ความต้านทานต่อภาวะแล้งและการเปลี่ยนแปลงโปรตีนที่ชักนำด้วยไคโตซานในข้าว *Oryza sativa* L.

นางสาววาสินี พงษ์ประยูร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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DROUGHT RESISTANCE AND PROTEIN CHANGES INDUCED BY CHITOSAN IN RICE *Oryza sativa* L.

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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วาสินี พงษ์ประยูร : ความต้านทานต่อภาวะแล้งและการเปลี่ยนแปลงโปรตีนที่ชักนำด้วยไคโตซานในข้าว *Oryza sativa* L. (DROUGHT RESISTANCE AND PROTEIN CHANGES INDUCED BY CHITOSAN IN RICE *Oryza sativa* L.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.ศุภจิตรา ชัชวาลย์ , อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.สิทธิรักษ์ รอยตระกูล , Prof. Luca Comai, Ph.D., 215 หน้า.

งานวิจัยครั้งนี้มีจุดประสงค์เพื่อตรวจหาชนิดและเข้มข้นของไคโตซานที่เหมาะสมในการต้านทานต่อภาวะแล้งในข้าว บนสมมุติฐานที่ว่า เป้าหมายหนึ่งของ ไคโตซานมีผลต่อระบบแอนติออกซิแดน ท์ และการศึกษาทางโปรติโอมิคส์นำมาใช้เพื่อ ระบุชนิดของ โปรตีนที่เกิดจากการใช้ไคโตซานในการต้านทานต่อภาวะแล้งในระบบอื่นรวมทั้งระบุยีนบางตัวที่ตอบสนองต่อไคโตซานในการต้านทานต่อภาวะแล้งในข้าวสายพันธุ์กลายด้วยเทคนิคของ TILLING

ข้าวสองพันธุ์ที่ใช้ในการศึกษาครั้งนี้คือข้าว สายพันธุ์เหลืองประทิว 123 ('LPT123') และ 'LPT123-TC171' ซึ่งมีลักษณะทางพันธุกรรมที่ใกล้เคียงกันแต่มียีนที่ตอบสนองต่อ ภาวะเค็มและแล้งแตกต่างกัน เพื่อศึกษาหาชนิดและความเข้มข้นของไคโตซานที่เหมาะสมในการต้านทานต่อภาวะแล้ง ใช้ไคโตซาน 4 ชนิด คือโพลิเมอร์ (P-80) โอลิโกเมอร์ (O-80) โพลิเมอร์ 90 (P-90) และ โอลิโกเมอร์ 90 (O-90) ความเข้มข้นที่ 20 หรือ 40 มิลลิกรัมต่อลิตร ภายใต้ภาวะแล้งและหลังจากภาวะแล้ง พบว่าไคโตซาน O-80 ความเข้มข้น 40 มิลลิกรัมต่อลิตร สามารถชักนำการเพิ่มขึ้นของน้ำหนักสด น้ำหนักแห้งในใบอย่างมีนัยสำคัญและมีแนวโน้มเพิ่มปริมาณน้ำในใบ (shoot water content) ใน 'LPT123' แต่ไคโตซานไม่มีผลต่อการเจริญเติบโต ของ 'LPT123-TC171' การใช้ไค โตซาน O-80 ก่อนได้รับภาวะแล้ง สามารถเพิ่มรงควัตถุที่ใช้ในการกระบวนการสังเคราะห์ด้วยแสง ในข้าวทั้งสองสายพันธุ์ หลังจากได้รับ ภาวะแล้งเป็นเวลา 7 วันใน 'LPT123' พบว่าการใช้ไคโตซานสามารถรักษาจำนวนรงควัตถุได้เท่ากับชุดควบคุมที่ไม่ได้รับไคโตซาน แต่ ไคโตซานเกิดผลในทางลบต่อ 'LPT123-TC171' ภาวะแล้งชักนำการ ผลิต H_2O_2 ในข้าวทั้งสองสายพันธุ์ แต่การใช้ไคโตซานในภาวะแล้ง แสดงให้เห็นถึงการลดลงของปริมาณ H_2O_2 ในข้าว 'LPT123-TC171' นอกจากนี้การตรวจสอบระบบของแอนติออกซิแดนท์ในภาวะแล้ง ปริมาณของแอสคอร์บิก GSH และ GSSG ไม่เกี่ยวกับการต้านทานต่อภาวะแล้งที่ชักนำด้วยไคโตซานใน 'LPT123' และพบการทำงานของเอนไซม์ ในรากมากกว่าใบ ของ 'LPT123' ซึ่งผลตรงข้ามกับ ที่พบใน 'LPT123-TC171' ไปสนับสนุนที่ว่าผลผลิตของ H_2O_2 ที่ต่ำลงในภาวะแล้ง ใน 'LPT123-TC171' ที่ได้รับไคโตซานก่อให้เกิดการ เจริญเติบโตและการต่อต้านต่อภาวะแล้งที่น้อยลงเมื่อเปรียบเทียบกับ 'LPT123' แสดงให้เห็นว่า H_2O_2 อาจจะเป็นตัวสัญญาณที่ต้องการในการตอบสนองต่อไคโตซานในข้าว นอกจากนี้ พบว่า การใช้ไคโตซานไม่มีผลต่อการเพิ่มขึ้นใน ทำงานของเอนไซม์ APTase ทั้งในใบและรากของ ข้าวทั้งสองสายพันธุ์ ภายใต้ภาวะแล้ง การศึกษาเชิงโปรติโอมิกส์ด้วย LC/MS/MS นำมาใช้ในการวิเคราะห์การเปลี่ยนแปลงของโปรตีนในการตอบสนองต่อไคโตซาน ในภาวะแล้ง จากผลการทดลอง พบการเปลี่ยนแปลงของโปรตีนทั้งหมด 168 โปรตีนในใบ และ 92 โปรตีนในราก โปรตีนที่มีการแสดงออกอย่างมีนัยสำคัญในใบและรากของ 'LPT123' โปรตีนที่แสดงออกน้อยลงจำนวน 20 และ 21 โปรตีน แต่พบโปรตีนที่มีการแสดงออกมาก ขึ้นจำนวน 15 และ 7 โปรตีน ตามลำดับ ส่วน 'LPT123-TC171' พบโปรตีนที่แสดงออกน้อยลงจำนวน 49 และ 12 โปรตีน และพบโปรตีนที่มีการแสดงออกมากขึ้นจำนวน 4 และ 8 โปรตีน ตามลำดับ การเปลี่ยนแปลงของโปรตีนอย่างมีนัยสำคัญในภาวะแล้งพบในกระบวนการต่างๆ เช่น กระบวนการเมตาโบลิซึม การส่งสัญญาณ กระบวนการถอดรหัส การลำเลียงสาร การต้านทานต่อโรค การเจริญเติบโตและการสลายของโปรตีน ชี้ให้เห็นถึงศักยภาพในการระบุชนิดของโปรตีนใหม่ที่เกี่ยวข้องในการต้านทานต่อภาวะแล้งในข้าว นอกจากนี้นำโปรตีนที่มีการแสดงออกมากขึ้น ในภาวะแล้งใน ใบของ 'LPT123' มาทำนายการทำงานการจับกับกรดนิว คลีอิคโดยใช้วิธีการ ของ qPCR พบการแสดงออกของยีน *Os12g23700* and *Os02g58440* ที่เกี่ยวข้องกับการส่ง สัญญาณและกระบวนการถอดรหัส ที่ได้รับไคโตซานเพิ่มมากขึ้นในช่วงเวลาเริ่มต้นเมื่อ เปรียบเทียบกับชุดควบคุม แสดงให้เห็นว่ายีนทั้งสองตัวน่าจะมีบทบาทในการตอบสนองต่อไคโตซา นในภาวะแล้งใน 'LPT123' และการระบุชนิดของการเกิดมิวเทชันในยีนที่สนใจจำนวน 5 ยีนเพื่อที่จะศึกษาลักษณะสมบัติ ต่อไปด้วยเทคนิค TILLING พบการเปลี่ยนแปลงของนิวคลีโอไทด์จำนวน 66 นิวคลีโอไทด์ในข้าวสายพันธุ์ Nipponbare ที่ได้รับ sodium azide and methyl nitrosourea (Az-MNU) และพบมิวเทชันต่างๆ เช่น silent non-severe mutation (NSM) possibly-severe mutation (PSM) truncation splicing และมิวเทชันในส่วนอินตรอน

สาขาวิชา....วิทยาศาสตร์ชีวภาพ.... ลายมือชื่อนิสิต.....

ปีการศึกษา..2554..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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This research aims to determine the appropriate chitosan types and concentrations for drought resistant induction in rice based on the hypothesized that the antioxidant system should be one of chitosan targets. Then, the proteomic approach was used to identify the other systems that resulted from chitosan treatment for drought resistant induction. Finally, the genes responsible for drought resistance induced by chitosan was identified and the mutant lines in some chitosan responsive genes were identified by TILLING method.

Two rice lines, 'Leung Pratew 123' ('LPT123') (*Oryza sativa* L. 'Leung Pratew123') and 'LPT123-TC171' rice lines, which have the similar genotype, except the genes responsible for salt and drought resistance were used in this study. To determine of the appropriate chitosan types and concentrations for drought resistance induction, four types of chitosan molecules, P-80, O-80, P-90 and O-90 at 20 or 40 mg/L were applied during and after drought stress. O-80 chitosan at the concentration of 40 mg/L significantly increased of SFW and SDW in 'LPT123', while shoot water content tended to increase, but chitosan did not affect 'LPT123-TC171' growth under drought stress. Application of oligomeric chitosan (O-80) before drought stress could increase photosynthetic pigments in both rice lines. After drought stress for 7 days, 'LPT123' could maintain the pigment contents at the same level on control without chitosan application, while it showed the negative effects on 'LPT123-TC171'. Drought induced H_2O_2 production was detected in both rice lines, but chitosan treatment clearly showed the reduction of H_2O_2 content only in 'LPT123-TC171' rice during drought stress. Moreover, antioxidative systems were quantified during drought stress. Ascorbic acid, GSH and GSSG contents did not seem to be involved in drought resistance, induced by chitosan in 'LPT123'. In 'LPT123', more antioxidant enzyme activities are found in roots than shoots, which is opposite to what found in 'LPT123-TC171, this supports lower H_2O_2 production during drought stress in 'LPT123-TC171' resulted in less growth and drought resistant enhancement by chitosan, when compared to 'LPT123'. This suggested that H_2O_2 might be the required signal for chitosan responses in rice. Furthermore, chitosan treatment did not affect the increasing of ATPase activities in leaves and roots of both lines under drought stress. The proteomics approach using LC-MS/MS was employed to analyze protein changes in response to chitosan during drought stress. The results showed the changing of total of 168 proteins in leaves and 92 proteins in roots. Within the significant protein expression in leaves and roots of 'LPT123', 20 and 21 proteins were found to be down-regulated, whereas 15 and 7 proteins were up-regulated, respectively. On the other hand, in leaves and roots of 'LPT123-TC171', 49 and 12 proteins were found to be down-regulated, while, 4 and 8 proteins were up-regulated, respectively. The significant changes in abundance of proteins during drought stress indicated that several pathways including metabolic process, signal transduction, transcription, transport, disease resistance/defense, growth and protein degradation. These data suggest the potential for identification of the novel proteins involving in drought resistance in rice. Moreover, the up-regulated proteins during drought stress in leaves of 'LPT123' which were predicted to have the nucleic acid binding activity, was investigated by qPCR method. The expression of the genes; *Os12g23700* and *Os02g58440* involving in signal transduction and transcription, respectively, when subject to chitosan were higher than the control especially in the early phase. This suggests that both genes may play a role in chitosan response in 'LPT123' during drought stress. Finally, TILLING was used to identify the mutant in the 5 genes of interest for further characterization. The 66 nucleotide changes were identified in the sodium azide and methyl nitrosourea (Az-MNU) treated 'Nipponbare' population. The various mutations were silent mutation, non-severe mutation (NSM), possibly-severe mutation (PSM), truncation, splicing and mutation in intron.

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LIST OF ABBREVIATIONS

A	adenine
AA	ascorbic acid
ABA	abscisic acid
ACN	acetonitrile
APS	ammonium persulfate
APx	ascorbate peroxidase
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
°C	degree Celsius
C	cytosine
Ca^{2+}	calcium ions
CAT	catalase
CDNB	1-chloro-2, 4-dinitrobenzene
CH_3COONa	sodium acetate
CRD	completely randomized design
Da	dalton
DCIP	dichlorophenolindolphenol
DD	deacetylation
DEPC	diethyl pyrocarbonate
DHAsA	dehydroascorbate
DMRT	Duncan's Multiple Range Test
DNPH	dinitrophenylhydradzine
DOC	deoxycholic acid
DP	degree of polymerization
DREB	Dehydration Responsive Element Binding
DTNB	dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
ESI-MS/MS	electrospray ionization tandem mass spectrometry

EST	expressed sequence tag
EtOH	ethanol
g	gram
G	guanine
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
GO	gene ontology
GR	glutathione reducatse
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione-*S*-transferase
h	hour
H_2O_2	hydrogen peroxide
ha	hecture
HCl	hydrochloric acid
kb	kilo base pair
LEA	Late Embryogenesis Abundant
LPT123	Leung Pratew 123
M	molar
MDAR	monodehydroascorbate reductase
MDAsA	monodehydroascorbate
MEV	Multi Experiment Viewer
mg	milligram
min	minute
ml	millilitre
MNU	methyl nitrosourea
N	north
Na_2CO_3	disodium carbonate
$Na_2S_2O_3$	sodium thiosulfate
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide

NCBI	National Center for Biotechnology Information
NE	northeast
nm	nanometer
O	oligomer
$^1O_2$	singlet oxygen
O_2^-	superoxide anion
O-80	oligomeric chitosan with 80% of deacetylation
OH^-	hydroxyl ion
P	polymer
PCR	polymerase chain reaction
PEG6000	polyethylene glycol 6000
pg	pictogram
PLBs	protocorm like bodies
POD	peroxidase
ppm	parts per million
PSI	photosystem I
PVP	polyvinylpyrrolidone
ROS	reactive oxygen species
rpm	revolutions per minute
s	second
S.E.	standard error
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis
SOD	superoxide dismutase
T	thymine
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethyl-ethane-1, 2-diamine
TILLING	Targetting Induced Local Lesions IN Genomes
µl	microlitre

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important crops in the world and it is the staple food for more than half of the world's population (Wu et al., 2004). Since rice has a genome significantly smaller than those of other cereals, it is a model to for study the genetic and molecular biology study. About ~130 million ha of the rice growing area threatened by abiotic stresses, especially drought (Ali and Komatsu, 2006). These areas consist of irrigated and rainfed lowlands, which are approximately 85% of total rice production area in the world. Drought stress causes the effect on the rice yield production (Gorantla et al., 2007). The method to solve the problem of drought stress-induced yield losses is to develop the conventional breeding for drought-tolerant rice lines. However, this method is slow, because of the lack of genetic and molecular informations on related genes and regulation.

Alternatively, an exogenous application of osmotic solutes such as putrescine, spermine (Ndayiragije and Lutt, 2006) and spermidine (Roy et al., 2005) mentioned to induce drought tolerance. ABA and glucose exogenous applications pretreated or acclimatized the plant before exposing to extreme environmental stresses, such as drought and salinity (Wang, Huang and Xu, 2003; Cha-um et al., 2007). Subjected to drought stress, plants change in a wide range of physiochemical and biochemical processes from photosynthesis to solute accumulation and protein synthesis (Mundree et al., 2002).

Chitosan is a derivative of chitin (Vander et al., 1998). Chitosan treatment in rice induces various defense-related cellular responses (Zhang et al., 2002). It was shown to be a chemical use for improvement of water use efficiency in pepper (*Capsicum* sp.) (Bittelli et al., 2001). Previously report indicated that chitosan could induce in stomatal closure via Ca^{2+} and hydrogen peroxide (H_2O_2) signals (Lee et al., 1999). Therefore, it may result in transpiration decrease. Chitosan application in pepper leaves could reduce water use efficiency by approximately 26–43%, while the plants could remain biomass and yield. It was suggested the chitosan as an antitranspirant by water use (Bittelli et al., 2001).

Molecular types and concentrations of chitosan application affect plant responses. Oligochitosan increases disease resistance and stimulates growth in plants (Chmielewski et al., 2007). Limpanavech et al. (2008) reported that oligomeric chitosan with 80% deacetylation (O-80) was appropriate chitosan type to stimulate flowering and increased the accumulative inflorescence number in *Dendrobium* 'Eiskul'. Too high concentration of chitosan showed the negative effects on *Dendrobium* 'Eiskul' growth *in vitro* (Pornpienpakdee et al., 2010). Boonlertnirun, Boonraung and Suvanasara (2008) indicated that polymeric chitosan increased the total rice yield, panicals per plant and dry matter in rice cv. 'Suphanburi 1'. As the appropriate chitosan application is required for the positive effect on plant growth, and different plant species respond differently to each chitosan type, the determination of the appropriate chitosan molecule and concentration for drought induction in rice is the priority determination. However, up to now, there have been no clear pathways for chitosan response in growth stimulation or stress tolerant induction. In this research, we reported the different types and concentrations of chitosan effects on drought resistance induction in Leung Pratew 123 ('LPT123') and its mutated line, Leung Pratew 123-TC171 ('LPT123-TC171') which leads to investigate the genetic evidence for chitosan responses in rice.

Dzung and Thang (2004); Dzung (2005) reported that theoligomeric chitosan at 30 mg/L was appropriate to increase chlorophyll content in soybean and peanut as well as plant growth. Moreover, in coffee seedling, application of oligomeric chitosan at 60 mg/L in field conditions increased total chlorophyll contents up to 15.36% compared to the control (Dzung, Khanh and Dzung, 2011). Khan et al. (2004) proposed that total phenolic contents and enzyme activities (phenylalanine ammonia-lyase and tyrosine ammonia-lyase) increased after treating with oligomeric chitosan and chitin in soybean leaves.

Reactive oxygen species (ROS) caused the damage to plant cell during drought stress (Mano, 2002). Singha et al. (2008) reported that chitosan increased in the amount of H_2O_2 accumulation in cultures of *S. sapinea*, while it decreased in superoxide formation. However, Srivastava et al. (2009) demonstrated that chitosan induced catalase (H_2O_2 scavenger) to prevent stomatal closure. Agrawal et al. (2002) revealed that chitosan could induce ascorbate peroxidase (APx) in rice. Moreover, the antioxidants and antioxidative enzymes such as reduced glutathione (GSH), glutathione-*S*-transferase (GST), glutathione reducatse (GR), and catalase (CAT) were measured after chitosan application for 2, 4, and 7 days in *Tubifex tubifex* (Mosleh et al., 2007). With these evidences in various plant species responding to chitosan, it is suggested that chitosan action involves in antioxidant systems, regardless of the positive or negative actions on plant growth.

Proteomic approach is a tool to separate protein mixtures (Zang and Komatsu, 2007). This method applied to analyze protein and gene changes. Ali and Komatsu (2006) used proteomics to study the proteins changes during drought stress in rice. Moreover, the increasing of the S-like RNase homologue, an actin depolymerizing factor and RuBisCO activase, and the decreased of an isoflavone reductase-like protein were observed in the rice leaves (Salekdeh et al., 2002b). The others approach, various genes responded to drought stress in rice such as glutathione

reductase (Kaminaka et al., 1998), a translation elongation factor (Li and Chen, 1999), an endo-1, 3-glucanase (Akiyama and Pillai, 2001), calcium-dependent protein kinase (Saijo et al., 2001), MAP kinase (Agrawal et al., 2003) and *DREB* genes (Dubouzet et al., 2003). However, there were reports on molecular investigation of rice leaf and root proteins via proteomics analysis involving in chitosan response under drought stress in rice plant. To further fulfill the lacking information in the literature, leaf and root proteins that are putative inductively expressed by chitosan under drought stress in two rice lines were investigated in this study using a proteomics approach.

Genetic mutation is a method for study the gene product functions and the role by mutagenesis to identify genes products and functions. Therefore, several approaches can be used for gene characterization, for example, the overexpression or reductive of gene expression of the target gene, or characterization of the mutant line containing the mutation in gene. TILLING (Targeting Induced Local Lesions IN Genomes) is a reverse genetic approach to detect natural DNA polymorphisms in the interested genes (McCallum et al., 2000a; Till et al., 2007). The TILLING technique is suitable for the detection of both induced and natural variation in plant species. This method affects to allelic series of point mutations in the essential genes, which are required for phenotypic analysis (Silme and Cagirgan, 2007). In this study, TILLING was used to identify the mutant in 'Nipponbare' rice containing the mutant in the gene of interest for further characterization.

For this research the appropriate chitosan types and concentrations for drought resistant induction in rice were determined. Then, as we hypothesized that the antioxidant system should be one of chitosan targets, this system was investigated after drought stress and chitosan application. The proteomic approach was used to identify the other systems that resulted from chitosan treatment and drought stress. Finally, the 5 genes responsible for drought resistance induced by chitosan was identified and characterized for their functions via TILLING method.

The objectives of this study are:

1. To determine the appropriate chitosan types and concentrations for drought resistance induction in ‘LPT123’ and ‘LPT123-TC171’ rice lines.

2. To investigate the effects of chitosan on physiological changes, antioxidative systems and ATPase activity after chitosan application under drought stress.

3. To examine the effects of chitosan on protein profiles after chitosan treatment under drought stress.

4. To identify and characterize some genes responsible for drought resistance induced by exogenous chitosan.

CHAPTER II

LITERRATURE REVIEW

1. Rice (*Oryza sativa* L.)

Rice is an important crop in Thailand and the main food of Asia, Latin America and Africa (Salekdeh et al., 2002a). Since rice has a genome significantly smaller than those of other cereals, it is a good model for genetic and molecular studies in plants (Ali and Komatsu, 2006). In worldwide, there are rice areas more than 100 million ha approximately 89% in Asia. Forty-five percent of the rice area is rainfed, 25% is upland. Asia has large rainfed rice growth area in eastern India, Bangladesh, northeast of Thailand, Cambodia, and the island of Sumatra in Indonesia (Serraj et al., 2009). Two species of rice which consist of *Orysa sativa* and *Oryza glaberrima* are grown worldwide and in parts of West Africa. The world‟s largest collection of rice diversity of The International Rice Genebank collects the various types of rice such as wild rice, the ancestors of rice, traditional and heirloom varieties as well as modern varieties (http://irri.org/about-rice/rice-facts/rice-basics).

1.1 Rainfed lowland rice environments in Thailand

In Thailand, the total areas approximately 9.2 million ha were used to grow rainfed rice accounting for 76% of the whole agricultural area (Jongdee et al., 2006). Rainfed rice ecosystems consist of three types; upland, lowland and deep water. Rainfed lowland covers about 6.8 million ha approximately 75% of the total rice growing areas (OAE, 2001).

In the North (1.4 million ha) and Northeast (4.8 million ha) of Thailand are the rainfed lowland areas. The production of rice in two regions is low in each year between 1.5 and 2.2 ton/ ha (Jongdee et al., 2006).

1.2 Rice under drought stress in Thailand

Drought is the serious problem in agriculture worldwide which affects to the rice yield (Gorantla et al., 2007; Cattivelli et al., 2008). In Northeast (NE) and North (N) of Thailand, rainfed lowland rice consists of shallow favourable and shallow drought-prone (Khush, 1984; Jongdee et al., 2006). Normally, the rainy season in Thailand starts in May and ending in October. Drought may occur at any time during growing season. Early season of drought affects to seedlings and the rice seed germination (Jongdee et al., 2006). Late season of drought develops before rice maturation at the end of the rainy season (Jongdee, 2001). Drought causes the loss of rice yield approximately 45% in the upper position of the toposequence (Jongdee, 2003).

1.3 'LPT123' and 'LPT123-TC171' rice lines

Thikart et al. (2005) reported that „LPT123-TC171", the mutant salt-tolerant Leung Pratew („LPT123") rice line obtained from *in vitro* somaclonal variation under salt stress condition for 10 generations. Rice plants were grown in WP nutrient solution, adding PEG 6000 200 g/L for 6 weeks. Then, the growth of the original and the resistant rice lines was determined. The higher shoot and root dry weights as well as shoot height were detected in „LPT123-TC171" rice line. Moreover, genetic variation in „LPT123" and „LPT123-TC171" were determined by RAPD approach showing the difference at molecular level in two rice lines. From Southern blot analysis indicated the different genomic expression patterns suggesting the differences in two rice lines at DNA level. Northern blot study showed the low gene expression in all samples.

2. Chitosan and chitin

Chitosan and chitin are aminoglucopyrans consisting of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues (Figure 2.1) (Rinaudo, 2006; Mourya and Inamdar, 2008). The properties of chitin are ß-1, 4-linkage, the degree of deacetylation as low as <10% and molecular weight as high as 1–2.5 x 10^6 Da (Prashanth and Tharanathan, 2007; Mourya and Inamdar, 2008). Chitosan produces from chitin by eliminating acetyl group called "deacetylation" which ranges from 40% - 98% using hot alkali (Kim, 2011), while molecular weight ranges between 5 x 10^4 -2 x 10^6 Da. The degree of deacetylation and polymerization (DP) are parameters to use chitosan for various applications (Mourya and Inamdar, 2008).

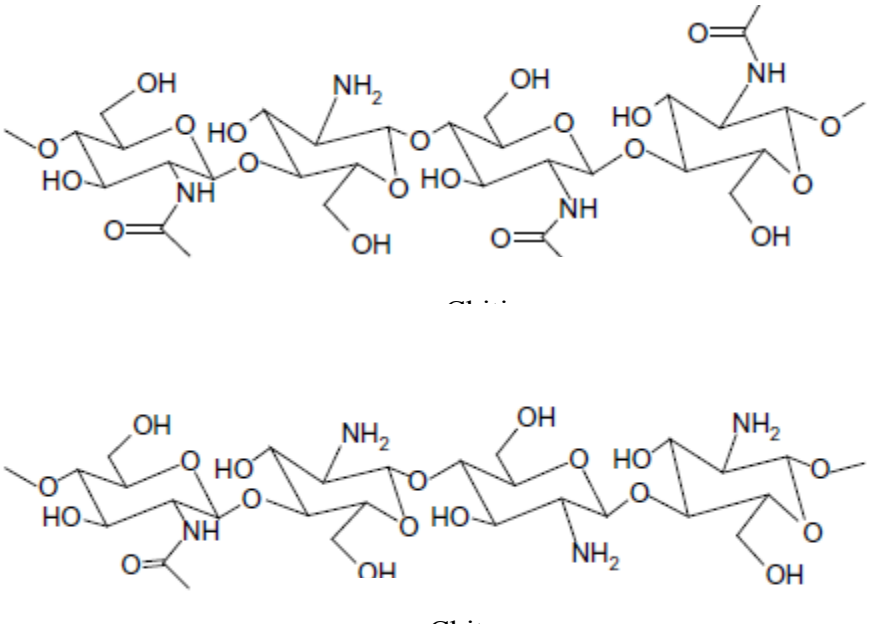


Figure 2.1 Primary structures of (A) chitin and (B) chitosan (Mourya and Inamdar, 2008).

Table 2.1 Source of chitin and chitosan (Rinaudo, 2006)

Sea animals	**Insects**	**Microorganisms**
Annelida	Scorpions	Green algae
Mollusca	Spiders	Yeast (ß-type)
Coelenterata	Brachiopods	Fungi (cell walls)
Crustaceans	Ants Mycelia	Penicillium
Lobster	Cockroaches	Brown algae
Crab	Beetles	Spores
Shrimp		Chytridiaceae
Prawn		Ascomydes
Krill		Blastocladiaceae

2.1 Application of chitosan

Chitosan is a positive charge, attracting negatively charged molecules (Uthairatanakij et al., 2007). It has the unique physiological and biological properties leading to the application in several industries such as removing dyes, eliminating metal ions, food preservation, controlling the release of drugs and controlling blood cholesterol as well as adding into cosmetic products (Shahidi et al., 2001; Uthairatanakij et al., 2007). Moreover, chitosan is applied in agriculture such as coating on fruits, seeds and vegetables (Zhang and Quantick, 1998; Jiang and Li, 2001; Lee et al., 2005; Photchanachai et al., 2006), controlling the releasing of fertilizers (Sukwattanasinitt et al., 2001), stimulating immune systems in plant, plant

growth and plant production and protecting plants from microorganisms (El Ghaouth, 1994; Hadwiger et al., 2002; Nge et al., 2006).

2.2 Effects of chitosan on rice responses

Chitosan was applied to stimulate growth and used to detoxify metal ion in rice (Boonlertnium et al., 2008; Tham et al., 2001). Boonlertnirun, Sarobol and Sooksathan (2006) applied polymeric chitosan on rice (*Oryza sativa* L. cv. „Suphanburi 1‟) by seed soaking before planting and foliar sprayings for four times during planting period. It was revealed that the rice yield tended to increase. The chitosan with different molecular weights and different methods did not affect plant height, thousand-grain weight and filled grains. Boonlertnirun et al. (2007) revealed that the application polymeric chitosan (degree of deacetylation = 96.62%, molecular weight ~ 100,000 kDa) before drought showed the highest yield, yield components and good recovery. Furthermore, the percentage of damaged leaves was less than the other treatments. Boonlertnirun et al. (2008) reported that polymeric chitosan at 80 mg/L by seed soaking and soil mixing for four times tented to stimulate growth and significantly increased rice yield. Moreover, seed soaking before planting and foliar spraying tended to induce in disease control. Tham et al. (2001) showed that the 10–200 mg/g of irradiated chitosan promoted growth of rice without chlorosis when grown under vanadium stress. Chitosan irradiated at 100 and 200 mg/ml could stimulate rice growth by 8% and suppress vanadium toxicity.

The chitosan stimulated two proteins and mRNAs of pathogenesis-related (PR) proteins, OsPR5 and OsPR10 and induced the accumulation of the *OsPR5* and *OsPR10* mRNAs. Moreover, the concentration and exposure time after chitosan treatment affected the expression and changes in diterpenoid lactone momilactone A, “phytocystatins” (cysteine proteinase inhibitors) and the flavonoid sakuranetin (Agrawal et al., 2002). Chitosan could induce the expression of the *REK* mRNA in responding to defense/stress of photosynthetic tissues in rice (Jwa et al.,

2002). Moreover, in cultured cells, chitosan also stimulated the hydrogen peroxide (H_2O_2), increased the activities of phenylalanine ammonialyase (PAL) and induced the expression of defense-related genes, *β-1,3-glucanase* and *chitinase* and pathogen-related protein (PR1) (Lin et al., 2005). The response of chitosan involved in the octadecanoid pathway in rice by increasing of 12-oxo-phytodieonic acid and jasmonic acid, the key biologically active regulators of plant self-defense responses (Rakwal et al., 2002). Kim et al. (2003) reported that signals molecules including chitosan induced *OsEDRI* expression which is a MAPK kinase kinase in defense/stress signaling and developmental pathways.

