its putrescine level was increased by 40% upon LT treatment. However, SPTC80182 exhibited the most increase in putrescine level after exposed to LT (78% higher than that of the control). LT also provoked the increase in spermidine levels in almost all varieties especially Supanburee1, which showed the most significant enhance, and KDML105 (67 and 60% increase, respectively). By contrast, LT treatment resulted in the decrease in spermidine content in SPTC80182, which contained only 84% of that in the control (Figure 4.7B).

The contents of spermine were profoundly lowered in SMGC02002 after LT treatment, exhibiting only 71% of that in the control. On the contrary, the different response was found in Supanburee1, which accumulated 43% higher. However, LT only slightly affected the levels of spermine in KDML105, SPTC80182 and Numroo (Figure 4.7C).

Interestingly, these results indicated the differential response in polyamine levels. While the most increased polyamine in LT tolerant varieties was putrescine, that in LT-sensitive varieties was spermidine. This pattern suggests that the metabolic flux of polyamine biosynthesis pathway may involve the response to LT and/or LT tolerance in Thai rice.

4.3.3 Effects of LT on flavonoid levels

The increase in lipid peroxidation (Figure 4.4) suggested that rice plants suffered from oxidative stress that was induced by LT. To prevent oxidative damages, 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* (Bors *et al.*, 1990), and their contents are changed in response to LT stress (Graham, 1998). Therefore, the involvement of flavonoids and LT tolerance of Thai rice was studied by measuring 3 major flavonoids, i.e. anthocyanins, flavonols and flavones.

LT treatment provoked the changes to flavonoid contents in all varieties although some changes were not significant (Figure 4.8). In spite of the distinct responsive patterns observed in the former experiments, there was the ambiguous correlation between LT-induced changes in flavonoid levels and LT tolerance.

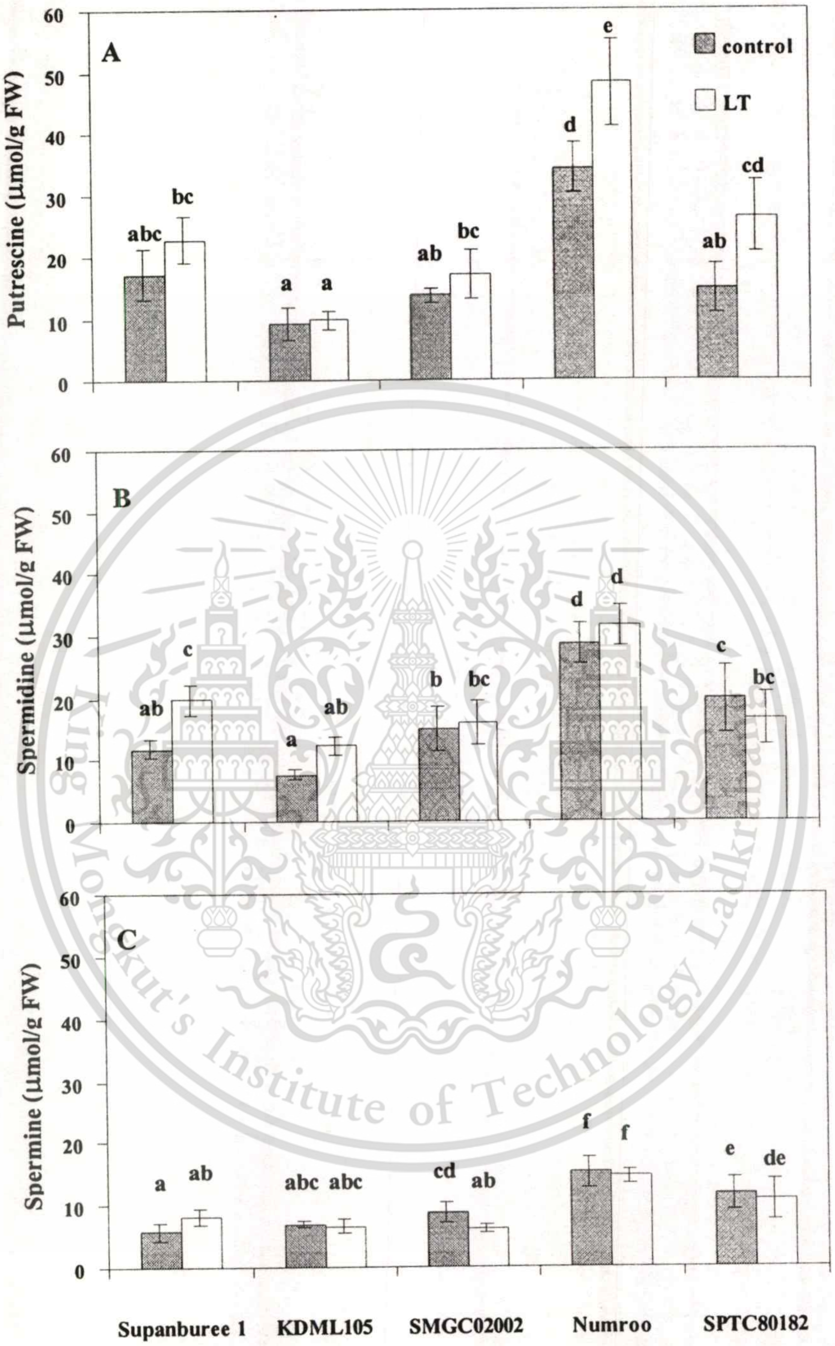


Figure 4.7 Polyamine contents of control and LT-treated rice seedlings

The contents of putrescine (A), spermidine (B) and spermine (C) were assayed as free compounds from the whole seedlings after LT treatment at 10°C in the dark period for 7 days. The data represent means and SD of 6 replicates. Different letters above the bars indicate statistically significant difference ($P \leq 0.05$) for each experiment.