2.3 Effects of chitosan on other plants

Chitosan could enhance shoots and roots growth. Wanichpongpan et al. (2001) reported that chitosan stimulated growth parameters such as flower-stem and leaves length, leaves and flower per bush numbers, leaves width in gerbera plants. Chitosan increased the growth in several plant species consisting of cabbage (*Brassica oleracea* L. var. „Capitata") (Hirano, 1988), soybean (*Glycine max* L.) sprouts (Lee et al., 2005) and sweet basil (*Ocimum basilicum* L.) (Kim, 2005). Moreover, chitosan enhanced in developing flower buds and induced the blooming of lisianthus (*Eustoma grandiflorum*) (Ohta et al., 1999; Jamal Uddin et al., 2004) as well as increased the number of inflorescences in *Dendrobium* „Eiskul" (Limpanavech et al., 2008).

The results of concentration and frequency of chitosan were studied in several plant species such as chilli, celery, bitter cucumber and Chinese cabbage showing that chitosan significantly increased the growth of chilli and yield of Chinese cabbage (Chandrkrachang et al., 2003).

Sizes and % of deacetylations of chitosan affected the plant responses. The appropriate chitosan types and concentrations could increase *Dendrobium* „Eiskul" and enhance plant growth *in vitro* and after transplanting (Pornpeanpakdee et al., 2010). However, different orchid species responded to chitosan differently. Kananont et al. (2010) revealed that two species of *Dendrobium* orchids also showed the different chitosan specificity for growth enhancement. In orchid plant, Limpanavech et al. (2008) studied the effects of chitosan on *Dendrobium* „Eiskul" using the oligomeric and polymeric chitosan molecules consisted of deacetylation percentage of 70, 80, and 90. Oligomeric chitosan (O-80) at 1, 10, 50 and 100 mg/L induced early flowering and increased the inflorescence number. Nge et al. (2006) showed that shrimp chitosan molecular weight at 1 kDa could promote growth of protocorm like bodies (PLBs) in *Dendrobium phalaenopsis*. Khan et al. (2004) applied of chitin and oligomeric chitosan in soybean leaves resulting in activity of phenylalanine ammonia-lyase and tyrosine ammonia-lyase enzymes.

3. Antioxidant systems

Plants subjected to various abiotic stresses resulting in producing reactive oxygen species (ROS) which were highly reactive and toxic substances. The ROS caused the damage to proteins, lipids, carbohydrates and DNA (Gill and Tuteja, 2010). In order to cope with various stresses, plants produced enzymatic and non-enzymatic antioxidative systems to eliminate ROS (Liu et al., 2011). The antioxidant enzymes were superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT) and peroxidase (POD). SOD converted O_2^- into H_2O_2 and O_2, CAT and POD changed H_2O_2 to H_2O (Reddy et al., 2004). Moreover, carotenoids which consisting of carotene and xanthophylls could scavenge ROS and stabilize photosynthetic systems (Liu et al., 2011).

3.1 Drought and antioxidative systems

Reactive oxygen species (ROS) caused the cellular damage during drought stress. When plant tissues subjected to drought stress affected the increasing of ROS and oxidizing target molecules, the induction of genes expression related to ROS scavenging, the increasing of antioxidative systems levels to scavenge ROS to protect plants from drought stress (Mano, 2002). In plant species, the enzymatic components for ROS scavenging systems are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx), peroxidase (POD), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR). Non-enzymatic components included flavanones, anthocyanins, carotenoids and ascorbic acid (AA) (Mittler et al., 2004). The level and amount of antioxidative enzymes activities depended on plant species under drought stress (Reddy et al., 2004).

3.2 Enzymatic antioxidant system

In plant cells, the mechanism to scavenge H_2O_2 content found in chloroplast and cytosol, called "ascorbate-glutathione pathway" (Figure 2.2) (Asada, 1999). Mechanism of detoxification H_2O_2 was reduced to H_2O by ascorbate peroxidase (APx) and ascorbate including monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase in "ascorbate-glutathione cycle" (Endogan, 2003). Ascorbate is first conversed to monodehydroascorbate by APx. The enzyme changes monodehydroascorbate to ascorbate does not take place quickly and then monodehydroascorbate will spontaneously disproportionate into ascorbate and dehydroascorbate. Dehydroascorbate recycle ascorbate with the contribution reduced glutathione that is generated through the action of glutathione reductase in a NADPH dependent reaction (Endogan, 2003; Ferman et al., 2003).

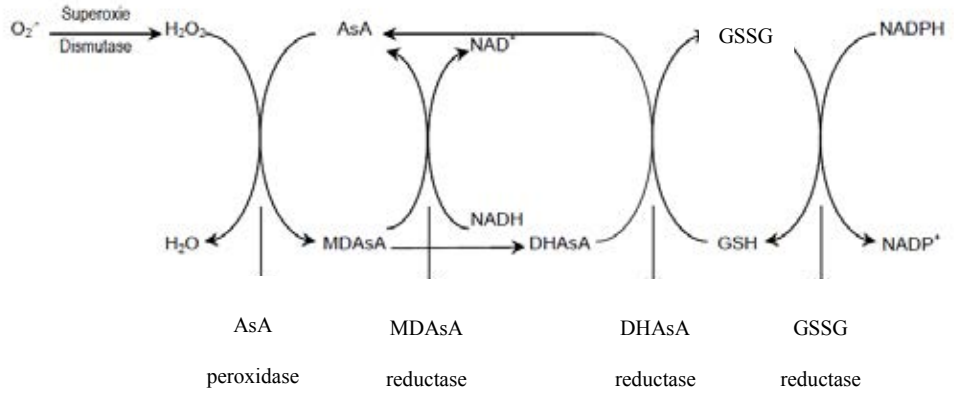


Figure 2.2 The ascorbate-glutathione pathway to detoxified H_2O_2; MDAsA; monodehydroascorbate, DHAsA; dehydroascorbate, GSSG; oxidized glutathione; GSH; reduced glutathione (Endogan, 2003).

3.3 Non-enzymatic antioxidative system

Plants activate non-enzymatic antioxidants involving in the ascorbate-glutathione cycle such as ascorbate (vitamin C), glutathione, α-tocopheral, carotenoids, flavonoid and phenolic compounds to decrease the damage resulting from ROSs (Jimenez et al., 2002; Vijayakumar et al., 2008). These compounds can directly react with ROS and remove them, or contribute to enzyme-catalyzed detoxification reactions (Noctor and Foyer, 1998).

3.3.1 Ascorbate or ascorbic acids (AA)

Ascorbic acid (vitamin C) acts as the antioxidant reacting with H_2O_2 via the activity of ascorbate peroxidase (APx) in ascorbate-glutathione cycle. Ascorbate donates hydrogen and changes to dehydroascorbic acid (DHAsA) (Conklin, 2001; Liu et al., 2006; Gill and Tuteja, 2010).

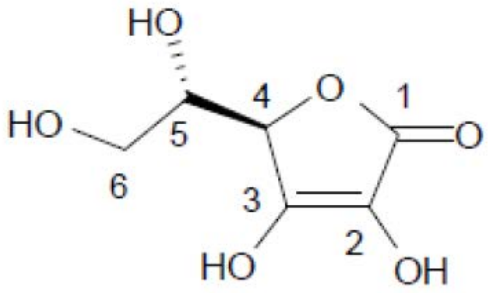


Figure 2.3 The structure of ascorbic acid (Liu et al., 2006).

3.3.2 Carotenoids

The structure of carotenoids consist of isoprenoid molecules. They are classified into two types, xanthophylls and carotene (Figure 2.4) (Moskalenko and Karapetyan, 1996; Bramley, 2002). The functions of carotenoids in plants are proposed. First, they absorb light at wavelength 400-550 nm and transfer to the chlorophyll (an accessory light harvesting role) (Sieferman-Harms, 1987; Niyogi, 1999; Arora et al., 2002). Second, they eliminate triplet sensitizer (^{3}Chl), $^1O_2$ and other free radicals to protect the photosynthetic system (Collins, 2001).

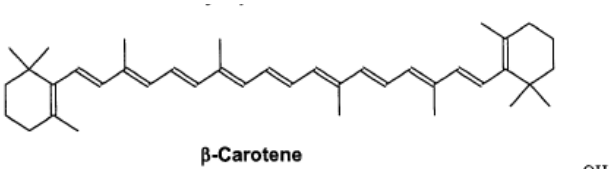


Figure 2.4 Structures of typical carotenoids (Bramley, 2002).

3.3.3 Glutathione

The glutathione is one of the non-enzymatic antioxidants, it acts an important role in intracellular to protect plants from reactive species of oxygen and a source of amino acid for protein synthesis (Meister and Anderson, 1983; Mosleh et al., 2007). It cooperated with ascorbate by changing from dehydro-ascorbate via the enzyme dehydro-ascorbate reductase (Suntres, 2002; Lei et al., 2003). Glutathione consists of reduced (GSH) and oxidized (GSSG) form of glutathione. GSH is regenerated by glutathione reductase (GR) in a NADPH-dependent reaction (Gill and Tuteja, 2010). The glutathione is used in the reaction of GST and others antioxidants such as glutathione peroxidase (GPx), and glutathione reductase (GR) (Suntres, 2002; Lei et al., 2003). Glutathione is capable to detoxify $^1O_2$ and OH^- and protects thiol groups in enzymes involving in alpha-tocopherol and ascorbate regeneration through the glutathione-ascorbate cycle (Endogan, 2003; Foyer and Noctor, 2005).

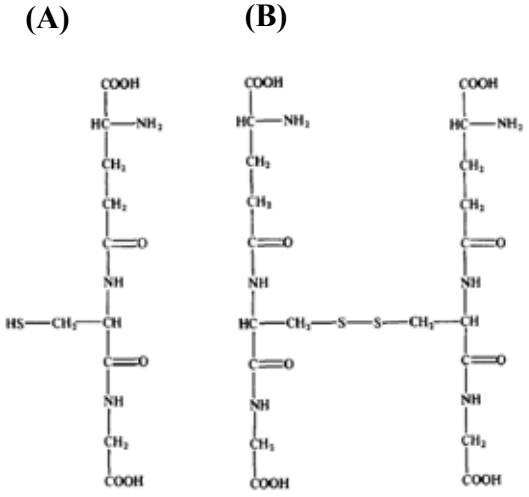


Figure 2.5 Structure formulas of reduced glutathione (A) and oxidized glutathione (B) (Gennaro and Abrigo, 1992).

3.3.4 Phenolic compound

Phenolics are one of the antioxidants in many plants to scavenge free radicals, break radical chain, and chelate metals (Bonoli et al., 2004). One type phenolic compound is gallic acid which is a trihydroxybenzoic acid in plants. It can be use as a standard to determine the phenolic content with the Folin-Ciocalteu reagent (Choi et al., 2006).

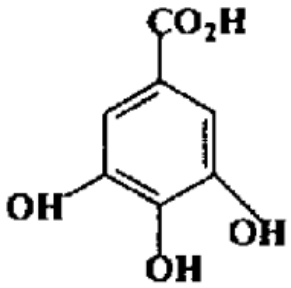


Figure 2.6 Structure of gallic acid (Salah et al., 1995).

3.4 Effects of chitosan on enzymatic and non-enzymatic ROS scavenging system

H_2O_2 was proposed to be a signal molecule responding to chitosan in plants. Singha et al. (2008) reported that chitosan caused an increase in H_2O_2 accumulation in *Sphaeropsis sapinea*, culture and decreased in superoxide formation. However, Srivastava et al. (2009) demonstrated that chitosan induced catalase and involved in reactive oxygen species (ROS) and calcium to protect stomatal closure. Agrawal et al. (2002) revealed that the chitosan could induce ascorbate peroxidase in rice. Moreover, the antioxidant enzymes such as catalase (CAT), glutathione (GSH) and glutathione-*S*-transferase (GST) as well as glutathione reducatse (GR) were measured after chitosan application for 2, 4, and 7 d in *Tubifex tubifex* (Mosleh et al., 2007). Agrawal et al. (2002) showed that 0.1% oligochitosan stimulated the production of reactive oxygen species (ROS) and phenolic secondary metabolites in rice leaves. Lee et al. (1999) mentioned that chitosan induced H_2O_2 evolution,

catalase activity and ascorbic acid contents. Khan (2003) applied of chitin and oligomeric chitosan to soybean and it was resulted in the decrease of total phenolic content in leaves. With these evidences in various plant species responding to chitosan, it is suggested that chitosan action involves in antioxidant systems, regardless of the positive.

4. Proteomics study of drought stress response in rice and other plants

Total set of encoding proteins in genome is called “proteome”, so the study of the proteome including the changing of structure and abundance in response to developmental and environmental is called “proteomics” (Wilkins et al., 1996; Salekdeh et al., 2002a).

Proteomics is a molecular tool to analyze protein changes in response to drought stress and other environmental changes (Salekdeh et al., 2002b; Zang and Komatsu, 2007). Ali and Komatsu (2006) reported that actin depolymerizing factor expressed in leaf blades, leaf sheaths, and roots under drought stress. The light harvesting complex chain II and the actin depolymerizing factor showed a high level under normal condition in drought-tolerant rice cultivar. Moreover, up-regulated S-like RNase homologue, an actin depolymerizing factor and RuBisCO activase including down-regulated isoflavone reductase-like protein were found in rice leaves (Salekdeh et al., 2002a). Ke et al. (2009) reported that late embryogenesis abundant (LEA)-like protein and chloroplast Cu–Zn superoxide dismutase (SOD) were up-regulated during drought stress, whereas Rieske Fe–S precursor protein was down-regulated. Xiong et al. (2010) showed the decrease in protein levels involving in redox metabolism, photosynthesis, cytoskeleton stability, defense, protein metabolism, and signal transduction. Salekdeh et al. (2002b) revealed 42 protein spots showed significant changes in two rice cultivars (*Oryza sativa* L. cv „CT9993“ and cv. „IR62266“). In „CT9993“ cultivar was up-regulated during drought stress. Moreover, various genes responded to drought stress in rice such as glutathione

reductase (Kaminaka et al., 1998), a translation elongation factor (Li and Chen, 1999), an endo-1, 3-glucanase (Akiyama and Pillai, 2001), calcium-dependent protein kinase (Saijo et al., 2001), MAP kinase (Agrawal et al., 2003), and *DREB* genes (Dubouzet et al., 2003) were discovered. These genes are involved in membrane integrity (LEA protein) (Xu et al., 1996; Rohila et al., 2002; Babu et al., 2004), signal molecule (kinases) (Saijo et al., 2001; Liu et al., 2003), water uptake (aquaporins) (Martre et al., 2002), and carbohydrate metabolism (TPS) (Jang et al., 2003).

Riccardi et al. (1998) investigated drought-responsive proteins in two maize lines. The result showed 78 out of 413 proteins in leaves and 38 proteins exhibited different expression in two maize cultivars. One protein was aldose/aldehyde reductase involving in cytotoxic lipid peroxide degradation. This enzyme resisted to various abiotic stress including H_2O_2 stress in transgenic tobacco (Oberschall et al., 2000).

5. Targeting Induced Local Lesions IN Genomes (TILLING)

TILLING is one of the techniques of gene functional study. This method is created while studying gene function of chromomethylase homologues in *Arabidopsis* (McCallum et al. 2000a). Several methods were used to study the functional inactivation of rice genes (Till et al., 2007). The TILLING approach is the choice to detect both induced and natural variation in plant species (Silme and Cagirgan, 2007). The other methods to find mutation such as tagging (Hirochika et al., 2004), introduced transposons (Kumar, Wing and Sundaresan, 2005) or T-DNA of *Agrobacterium tumefaciens* (An et al., 2005), RNA interference (Miki, Itoh and Shimamoto, 2005) were reported. Chemical mutagens such as ethyl methanesulfonate (EMS) and methyl nitrosourea (MNU) were applied for forward and reverse genetic screens to induce mutation by changing of a single nucleotide (Sega, 1984; Greene et al., 2003; Guenet, 2004). TILLING approach was applied in various organisms, including fruit fly, *Drosophila melanogaster* (Winkler et al., 2005), nematode

(*Caenorhabditis elegans*) (Gilchrist et al., 2006; Cuppen et al., 2007), rat (*Rattus norvegicus*) (Smits et al., 2006), zebrafish (*Danio rerio*) (Draper et al., 2004), maize (*Zea mays* L.) (Till et al., 2004), barley (*Houdeum vulgare* L.) (Talame, 2008), rice (*Oryza sativa* L.) (Wu et al., 2005; Till et al., 2007, Tsai et al., 2011), sorghum (*Sorghum bicolor* L.) (Xin et al., 2008), and wheat (*Triticum aestivum* L.) (Uauy et al., 2009).

5.1 TILLING method

The TILLING strategy is to mutagenize by using the chemicals for to discover high throughput mutation (McCallum et al., 2000b; Colbert et al., 2001). TILLING pipeline initiated from the process of mutagenesis, the elimination of a chimeric population, preparation of a germplasm stock, DNA extraction and pooling, discovering mutations, and evaluation of phenotype/ genotype mutants for breeding programs (Figure 2.7). Many mutagenized population were treated with various mutagens producing a chimeric plant in the first generation and caused single nucleotide polymorphisms (insertions/deletions) in genome (Till et al., 2007; Tadele, MBA and Till, 2010). Moreover, many germplasm stocks were prepared for long periods of mutant lines. Finally, DNAs was extracted from each individual mutant and they were pooled for the library preparation. The mutations were screen of interested genes in selected regions (Henikoft, Till and Comai, 2004; Tadele et al., 2010).

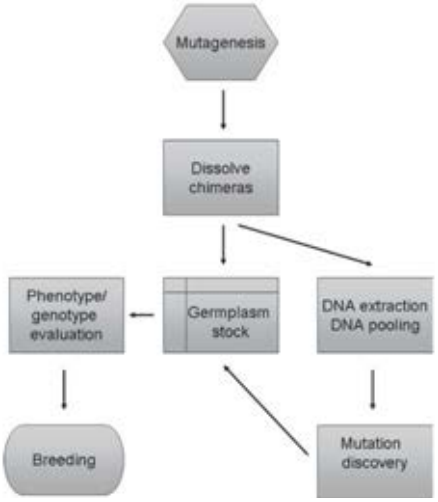


Figure 2.7 TILLING pipeline for gene function analysis and developing new crop varieties (Tadele et al., 2010).

5.1.1 Developing a mutagenized population

Ethyl methanesulfonate (EMS) caused a variety of lesions nonsense and missense mutations producing a high density of point mutations (Koornneef et al. 1982; Greene et al., 2003). EMS treatment induced mutations in most organisms and highly changed G:C->A:T transition (Greene et al., 2003, Slade et al., 2005). Typically, various plant seeds are mutated by soaking with chemical mutagen approximately 10–24 h (Till, Comai and Henikoff, 2007; Till et al., 2007). The M1 generation generated heterozygous mutation which was not suitable for TILLING screening. Therefore M2 progeny from a self cross of the M1 should segregate mutations according to Mendelian ratio (1: 2: 1). When mutations were identified, the M3 seed could be germinated for phenotypic analysis (Figure 2.8) (Till, Comai and Henikoff, 2007; Tadele et al., 2010).

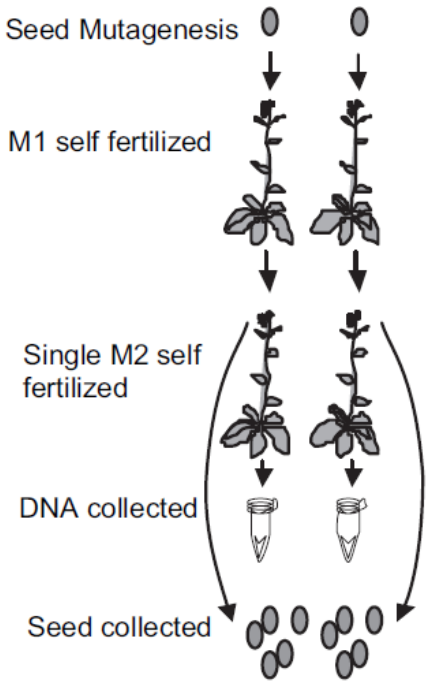


Figure 2.8 Seed mutagenesis for TILLING (Till, Comai and Henikoff, 2007).

5.1.2 DNA preparation and pooling

In each TILLING group different methods have been performed for screening. Two-, three-, four-, six-, and eight-fold pooling were tested. Typically, a one-dimensional pooling strategy was individual sample representing only one pool (Till, Comai and Henikoff, 2007). Two-dimensionally pooling presented two types of pools, eight "row pools" and 12 "column pools (Figure 2.9). Mutations in both of row and column pools were identified in eight individuals (Colbert et al., 2001; Tsai et al., 2011). Additionally, tridimensional pooling was used to pool sample to solve the problem of bidimensional pooling. Sixteen row pools (48 individuals), 16 column pools (48 individuals), and 12 "dimensional" (or "D") pools (64 individuals) were designed in the total of 768 rice individuals in terms of tridimensional pooling (Figure 2. 10) (Tsai et al., 2011).

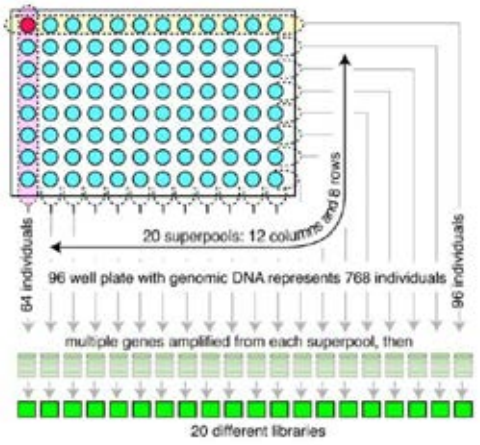


Figure 2.9 Bidimensional pooling methods for discovering mutation on 96-well plates (Tsai et al., 2011).

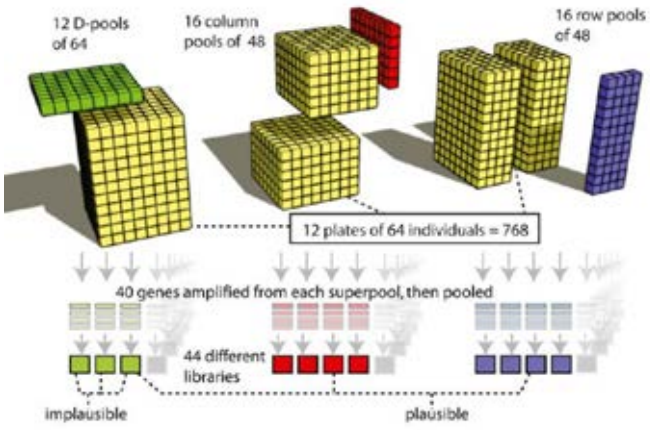


Figure 2.10 Tridimensional pooling methods for mutation discovery. Eight individuals arrayed on 96-well plates (Tsai et al., 2011).

5.1.3 Mutation discovery

In the past, TILLING strategy could discover mutations base on PCR products and digested with CELI endonuclease at mismatched sites in heteroduplexes (Oleykowski et al., 1998) or with enzyme from celery (*Apium graveolens*) extracts (Till et al., 2004). High-resolution melting detected the

heteroduplexes (Dong et al., 2009). Although, these methods are effective, but need to use labor intensive and challenge to pools deeper than eight individuals. If mismatches have been detected, are needed to be sequenced. Nowadays, sequencing uses for discovering the mutations and polymorphisms by comparing of a putative new allele with a reference sequence. Bioinformatics analysis cooperated with sequencing technique to discriminate of sequencing errors from real changes and increased the sufficient sequencing coverage (Druley et al., 2009; Koboldt et al., 2009).

CHAPTER III

MATERIALS AND METHODS

I. MATERIALS

1. Plant materials

1.1 Rice cultivar/line Leung Pratew 123 (*Oryza sativa* L. cv. 'LPT123') (a salt-sensitive line or original line).

1.2 Rice cultivar/line Leung Pratew 123-TC171 (*Oryza sativa* L. cv. 'LPT123-TC171') (a salt-resistant line or isogenic mutant line).

1.3 Rice cultivar/line 'Nipponbare' (*Oryza sativa* cv. 'Nipponbare') for the TILLING (Targeting Induced Local Lesions IN Genomes) study at UC Davis, CA, US.

The Thai indica rice cultivar 'LPT123' was obtained from the Agriculture Department, Ministry of Agriculture and Cooperation, Thailand. 'LPT123' and 'LPT123-TC171' rice have the similar genotype as 'LPT123-TC171' is the mutated line isolated with salt resistant phenotype from the somaclonal variation *in vitro* of 'LPT123' (Vajrabhaya and Vajrabhaya 1991; Thikart et al., 2005; Udomchalothorn et al., 2009) and then 'LPT123-TC171' was self-fertilized and screened for salt resistance characters for ten generations. The selection of salt resistance characters was performed in 3 week-old seedlings. 'LPT123' rice and the salt-resistant mutated line, 'LPT123-TC171' were used in all experiments. Plants were grown in modified WP as under natural light. These rice cultivars were used for all experiments, except in part of TILLING (Targeting Induced Local Lesions IN Genomes) study.

2. Instruments

2.1 Equipment for plant growing

-Glass bottle 150 ml

-Plastic tray 32x11x9 $inch^2$

-Sand

-Sponges

-Future board

2.2 Equipment for plant growth study

-Aluminium foil

-Eppendorf 1.5 ml

-Forceps

-Hot air oven

-Ruler

-Scissors

-Balance: Sartorius CP423s (Scientific Promotin Co. USA)

2.3 Equipments for enzymatic and non-enzymatic extraction and analysis

-Motars and pestles

-Spatula

-Refrigerated centrifuge (Universal 32R, Hettich, Germany)

-Spectrophotometer (Agilent Technology, USA)

-Micro plate reader (VERSA max, USA)

-Centrifuge (Universalt6, Hettich, Germany)

2.4 Equipment for proteomics study

-Mortars and pestles

-Spectrophotometer (Agilent Technology, USA)

-Incubator (Gemmyco, USA)

-Orbital shaker (Biosan, USA)

, -Protein electrophoresis (Bio-Rad, USA)

-Camera

-Ultimate 3000 LC system (Dionex, USA)

-ESI ion Trap MS (HCT ultra PTM Discovery System, Bruker Daltonik)

-Pre-column (Monolithic Trap Column, 200 µm i.d. x 5 cm)

-Nano column (Monolithic Nano Column, 100 µm i.d. x 5 cm)

2.5 Equipment for RNA extraction

-Deep freezer –80ºC

-DNA-RNA horizontal gel eletrophoresis apparatus

-Eppendorf tubes 1.5 ml

-Gel document (Gel DocTM 2000, BIO-RAD)

-Microwave oven

-Motar and pestle

-Refrigerated centrifuge (Universal 32R, Hettich, Germany)

-Spatula

-Spectrophotometer (Agilent Technology, USA)

-Vortex mixer

-Water bath (DAIHAN LABTECH CO., LTD)

2.6 Equipment for determination of gene expression by quantitative polymerase chain reaction (qPCR)

-Pipette tips 10, 100 and 1000 µl

-Individual PCR tube ™

-PCR tube strips, flat cap strips (Bio-Rad, USA)

-Low tube strip, WHT (Bio-Rad, USA)

-CFX96TM real- time system (Bio-Rad, USA)

2.7 Equipment for TILLING (Targeting Induced Local Lesions IN Genomes) technique

-Beckman Biomek 2000 (Biodirect, USA)

-PCR machine (GeneAmp®PCR System 9700 Base Module, Applied Biosystems, USA)

-UVP Imaging (Labworks Software)

-Bio analyzer (Agilent Technogies, USA)

-Microlab 4200 pipetting robot (Hamilton, USA)

-Analyst plate reader (Bio-Tek Synergy HT, USA)

-Illumina ultra high throughput sequencing (GAII, USA)

3. Chemicals and reagents

3.1 Chemicals for chitosan treatment and drought stress

-Oligomeric (O) and polymeric (P) chitosan with deacetylation percentage of 80 and 90

-WP No.2 nutrient solution (Vajrabhaya and Vajrabhaya, 1991) (see in Appendix A)

-10% polyethylene glycol 6000 (PEG6000)

-Distilled water

-0.1% acetic acid

3.2 Chemicals for H_2O_2 determination

-Phosphate buffer (pH 6.5) (see in Appendix A)

-Hydroxylamine

-Titanium sulphate

-Sulfuric acid

3.3 Chemicals for chlorophyll and carotenoid contents analysis

-95.5% acetone

3.4 Chemicals for ascorbic acid content analysis

-2% dinitrophenylhydradzine (DNPH) in 4.5 M sulfuric acid

-6% metaphosphoric acid in 2 M acetic acid

-2% 2, 6-dichlorophenolindolphenol (DCIP)

-2% thiourea in 5% metaphosphoric acid

-90% sulfuric acid

3.5 Chemicals for glutathione content analysis

-Potassium phosphate buffer (pH 7.0) (see in Appendix A)

-5, 5 dithiobis-2-nitrobenzoic acid (DTNB)

-Nicotinamide adenine dinucleotide phosphate (NADPH)

-Glutathione reductase (60 units/ml)

3.6 Chemicals for phenolic compound analysis

-80% ethanol (EtOH)

-4 N sodium hydroxide (NaOH)

-6 N hydrochloric acid (HCl)

-Ethyl acetate

-Distilled water

-Folin-Ciocalteu's phenol reagent (Fluka, Switzerland)

3.7 Chemicals for enzymatic extraction and assay

Extraction buffer;

-0.1 M sodium phosphate buffer (pH 7.0)

-2 mM ethylenediaminetetraacetic acid (EDTA)

-4 mM dithiothreitol (DTT)

-6 mg polyvinylpyrrolidone (PVP)

-Standard protein; bovine serum albumin (BSA) (2 µg/µl)

3.7.1 Ascorbate peroxidase (APx) activity assay

-50 mM sodium phosphate buffer (pH 7.0)

-100 mM EDTA

-11.6 mM H_2O_2

-100 mM ascorbic acid

3.7.2 Glutathione-*S*-transferase (GST) activity assay

-50 mM sodium phosphate buffer (pH 7.0)

-20 mM reduced glutathione (GSH)

-20 mM 1-chloro-2, 4-dinitrobenzene (CDNB)

-95% ethanol

3.7.3 Guaiacol peroxidase (GPx) activity assay

-50 mM sodium phosphate buffer (pH 7.0)

-11.6 mM H_2O_2

-117.9 mM guaiacol

3.7.4 ATPase activity assay

-100 mM Tris-HCl buffer (pH 6.5)

-50 mM magnesium sulfate ($MgSO_{4)}$

-50 M ammonium molybdate

-15 mM adenosine-5'-triphosphate (ATP)

-1.15 M perchloric acid

-1.5% Tween-20

3.8 Chemicals for proteomic study

3.8.1 Protein extraction and precipitation

-0.1% sodium dodecyl sulfate (SDS)

-Bovine serum albumin (BSA) (2µg/µl)

-72% trichloroacetic acid (TCA)

-0.15% deoxycholic acid (DOC)

3.8.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) preparation

(see in Appendix A)

-40% (W/V) acrylamide

-1.5 M Tris HCl pH 8.8

-0.5 M Tris HCl pH 6.8

-10X sodium dodecyl sulfate (SDS)

-Distilled water

-10% ammonium persulfate (APS)

-N, N, N', N'-tetramethyl-ethane-1, 2-diamine (TEMED)

3.8.3 **Silver staining** (see in Appendix A)

-Methanol

-48% acetic acid

-37% formaldehyde

-35% ethanol

-0.2% silver nitrate

-Disodium carbonate **(**Na_2CO_3)

-0.02% sodium thiosulfate ($Na_2S_2O_3$)

-37% formaldehyde

-0.5M EDTA

-Protein ladder 10-250 kDa (New England Biolabs, USA)

3.8.4 In gel digestion for liquid chromatography–tandem mass spectrometry (LC-MS/MS)

-100% acetonitrile (ACN)

-10 mM dithiothreitol (DTT)

-10 mM ammonium biocarbonate

-10 ng trypsin

-0.1% trifluoroacetic acid (TCA)

-Steriled milli Q water

3.9 Chemicals for RNA extraction

-Liquid nitrogen (from TIG, Thailand)

-Plant RNA Purificaion Reagent (Invitrogen, USA)

-Phenol: Chloroform: Isoamyl alchohol (25:24:1) (v/v)

-Chloroform: Isoamyl alcohol (24:1) (v/v)

-Absolute ethanol (Liquid Distillery Organization Excise Dept, Thailand)

-5X TBE (see in Appendix A)

-Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich Co., USA)

-Hydrogen peroxide

-80% ethanol

-Sodium acetate (CH_3COONa)

-Sodium chloride (NaCl) (Sigma-Aldrich Co., USA)

-DNaseI (Takara Bio Inc., Japan)

-2-mercaptoethanol (Merck, Germany)

3.10 Chemicals for qPCR

-100 mM dATP, dCTP, dGTP, dTTP (Promega, USA)

-Oligo $(dT)_{15}$ (Merck, Germany)

-M-MLV reverse transcriptase (Promega, USA)

-2X Prime Q-Master Mix (GENET BIO, Korea)

3.11 Chemicals for TILLING (Targeting Induced Local Lesions IN Genomes)

3.11.1 DNA extraction

-1 mM sodium azide

-15 mM methyl nitrosurea

-FastDNA™ Kit (MP Biomedicals, USA)

3.11.2 PCR amplification

-1.5% agarose gel

-1X TAE

-Ethidium bromide (EtBr)

-Ex Taq DNA polymerase, hot-start version (Takara Bio Inc, Japan)

-$MgCl_2$ 1.25 mM (Takara Bio, Japan)

-Hypure ™ molecular Biology Grad Water (Hyclone, USA)

-dNTP (Takara Bio, Japan)

-Low DNA mass ladder (Invitrogen, USA)

3.11.3 Library preparation for illumina sequencing

-GeneRuler™ 1 kb DNA ladder plus (Fermentas, USA)

-Agencourt ampure kits (Beckman Coulter, USA)

-End it DNA end repair kit (Epicentre, USA)

-Klenow fragment (3→5 exonuclease) (NEB, USA)

-Fragmentase enzyme (NEB, USA)

-End repair module (NEB, USA)

II. METHODS

1. Determination of the appropriate chitosan types and concentrations for drought resistance induction in rice (*Oryza sativa* L.)

1.1 Growing condition and chitosan treatment

Rice seeds were soaked in oligomeric (O) and polymeric (P) chitosan with deacetylation percentage of 80 and 90 at concentration of 20 or 40 mg/L prepared as indicated in Limpanavech et al. (2008) for 24 h, then, seeds were germinated on sand for 2 weeks before transferring to the nutrient solution (see in Appendix A). Chitosan with 0.01% Triton X-100 addition was applied to the seedlings by spraying until fully soaked, when the plants were 2 weeks and 4 weeks old. The control treatment was performed by spraying with distilled water supplemented with 0.01% Triton X-100 at the same period of time. Two days after final chitosan application, plants were treated with 10% polyethylene glycol 6000 (PEG6000) by adding the PEG6000 to the nutrient solution for drought treatment for 2 weeks. Then, the plants were transferred to the freshly prepared nutrient solution without PEG6000, which is called the re-water treatment for 1, 2 and 3 weeks. The nutrient solution was refreshed every 7 days for the whole experiment.

1.2 Data collection

Plants were collected after 2 weeks of drought treatment and 1, 2 and 3 weeks after re-watering for shoot fresh weight, shoot dry weight and shoot water content determination. For the water content was calculated according to the following equations:

$$\text{Water content} = \frac{\text{(Fresh weight- Dry weight)}}{\text{Fresh weight}}$$

1.3 Experimental design, statistical analysis

The experiment was performed with completely randomized design (CRD) with 4 replicates. For statistical analysis, the data were subjected to analysis of variance and the mean comparison was done using Duncan's multiple range test (DMRT) with $p<0.05$ using SPSS software (IBM SPSS Modeler). The data were shown as mean ± S.E. (standard error)

2. Investigation of chitosan effects on physiological changes during drought stress

2.1 Chitosan treatment

For chitosan treatment, rice plants were sprayed with oligomeric chitosan with 80% degree of deacetylation (O-80) at 40 mg/ L solution, which was revealed as the best condition from *Experiment 1*.

2.2 Data collection

For photosynthetic pigment analysis, plant leaves were collected from 4 week-old plants before drought stress and the plants after 7 days of drought stress. For determination of H_2O_2, ascorbic acid, reduced glutathione (GSH) and its oxidized

form (GSSG), including the enzyme activity determination, plant leaves were collected on day 0, 1, 2, 4 and 7 days after drought stress.

2.3 Experimental design and statistical analysis

The experiment was performed with completely randomized design (CRD) with 4 replicates. For statistical analysis, the data were subjected to analysis of variance and the mean comparison was done using Duncan's multiple range test (DMRT) with $p<0.05$ using SPSS software (version 20). The data were shown as mean ± S.E. (standard error).

2.4 Measurement of some physiological changes

2.4.1 Photosynthetic pigments

Chlorophyll a (Chl*a*), chlorophyll *b* (Chl*b*) and carotenoid were analyzed following the method of Lichtenthaler (1987) and Shabala et al., (1998). The Chl*a* and Chl*b* concentrations were investigated using an UV-visible spectrophotometer (Agilent Technology, USA) at wavelengths 662 and 644 nm. And carotenoid content was quantified at 470 nm. A solution of 95.5% acetone was used as a blank. The Chl*a*, Chl*b* and carotenoid contents in the leaves were calculated according to equations:

$$[\text{Chl}a] = 9.784\ D_{662} - 0.99\ D_{644}$$

$$[\text{Chl}b] = 21.42\ D_{664} - 4.65\ D_{662}$$

$$[\text{Carotenoid}] = \frac{(1000\ D_{470} - 1.90[\text{Chl}a] - 63.14[\text{Chl}b])}{214}$$

Where D_i is the optical density at wavelength i

2.4.2 H_2O_2 content

One hundred milligrams of leaf tissues were extracted and were added with 1 ml phosphate buffer (pH 6.5) containing 1 mM hydroxylamine. After centrifuge at 13,000 rpm for 25 min, 0.5 ml of 0.2% titanium sulphate in 20% H_2SO_4 (v/v) (freshly preparation) was added to 1 ml of supernatant and then centrifuged at 10,000 rpm for 15 min. To determine H_2O_2 content, the supernatant was measured at 410 nm as described by Jana and Choudhuri (1982). The determination of H_2O_2 concentration was performed by using the standard curve (see in Figure C.1).

3. Determination of chitosan effects on antioxidative systems and ATPase during drought stress

3.1 Determination of non-enzymatic antioxidants

3.1.1 Free phenolic compound

Free phenolic compound extracts from rice leaves were preceded according to the method of Choi et al. (2006) with some modifications. One hundred mg of rice leaves were ground with liquid nitrogen to fine powder, and then 5 ml of 80% ethanol were added. The homogenate were vortexed for 1 min and centrifuged at 9,000 rpm for 5 min at 25 ° C. Then, the supernatants were collected for free extract analysis. Fifty microlitre of the extract solutions were mixed with 25 µl of a 50% Folin-Ciocalteu's phenol reagent and 500 µl of 2% Na_2CO_3 solution. After incubation at room temperature for 30 min, the absorbance at 750 nm was measured. Phenolic concentrations represented as µg gallic acid equivalents per 1 g of leaf fresh weight (Standard curve see in Figure C.2).

3.1.2 Ascorbic acid (AA)

Total AA contents were determined using the dinitrophenylhydradzine (DNPH) method with some modifications followed by Shin

et al. (2007). Leaf tissue samples (100 mg) were extracted with 1 ml of 6% metaphosphoric acid in 2 M acetic acid. The extracted was filtered through #1 Whatman filter paper. After that, 1 ml of supernatant and 0.1 ml of 2% 2, 6-dichlorophenolindolphenol (DCIP) were mixed and incubated at room temperature for 1 h. Then, 1 ml of 2% thiourea in 5% metaphosphoric acid and 1 ml of 2% dinitrophenylhydrazine (DNPH) in 4.5 M sulfuric acid were added, incubated at 60 °C for 3 h. To stop the reaction, the tubes were placed in an ice bath and 3 ml of 90% sulfuric acid (on ice) were slowly added. Total AA was quantified by measurement of the absorbance at 540 nm and compared to the standard curve. The concentration was expressed as ascorbic acid on a fresh weight basis (see in Figure C.3).

3.1.3 Glutathione

Glutathione including reduced glutathione (GSH) and oxidized glutathione (GSSG) were assayed using the sensitive and specific enzyme method as described by Castillo and Greppin (1988). Leaf tissue samples, 100 mg, were homogenized with 1 ml of cold potassium phosphate buffer (60 mM KH_2PO_4, pH 7.0), containing 0.1 M KCl and 2.5 mM EDTA. After centrifuge, 200 µl of the supernatant were added to the reaction mixture for each measurement. For determination of total glutathione (GSH and GSSG), the supernatant was added to the reaction mixer, containing 1 ml of 60 mM K-phosphate buffer containing 2.5 mM EDTA (pH 7.5) and 0.66 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 20 µl of 20 mM NADPH and 20 µl of 60 units/ml glutathione reductase. After incubation for 10 min, the mixtures were measured the absorbance at 412 nm to determine the concentration of reduced DTNB (which is proportional to GSH or GSSG). The total glutathione were calculated according to GSH standard curve (see in Figure C.4).

3.2 Determination of enzymatic antioxidants

3.2.1 Extraction

For enzyme extraction, 100 mg of leaf and root tissues were homogenized with 800 µl of extraction buffer (0.1 M sodium phosphate buffer (pH 7.0), 2 mM EDTA, 4 mM DTT, and 6 mg of polyvinylpyrrolidone) in the microcentrifuge tube by using micro pestle. The whole extraction carried on ice. The homogenate was incubated in sonicator for 15 min and centrifuged at 12,000 rpm for 15 min. The supernatant was used for the assays of enzyme activity. Protein contents were measured by the method of Lowry (1951) using bovine serum albumin (BSA) as a standard (see in Figure C.5).

3.2.2 Enzymatic activity assay

3.2.2.1 Ascorbate peroxidase (APx)

Ascorbate peroxidase activity was determined according to the modified method of Nakano and Asada (1981). The substrate solution (50 ml) containing 48.6 ml of 50 mM sodium phosphate buffer (pH 7.0), 0.4 ml of 100 mM EDTA, 0.75 ml of 11.6 mM H_2O_2, 0.25 ml of 100 mM ascorbic acid was prepared. The oxidation of ascorbate was started by adding 200 µl of substrate solution into enzyme extract in microtiterplate. The decrease in absorbance at 290 nm due to the oxidation of ascorbate was monitored. One unit of enzyme activity was defined as 1 µmol of ascorbate oxidation per min.

3.2.2.2 Glutathione-*S*-transferase (GST)

Glutathione-*S*-transferase activity was measured according to the modified method of Mannervik and Guthenberg (1981). The substrate solution (50 ml) containing 45 ml of 50 mM sodium phosphate buffer (pH 7.0), 2.5 ml of 20 mM glutathione (GSH), 2.5 ml of 20 mM 1-chloro-2, 4-

dinitrobenzene (CDNB) in 95% ethanol. The reaction was initiated by adding 200 µl of substrate solution into enzyme extract in microtiterplate. The kinetic of the formation of 2, 4-dinitrophenylglutathione was measured by monitoring changes in absorbance at 340 nm for 3 minutes. One unit of enzyme activity was defined as the amount enzyme that causes the formation of 1 nmol of 2, 4-dinitrophenylglutathione per min.

3.2.2.3 Guaiacol peroxidase (GPx)

Guaiacol peroxidase activity was measured according to the modified method of Nakano and Asada (1981). The substrate solution (50 ml), containing 48.25 ml of 50 mM sodium phosphate buffer (pH 7.0), 0.75 µl of 11.6 mM H_2O_2, and 1 ml of 117.9 mM guaiacol, was prepared. The reaction was started by adding 200 µl of substrate solution into enzyme extract in microtiterplate. The kinetic of the formation of tetraguaiacol was measured by monitoring changes in absorbance at 436 nm for 3 min. One unit of peroxidase was defined as the amount enzyme that causes the formation of 1 nmol tetraguaiacol per min.

4. Determination of chitosan effects on ATPase activity (*P-type* and *V-type* ATPase) during drought stress

ATPase activity was measured according to the modified method of Wortsman et al. (2001). The substrate solution (20 ml) contained 13.92 ml of 100 mM Tris-HCl buffer (pH 6.5), 2 ml of 50 mM $MgSO_4$, 80 µl of 50 mM ammonium molybdate, 4 ml of 15 mM ATP. The reaction was initiated by adding 5µl of substrate solution into 5 µl of enzyme extract in microtiter plate and incubated 37 ^oC for 30 minutes. The reaction was stopped by adding 30 µl of 1.15 M perchloric acid and mixing. Then, 200 µl of color reagent and 10 µl of 1.5% Tween-20 were added into the mixture respectively. After mixing, the mixture was measured the absorbance at 660 nm and compare to standard curve (see in Figure C.6). One unit of ATPase activity is defined as the amount of enzyme leasing 1 µmol of Pi released per min.

5. Determination of chitosan effects on protein profiles after drought and chitosan application

5.1 Plant tissue collection

The tissue samples were collected after drought treatment for 2, 6, 24, 48 or 96 h. Leaf and root tissues were separated and immediately frozen in liquid nitrogen and stored at -80 ^{o}C prior to protein extraction.

5.2 Protein extraction and separation by one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE)

The protein was extracted from 300 mg of leaf and root tissues with 0.1% SDS. Samples were incubated at 37ºC for 3 h followed by centrifugation at 13,000 rpm for 15 min. The protein contents were determined according to Peterson (1983) using BSA as standard protein. The isolated proteins were analyzed by 12.5% polyacrylamide gels one-dimensional SDS-PAGE (Laemmli et al., 1970) and visualized by silver stain (see in Appendix D.1-D.4) (Blum et al., 1987).

5.3 In-gel digestion

After protein bands were excised according to protein ladder (see in Appendix D). The gel pieces were digested using an in-house method developed by Jaresitthikunchai et al. (2009). The gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10 mM DTT in 10mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10 mM ammonium bicarbonate) was added and incubated for 20 min at room temperature, then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated for a few h or overnight at room

temperature. The tryptic peptides were extracted by using 30 µl of 50% ACN in 0.1% formic acid (FA) and kept at -80°C for mass spectrometric analysis.

5.4 Electrospray ionization tandem mass spectrometry (ESI-MS/MS)

The protein digests were injected to Ultimate 3000 LC system (Dionex) coupled with ESI-Ion Trap MS (HCT ultra PTM Discovery System, BrukerDaltonik) with electrospray at a flow rate of 20 µl/min to µ-precolumn (Monolithic Trap Column, 200 µm i.d. x 5 cm). The sample was separated on a nano column (Monolithic Nano Column, 100 µm i.d. x 5 cm) at a flow rate of 1 µL/min. A Solvent gradient (Solvent A: H_2O, 0.1% formic acid; solvent B: 20% H_2O, 80% acetronitrile, 0.1% formic acid) was started as being 10% - 70% B at 0-13 min, 90% B at 13-15 min and 10% B at 15-20 min.

5.5 Protein quantitation and identification

DeCyder MS Differential Analaysis software (DeCyderMS, GE Healthcare) was used for proteins quantitation (Johansson et al., 2006; Thorsell et al., 2007). The raw data from LC-MS were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ions signal intensities in MS mode. The MS/MS data from DeCyderMS were submitted to search on database using the Mascot software (Matrix Science, London, UK, (Perkins et al., 1999)). Protein identification was performed by searching against the *Oryza sativa* L. (rice) subset of the US National Center for Biotechnology Information (NCBI) non redundant. The following parameters were used for searching: peptide tolerance, ±2 Da; fragment mass tolerance, ±2 Da; peptide charge, 1+, 2+ and 3+; maximum allowed missed cleavage, 1; instrument type, ESI-TRAP. Protein scores were derived from ion scores as a non-probabilistic ranking protein hits and obtained as the sum of peptide scores. The score threshold was set at $p < 0.05$ by Mascot algorithm.

5.6 Gene ontology and expression clustering

The proteins identified were used for gene ontology (GO) using gene ontology categorizer (http://eagl.unige.ch/GOCat/) or rice genome annotation project (http://rice.plantbiology.msu.edu). The protein significantly expression in the hierarchical clustering was generated by Multi Experiment Viewer (MEV) software (Saeed et al., 2003) using t-test p value < 0.05 and Pearson correlation.

6. Detection of responding gene expression responds to drought stress after chitosan

6.1 Sample collection

Leaf and root samples were collected after drought treatment for 2, 6 and 24 h. The rice leaves of 'LPT123' were stored at -80^{o}C prior to RNA extraction.

6.2 Determination of gene expressions by qPCR

Total RNA was isolated from of rice leaves using hot phenol method according to Thikart et al. (2005) (see in Appendix B). Contaminated genomic DNA was eliminated by DNase I (RNase-free) digestion according to manufacturer's instructions (Takara Bio, Japan) (see in Appendix B). To synthesis the first-strand cDNA, 2 µg of total RNA were reversed transcribed into cDNA in 20 µl reaction containing 1X M-MLV reaction buffer, 1 mM of dNTPs mixture, 0.5 µg oligo$(dT)_{15}$ (Promega, Madison, NI, USA) and 200 units M-MLV reverse transcriptase (Promega, Madison, NI, USA). The quantitative real-time PCR was performed in a final volume of 20 µl containing a 1 µl of the first strand cDNA reaction, 0.25 µM of each of the gene-specific primers, 1X of Prime Q-Master Mix consisting of 1 unit HS Prime Taq DNA Polymerase, 5 mM $MgCl_2$, 2 mM dNTPs mixture and 2X SYBR Green I. The qPCR amplifications of the 5 genes including the reference gene (*OsEF1α*) were performed at an initial denaturation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s. The specific primer pairs for amplification

were designed manually, and then confirmed with the OligoAnalyzer 3.1 software (www.idtdna.com) and NCBI Primer-BLAST tool. Gene specific primers were given in Table 3.1. At least three independent qPCR reactions were performed.

Table 3.1 Primers for qPCR

Gene name	**Sequence (5'⟶ 3')**	**Tm (°C)**	**Product size (bp)**
Os02g58440-F	TCAGCAGAATGCACCTCCTG	59.3	179
Os02g58440-R	TTGAGGGTGGAGGTCGGTAA	59.3	
Os12g23700-F	ATTCTGGGGTCGCAGGAGTA	59.3	162
Os12g23700-R	CCACGACTTCATGTCCTGCA	59.3	
Os01g2260-F	TTCTGCACCATCAACGCCTT	57.3	166
Os01g2260-R	TAGCAAACGATCCACGTCAG	57.3	
Os05g40220-F	GTCTGTGCCTGCTATGAGTC	59.4	153
Os05g40220-R	CCATTATCTGTCCCTGTGGC	59.4	
Os04g50204-F	GCACGAAATGTCAGCTCAAC	57.3	209
Os04g50204-R	CACTCTTGGTTTCTGCTTCC	57.3	
OsEF1α-F	ATGGTTGTGGAGACCTTC	58.2	116
OsEF1α-R	TCACCTTGGCACCGGTTG	58.7	

7. Targeting Induced Local Lesions IN Genomes (TILLING)

The TILLING technique consisted of 7 steps following the method of Tsai et al. (2011). The 5 primers of target genes (Table 3.2) were designed using the Codons Optimized to Discover Deleterious Lesions (CODDLe) program (http://www.proweb.org/coddle/).

Table 3.2 Primers for TILLING study

Gene name	**Sequence (5' ⟶ 3')**	**Tm (°C)**	**Product size (bp)**
Os01g42260-F	CAGATCCACCACAAGCAGGTAATTCCA	69.761	1499
Os01g42260-R	TCGCCTTGGAGGTAAGCATGTTAGGAA	70.049	
Os05g40220-F	AATTGAAGACCACCCAAACCAGGGAGT	70.135	1493
Os05g40220-R	AGAAAAGGCACTGCAACACAGTCATGG	70.156	
Os05g33890-F	CCCCTGATGATAATCTCGGTTCCCTCT	69.796	1459
Os05g33890-R	AATAACATGCAAATCACCAGCGAAGCA	69.919	
Os12g42760-F	CGTACCAGCCGCGAGTATATCAGAGGT	69.776	1500
Os12g42760-R	TCCTCCAAATCAGTCCTCACTGGCTTC	70.412	
Os04g50204-F	ATTCCGAGGTAGGGAGCTCGTCAAAAG	69.945	1485
Os04g50204-R	GCAGACCACCAAATTTCTTCAGCAAGG	70.317	

CHAPTER IV

RESULTS AND DISCUSSIONS

I. RESULTS

1. Determination of the appropriate chitosan types and concentrations for drought resistance induction in rice (*Oryza sativa* L.)

To investigate the suitable chitosan types and concentrations in 'LPT123' and 'LPT123-TC171' were performed for drought stress response in 4 week-old plants.

Four types and two concentrations of chitosan; O-90, P-90, O-80, P-80 at 20 or 40 mg/L were tested for drought stress response in 4 week-old of 'LPT123' and 'LPT123-TC171' seedlings. Chitosan could enhance growth in 'LPT123' under drought stress and after re-watering but no effect on 'LPT123-TC171' growth. Under drought stress, 40 mg/L O-80 chitosan significantly increased of shoot fresh weight (SFW) (Figure 4.1) and shoot dry weight (SDW) (Figure 4.2) in 'LPT123', but it did not clearly affect leaf water content (Figure 4.3). It increased 2.2 and 3.1 fold of SFW and SDW, when compared chitosan to the non-treated ones. On the other hand, no positive effects of chitosan induced drought resistance were found in 'LPT123-TC171'.

After rewatering, the highest SFW and SDW of 'LPT123' were found in two plants treated with 40 mg/L O-80 chitosan (Figure 4.4 A, 4.5 A and 4.6 A). The significantly higher SFW and SDW, when compared to the non-chitosan treated control were found after 3 weeks of rewatering (Figure 4.6 A). Contrastly, in 'LPT123-TC171' significant growth enhancement effects by chitosan treatment did not found (Figure 4.4, 4.5 and 4.6). Moreover, the significant retard and effect by chitosan application was detected in 'LPT123-TC171' after rewatering. After 1 week

of rewatering, the non-chitosan treated ‘LPT123-TC171’ had the highest SFW and SDW (Figure 4.4 A, B). SFW of control was significantly higher than SFW and SDW of other treatments. For SDW, the SDW of control, was significantly higher than the ‘LPT123-TC171’ treated with 40 mg/L, 20 mg/L P-90, 40 mg/L P-80, and 20 mg/L O-80. The ‘LPT123-TC171’ control had the highest SFW and SDW until after 3 weeks of rewatering (Figure 4.4, 4.5 and 4.6).

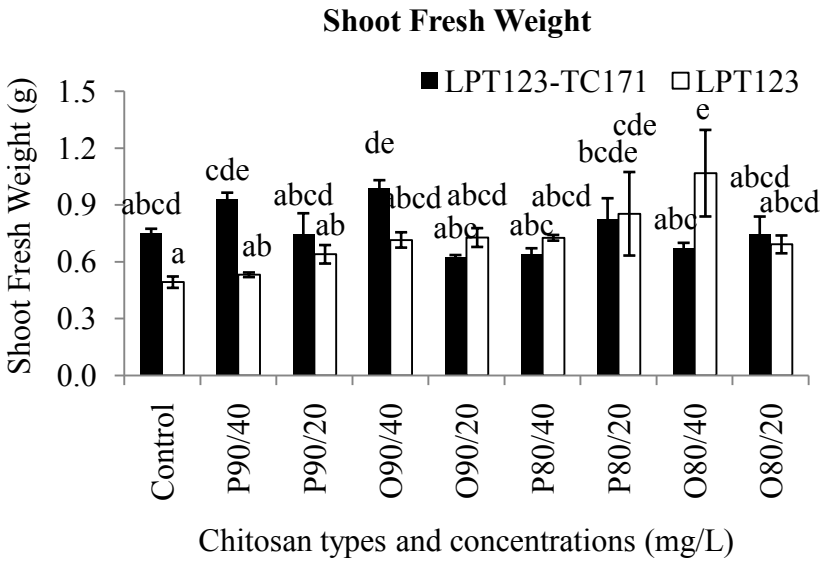


Figure 4.1 The chitosan types and concentrations affected shoot fresh weight of'LPT123' and 'LPT123-TC171' under drought condition for 2 weeks. Error bars indicated S.E. Means with different letters are significantly different at $p<0.05$ (Duncan's multiple range tests).

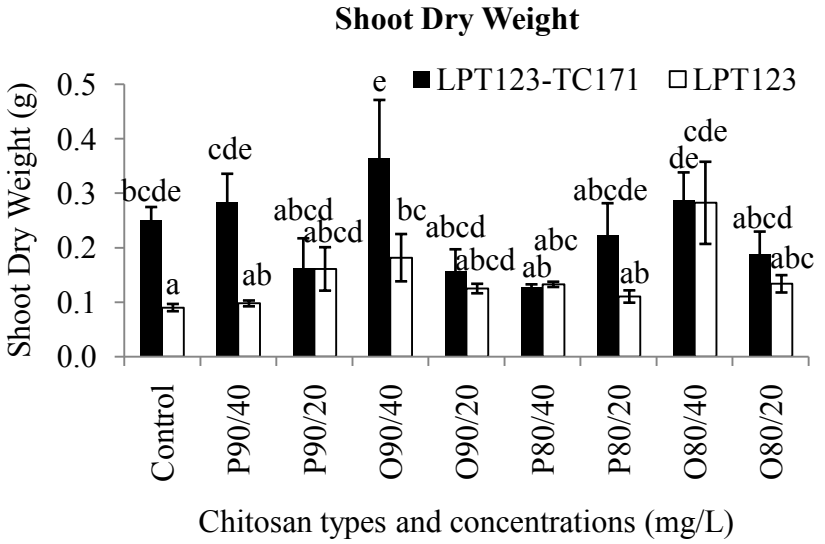


Figure 4.2 The chitosan types and concentrations affected shoot dry weight of 'LPT123' and 'LPT123-TC171' under drought condition for 2 weeks. Error bars indicated S.E. Means with different letters are significantly different at $p<0.05$ (Duncan's multiple range tests).

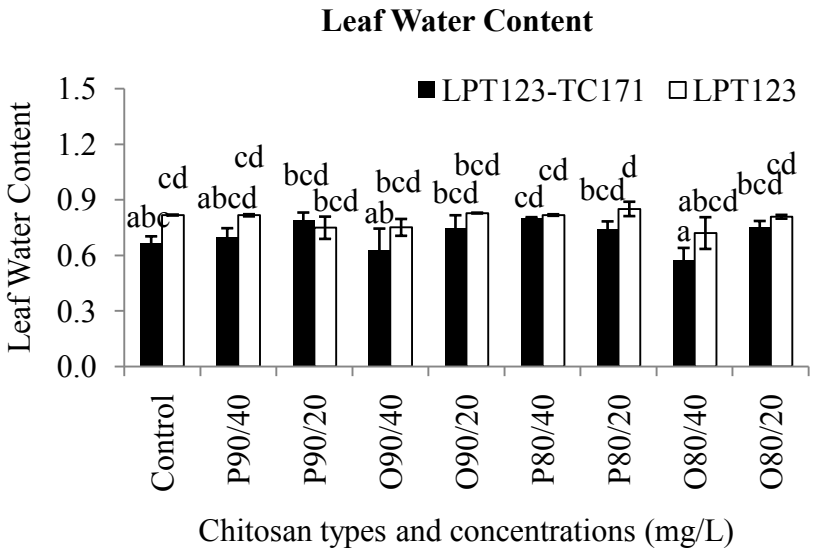
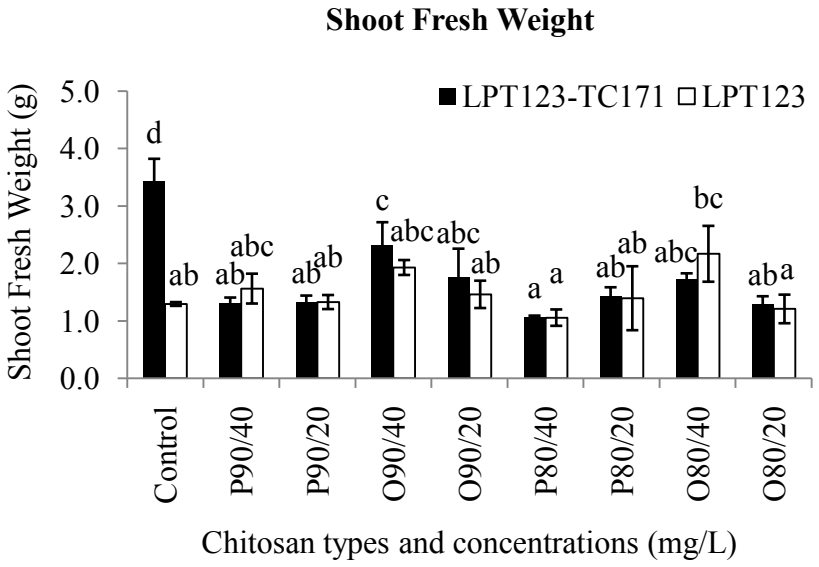


Figure 4.3 The chitosan types and concentrations affected shoot water content of 'LPT123' and 'LPT123-TC171' under drought condition for 2 weeks. Error bars indicated S.E. Means with different letters are significantly different at $p<0.05$ (Duncan's multiple range tests).