After exposed to LT, the decline in levels of all detected anthocyanins (delphinidin, cyanidin and pelargonidin) was observed in 2 LT-tolerant varieties, namely, SMGC02002 and SPTC80182. LT-treated SPTC80182 accumulated all anthocyanins about 65% of the control. SMGC02002 seedlings also exhibited the similar responsive pattern although the magnitude of difference was not remarkable. By contrast, LT induced the elevation of anthocyanin contents in Numroo, the other LT-tolerance rice, which were up to 28% higher than those in the control. LT was also responsible for enhanced anthocyanin levels in LT-sensitive varieties. Both Supanburee1 and KDML105 exhibited about 30 and 60% higher contents of anthocyanins in response to LT (Figure 4.8A, B and C).

LT did not caused striking changes to flavonol levels. Among all varieties, only SMGC02002 showed the remarkable increase in all detected flavonols (myricetin, quercetin and kaempferol), accounting for about 20% induced by LT. However, the content of flavonols in LT-treated SPTC08182 was also increased for about 6% compared to the control. By contrast, the decrease trend was found in LT-treated Numroo, Supanburee1 and KDML105, whose flavonol contents were about 6-9% lower than those in the controls (Figure 4.8D, E and F).

The changes in the amount of flavones followed the similar pattern to those of flavonols, but the magnitudes were more pronounced. Paralleling with the increase in flavonol levels, SMGC02002 also exhibited 16% higher contents of all examined flavones (luteolin and apigenin) in response to LT. On the other hand, the levels of flavones in LT-treated Supanburee1 and KDML105 were significantly decreased for up to 10%. Compared to the control, LT did not lead to the obvious changes in luteolin level in Numroo seedlings. However, 10% decrease in apigenin was found in this variety after exposed to LT (Figure 4.8G and H).

4.4 Effects of LT on the antioxidative ability of rice seedlings

In addition to flavonoids, plants also synthesize other antioxidants, such as ascorbic acid, tocopherols, carotenoid, glutathione and other phenolic compounds. Most of these agents are water-soluble and they may be part of a complex mechanism of LT tolerance that involves both the avoidance of ROS overproduction and the protection from ROS produced (Møller *et al.*, 2007). In this experiment, the effects of LT on antioxidative ability were assessed by determining DPPH$^{\bullet}$-scavenging activity of water/methanol extracts of Thai rice seedlings.

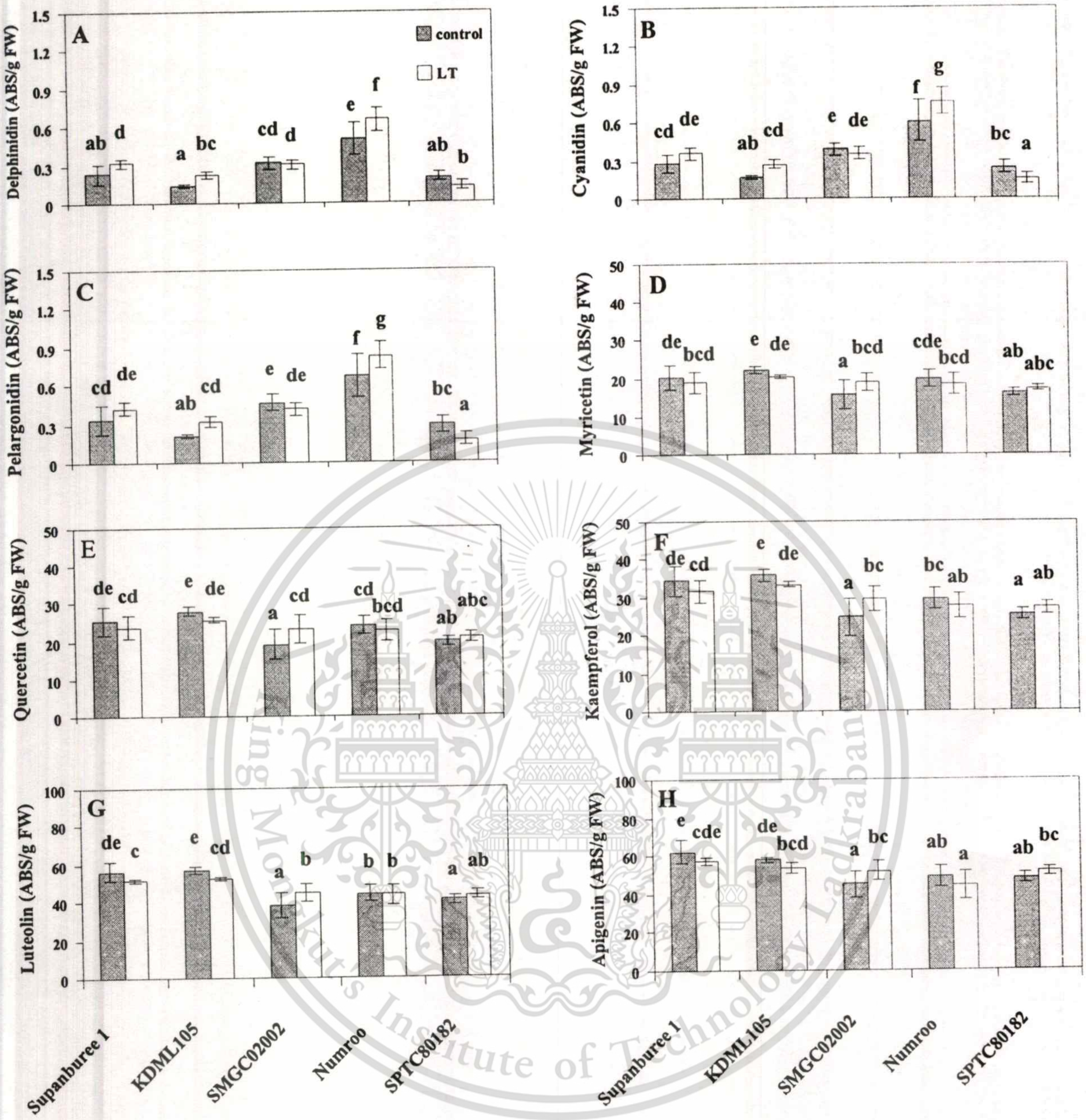


Figure 4.8 Effects of LT on flavonoid contents in comparison with the control seedlings

Flavonoids were extracted from the whole seedlings after LT treatment at 10°C in the dark period for 7 days. The level of each flavonoid was determined as the absorbance at the specific wavelength. The detected flavonoids included anthocyanins (A, B and C), flavonols (D, E and F), as well as flavones (G and H). The data represent means and SD of 6 replicates. Different letters above the bars indicate statistically significant difference ($P \leq 0.05$) for each experiment.

EC_{50} of LT-treated SPTC80182, Numroo and SMGC02002 were decreased by 10% than their controls (Figure 4.9), indicating that the free radical-scavenging activities in these varieties were induced by LT. On the other hand, the antioxidative ability of LT-sensitive varieties remained unchanged after LT treatment. These results suggested that LT tolerance of Thai rice correlated with the increased antioxidative ability in response to LT.

4.4 Effects of LT on the expression of polyamine biosynthetic genes

Genes encoding polyamine biosynthetic enzymes have been isolated from a variety of plant species. Moreover, the expression of several genes is induced by environmental stresses including LT (Takahashi *et al*., 2003). In this study, the differential metabolic fluxes of polyamine biosynthetic pathway between LT-tolerant and LT-sensitive groups were observed after LT treatment. To test whether the changes in gene expression were responsible for this evidence, the expression of genes encoding SAMDC (*OsSAMDC1* and *OsSAMDC2*) as well as SPDS (*OsSPDS*) were analyzed using RT-PCR. These enzymes regulate the synthesis of spermidine, the most accumulated polyamine in LT-sensitive varieties in response to LT.

The results from RT-PCR indicated that LT treatment had little effects on the expression of *OsSPDS* in all varieties. The significant accumulation of *OsSAMDC1* in response to LT was observed in almost all varieties except Numroo, whose *OsSAMDC1* mRNA level were not affected by LT. However, the increase in *OsSAMDC2* mRNA levels was detected in only 2 varieties, i.e. Supanburee1 and SMGC02002. LT did not induce the significant changes in transcript levels of *OsSAMDC2* in KDML105 and SPTC80182. On the other hand, LT-treated Numroo exhibited the decrease trend in *OsSAMDC2* expression (Figure 4.10 and 4.11).

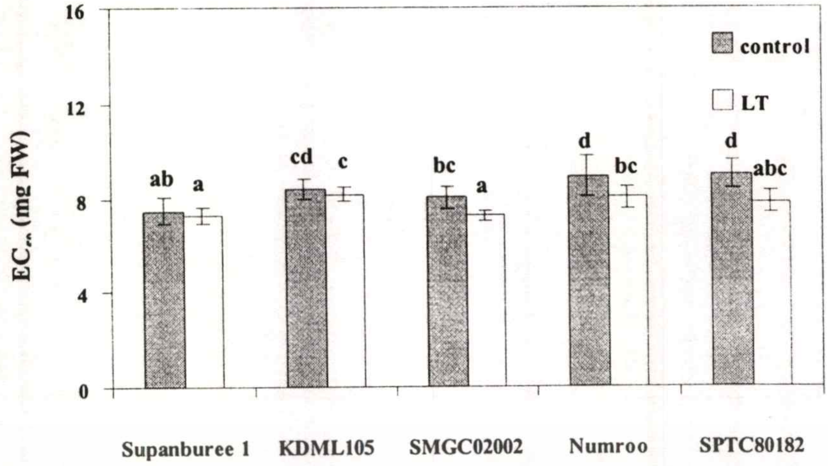


Figure 4.9 DPPH$^{\bullet}$-scavenging activity of control and LT-treated rice seedlings

The ability to scavenge DPPH$^{\bullet}$ was assayed using water/methanol extracts from the whole seedlings after LT treatment at 10°C in the dark period for 7 days. The activity was reported as the amount of seedlings needed to decrease the initial DPPH$^{\bullet}$ concentration by 50% (EC_{50}). The data represent means and SD of 6 replicates. Different letters above the bars indicate statistically significant difference ($P \leq 0.05$) for each experiment.

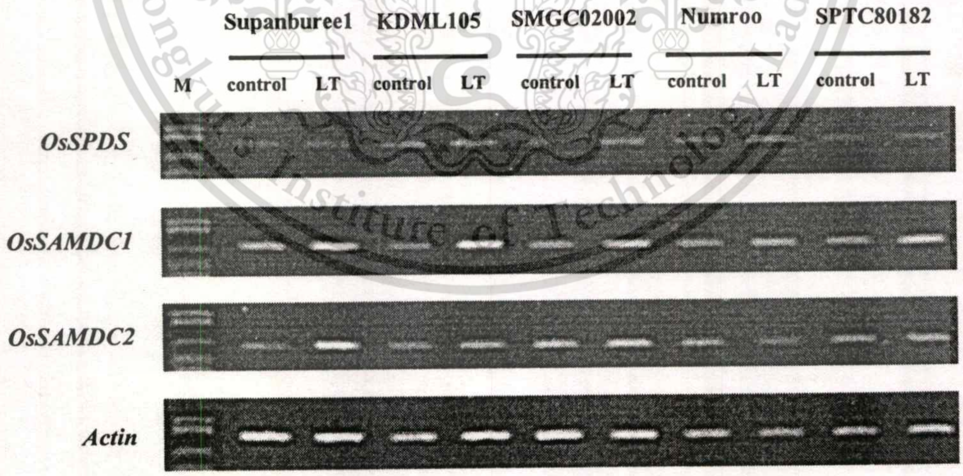


Figure 4.10 Effects of LT on the expression of *OsSPDS*, *OsSAMDC1* and *OsSAMDC2*

Total RNA was extracted from the whole seedlings after LT treatment at 10°C in the dark period for 7 days. RT-PCR products were separated in 1.5% agarose gel and stained with ethidium bromide.