(A)



(B)

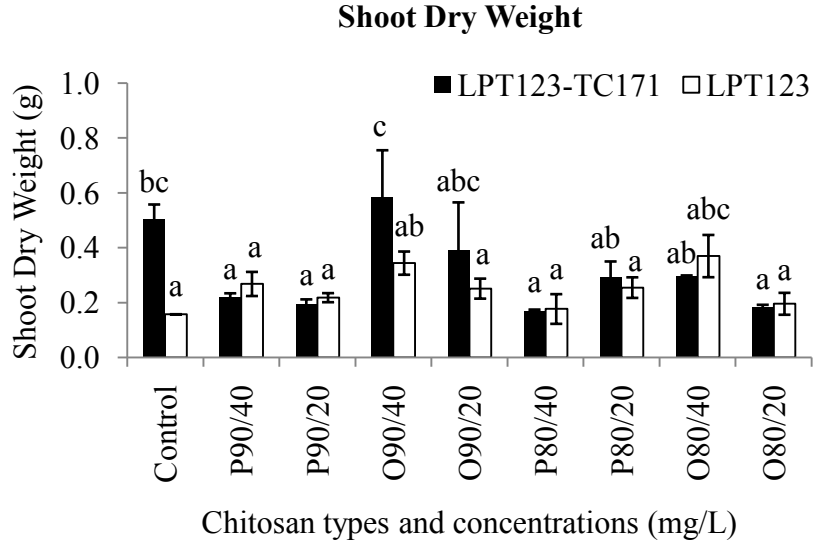
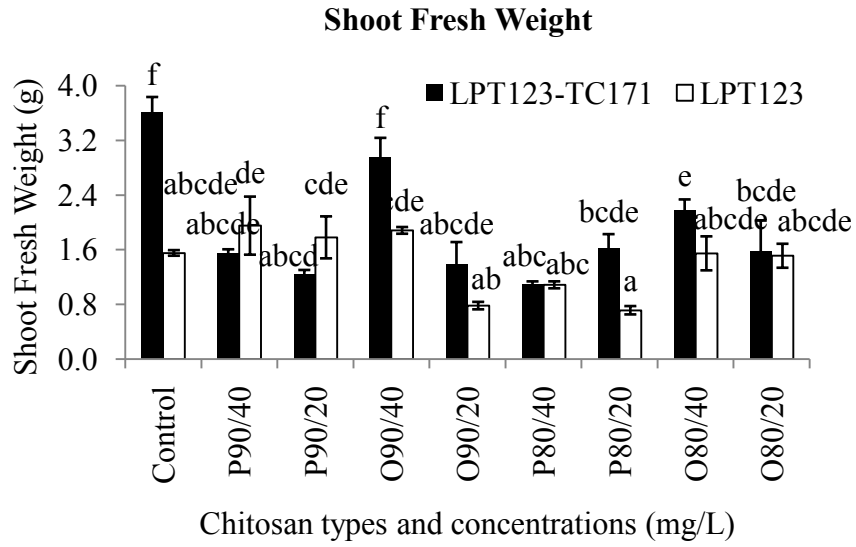


Figure 4.4 The chitosan types and concentrations affected shoot fresh weight (A) and shoot dry weight (B) of 'LPT123' and 'LPT123-TC171' during re-water for 1 week. Error bars indicated S.E. Means with different letters are significantly different at $p<0.05$ (Duncan's multiple range tests).

(A)



(B)

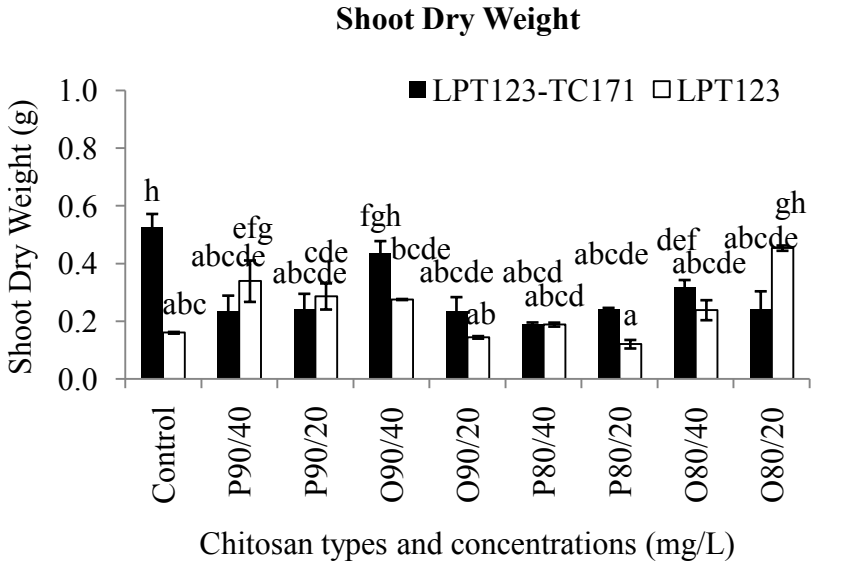
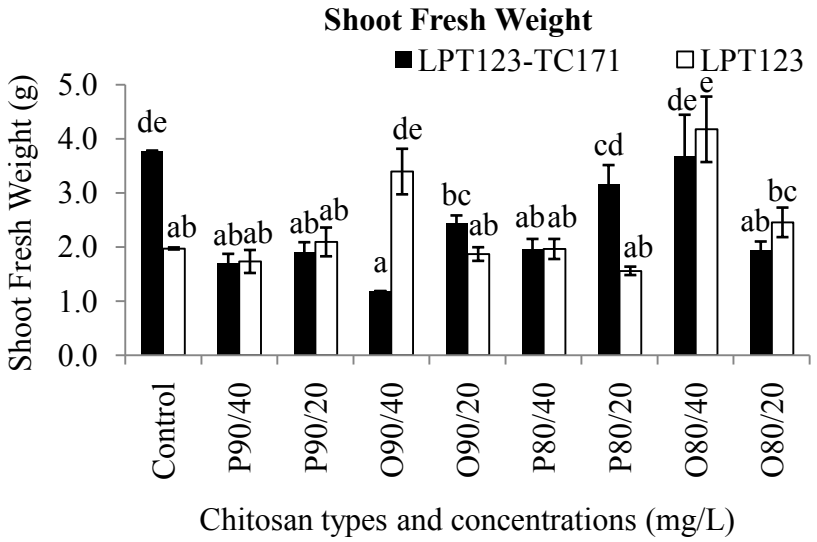


Figure 4.5 The chitosan types and concentrations affected shoot fresh weight (A) and shoot dry weight (B) of 'LPT123' and 'LPT123-TC171' during re-water for 2 weeks. Error bars indicated S.E. Means with different letters are significantly different at $p<0.05$ (Duncan's multiple range tests).

(A)



(B)

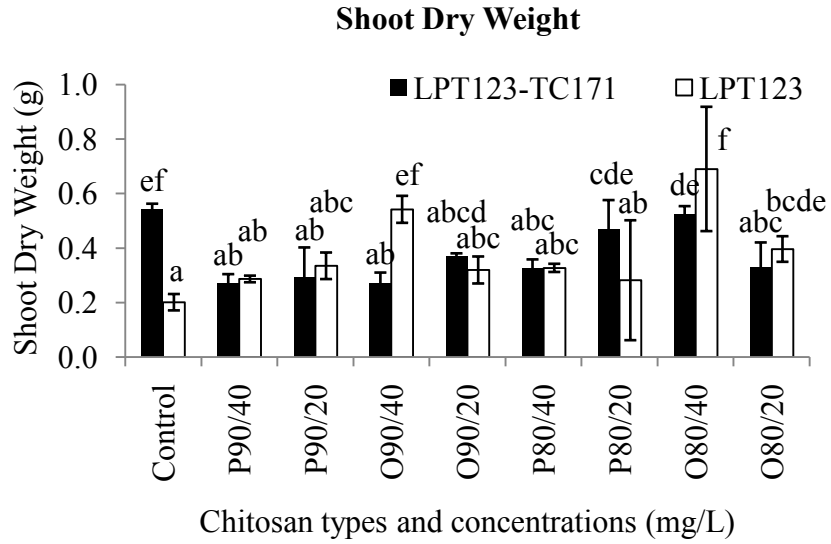


Figure 4.6 The chitosan types and concentrations affected shoot fresh weight (A) and shoot dry weight (B) of 'LPT123' and 'LPT123-TC171' during re-water for 3 weeks. Error bars indicated S.E. Means with different letters are significantly different at $p<0.05$ (Duncan's multiple range tests).

2. Investigation of chitosan effects on physiological changes during drought stress

2.1 Photosynthetic pigments

Drought stress and oligomeric chitosan effects were studied in rice leaves after 7 days of drought stress. When 4 week-old plants of both lines treated with 40 mg/L O-80, the significant higher content of photosynthetic pigments chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids than the non-chitosan treated plants were observed (Figure 4.7). After 7 days of drought stress without chitosan treatment, the significant increase of photosynthetic pigments was found in both rice lines. Drought-chitosan treated 'LPT123' plant showed similar level of photosynthetic pigments after drought stress when compared with the non chitosan-treated ones (Figure 4.7 A). Contrastingly, chitosan inhibited the increase of pigment content after 7 days of drought stress in 'LPT123-TC171' rice plant (Figure 4.7 B).

2.2 H_2O_2 content

Chitosan application showed less H_2O_2 production under drought stress in 'LPT123' than in 'LPT123-TC171'. Up to 50 μmol/g FW H_2O_2 was induced in chitosan and non chitosan application in 'LPT123' after 4 days of drought stress. Then, the H_2O_2 level was equal to the level of the initiation time. The level of H_2O_2 in drought-treated 'LPT123' plants with or without chitosan treatment was similar in all time point tested, except on day 4 after drought, when the chitosan-treated plant had the significant lower level of H_2O_2. Chitosan treatment cause 16% reduction of H_2O_2 after day 4 of drought stress in 'LPT123' plants (Figure 4.8 A).

On the other hand, the highest H_2O_2 level at 30 μmol/g FW was detected in non chitosan-treated 'LPT123-TC171' on day 2 after drought stress, and it was down to the normal level on day 7 under drought treatment (Figure 4.8 B). Interestingly, chitosan application inhibited H_2O_2 production scavenging the

significant lower level up to 56 % reduction of H_2O_2 in plants under drought stress on day 2, 4 and 7 in ‘LPT123-TC171’ plants (Figure 4.8 B).

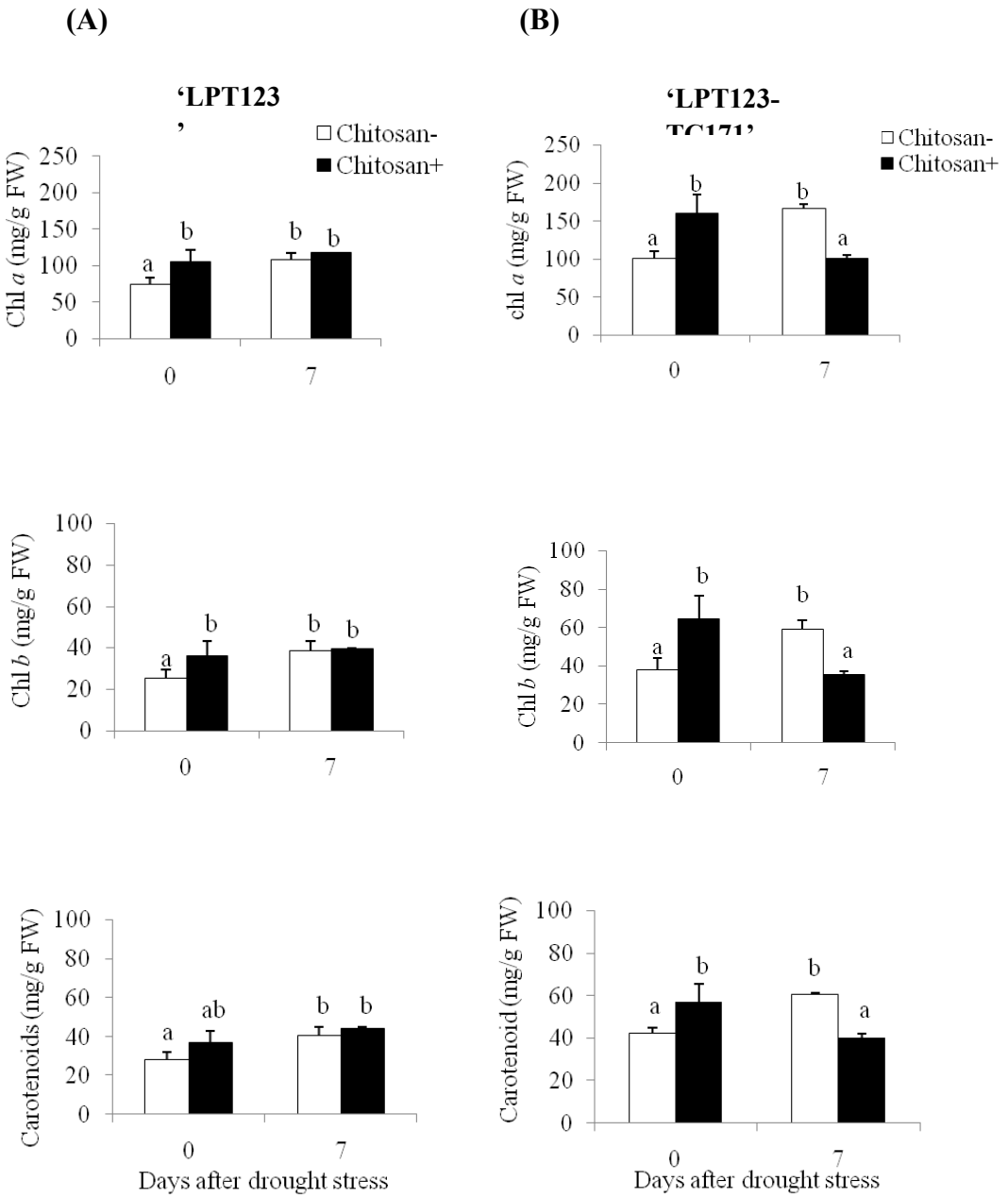
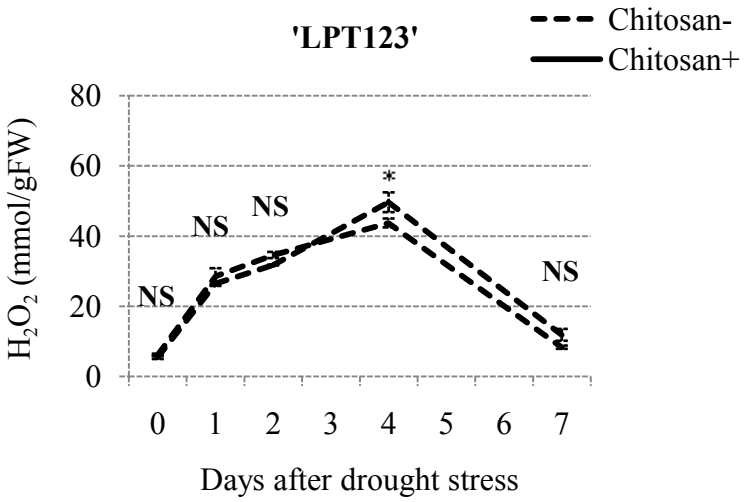


Figure 4.7 Photosynthetic pigments, chl *a* (chlorophyll *a*), chl *b* (chlorophyll *b*), and carotenoids, respectively in 'LPT123' (A) and 'LPT123-TC171' (B) leaves were treated or non-treated with chitosan before and after drought stress for 7 days. Error bars indicated S.E. Means with different letters are significantly different at $p<0.05$ (Duncan's multiple range tests).

(A)



(B)

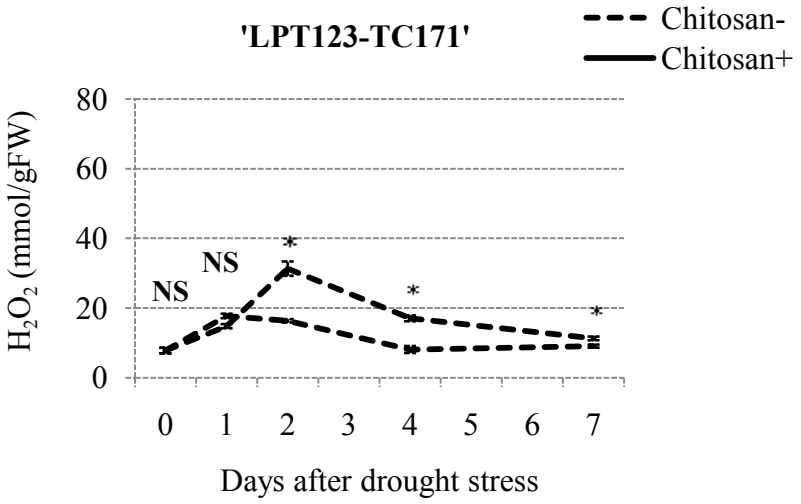


Figure 4.8 Hydrogen peroxide contents subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123' (A) and 'LPT123-TC171' (B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bars indicated S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

3. Determination of chitosan effects on antioxidative systems during drought stress

3.1 Determination of non-enzymatic antioxidants

3.1.1 Free phenolic compound content

Chitosan application significantly increased free phenolic contents in ‘LPT123’ rice after 2 days of drought stress, but it did not affect the free phenolic contents in ‘LPT123-TC171’ (Figure 4.9). In ‘LPT123’ rice, chitosan application exhibited the increase of phenolic contents on day 1, 2 and 4 after drought stress and the significantly highest level of free phenolic contents was found 2 days after drought stress. Without chitosan application under drought stress in ‘LPT123’ gradually increased the phenolic compound contents (Figure 4.9 A).

3.1.2 Ascorbic acid content

Chitosan showed the minor effect on ascorbic acid content (Figure 4.10). In ‘LPT123’, drought stress have the effect on ascorbic acid content in both plant treated with chitosan and without chitosan treatment (Figure 4.10 A). However, in ‘LPT123-TC171’, drought stress tended to increase the level of ascorbic acid with highest level on day 4 after drought stress in without chitosan treatment. The similar response was also detected in ‘LPT123-TC171’ treated with chitosan (Figure 4.10 B).

3.1.3 Glutathione

3.1.3.1 Reduced glutathione (GSH) content

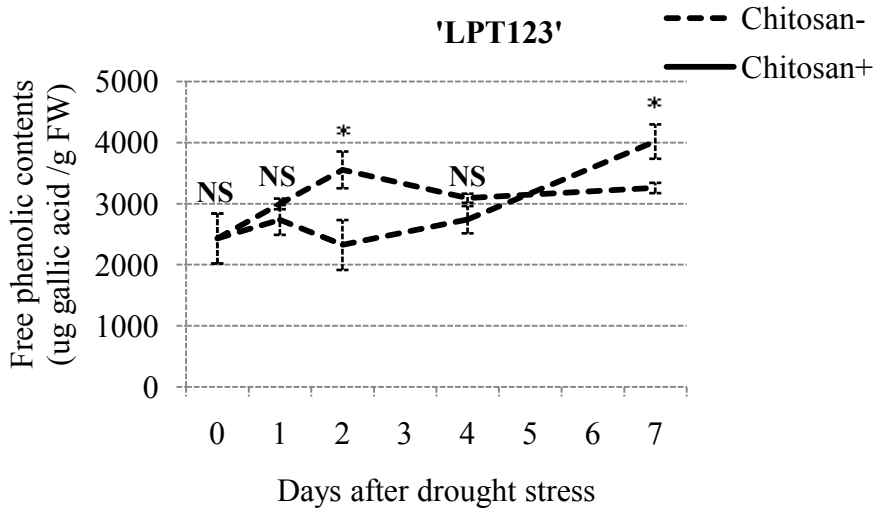
Chitosan decreased reduced glutathione (GSH) content during drought stress in ‘LPT123-TC171’, but not in ‘LPT123’. Drought stress increased GSH in both ‘LPT123’ (Figure 4.11 A) and ‘LPT123-TC171’ (Figure 4.11 B). However, GSH in ‘LPT123’ was more than 20 times lower than the GSH content found in ‘LPT123-TC171’. Chitosan application did not showed the effects on GSH

content in ‘LPT123’ (Figure 4.11 A), but it significantly decreased the accumulation of GSH in ‘LPT123-TC171’ (Figure 4.11 B).

3.1.3.2 Oxidized glutathione (GSSG) content

The oxidized glutathione (GSSG) was increased after drought stress in both rice line (Figure 4.12). Chitosan caused the slightly changes in GSSG content of ‘LPT123’ during drought stress (Figure 4.12 A). In chitosan-treated ‘LPT123-TC171’, the significant increase of GSSG was detected on day 7 after drought stress, while in the non chitosan treated ones, GSSG tended to increase since after 4 days of drought stress (Figure 4.12 B). After 7 days in drought stress, the GSSG content in chitosan-treated ‘LPT123-TC171’ was higher than the non chitosan treated ones, which is opposite to what found in ‘LPT123’. However, the difference of GSSG content between the non chitosan and the chitosan-treated ‘LPT123’ was less than the difference of GSSG content found in non treated chitosan and the chitosan-treated ‘LPT123-TC171’.

(A)



(B)

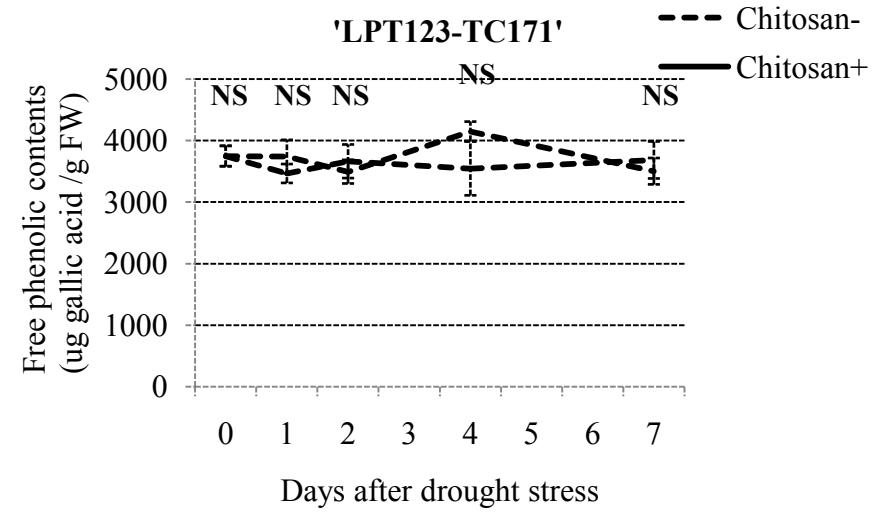


Figure 4.9 Free phenolic contents subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123' (A) and 'LPT123-TC171' (B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bars indicated S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

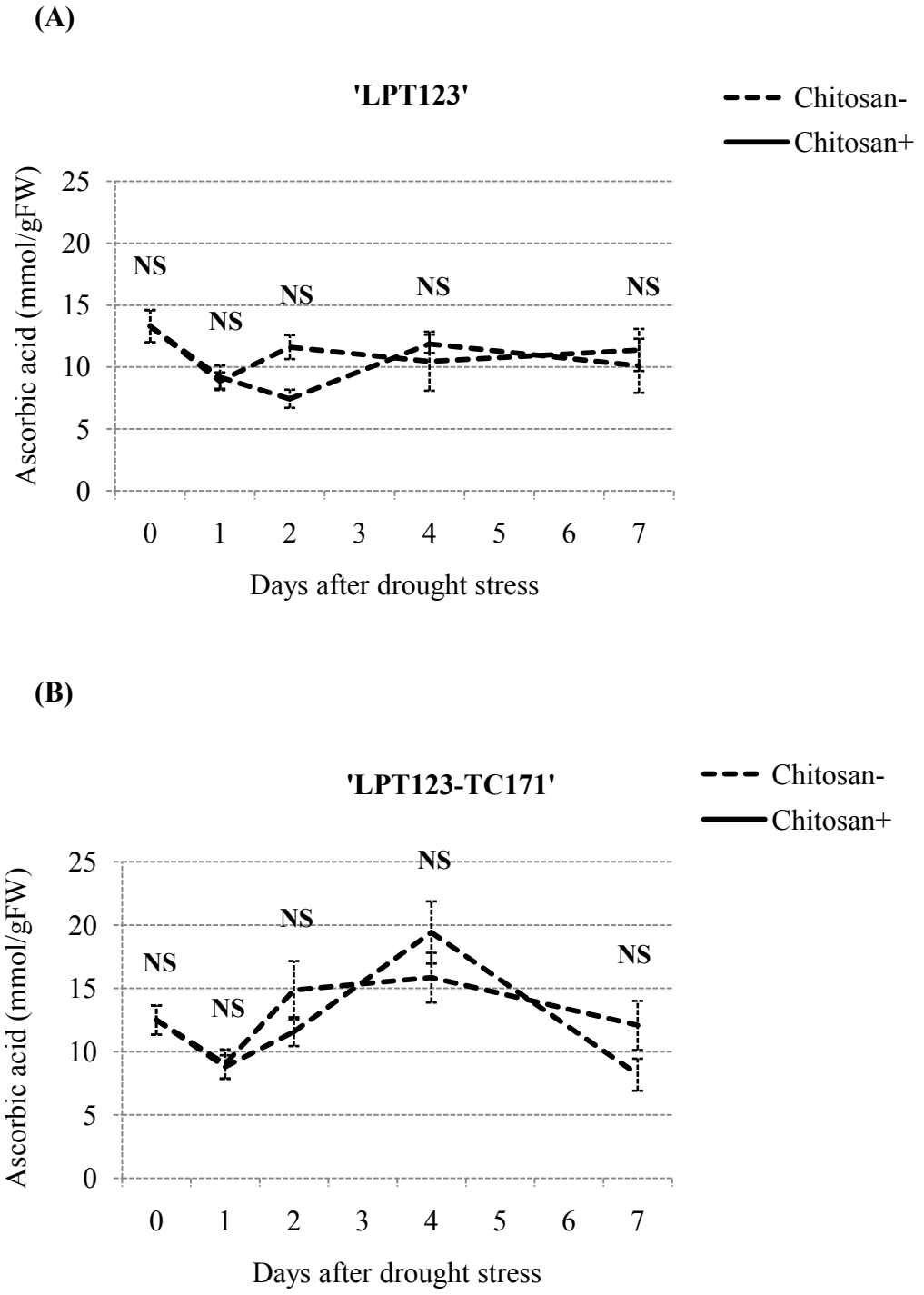
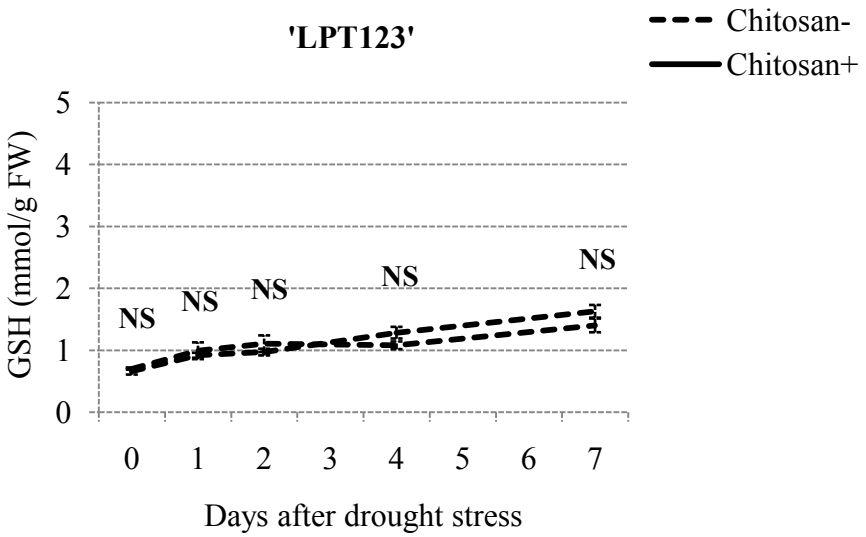


Figure 4.10 Ascorbic acid contents subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123' (A) and 'LPT123-TC171' (B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. NS is non significantly different of means at $p<0.05$.

(A)



(B)

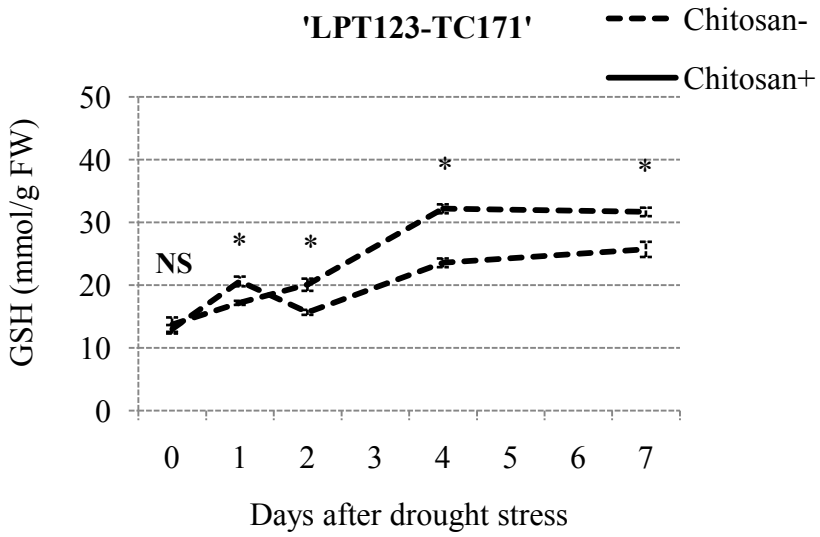
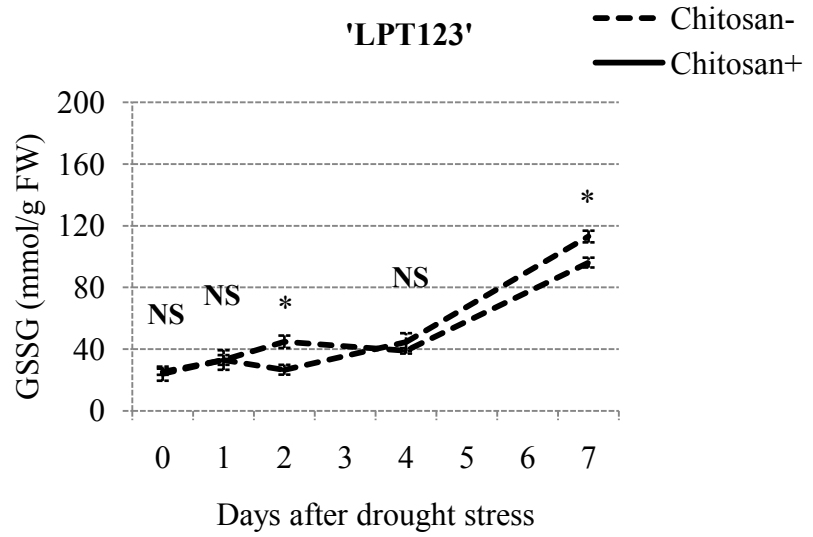


Figure 4.11 Reduced glutathione contents subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123' (A) and 'LPT123-TC171' (B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

(A)



(B)

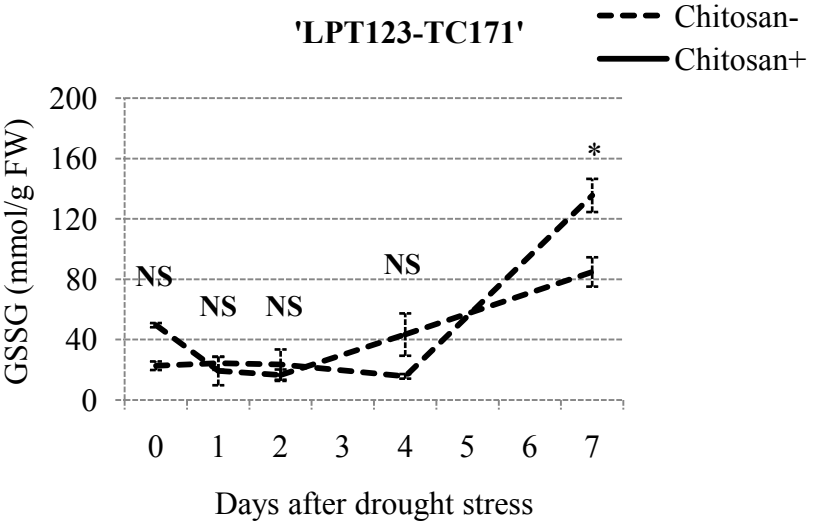


Figure 4.12 Oxidized glutathione contents subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123' (A) and 'LPT123-TC171' (B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

3.2 Determination of enzymatic antioxidants

3.2.1 Guaiacol peroxidase (GPx) activity

3.2.1.1 Acidic peroxidase

Acidic peroxidase activity represents the vacuole peroxidase activity (Csiszár et al., 2012). Chitosan treatment did not significantly affect the increasing of acidic GPx activity in both rice lines in leaves and roots under drought stress condition in both rice lines (Figure 4.13 and 4.14).

3.2.1.2 Neutral peroxidase

Neutral peroxidase activity represents the cytoplasmic peroxidase activity (Csiszár et al., 2012). It was found that chiosan treatment did not change the cytoplasm peroxidase activity in 'LPT123' leaves (Figure 4.15 A), while it increased peroxidase activity in 'LPT123-TC171' leaves after 1 day of drought stress (Figure 4.15 B). In 'LPT123' leaves, neutral GPx activity did not change by drought stress, while in 'LPT123-TC171', chitosan could induce the neutral GPx activity after a day of drought treatment.

In roots, the higher level of neutral GPx activity was found chitosan treated 'LPT123' when starting the drought treatment (Figure 4.16 A), which was opposite to the chitosan treated 'LPT123-TC171' that the level of neutral GPx activity was significantly lower. However, neutral GPx activity in roots did not show any response after drought stress (Figure 4.16 B).

3.2.1.3 Basidic peroxidase

Basidic peroxidase activity represents the GPx activity in membrane (Csiszár et al., 2012). Chitosan and non-chitosan plants did not change of the basidic GPx activity of leaves in both rice lines (Figure 4.17). Conversely, in root, chitosan application afterward basic GPx activity in both rice lines (Figure 4.18). In 'LPT123', the roots of chitosan treated 'LPT123' showed the significant higher level of basidic GPx activity after 2 days of drought stress, when compared to non-chitosan treated ones (Figure 4.18 A). On the other hand, in 'LPT123-TC171' root of chitosan treated plants had the significant lower basidic GPx activity than the non-chitosan treated ones (Figure 4.18 B). Although drought stress could increase GPx activity in roots of chitosan treated 'LPT123-TC171', the level of GPx activity was significant lower than the activity found in non-chitosan treated ones, until 7 days after drought stress, when the GPx activity in the chitosan treated plants was higher (Figure 4.18 B).

3.2.2 Ascorbate peroxidase (APx) activity

Before drought stress, chitosan treatment had no effect on APx activity in 'LPT123' leaves, but significantly increase APx activity in 'LPT123-TC171' leaves (Figure 4.19 A and B). Chitosan application did show the effect on APx activity in roots of both lines (Figure 4.20 A and B). Drought stress did not change the APx activity in non-chitosan treated 'LPT123', but with chitosan treatment, the tendency of APx induction was found after a day of drought treatment. However, it was decreased to the lower level after 2 days of drought stress, when compared to the non-chitosan treated plants. After 7 days of drought stress APx activity in non-chitosan and chitosan treated 'LPT123' was similar (Figure 4.20 A). In chitosan treated 'LPT123-TC171', 50-80% reduction in APx activity was found in after 1 day of drought stress (Figure 4.20 B). However, the APx activity was about 20-50 U/mg protein, which was in the range of APx activity found in 'LPT123' (Figure 4.20 A).

3.3.3 Glutathione-*S*-transferase (GST) activity

Chitosan could not induce the GST activity in leaf tissues of both rice lines under drought stress (Figure 4.21). Conversely, chitosan application increased the GST activity in roots of 'LPT123' after 1 day of drought treatment (Figure 4.22 A) and decrease GST activity in roots of 'LPT123-TC171' after 2 to 4 days of drought stress (Figure 4.22 B).

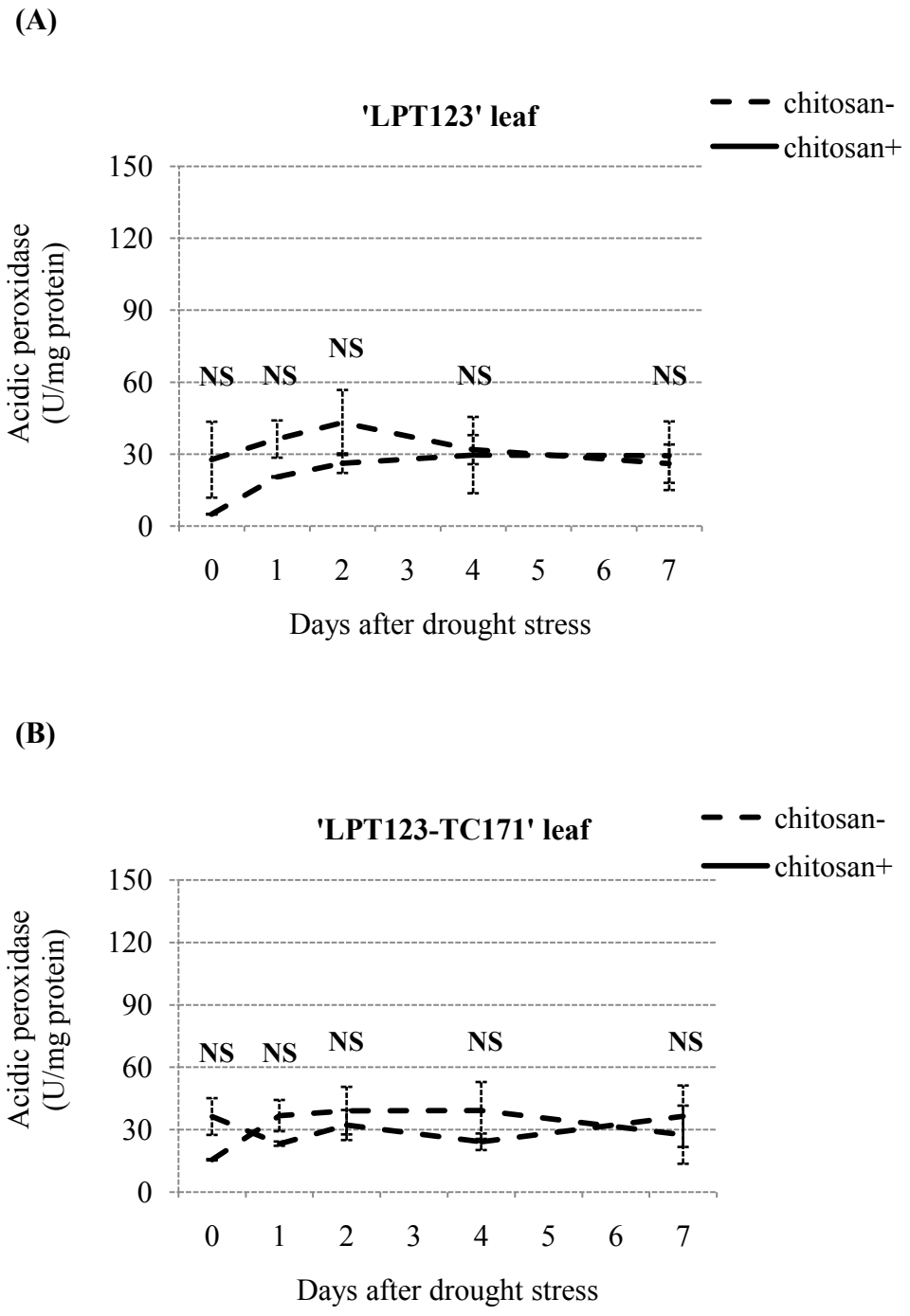


Figure 4.13 Acidic peroxidase (GPx) activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123'(A) and 'LPT123-TC171'(B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. NS is non significantly different of means at $p<0.05$.

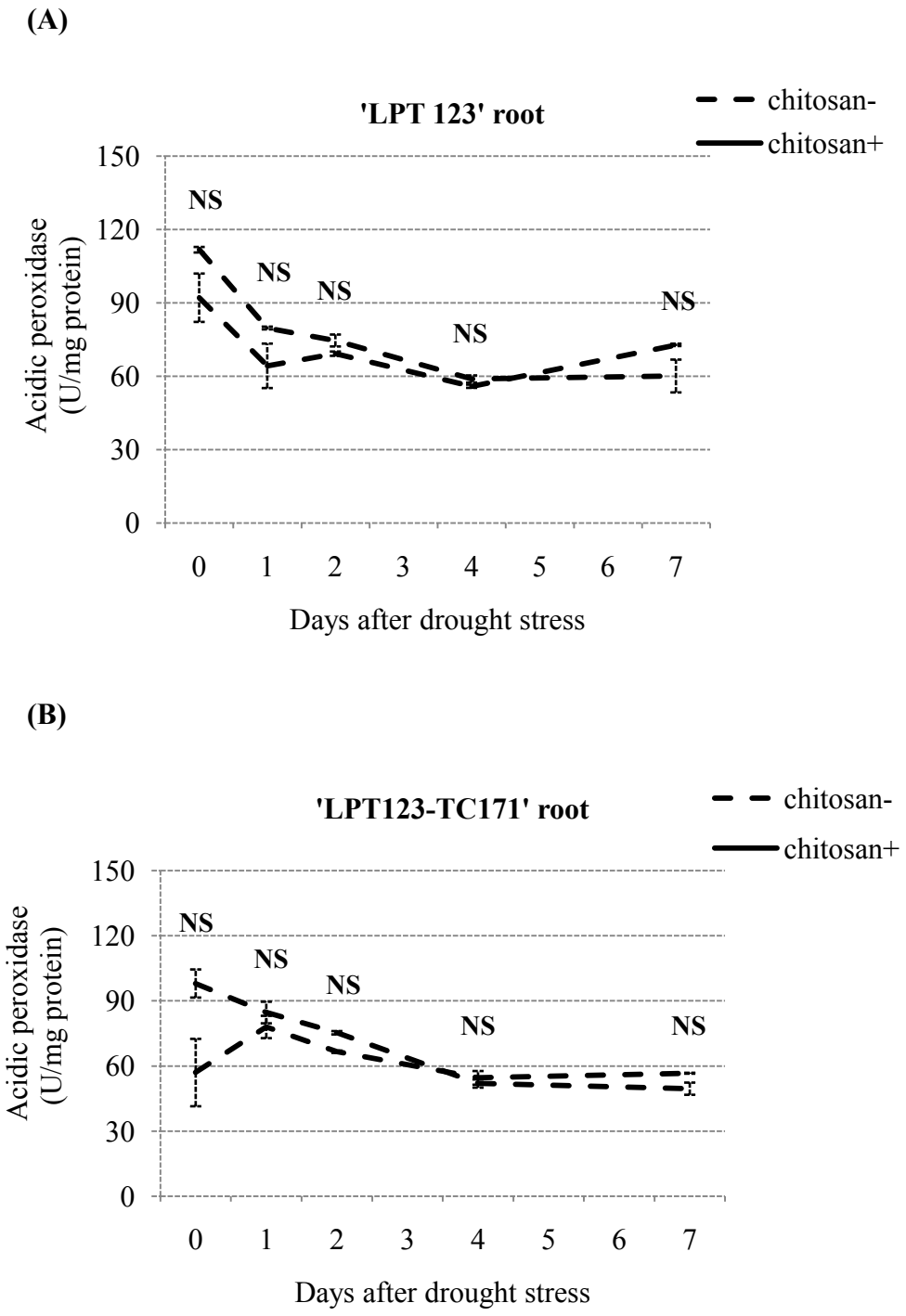


Figure 4.14 Acidic peroxidase (GPx) activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123'(A) and 'LPT123-TC171'(B) roots. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. NS is non significantly different of means at $p<0.05$.

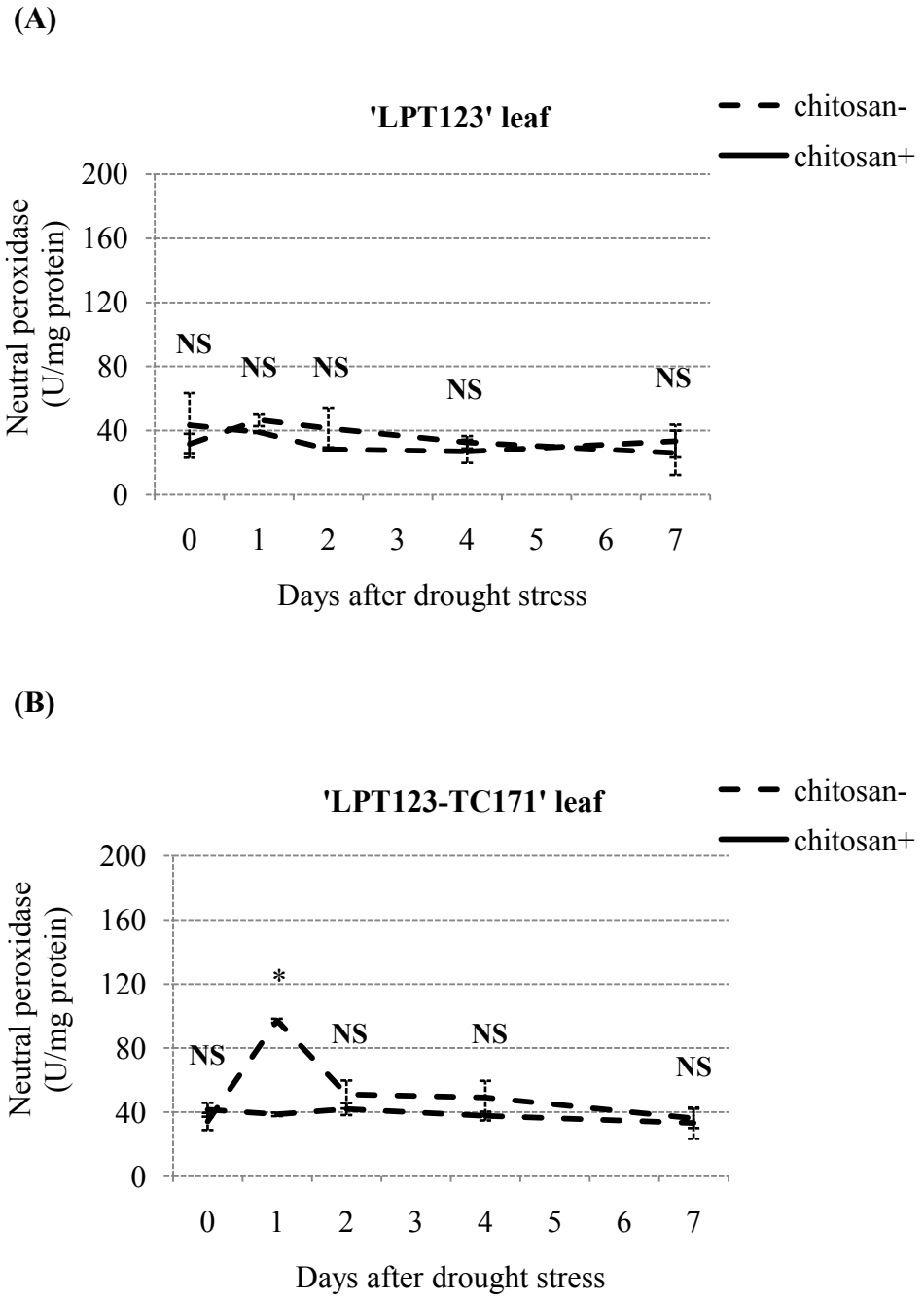
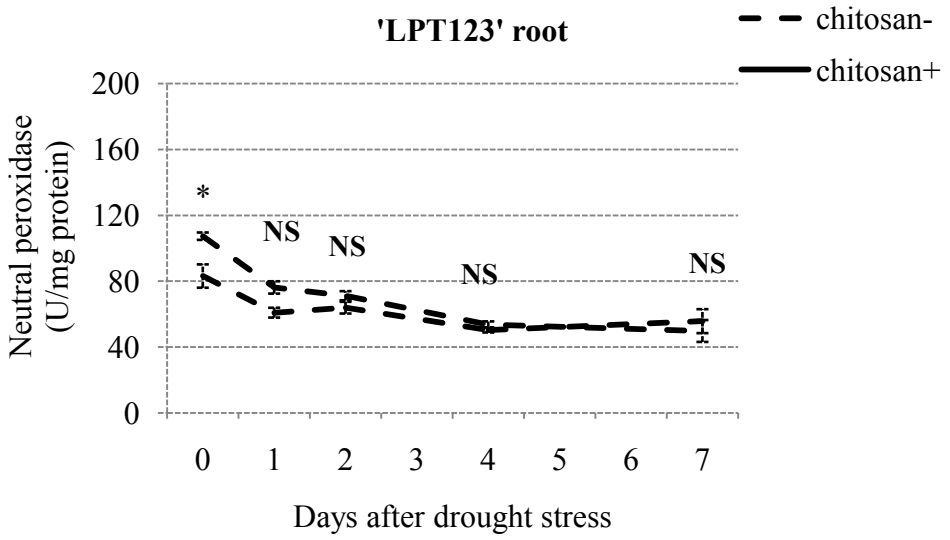


Figure 4.15 Neutral peroxidase (GPx) activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123'(A) and 'LPT123-TC171'(B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

(A)



(B)

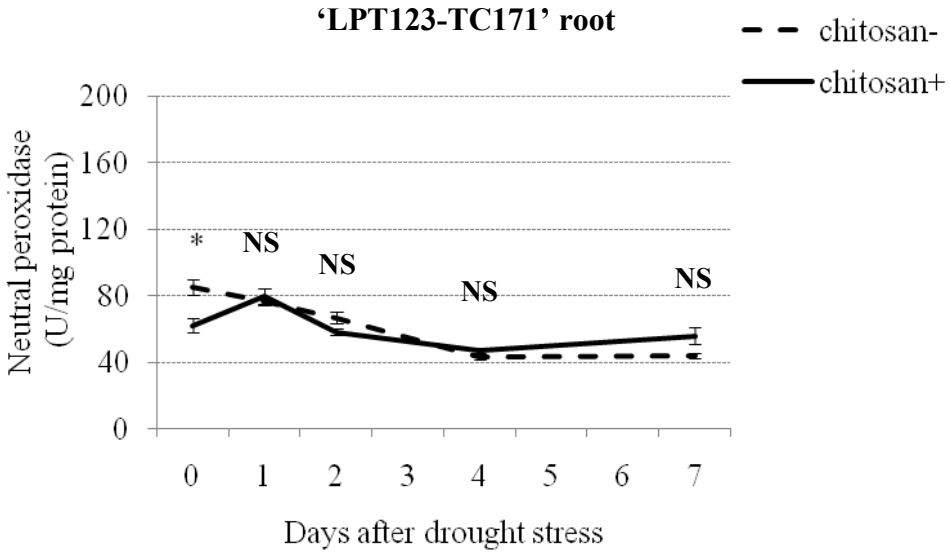


Figure 4.16 Neutral peroxidase (GPx) activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123'(A) and 'LPT123-TC171'(B) roots. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

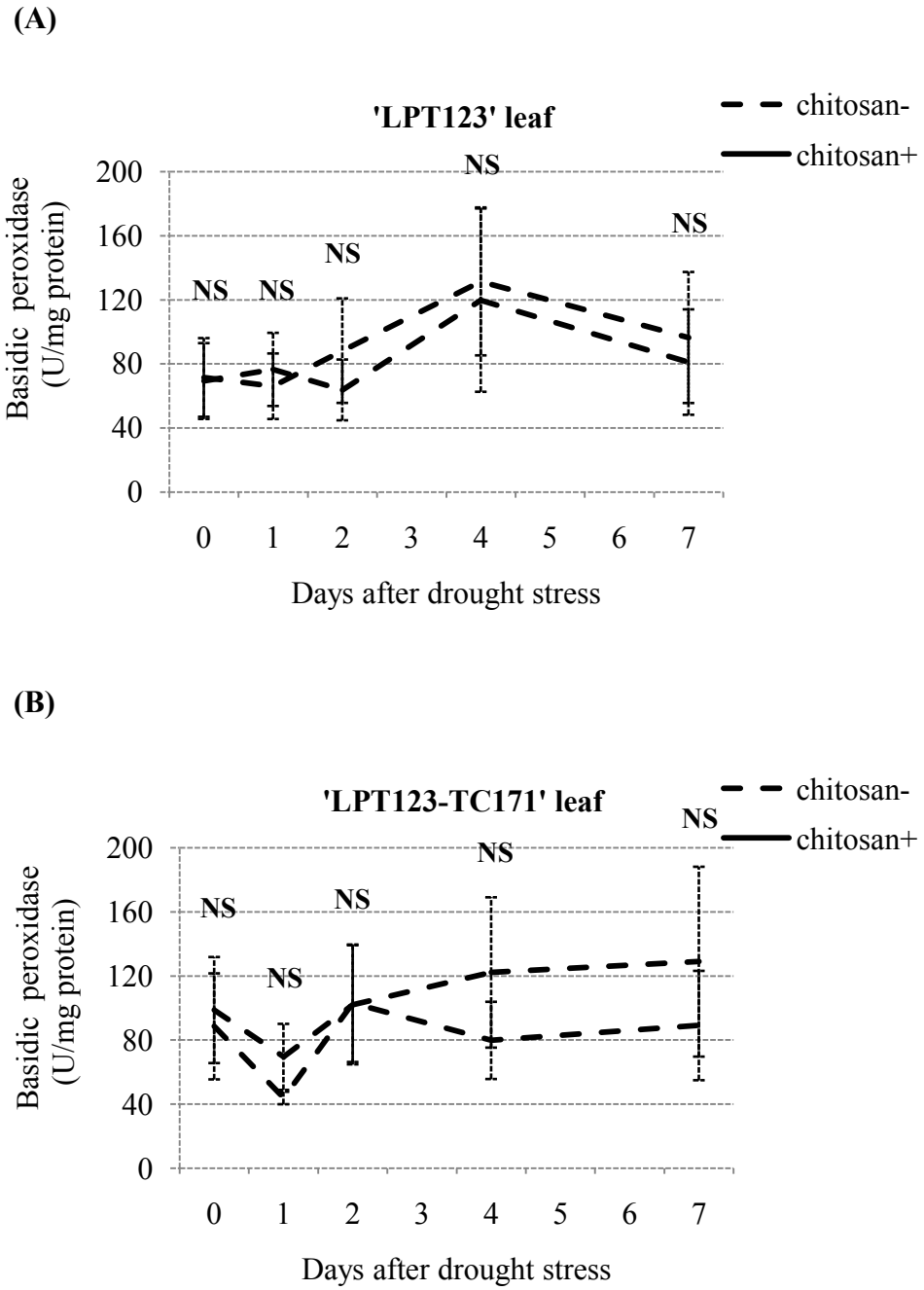


Figure 4.17 Basidic peroxidase (GPx) activity subjected to drought stress for 1, 2, 4 or 7 days in leaves of 'LPT123'(A) and 'LPT123-TC171'(B). Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

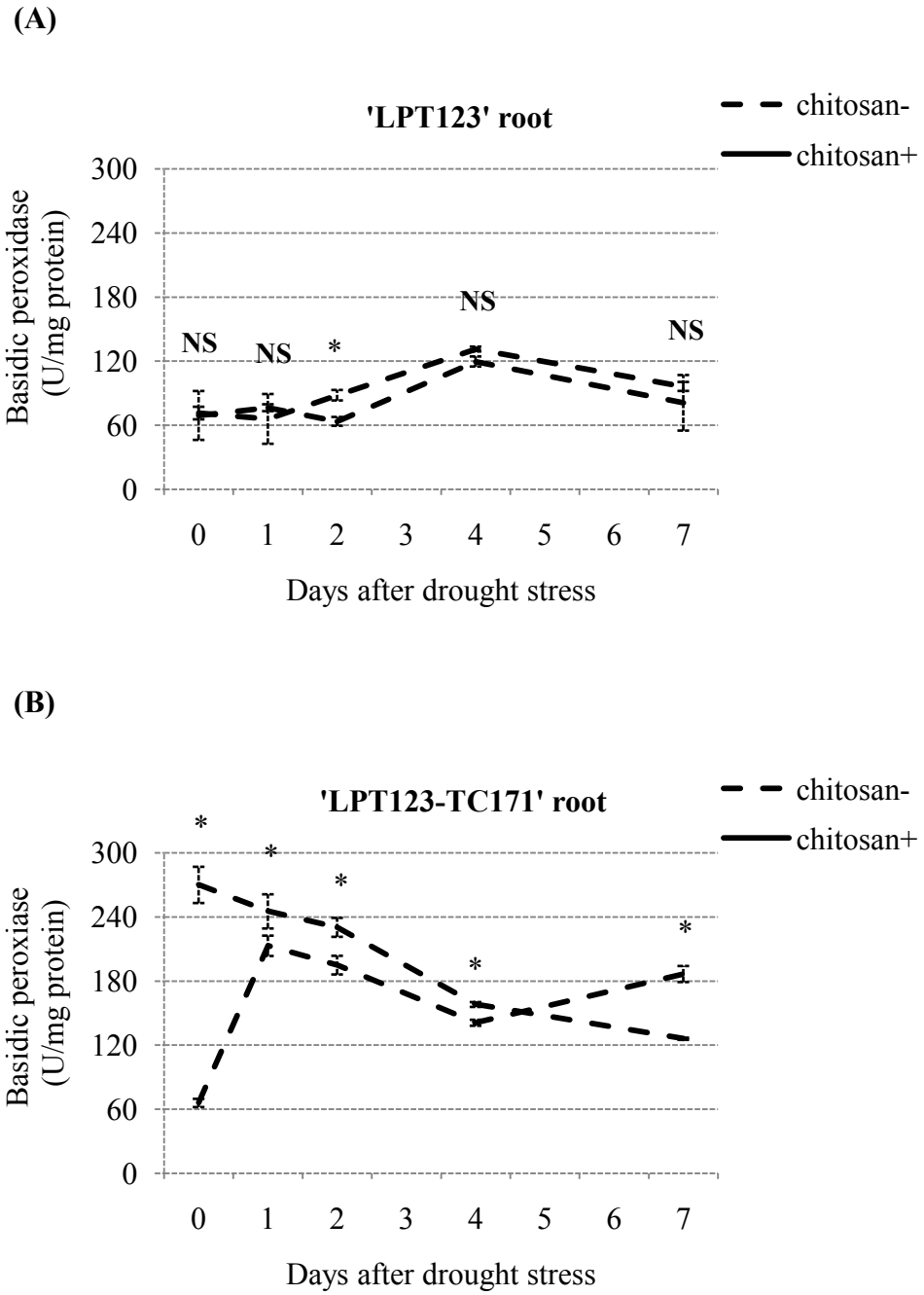
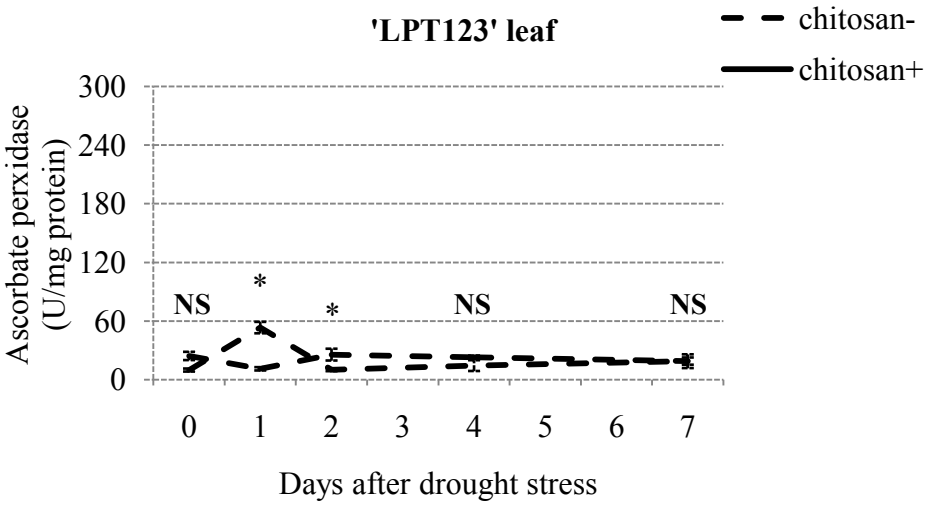


Figure 4.18 Basidic peroxidase (GPx) activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123'(A) and 'LPT123-TC171'(B) roots. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

(A)



(B)

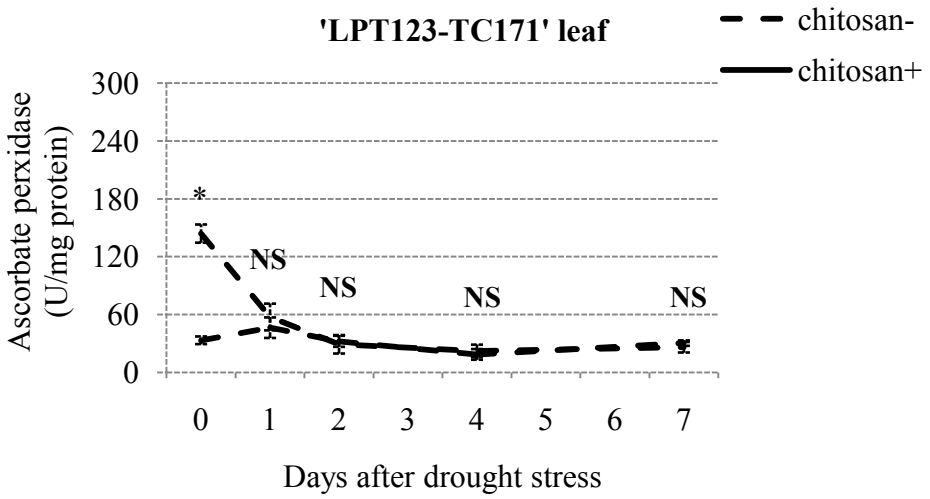


Figure 4.19 Ascorbate peroxidase (APx) activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123'(A) and 'LPT123-TC171'(B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