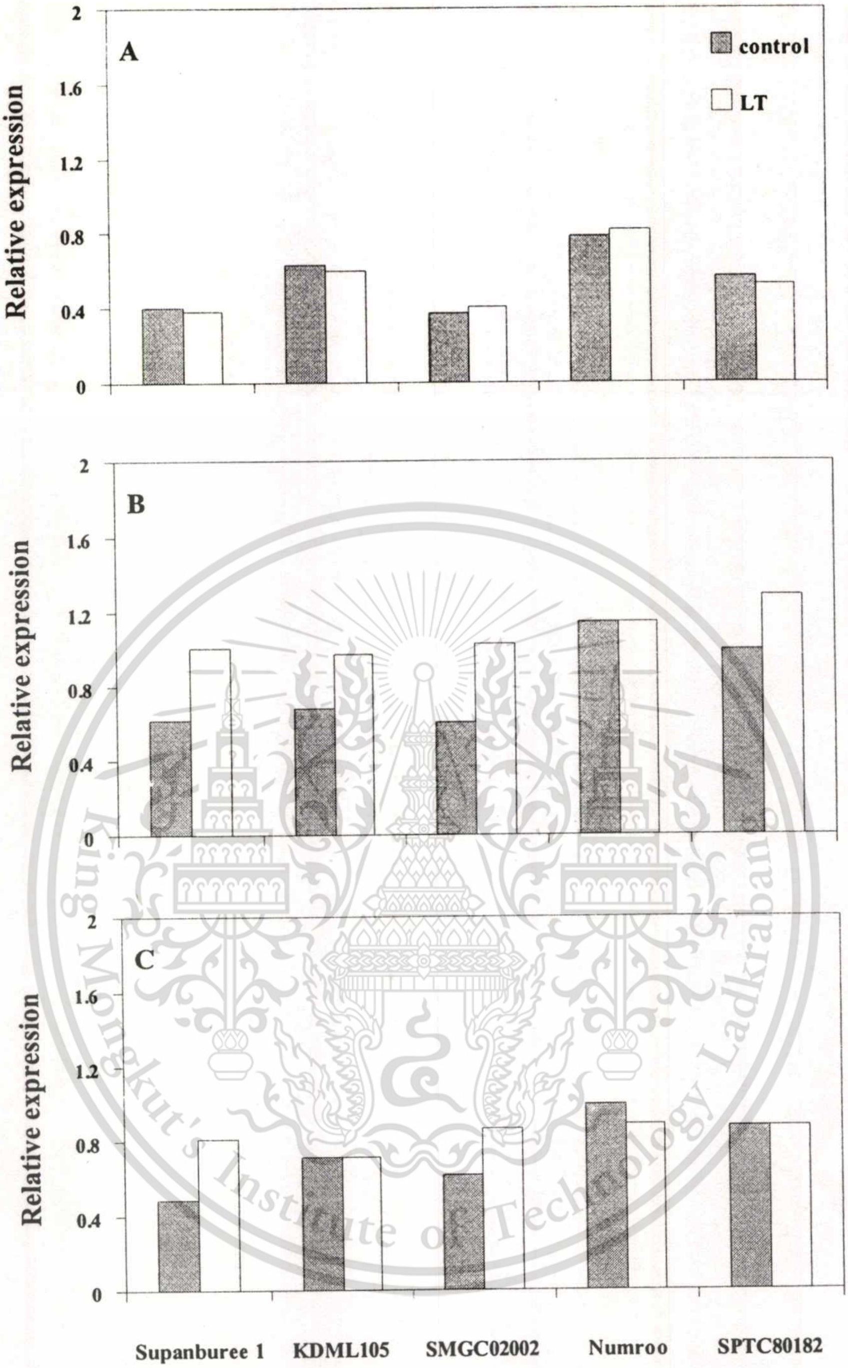


Figure 4.11 Expression of polyamine biosynthesis genes in response to LT

Relative expression of *OsSPDS* (A), *OsSAMDC1* (B) and *OsSAMDC2* (C) represents the ratio of transcript level of each gene to that of Actin from the same sample.

CHAPTER 5

DISCUSSSION

5.1 Categorization of Thai rice varieties based on physiological changes in response to LT

LT has been reported to bring about the dysfunction in nutrient uptake, source-sink translocation, stromal closure and metabolism (Allen and Ort, 2001); thus, exposure to LT leads to growth retardation in the level of whole plant. In general, rice is susceptible to LT at any stage of development. In case of rice seedlings, exposure to LT not only retards the growth but also causes poor establishment and delayed maturation (Zhang *et al.*, 2005). LT also affects photosynthesis by causing the depletion of chlorophyll. Yoshida *et al.* (1996) reported that LT-induced chlorosis of developing rice leaves was related to the disturbance of chloroplast development. Moreover, the level of chlorosis was more severe in Indica than Japonica variety.

Regarding to this rationale, screening based on physiological changes in response to LT has been employed to search for LT-tolerant varieties from nature. This technique includes determining germination rate and greening ability (Sthapit and Witcombe, 1998) as well as measuring the levels of chlorosis and growth rates (IRRI, 2002). Bertin *et al.* (1996) adopted the survival tests to evaluate LT sensitivity in rice seedlings at 2-leaf stage. They suggested that the screening was most effective if performed at 10°C.

In this study, LT-tolerance of 5 Thai rice varieties was determined *in vitro* based on physiological characteristics, along with the changes in relative growth rates and chlorophyll contents (Figure 4.2, 4.3 and 4.4). The rice varieties were categorized into LT-tolerant (SPTC80182, Numroo and SMGC02002) and LT-sensitive (Supanburee1 and KDML105) groups. This categorization was in agreement with the field-based data from Rice Research Center at Sanpatong, Chiangmai, Thailand (Personal contact), suggesting the validity of the criteria used.

5.2 LT-induced lipid peroxidation and effects of LT on antioxidative ability of Thai rice

It is well documented that LT causes the perturbation of normal metabolisms, thus leading to the over accumulation of ROS (Kaplan *et al.*, 2004). The study of Fadzillah *et al.* (1996) indicated the increase in H_2O_2 level in rice shoot cultures upon LT treatment. A correlation between H_2O_2 accumulation and LT stress has also been reported in maize (Kingston-Smith *et al.*, 1999) and winter wheat (Okuda *et al.*, 1991). One of the major targets of ROS is membrane lipids. The peroxidation of lipid consequently results in the accumulation of MDA (Mittler *et al.*, 2004). It was found that the accumulation of H_2O_2 in arabidopsis callus was accompanied by the increase in lipid peroxidation during the course of LT (4°C) treatment (O'Kane *et al.*, 1996). Parelling with the formers reports, all Thai rice varieties in this study exhibited an increase in MDA levels in response to LT (Figure 4.4), indicating that they suffered from oxidative stress.

The study of Campos *et al.* (2003) indicated that the amount of LT-induced lipid peroxidation correlated with LT sensitivity. Likewise, the LT-sensitive rice varieties exhibited the more increase in MDA levels after treated with LT. Since the content of MDA relates to the extent of oxidative damages, this result suggested that LT-tolerant rice varieties possessed the more effective mechanisms to prevent the deleterious effects of ROS produced and/or to repair the damage from ROS.

The data on DPPH$^{\bullet}$-scavenging activity (Figure 4.9) indicated that the increased antioxidative ability of LT-tolerant varieties was more pronounced than that of LT-sensitive varieties. Because water/methanol extracts were used in the assay, this result suggested that the accumulation of water-soluble antioxidants was important for LT tolerance of Thai rice and it may account for the less extent of lipid peroxidation observed in LT-tolerant varieties.

Guo et al. (2006) reported that the higher levels of ascorbate and glutathione were observed in LT-tolerant rice varieties than LT-sensitive varieties during the course of LT treatment. Moreover, the increase in those antioxidants coincides with higher activities of antioxidative enzymes, including superoxide dismutase, catalase and ascorbate peroxidase. Therefore, the accumulated water-soluble antioxidants observed in this study might directly scavenge ROS or be part of enzymatic antioxidative systems. In addition, some lipid-soluble antioxidants such as tocopherols were reported to play a role in ROS scavenging (Havaux *et al.*, 2005). This evidence suggests that Thai rice may also adopt other mechanisms, such as the synthesis of lipid-soluble antioxidants to prevent oxidative damages.

Besides their toxicity, ROS, especially H_2O_2, were used as signaling molecules by plants. H_2O_2 treatment could trigger the expression of genes that are of potential importance to stress response (Desikan *et al.*, 2001). Exogenous H_2O_2 could activate ROS-bZIP1 transcription factor, which involved in LT-response transcriptional network of rice (Cheng *et al.*, 2007). The early and sustainable expression of ROS-bZIP1 regulons was observed in LT-tolerant but not in LT-sensitive varieties. According to this evidence, one of the important factors that provide LT tolerance to Thai rice is probably the ability to control levels of ROS in order to prevent oxidative damages and use them as signaling molecules that trigger cellular defense against LT.

5.3 LT induced changed in levels of stress-related metabolites

5.3.1 Effects of LT on the contents of soluble sugars

LT can cause the depletion of cellular water, leading to the destruction of membrane integrity and protein degradation. While encountering dehydration, many plants accumulate compatible solutes, such as saccharides, polyols, amino acids and quaternary ammonium compounds (Ashraf and Fooland, 2007). The increase in cellular osmolarity that results from the accumulation of these compounds is accompanied by the influx of water into cells or the reduced efflux from cells, thus preventing plants from dehydration (Hare *et al.*, 1998). Under LT stress, *β*-amylase is responsible for the increase in soluble sugars in arabidopsis by activating the degradation of starch into maltose. Maltose can either act as compatible solutes or a substrate for the synthesis of other sugars (Kaplan *et al.*, 2006). Moreover, the degradation of Rubisco large subunit can be observed in LT-treated rice plants (Yan *et al.*, 2005), leading to the limited photosynthesis. Therefore, the catabolism of starch might account for the accumulation of soluble sugars in response to LT observed in Thai rice (Figure 4.5).

In addition, LT-sensitive varieties exhibited higher levels of accumulated sugars after LT treatment. It has been reported that drought stress is accompanied by a decrease in starch biosynthesis in favor of soluble sugars (Valladsen *et al.*, 2005). Taken together, this result may indicate that LT-sensitive varieties encountered more severe dehydration during dehydration stress.

Another possible reason for the increased levels of soluble sugars is based on the rationale that LT can inhibit the transport of photoassimilates from sources, thus preventing the use of sugars by sink tissues. In this case, the less extent of inhibition would occur in LT-tolerant varieties under LT condition. The study of Maeda *et al.* (2006) indicated the roles of tocopherols in phloem loading. Tocopherol-deficient arabidopsis mutant displayed structural changes in phloem parenchyma transfer cells and the accumulation of sugars in source leaves even without the induction of stress. Further study is needed to elucidate whether tocopherol prevents the block of sugar export by LT in Thai rice.