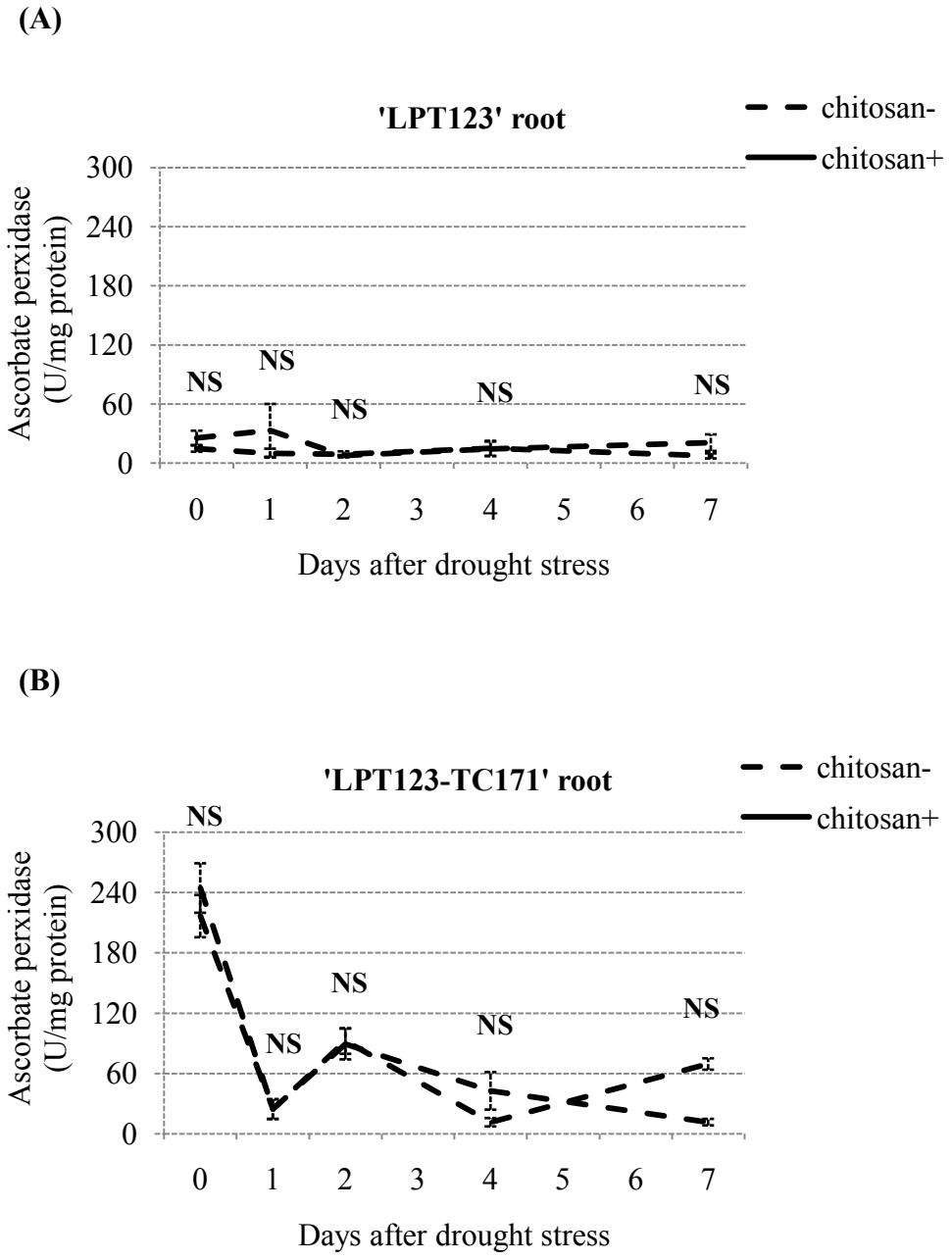
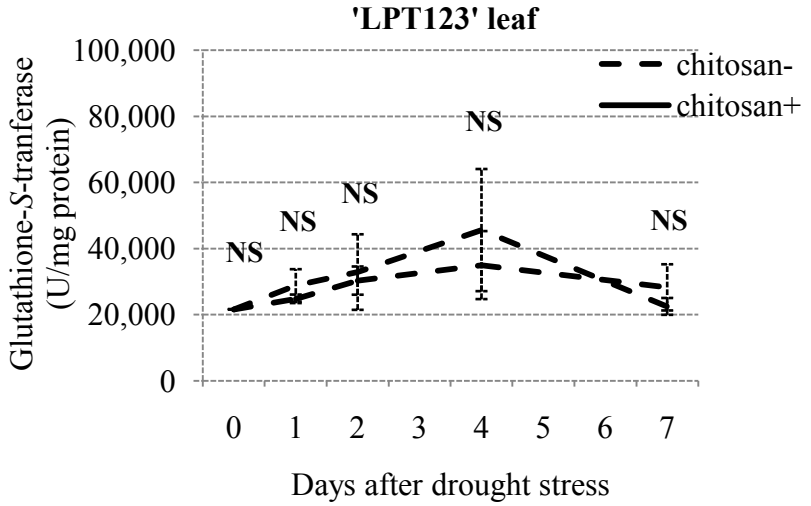


Figure 4.20 Ascorbate peroxidase (APx) activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123'(A) and 'LPT123-TC171'(B) roots. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

(A)



(B)

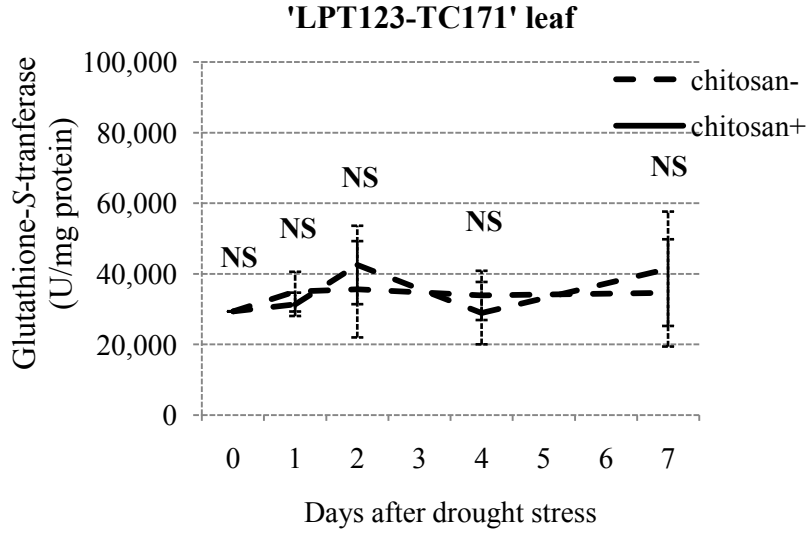


Figure 4.21 Glutathione-*S*-tranferase activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123'(A) and 'LPT123-TC171'(B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

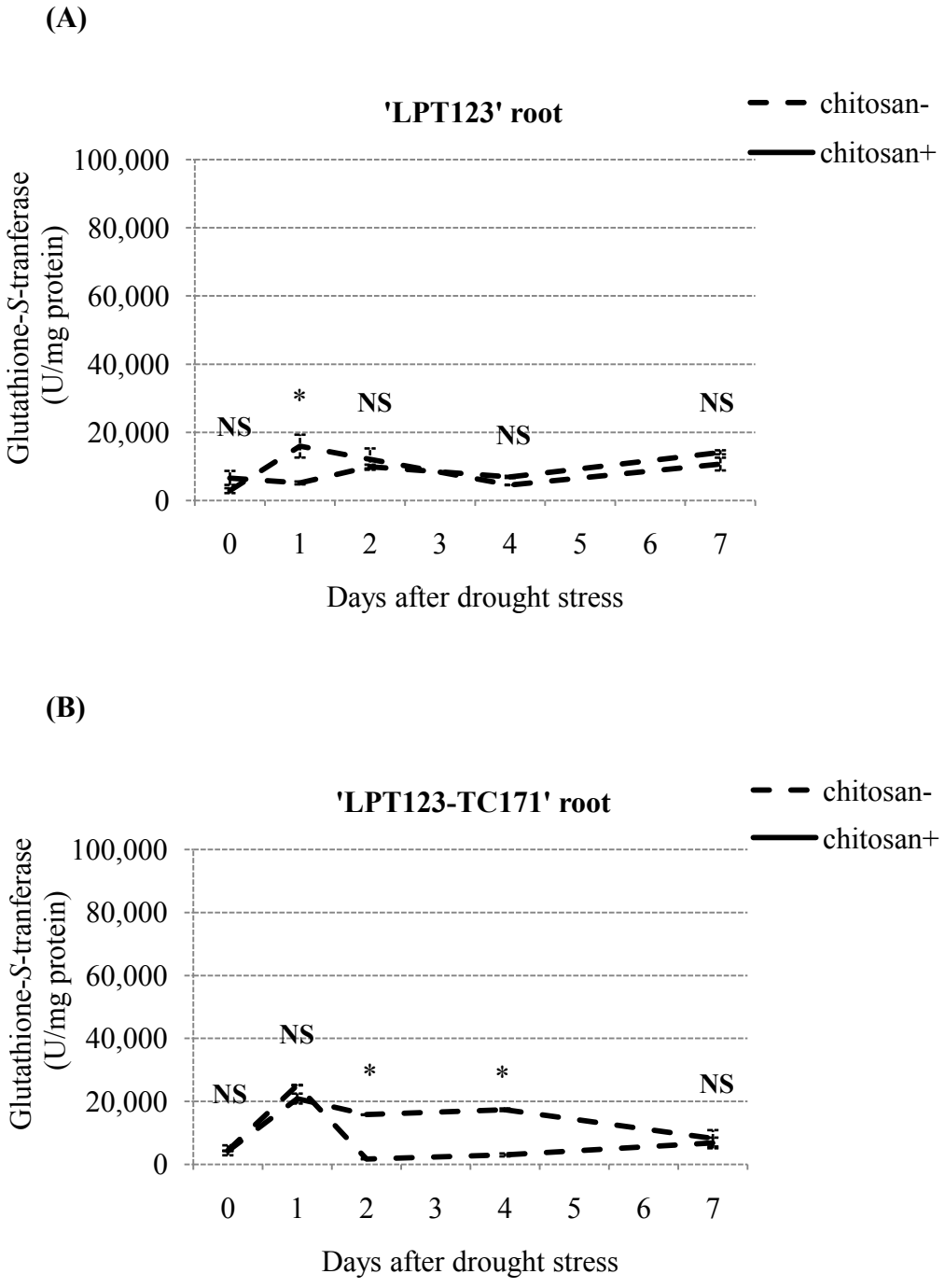


Figure 4.22 Glutathione-*S*-tranferase activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123' (A) and 'LPT123-TC171' (B) roots. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

4. Determination of chitosan effects on ATPase activity (*P-type* ATPase and *V-type* ATPase) during drought stress

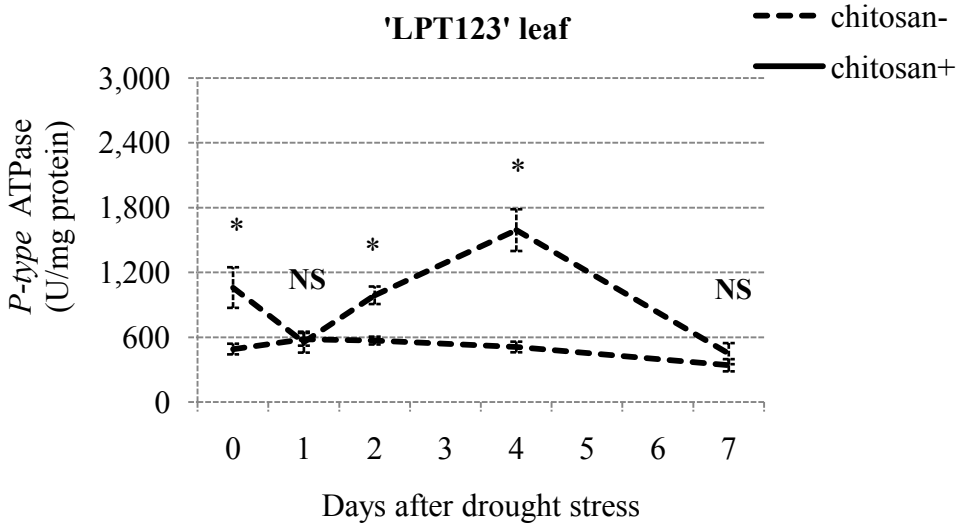
4.1 *P-type* ATPase activity

The chitosan application affected on the ATPase activities in leaves and roots in both lines (Figure 4.23-4.26). Drought stress caused the induction of *P-type* ATPase in leaf tissues of both lines (Figure 4.23 A and B). Chitosan treatment significant lowered *P-type* ATPase activity in leaves during drought stress (Figure 4.23). The opposite response was found in roots (Figure 4.24). Drought lowered *P-type* ATPase activity in roots, and chitosan treatment did affect *P-type* ATPase activity in root tissues during drought stress (Figure 4.24 A and B).

4.2 *V-type* ATPase activity

For vacuolar (*V-type* ATPase) drought stress caused the reduction in *V-type* ATPase activity in 'LPT123' leaves (Figure 4.25 A), but increase it in 'LPT123-TC171' ones (Figure 4.25 B). Chitosan treatment resulted in the unchanged activity during drought stress in roots of both rice lines (Figure 4.26). These resulted in the higher level of *V-type* ATPase in leaves of non-chitosan treated plants when compared to the treated ones (Figure 4.25). For *V-type* ATPase in roots, drought stress caused the reduction in *V-type* ATPase similar to what found in *P-type* ATPase activity. However, in 'LPT123' roots, chitosan showed no effects on *V-type* ATPase activity (Figure 4.26 A). On the contrary, in 'LPT123-TC171' chitosan application decreased the enzymatic activity before drought stress. As the drought stress caused the reduction of enzymatic activity, no difference was found between the chitosan treated and non-chitosan treated plants (Figure 4.26 B).

(A)



(B)

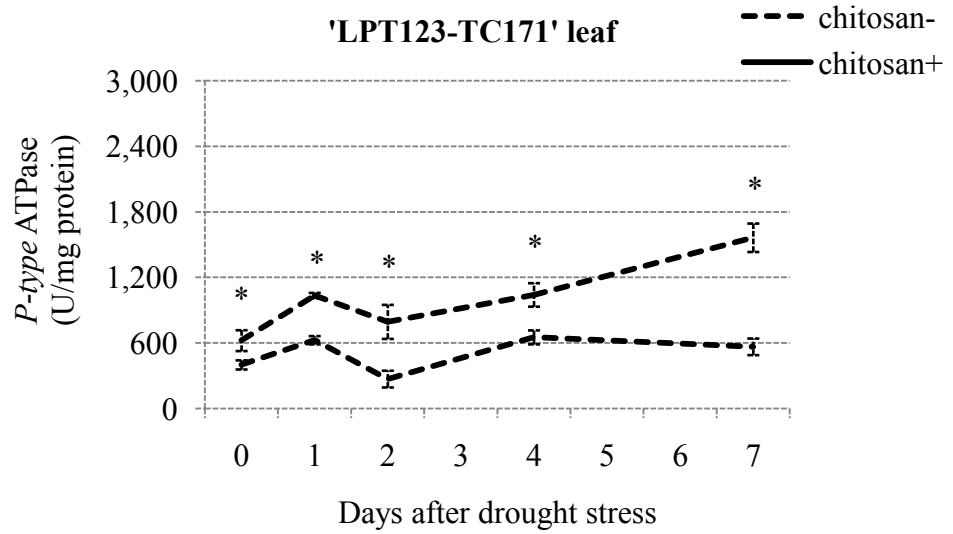


Figure 4.23 *P*-type ATPase activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123' (A) and 'LPT123-TC171' (B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

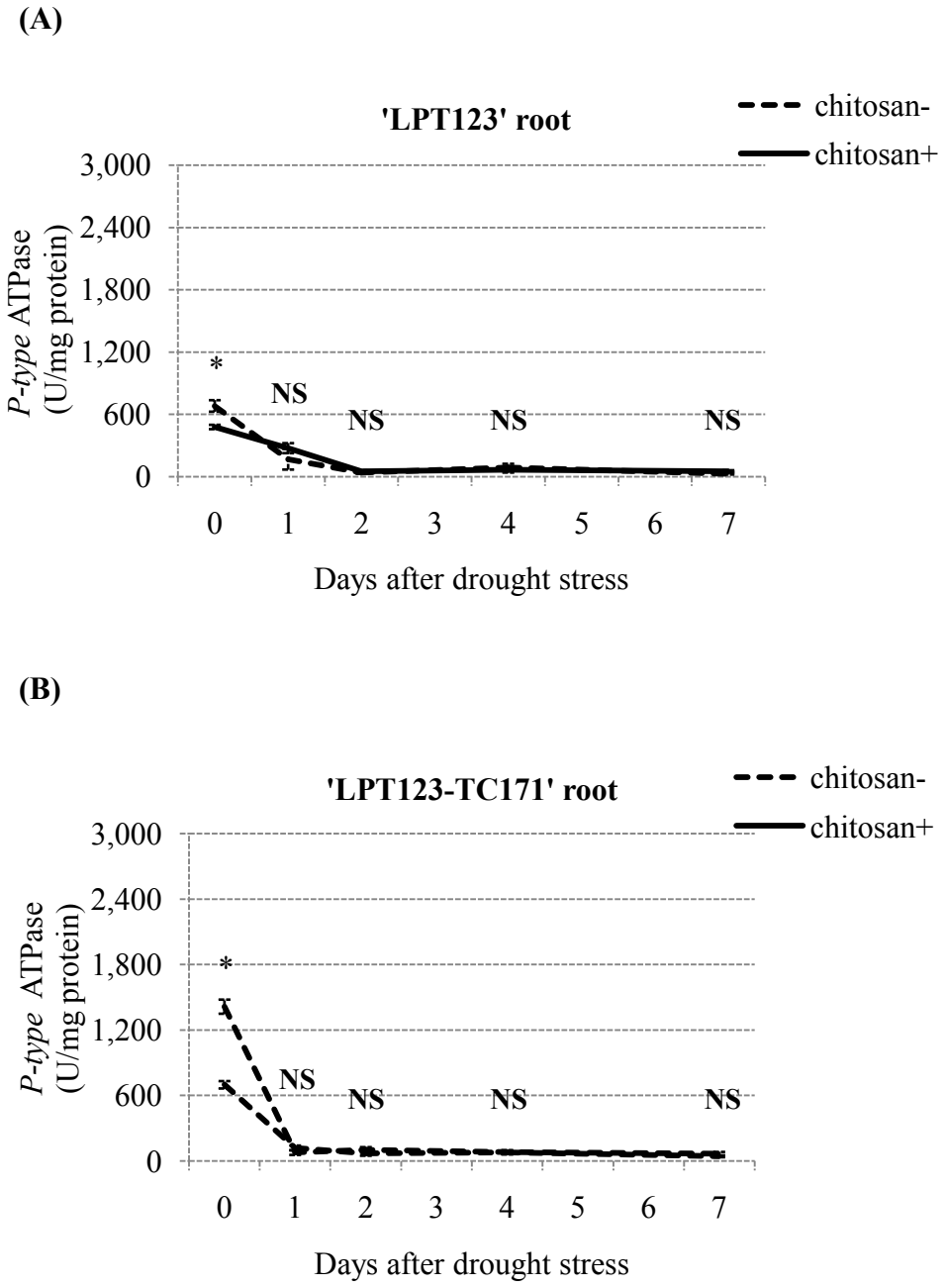


Figure 4.24 *P*-type ATPase activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123' (A) and 'LPT123-TC171' (B) roots. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

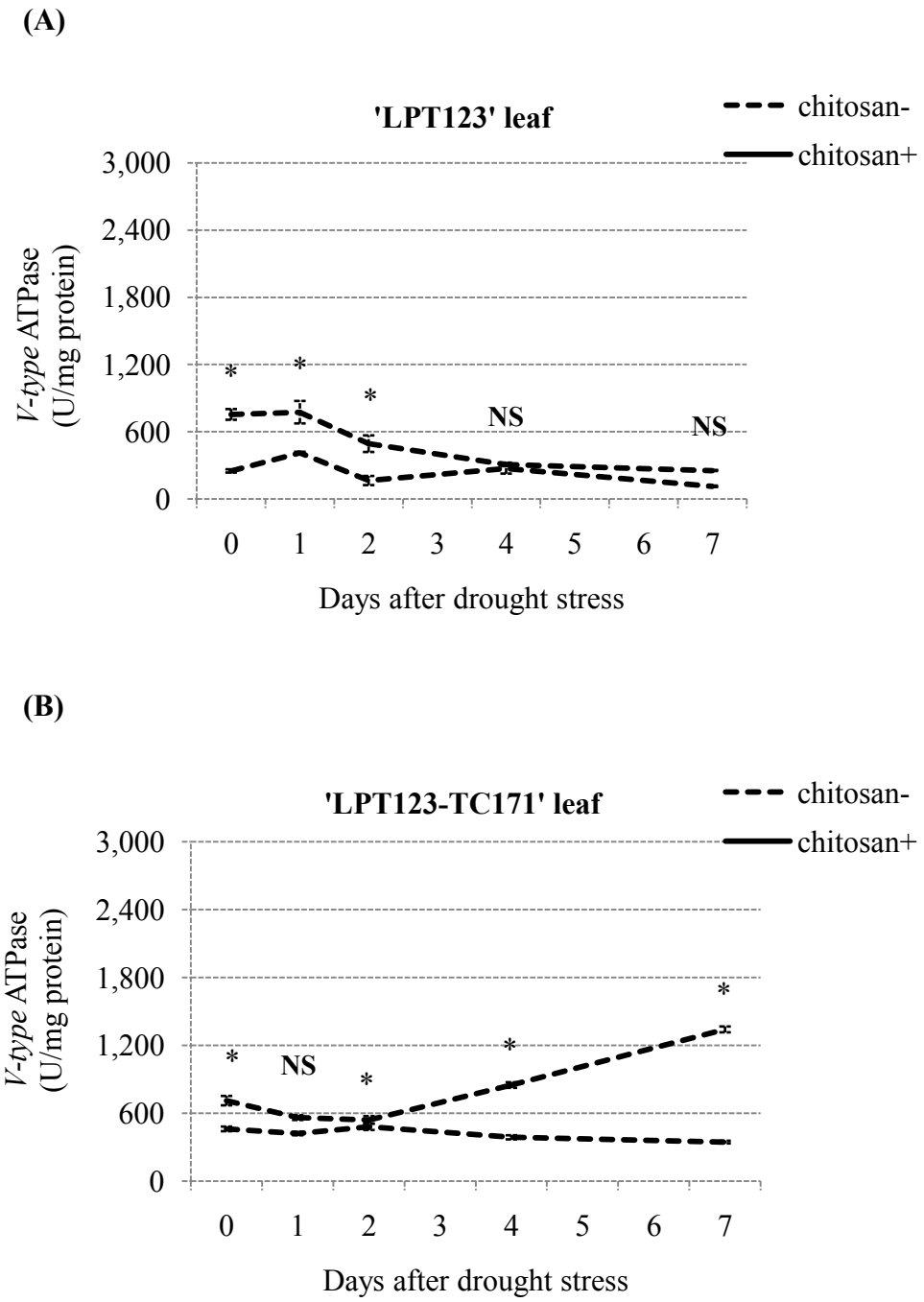


Figure 4.25 *V*-type ATPase activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123' (A) and 'LPT123-TC171' (B) leaves. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

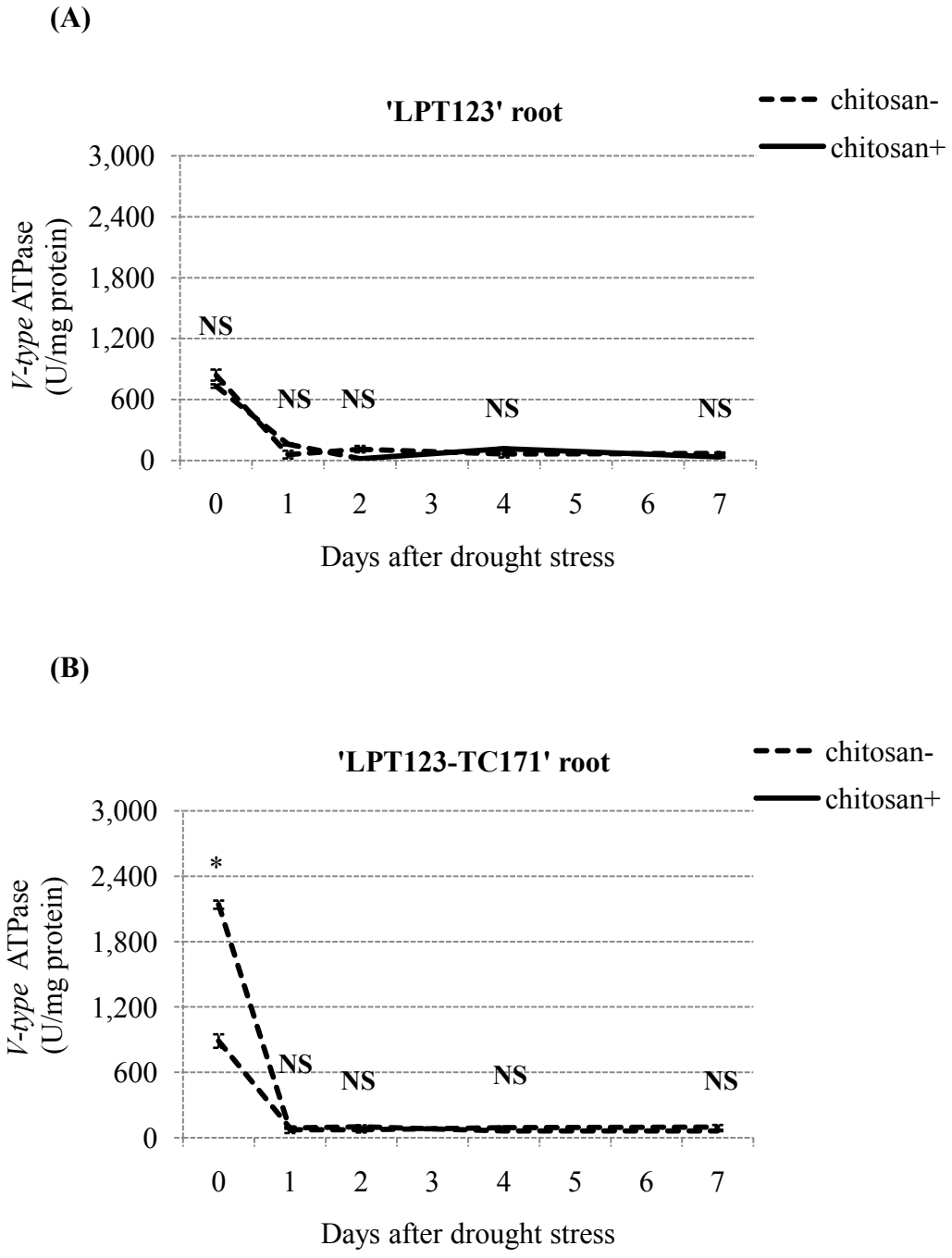


Figure 4.26 *V*-type ATPase activity subjected to drought stress for 1, 2, 4 or 7 days in 'LPT123' (A) and 'LPT123-TC171' (B) roots. Rice were treated (solid line) or non-treated (dash line) with chitosan. Error bar indicates S.E. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

5. Proteomic analysis of chitosan-responsive proteins in 'LPT123' and 'LPT123-TC171' rice lines

After four-week-old seedlings were subjected to oligomeric chitosan O-80 at 40 mg/L for 2, 6, 24, 48 or 96 h, total proteins were extracted and separated by one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE). The tryptic peptides from each gel plug were analyzed by LC-MS. The stress-sensitive ('LPT123') and stress-tolerant ('LPT123-TC171') genotypes revealed numerous constitutive and stress-induced differences in seedling proteome. By LC-MS analysis, 2768 and 1525 proteins were found in leaves and roots of both lines. However, only 11% in leaves and 9% in roots could be identified with known function or showed high homology with proteins existed in NCBI database. The total of 168 proteins in leaves of both rice lines were classified into 12 groups, including photosynthesis (1%), growth (1%), disease resistant protein (3%), metabolic process (13%), secondary metabolic process (2%), transcription (9%), cytoskeleton (1%), signal transduction (8%), transport (3%), translation (2%), transposon/retrotransposon (18%) , unknown/other (39%) (Figure 4.27 A). Furthermore, the 92 proteins in roots were divided into 8 groups, including metabolic process (14%), disease resistant protein (4%), signal transduction (9%), transcription (7%), transport (6%), photosynthesis (1%), transposon/retrotransposon (23%), unknown/other (36%) (Figure 4.27 B).

The overlapping of the identified proteins among leaves and roots was illustrated in Figure 4.28. Nine similar proteins were found in leaves and roots consisting of DNA-directed RNA polymerase III subunit RPC1 (LOC_Os04g41490), GDSL-like lipase/acylhydrolase (LOC_Os07g44780), phosphoribosyltransferase (LOC_Os07g30150), RGH1A (LOC_Os12g36720), transposon protein (LOC_Os06g24050), expressed protein (LOC_Os09g31458), retrotransposon protein (LOC_Os07g09990), abscisic stress-ripening (LOC_Os11g06720) and YT521-B (LOC_Os04g51940) (Figure 4.28). The lists of the similar proteins were shown in Table D.1.

5.1 Protein profiles in 'LPT123' rice line

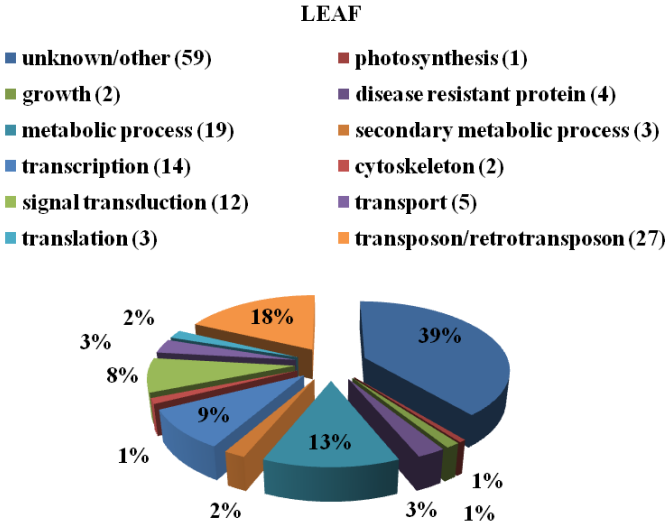
Among the total proteins, hierarchical clustering was performed to achieve the expression profile of the proteins that expressed at different time points in leaves and roots under drought stress with or without chitosan application representing as 123LD80, 123RD80, 123LD and 123RD, respectively. We used Student's *t*-test to select data sets from control and treatment groups with statistical significance ($p<0.05$). The data were taken in terms of fold expression with the unstressed expression value (0 h). Furthermore, the data sets were log-transformed to base 2 to level the scale of expression and to reduce the noise. According to the criteria, the 39 and 24 proteins were significantly differential expressed in leave and roots, respectively (Figure 4.29 A and B).