In addition to the osmoprotective role, some kinds of soluble sugars, such as mannitol, have been proven to confer protection against oxidative damages by scavenging ROS (Shen *et al.*, 1997). However, the accumulation of sugars negatively regulates the expression of Calvin cycle enzymes (Rolland *et al.*, 2002). This evidence provokes poor recycling of $NADP^+$ and excessive electron transfer that may contribute to ROS production. Therefore, the high concentration of soluble sugars observed in the LT-sensitive varieties may be excessive, thus promoting the overproduction of ROS.

5.3.2 Effects of LT on proline levels

Amino acid proline is also essential for plants in the period of osmotic stress. LT induces the expression of *OsP5CS* gene, which encodes Δ^1-pyrroline-5-carboxylate synthase enzyme for proline biosynthesis, leading to the increase in proline content (Hur *et al.*, 2004). In this study, LT elevated proline levels in all rice varieties, but the increase was more pronounced in LT-sensitive varieties (Figure 4.6). Paralleling with soluble sugars, proline plays a role in osmotic adjustment and drought stress induces the accumulation of proline (Bray, 1997). Therefore, the extent of dehydration in LT-sensitive varieties may be more severe than that in LT-tolerant rice under LT treatment.

Proline metabolism is responsive to the levels of sugars, especially when the internal concentration exceeds a critical level, especially during dehydration. The exogenous supply of several kinds of sugars, such as glucose, fructose and sucrose, induces proline accumulation (Kishor *et al.*, 2005). The study of Hellmann *et al.* (2000) indicated that endogenous glucose could reduce proline catabolism by repressing the expression of *ProDH* gene, which encoded proline degradation enzyme (proline dehydrogenase). Taken together, the high concentration of proline observed in LT-sensitive rice may be caused by high sugar levels in response to LT.

Although proline accumulation confers tolerance several stresses including LT, there are a number of reports indicating the deleterious impacts of excessive proline level. Arabidopsis *pdh* mutant, which defected in proline dehydrogenase, showed hypersensitivity to exogenous application of proline while wild type plants grew normally (Nanjo *et al.*, 2003). Hellmann *et al.* (2000) reported that the toxicity of proline was mediated by the accumulation of Δ^1-pyrroline-5-carboxylate, a product of proline degradation. They suggested that this compound might act as a signaling molecule that triggered apoptosis. Because proline serves as the energy source after stress, there may be the degradation of proline during the light period when rice plants in this study were grown in normal condition. The greater extent of accumulated proline in LT-sensitive rice may contribute to higher contents of Δ^1-pyrroline-5-carboxylate, thus leading to the more severe injuries observed in these varieties.

5.3.3 Effects of LT on polyamine levels and the expression of polyamine biosynthetic genes

Polyamines are implicated in a wide range of biological processes including stress response (Alcázar *et al.*, 2006). It has been reported that LT induces the accumulation of polyamines in many species, such as cucumber, zucchini, arabidopsis and spinach. The study of Lee *et al.* (1997) indicated the significant increase in putrescine, spermidine and spermine in LT-tolerant but not in LT-sensitive rice. Moreover, the increase in putrescine level was correlated with the ability to tolerate LT of rice plants (Lee *et al.*, 1995). Similarly to the reports, the LT-tolerant rice varieties in this study exhibited the increase in putrescine contents with the greatest extent among all detected polyamines (Figure 4.7). In rice seedlings grown at LT (5°C) condition, putrescine accumulation was observed at the third day, while the increase in spermidine and spermine levels was detected after 9 days of LT treatment (Lee, 1997). The similar trend was also presented in LT-treated of poplar (Renaut *et al.*, 2005). It has been proposed that putrescine contents usually react quickly to the changes in environment. However, its increase is often observed when the stress is relatively mild (Gropp and Benevides, 2007). Therefore, the metabolic flux toward spermidine presented in Supanburee1 and KDML105 may imply that these varieties encountered more sever damages after exposed to LT.

Another possible reason for this evidence relies on the physiological functions of putrescine and spermidine. It was found that the application of putrescine, but not spermidine could inhibit DNA degradation and prevent cell death of tobacco protoplast (Papadakis *et al.*, 2005). Moreover, the increase in putrescine is accompanied by the inhibition of DNA methylation. This permits the expression of genes, including those related to stress responses (Martin-Tanguy *et al.*, 1996). Regarding to these studies, the accumulation of putrescine may contribute to LT tolerance of Thai rice.

In plants, two enzymes, namely SPDS and SAMDC, regulate the synthesis of spermidine from putrescine. It has been reported that genes encoding these enzymes are presented as small gene families and the expression of several genes is induced by LT (Alcázar *et al.*, 2006). To date, 2 genes for spermidine synthase have been characterized in rice (*OsSPDS1*and *OsSPDS2*). Imai *et al.* (2004) reported that *OsSPDS2* mRNA significantly accumulated in roots during LT stress. However, only little increase in transcript level of *OsSPDS2* as well as its paralogous *OsSPDS1* was observed in shoot samples. In this study, LT did not induce significant changes in transcript level of *OsSPDS* in all rice varieties (Figure 4.10 and 4.11). This may be attributable to the different rice tissues as well as techniques for monitoring gene expression. In the experiment of Imai *et al.* (2004) , the expression of *OsSPDS1* and *OsSPDS2* was analyzed in shoot or root separately by using northern hybridization with specific probes for each gene. By contrast, whole seedlings were used in this study and gene expression was analyzed by using RT-PCR with degenerated primers that was designed for amplifying both *OsSPDS1* and *OsSPDS2* simultaneously. Therefore, the low proportion of roots (approximately ¼) in each sample as well as the specificity of technique for analyzing gene expression may account for these differences.

In rice genome, there are 2 genes encoding SAMDC, i.e. *OsSAMDC1* and *OsSAMDC2*. Pillai and Akiyama (2004) indicated that the expression of both *OsSAMDC1* and *OsSAMDC2* was induced by LT and it was responsible for the increase in spermidine in rice during LT stress. Similarly, the expression of *OsSAMDC1* was significantly increased in both Supanbureel and KDML105, suggesting that *OsSAMDC1* may be responsible for the accumulation of spermidine in LT-sensitive varieties. However, LT also induced the expression of *OsSAMDC2* in Supanbureel. *OsSAMDC2* has been proven to involved multi-stress response, such as ABA, drought and LT (Pillai and Akiyama, 2004). Moreover, spermidine was reported as the major polyamine accumulated in drought-stressed tissue (Liu *et al.*, 2000). Therefore, the expression of *OsSAMDC2* in Supanbureel may reflect the severe dehydration occurred during exposure to LT.

LT-induction of OsSAMDC genes was also observed in SPTC80182 and SMGC02002 seedlings although these varieties exhibited the metabolic flux toward putrescine. In plants, polyamines are present not only as free bases but also as conjugated forms. Moreover, the conjugation of spermidine to phospholipid head groups can maintain membrane integrity during stress (Takahashi *et al.*, 2003). Therefore, it is possible that the conjugations of spermidine involves in controlling its levels and provide protective roles against LT to SPTC80182 and SMGC02002 varieties.

By contrast, the level of spermidine in Numroo was still increased although the overall expression of *OsSAMDC* was decreased in response to LT. This is probably attributable to the lower degradation rate of spermidine. In plants, amine oxidases regulate the catabolism of polyamines, which leads to the production of H_2O_2 (Cona *et al.*, 2006). Thus, the decrease in spermidine degradation may provide advantages in controlling ROS levels in Numroo during exposed to LT.

5.3.4 Effects of LT on flavonoid levels

Flavonoids play a role in plant protection against LT stress through their antioxidative ability. It has been reported that the levels of flavonoids increase in response to LT in many plant species (Shirley, 1996). Moreover, LT treatment of arabidopsis is accompanied with the upregulation of at least 12 flavonoid biosynthetic genes (Hannah *et al.*, 2006). In this study, the changes in flavonoid levels were also observed in all rice varieties after LT treatment. However, the correlation between LT-induced changes in flavonoid levels and LT tolerance was unclear. While the metabolic flux toward flavonol and flavone was observed in LT-tolerant SMGC02002 and SPTC80182, the increase in anthocyanin was presented in Numroo, the other LT-tolerant variety, as well as in all LT-sensitive varieties (Figure 4.8). These results suggested that flavonols and flavones might contribute to LT tolerance of SMGC02002 and SPTC80182.

Anthocyanins have been reported to involve in LT protection in many plants, such as bilberry, winter oilseed rape and peach (Taulavuori *et al.*, 2004; Solecka and Kacperska, 1995; Leng and Qi, 2003). According to these studies, the increase in anthocyanin levels in Numroo may be a part of protective mechanisms against LT. However, Numroo may employ other mechanisms than the accumulation of flavonoids to cope with LT.

On the other hands, there are reports indicated that the accumulation of anthocyanins might be an indicator of stress and senescence in plants (Maeda *et al.*, 2006 and Winkel-Shirley, 2001). The accumulation of anthocyanins in Supanburee1 and KDML105, therefore, might imply the intolerance to LT of these varieties.

CHAPTER 6

CONCLUSION AND SUGGESTION

6.1 Conclusion

In this study, 5 varieties of Thai rice were categorized based on the physiological changes in response to LT. LT-tolerant group (SMGC02002, Numroo and SPTC80182) exhibited the better physiological characteristics, i.e. relative growth rates and chlorophyll contents, than LT-sensitive group (Supanburee1 and KDML105). Moreover, the contents of MDA, a product of lipid peroxidation, were increased in the less extent in LT-tolerant varieties.

The study of LT-induced changes in some stress-related metabolites and antioxidative ability revealed the differential responses between LT-tolerant and LT-sensitive groups. After LT treatment, the more increased levels of soluble sugars and proline were observed in LT-sensitive varieties, suggesting that they might encounter more severe dehydration. Differential responses in polyamine accumulation in response to LT were also detected. While the most increased polyamine in LT-tolerant group was putrescine, that in LT-sesitive group was spermidine. Moreover, it was found that LT tolerance related with the increase in antioxidative ability. However, the correlation between flavonoid accumulation under LT stress and the ability to tolerate LT was unclear, suggesting that the response in flavonoid levels may be species-specific.

The expression of 3 polyamine biosynthetic genes, namely *OsSPDS*, *OsSAMDC1* and *OsSAMDC2* was studied. The results indicated that *OsSPDS* was not responsive to LT. The changes in the expression of *OsSAMDC1* and/or *OsSAMDC2* were accompanied with the increase in spermidine levels in LT-sensitive varieties after exposed to LT. On the other hand, there was no correlation between the expression of *OsSAMDC* and levels of spermidine in LT-tolerant varieties. These results indicated that other mechanisms, such as polyamine conjugation or degradation, might also account for the changes in polyamine levels in response to LT.

6.2 Suggestion

LT stress causes the reconfiguration of plant metabolome as a result of changes in enzymatic activity. A number of metabolites are accumulated under this condition because they provide protective roles to plants. In this study, LT also induced the changes in levels of metabolites in Thai rice. Some metabolites such as putrescine and water-soluble antioxidants were more accumulated in LT-tolerant varieties than LT-sensitive ones. These metabolites may be used as markers for the selection of other LT-tolerant varieties form nature. Moreover, they may be the promising candidates for plant improvement via genetic engineering.

The osmoprotectants (soluble sugars and proline), however, are also induced in both LT-tolerant and LT-sensitive varieties, suggesting the importance of these compounds to Thai rice plants under LT condition. However, their accumulation should be optimized because the overproduction may bring about the deleterious effects to plants. In this regard, the utilization of stress-induced promoter may be advantageous.