Within the significant protein expression in leaves, 20 proteins were found to be down regulated, whereas the other ones were 15 up-regulated proteins (Figure 4.30 A and B). The main functional properties of the different protein expression in leaves are given in Figure 4.31-4.32. Based on the available gene ontology (GO) annotations of the differentially expressed proteins, down-regulated proteins included signal transduction (5%; jasmonate O-methyltransferase), disease resistant protein (10%; CHIT15 - chitinase family protein precursor and disease resistance protein, RPM1), growth (5%; expansin precursor), retrotransposon protein (10%), other (35%) and unknown (35%) (Figure 4.31). While, the metabolic process (27%; enolase, DNA topoisomerase 3 protein, decarboxylase and aspartokinase, chloroplast precursor), signal transduction (13%, protein kinase family proteinandcyclin-dependent kinase G-2), transcription (13%, zinc finger, C_3HC_4 type domain containing protein and zinc finger C-x8-C-x5-C-x3-H type family protein), retrotransposon protein (27%) and unknown function (20%) were predominantly up-regulated (Figure 4.32).

Furthermore, within the significant protein expression in roots, 21 proteins were found to be down regulated, whereas the other ones showed 7 up-regulated proteins

after chitosan application (Figure 4.33). Twenty one down-regulated proteins in roots consisted of metabolic process (14%; peroxidase precursor, GDSL-like lipase/acylhydrolase and hhH-GPD superfamily base excision DNA repair protein), transport (10%; auxin efflux carrier and mitochondrial carrier protein), transcription factor (5%; KIP1), reprotransposon protein (14%), transposon protein (5%), other (24%) and unknown (29%) (Figure 4.34). While, four up-regulated protein were found including disease resistant protein (14%; powdery mildew resistance protein PM3b), signal transduction (14%; polygalacturonase inhibitor 2 precursor), retrotransposon protein (43%), transcription (14%; DNA-directed RNA polymerase III subunit RPC1) and other (14%) (Figure 4.35).

(A)



(B)

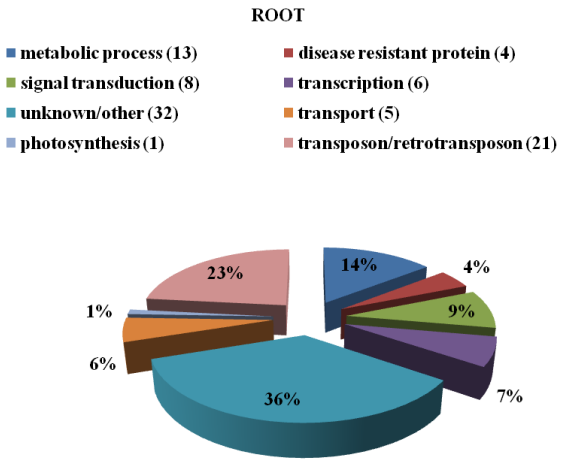


Figure 4.27 The functional categorization of the 168 and 92 proteins in 'LPT123' and 'LPT123-TC171' lines in leaves (A) and roots (B) during drought stress with or without chitosan application were performed through the rice genome annotation project database (http://rice.plantbiology.msu.edu/).

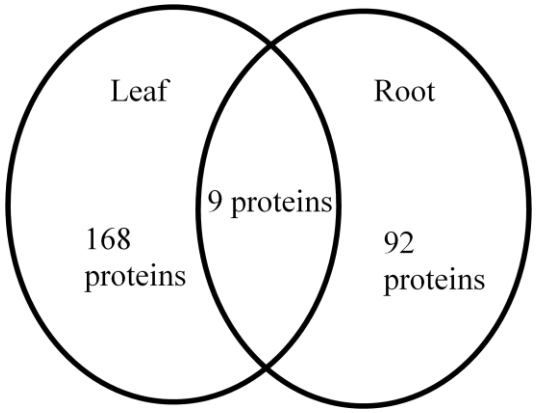
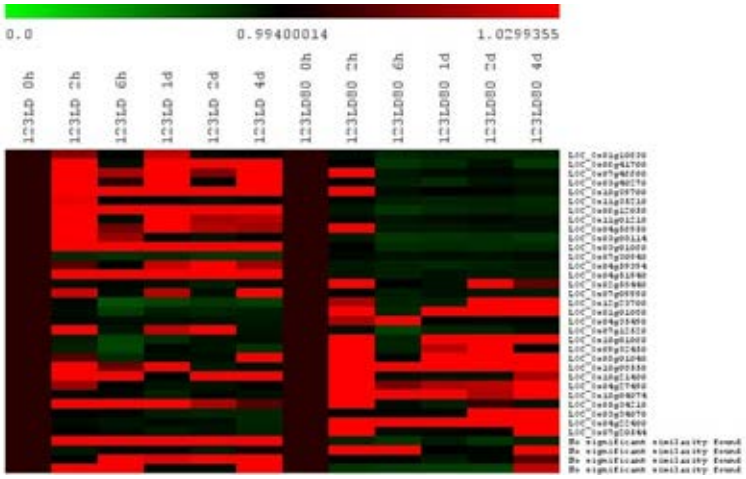


Figure 4.28 Number of proteins was identified in leaves and roots and the overlap of proteins between two groups. Nine similar proteins were LOC_Os04g41490, LOC_Os07g44780, LOC_Os07g30150, LOC_Os12g36720, LOC_Os06g24050, LOC_Os09g31458, LOC_Os07g09990, LOC_Os11g06720 and LOC_Os04g51940. Lists of all proteins were shown in Table D.1.

(A)



(B)

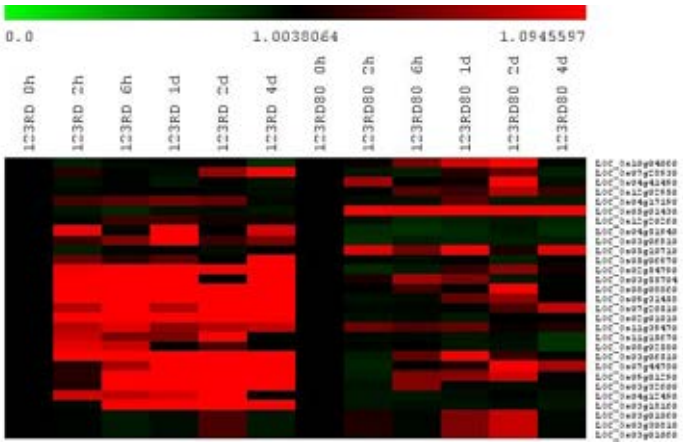
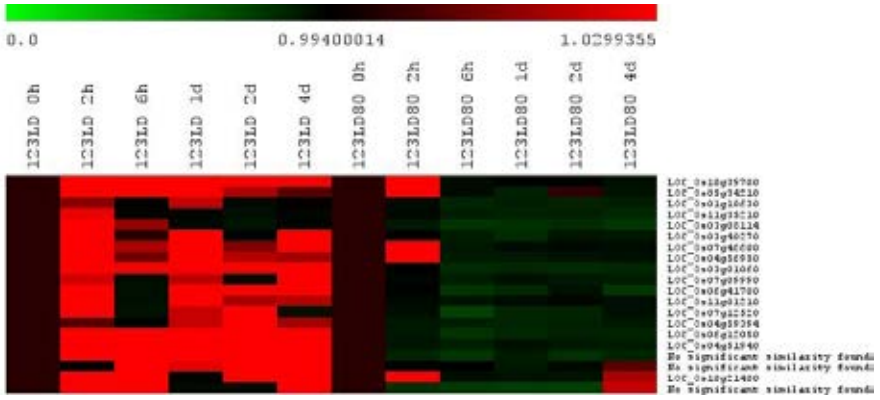


Figure 4.29 Significant expressed protein in leaves (A) and roots (B) during drought stress with or without chitosan in 'LPT123'. Controls; 123LD or 123 RD, treatments; 123LD80 or 123 RD80 in leaves and roots, respectively at 0 h, 2 h, 6 h, 1 d, 2 d or 4 d. The heat maps were generated using Multi experiment Viewer (MeV) software. The upper bar from pale green to red colors indicated low to high protein expression. Red is up-regulated; green is down- regulated; black is no change in protein expression.

(A)



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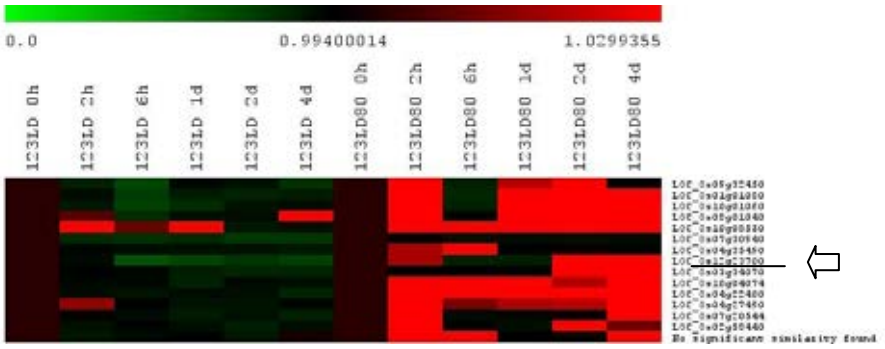


Figure 4.30 Hieratical clustering of expression profiles of drought-responsive proteins with or without chitosan application in 'LPT123' leaves. The significant differentially expressed proteins were divided into 2 groups; down- (A) and up- (B) regulated resulting from t-test analysis. The lists of all proteins were shown in Table D.2-D.3.

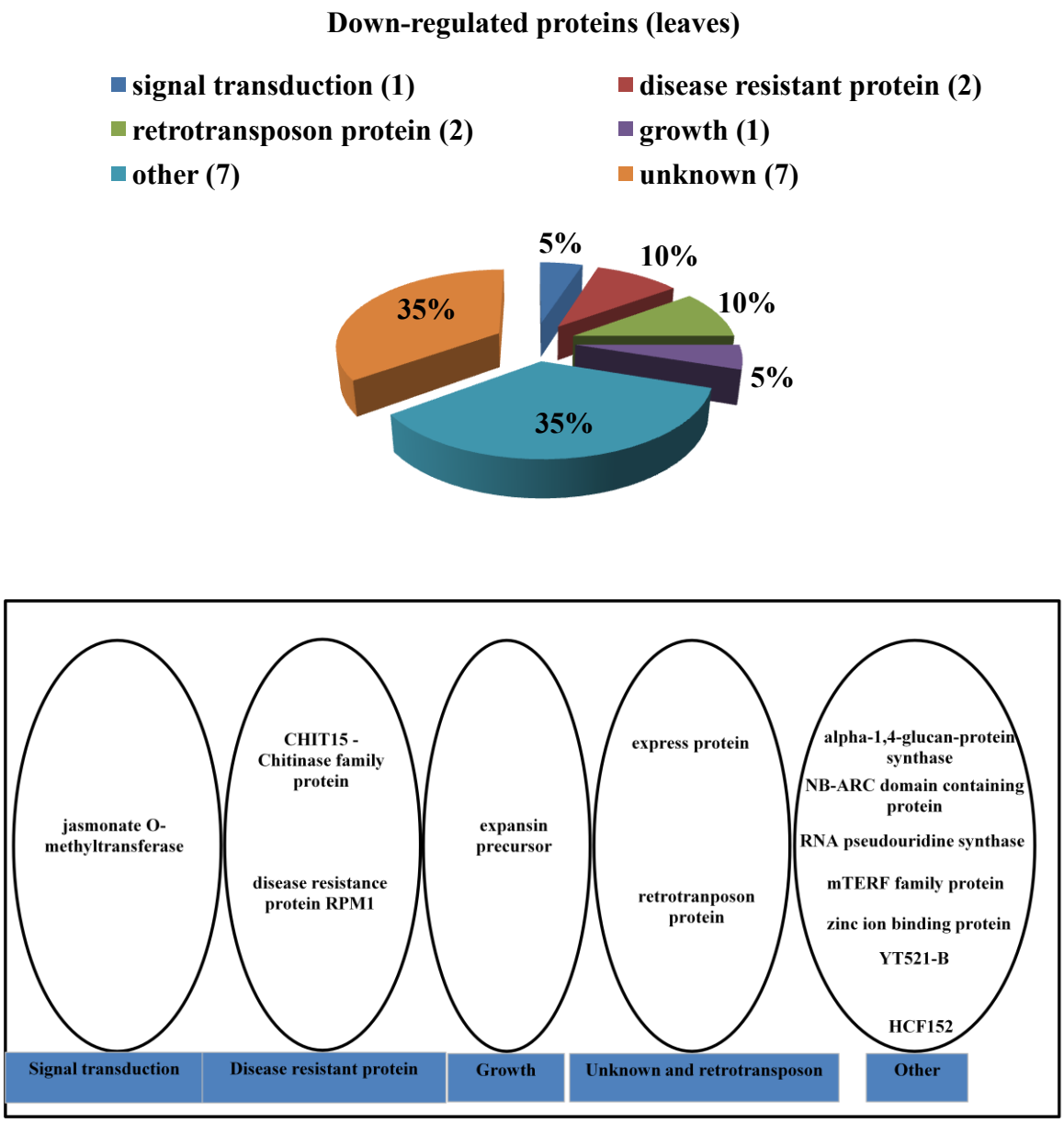


Figure 4.31 Functional clustering of the down-regulated proteins in 'LPT123' leaves were constructed through the rice genome annotation project database (http://rice.plantbiology.msu.edu/).

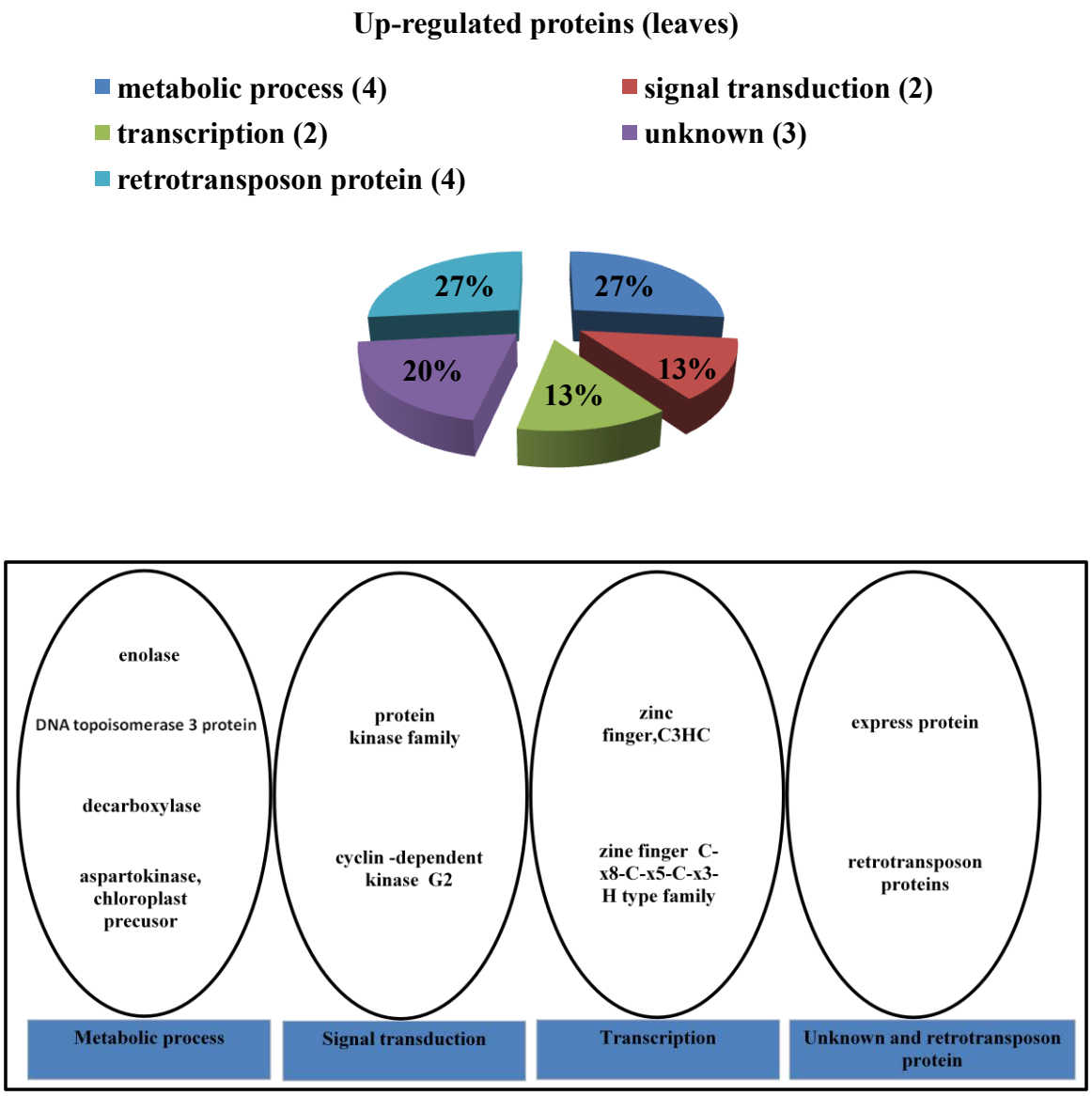
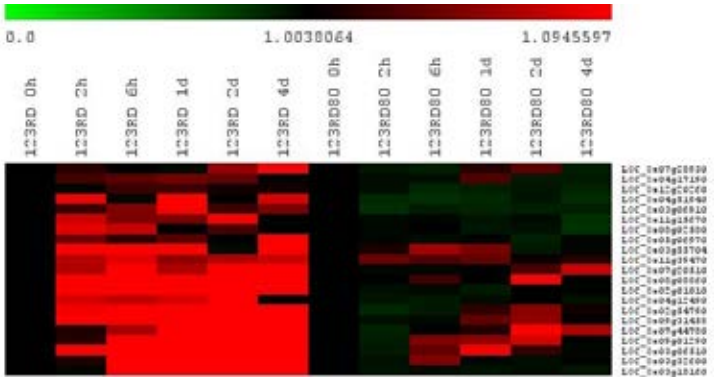


Figure 4.32 Functional clustering of the up-regulated proteins in 'LPT123' leaves were constructed through the rice genome annotation project database (http://rice.plantbiology.msu.edu/).

(A)



(B)

Figure 4.33 Hieratical clustering of expression profiles of drought-responsive proteins with or without chitosan application in 'LPT123' roots. The significant differentially expressed proteins were divided into 2 groups; down- (A) and up- (B) regulated from t-test analysis. The lists of all proteins were shown in Table D.4-D.5.

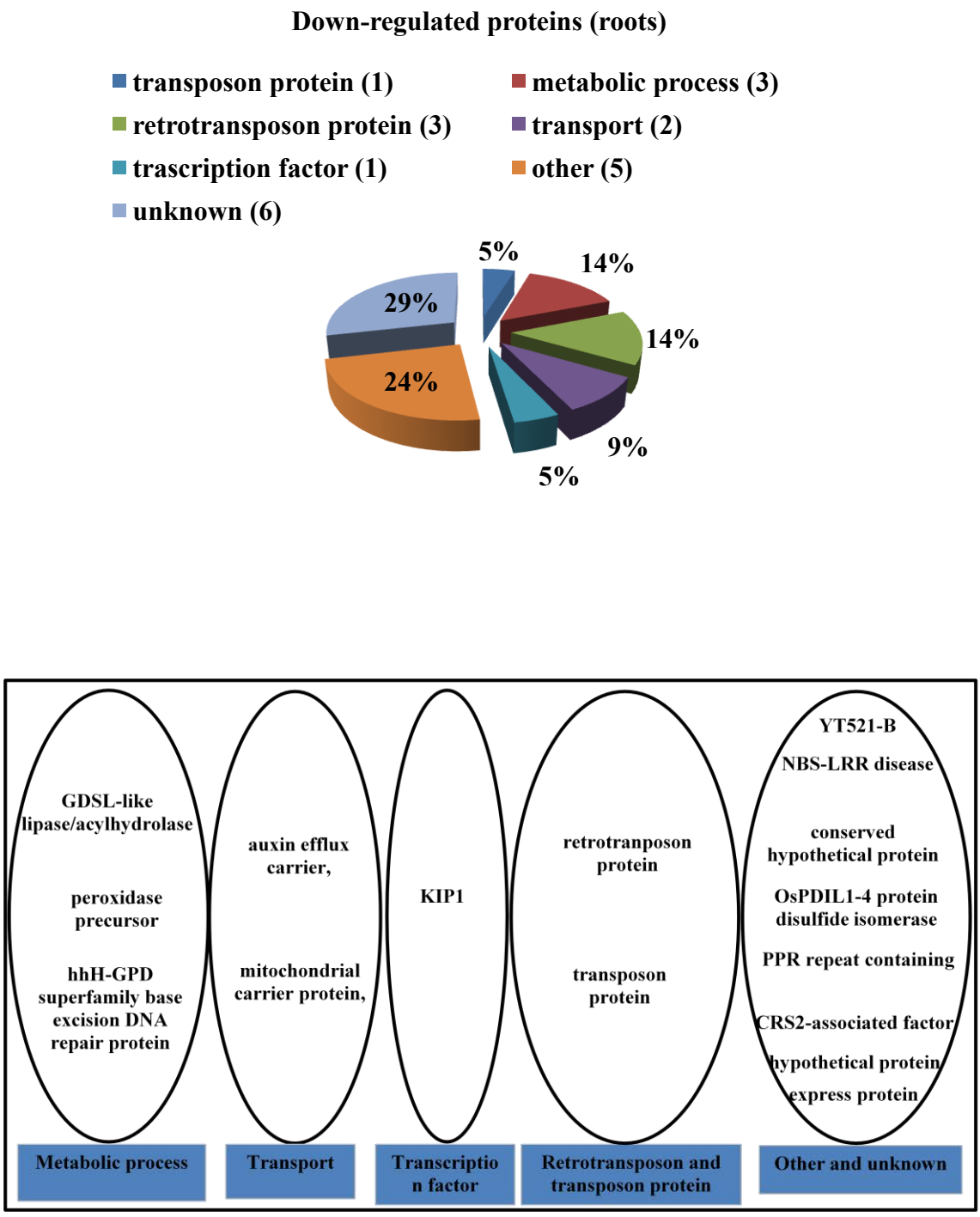


Figure 4.34 Functional clustering of the down-regulated proteins in 'LPT123' roots were constructed through the rice genome annotation project database (http://rice.plantbiology.msu.edu/).

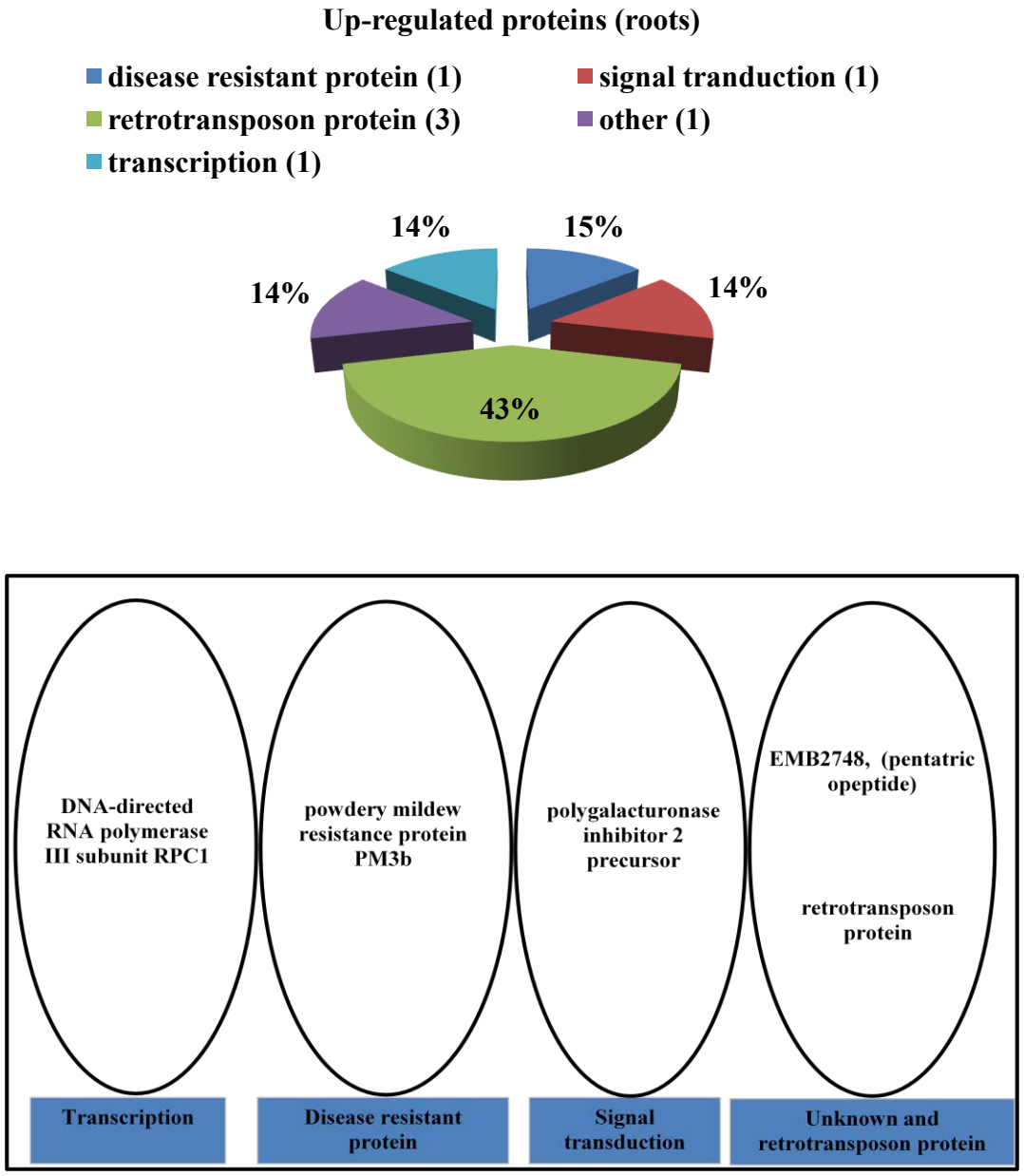


Figure 4.35 Functional clustering of the up-regulated proteins in 'LPT123' roots were constructed through the rice genome annotation project database (http://rice.plantbiology.msu.edu/).

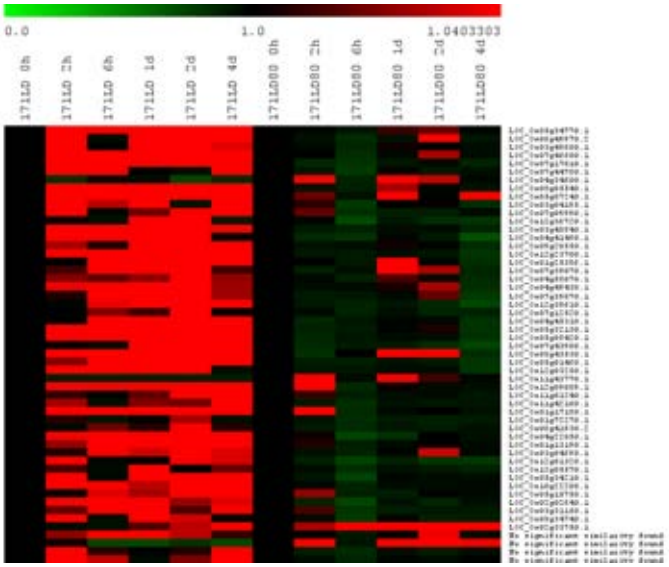
5.2 Protein profiles in 'LPT123-TC171' rice line

The number and mostly type of significantly expression proteins in 'LPT123-TC171' found differently from 'LPT123'. Among the total proteins, hierarchical clustering was performed to achieve the expression profile of the proteins that expressed at different time points in leaves and roots under drought stress treated with chitosan (171LD80 and 171RD80, respectively) or without chitosan (171LD and 171RD, respectively) application. The 53 proteins were significantly differentially expressed in leaves and, 20 proteins were differentially expressed in roots (Figure 4.36).

Within the significant protein expression in leaves, 49 proteins were down-regulated, the rest proteins were 4 up-regulated after chitosan application during drought stress (Figure 4.37 A and B). The main functional properties of the different protein expression in leaves are given in Figure 4.38-4.39. Based on the available gene ontology (GO) annotations of the differentially expressed proteins, down regulated-proteins were classified into 6 groups. First, transcription (14%) involved in OsFBX306 -F-box domain containing protein, zinc finger protein, cyclin-dependent kinase G-2, DNA-directed RNA polymerase III subunit RPC1, ribosomal L18p/L5e family protein, leucine rich repeat family, IBR domain containing protein and zinc-binding protein. Second, disease resistant proteins (5%) were multidrug resistance-associated protein and NBS-LRR disease resistance protein. Third, metabolic process (16%) included aspartic proteinase nepenthesin precursor, 5-formyltetrahydrofolate cyclo-ligase, cytochrome c, chalcone synthase, alpha-amylase precursor, cytochrome P450, GDSL-like lipase/acylhydrolase. Forth, protein degradation (5%) showed STIP1 homology and U box-containing protein 1, proteasome subunit. Fifth, signal transduction (6%) exhibited protein kinases, cyclin-dependent kinase G2. Last, 50% of retrotransposon/transposon and unknown functional proteins were mostly found in these groups (Figure 4.38). On the other hand, up-regulated proteins included transcription (25%; leucine rich repeat family protein), transposon (25%) and unknown (50%) proteins (Figure 4.39).

Furthermore, within the significant protein expression in roots, 12 proteins were found to be down regulated, whereas the other 8 proteins were up regulated after chitosan treatment (Figure 4.40 A and B) (List of proteins shown in Table D.8-D.9). Twelve down-regulated proteins in roots consisted of signal transduction (25%; polygalacturonase inhibitor 2 precursors, receptor kinase like protein), transport (17%; mitochondrial carrier protein) and retrotransposon (25%) including unknown/other (37%) proteins (Figure 4.41). While, 8 up-regulated protein expressions were found including metabolic process (25%; glucan endo-1, 3-*beta*-glucosidase precursor, lactose permease-related protein), defense (13%; stripe rust resistance protein Yr10) and unknown/other (62%) (Figure 4.42).