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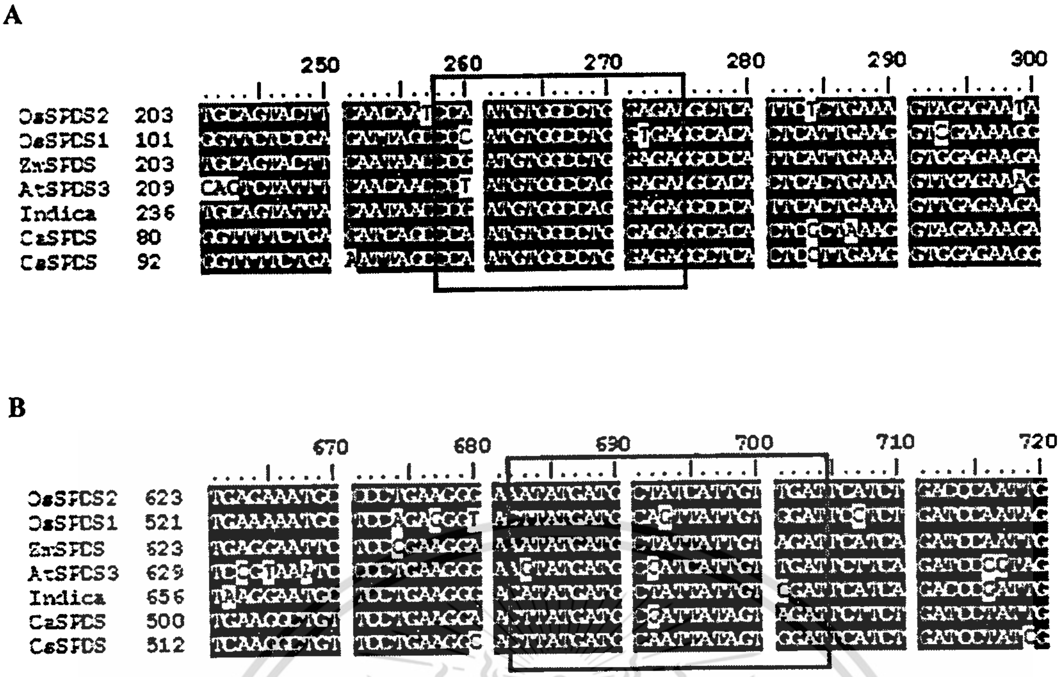
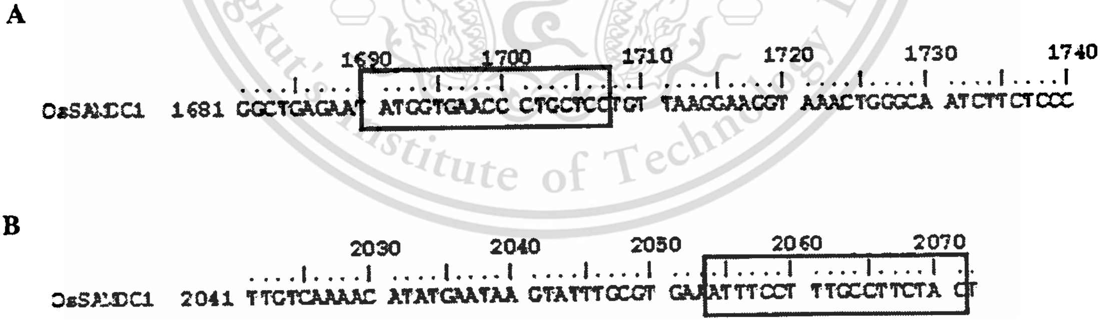
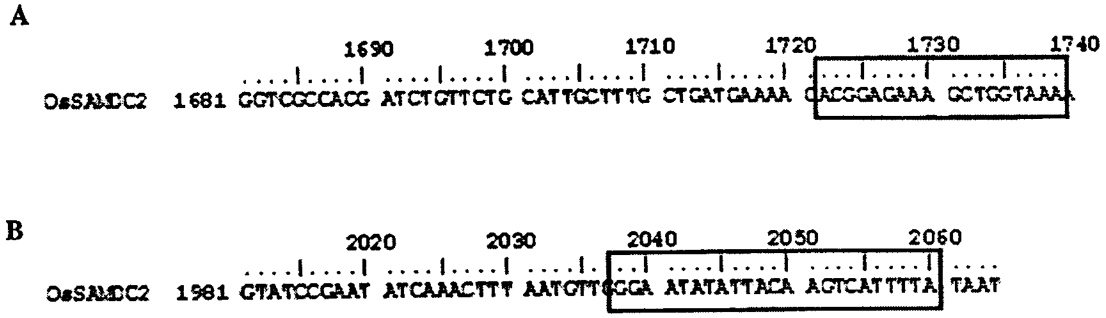
APPENDICES



Appendix 1. Multiple alignments showing the conserved regions of *SPDS*. Nucleotide sequences used to design forward primer (A) and reverse primer (B) for amplifying *OsSPDS* from Thai rice are shown in the boxes.



Appendix 2. Nucleotide sequences of *OsSAMDC1* used to design forward primer (A) and reverse primer (B) as shown in the boxes.



```
A
                    1690      1700      1710      1720      1730      1740
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
OsSAMDC2  1681 GGTCGCCACG ATCTGTTCTG CATTGCTTTG CTGATGAAAA CACGGAGAAA GCTGGTAAAA

B
                    2020      2030      2040      2050      2060
               ....|....| ....|....| ....|....| ....|....| ....|....| ....
OsSAMDC2  1981 GTATCCGAAT ATCAAACTTT AATGTTGGGA ATATATTACA AGTCATTTTA TAAT
```

Appendix 3. Nucleotide sequences of *OsSAMDC2* used to design forward primer (A) and reverse primer (B) as shown in the boxes

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