(A)



(B)

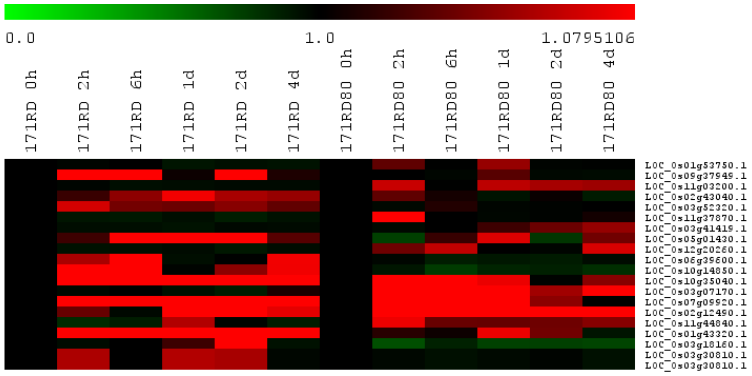
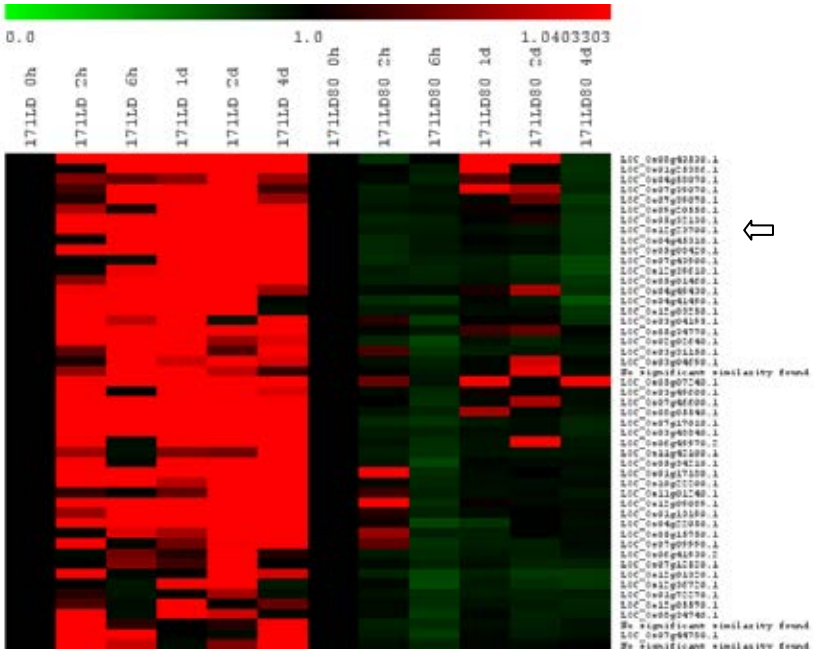


Figure 4.36 Significant expressed protein in leaves (A) and roots (B) during drought stress with or without chitosan in 'LPT123-TC171'. Controls; 171LD or 171RD, treatments; 171LD80 or 171RD80 in leaves and roots, respectively at 0 h, 2 h, 6 h, 1 d, 2 d or 4 d. The heat maps were generated using Multi experiment Viewer (MeV) software. The upper bar from pale green to red colors indicated low to high protein expression. Red is up-regulated; green is down-regulated; black is no change in protein expression.

(A)



(B)

Figure 4.37 Hieratical clustering of expression profiles of drought-responsive proteins with or without chitosan application in 'LPT123-TC171' leaves. The significant differentially expressed proteins were divided into 2 groups; down- (A) and up- (B) regulated from t-test analysis. The lists of all proteins were shown in Table D.6-D.7.

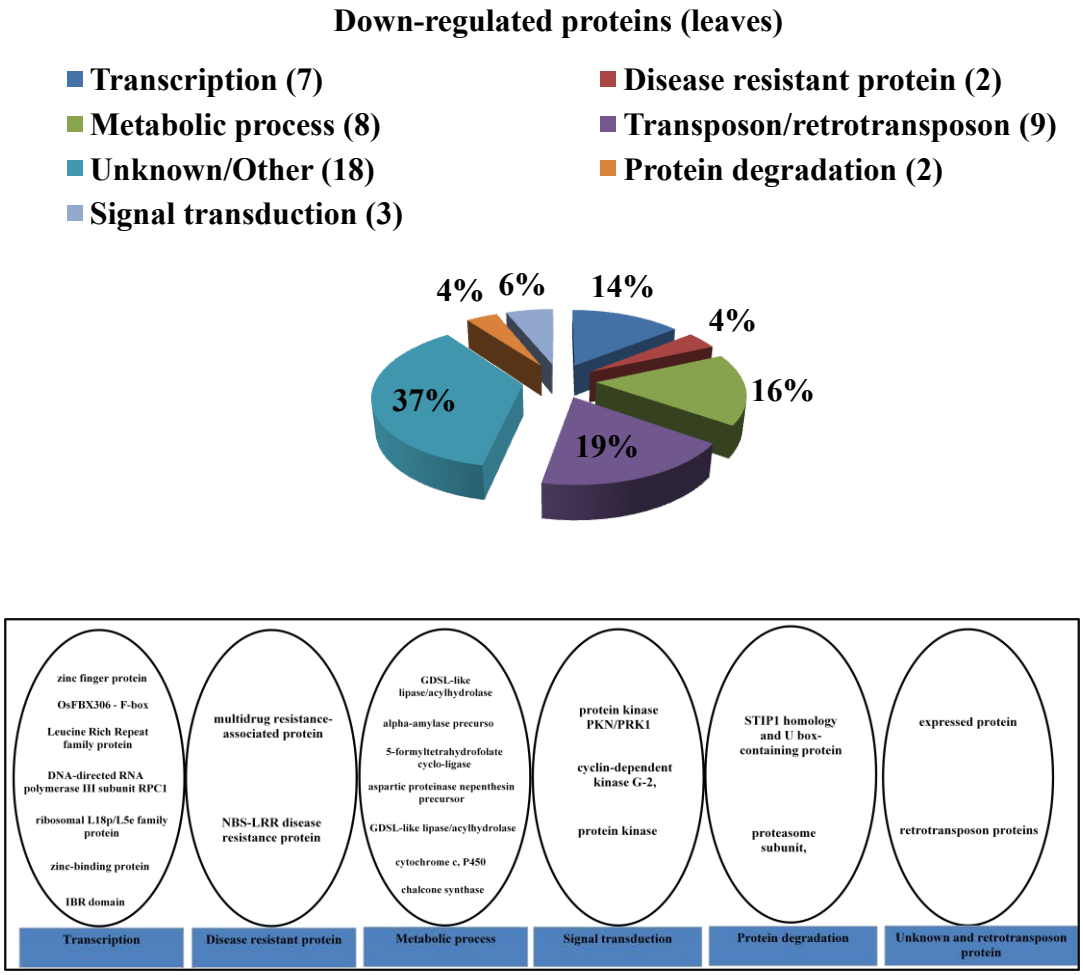


Figure 4.38 Functional clustering of the down-regulated proteins in 'LPT123-TC171' leaves were constructed through the rice genome annotation project database (http://rice.plantbiology.msu.edu/).

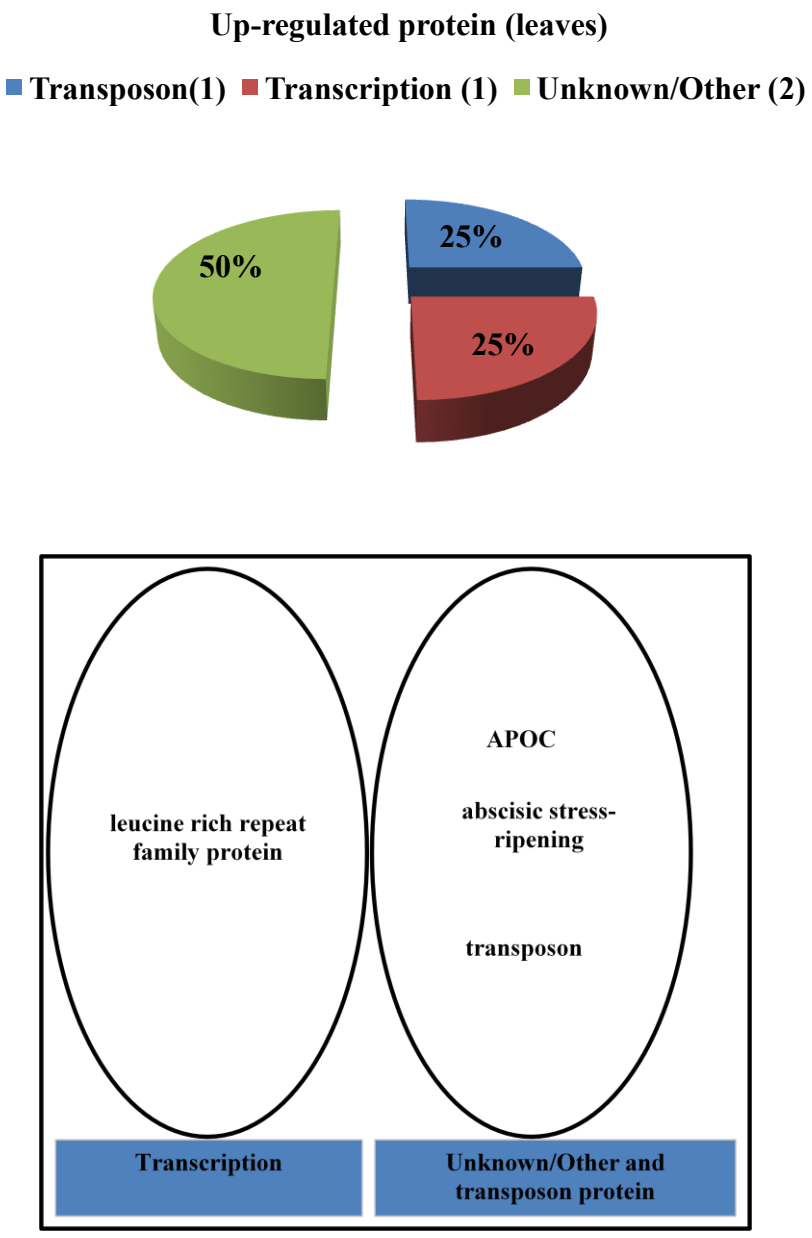
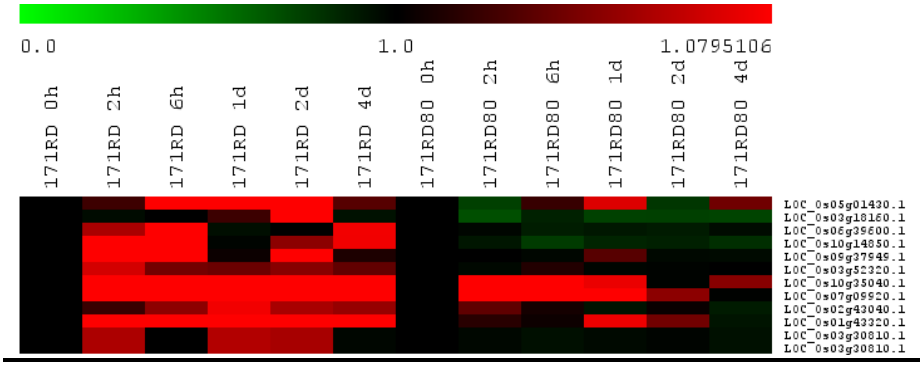


Figure 4.39 Functional clustering of the up-regulated proteins in 'LPT123-TC171' leaves were constructed through the rice genome annotation project database (http://rice.plantbiology.msu.edu/).

(A)



(B)

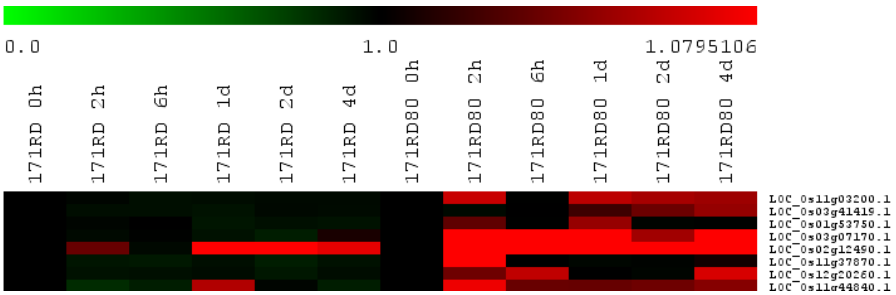


Figure 4.40 Hieratical clustering of expression profiles of drought-responsive proteins with or without chitosan application in 'LPT123-TC171' roots. The significant differentially expressed proteins were divided into 2 groups; down- (A) and up- (B) regulated from t-test analysis. The lists of all proteins were shown in Table D.8-D.9.

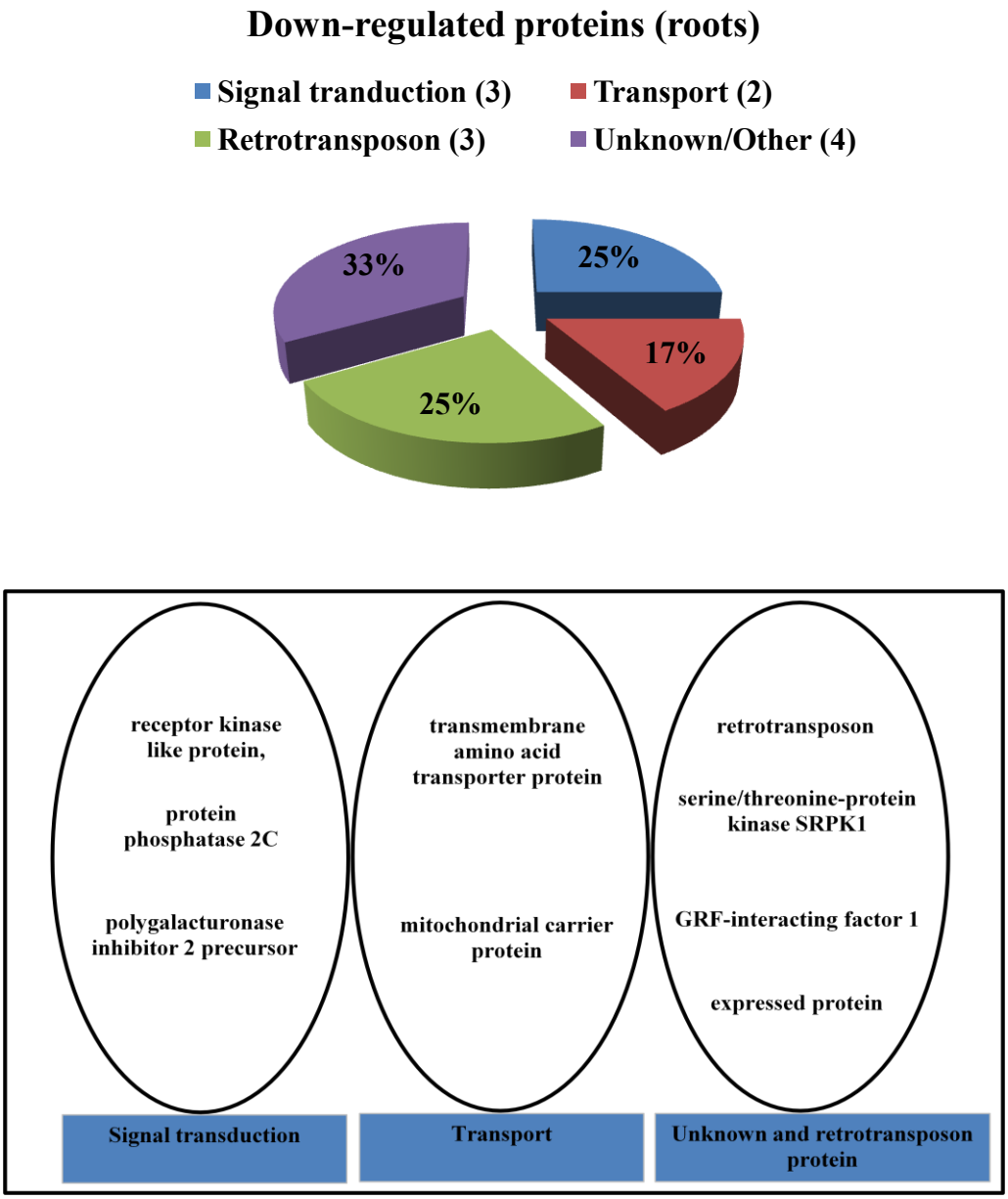


Figure 4.41 Functional clustering of the down-regulated proteins in 'LPT123-TC171' roots were constructed through the rice genome annotation project database (http://rice.plantbiology.msu.edu/).

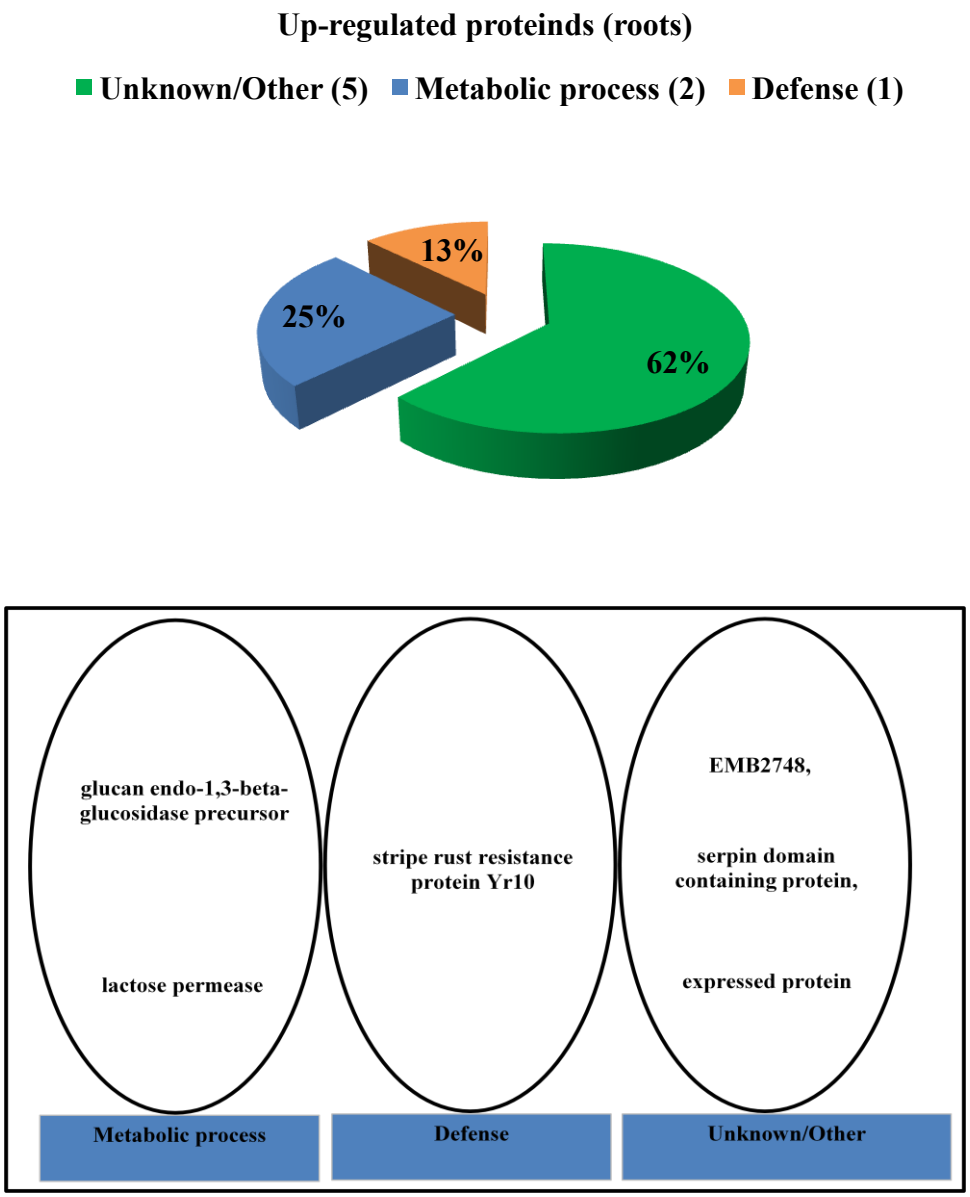


Figure 4.42 Functional clustering of the up-regulated proteins in roots were constructed through the rice genome annotation project database (http://rice.plantbiology.msu.edu/).

5.3 Similar 9 proteins found in ‘LPT123’ and ‘LPT123-TC171’

In leaves, five significant proteins were found in ‘LPT123’ and ‘LPT123-TC171’. Interestingly, the signal transduction cyclin-dependent kinase G-2 (gi|15128439) was found to be up-regulated protein in ‘LPT123’, whereas it was found to be down-regulated in ‘LPT123-TC171’. The rest proteins were down regulated in the both rice lines (gi|51038233, gi|115473865, gi|14718312 and gi|38345347). These proteins were retrotransposon or unknown proteins.

In contrast, in 2 root significant proteins (gi|50872417 and gi|115461607) were regulated in ‘LPT123-TC171’, contrastly, gi|77555031 protein was found to be up regulated in ‘LPT123-TC171’ after chitosan application. Moreover, the gi|115452405 protein expressed low level after chitosan application under drought stress condition in both lines. Interestingly, the gi|115461607 and gi|115452405 were signal transduction and transporter proteins, respectively (Table 4.1).

Table 4.1 The similar 9 proteins were found in both 'LPT123' and 'LPT123-TC171' lines

Protein	Gi number	'LPT123-TC171'	'LPT123'
-Leaves			
Os12g23700	gi|15128439	down	up
Os05g34210	gi|51038233	down	down
Os07g46600	gi|115473865	down	down
Os07g09990	gi|14718312	down	down
Os07g12520	gi|38345347	down	down
-Roots			
Os03g30810	gi|50872417	down	up
Os05g01430	gi|115461607	down	up
Os12g20260	gi|77555031	up	down
Os03g18160	gi|115452405	down	down

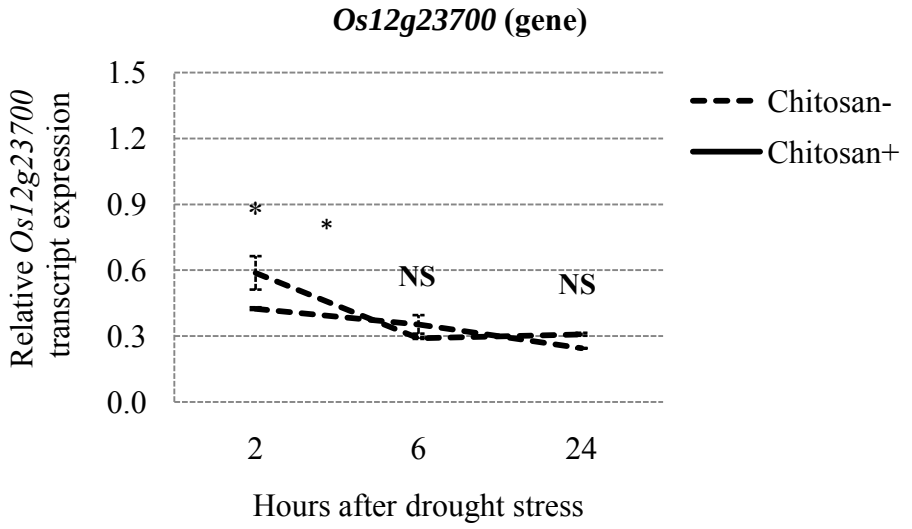
6. The expression of *Os12g23700* (gi|15128439) and *Os02g58440* (gi|46805523) which were up-regulated proteins (genes) in leaves

From rice database indicated that the Os12g23700 protein was cyclin-dependent kinase G-2 involving signal transduction protein. The Os12g23700 protein was found in both original 'LPT123' and mutant 'LPT123-TC171' lines. The Os12g23700 were found to be up-regulated in 'LPT123' (Figure 4.30 B), whereas down-regulated in 'LPT123-TC171' (Figure 4.37 A). The expression level of *Os12g23700* revealed the highest at 2 h after chitosan treatment under drought stress and then decreased at 6 h and 24 h. This gene expression level in chitosan spraying was higher than control, except at 6 h. When compare to control (absence of chitosan application), the expression level of Os12g23700 was unchanged during drought stress (Figure 4.43 A). On the other hand, Os12g23700 protein expression in chitosan treatment was higher than non-chitosan application (Figure 4.43 B). The cyclin-dependent kinase G-2 responded to chitosan in the early phase. From the result suggested that this signal

transduction gene may play a role in chitosan response in rice seedlings under drought stress in ‘LPT123’.

Moreover, zinc finger C-x8-C-x5-C-x3-H type (Os02g58440) was the up-regulated protein in the original ‘LPT123’ line involving transcription. The gene expression pattern of *Os02g58440* was quite similar to *Os12g23700* (Figure 4.42 A). Chitosan treatment induced the highest *Os02g58440* gene expression at 2 h and expressed higher than the control. When compared to non-chitosan application, *Os02g58440* gene did not change after chitosan application under drought stress (Figure 4.44 A). Furthermore, the Os02g58440 protein expression in chitosan application was higher than those controls under drought stress (Figure 4.44 B). Both gene and protein predominantly expressed at the early phase. The result suggested that transcriptional gene may play a role in chitosan response in rice seedlings during drought stress in the unique original ‘LPT123’ rice.

(A)



(B)

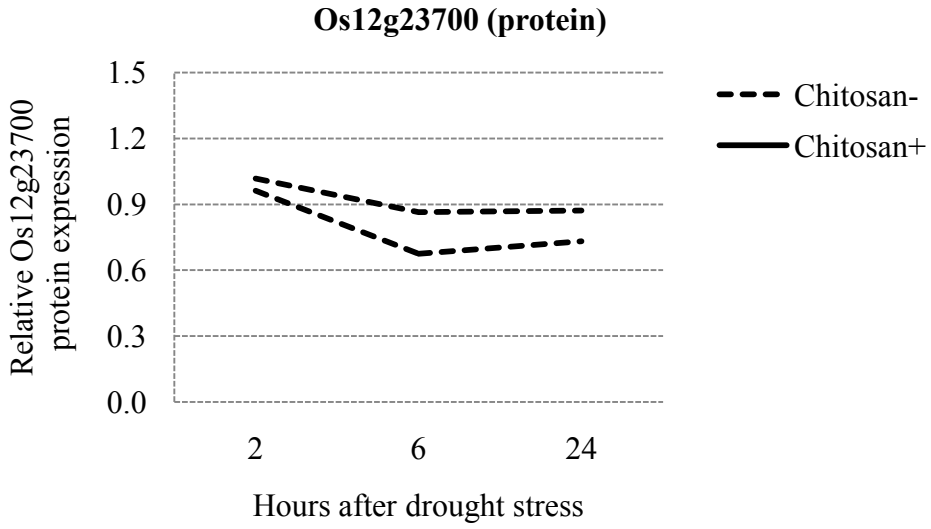
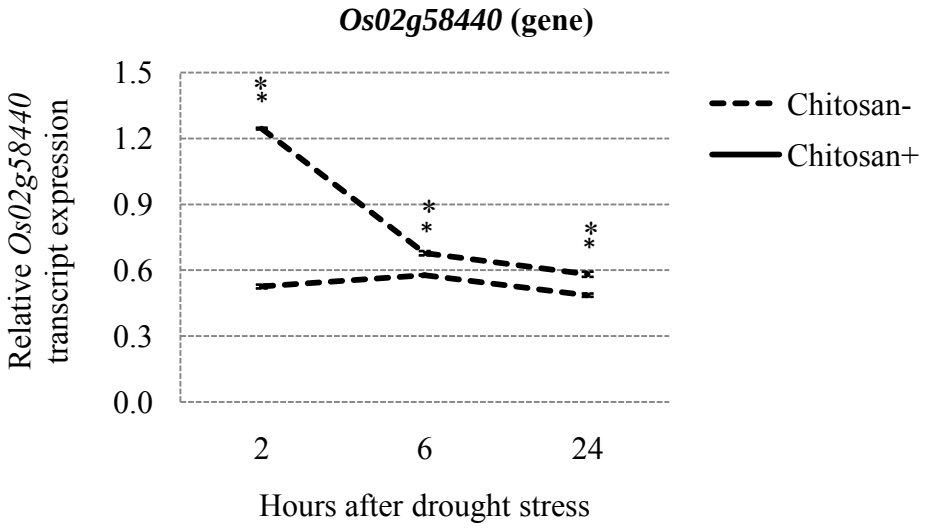


Figure 4.43 The relative *Os12g23700* gene expression (A) and the Os12g23700 protein expression (B) of up-regulated gene/protein in 'LPT123' leaves at 2, 6 or 24 h during drought stress. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

(A)



(B)

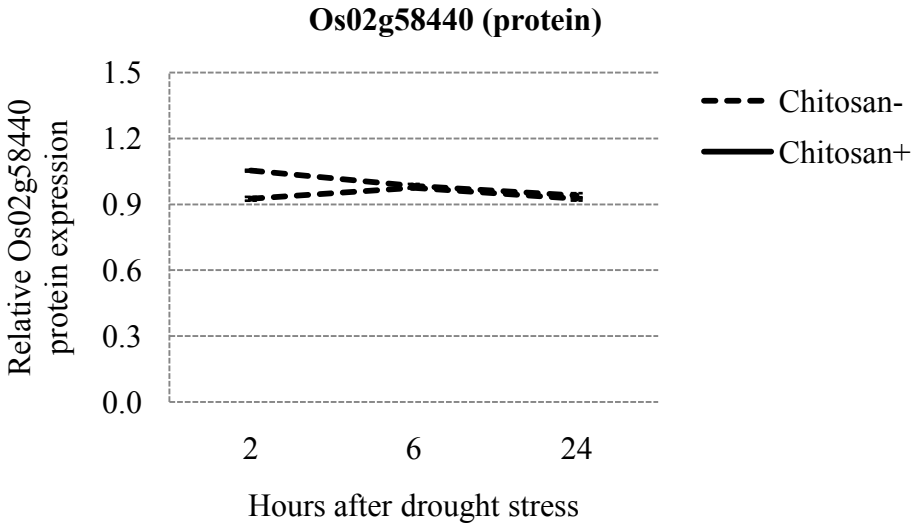


Figure 4.44 The relative *Os02g58440* gene expression (A) and the Os02g58440 protein expression (B) of up-regulated gene/protein in 'LPT123' leaves at 2, 6 and 24 h during drought stress. Bar is a standard error. Stars (*) represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non significantly different of means at $p<0.05$.

7. Targeting Induced Local Lesions IN Genomes (TILLING)

7.1 Selection the chitosan-responsive proteins for TILLING approach

The five proteins were selected for TILLING study consisting of Os01g42260, Os05g40220, Os05g33890, Os12g42760 and Os04g50204 proteins. These chitosan-responsive proteins expressed with different pattern under drought stress in 'LPT123' and 'LPT123-TC171' leaves. The protein intensity values were plotted in terms of fold expression with the unstressed expression value (0 h) at each time point after drought stress.

Os01g42260 protein was found in 'LPT123', but without the detectable expression in 'LPT123-TC171' (Figure 4.45). Without chitosan application, showed Os01g42260 protein expression level was increased after 6 h of drought stress in 'LPT123', while no relative expression was detected in 'LPT123-TC171' after 6 h of drought stress (Figure 4.45).

Os05g40220 protein level was significant changed due to the chitosan expression in 'LPT123-TC171', but it was decreased in 'LPT123' after 24 h of drought stress, if the plants were treated with chitosan (Figure 4.46).

Os05g33890 expression pattern was quite similar in both 'LPT123' and 'LPT123-TC171' rice and chitosan did not affect the level of protein expression during drought stress in both rice lines (Figure 4.47).

Os12g42760 was the down-regulated protein during drought stress in 'LPT123', but it was up-regulated in 'LPT123-TC171' when no chitosan was applied. However, with chitosan application even low level of protein was found in both rice lines (Figure 4.48).

Without chitosan, Os04g50204 protein was detected at the early phase of drought stress in 'LPT123', but reduction of this protein was found in 'LPT123-TC171'. With chitosan treatment, the response in 'LPT123' was similar to the in non-chitosan treatment. On the contrary, chitosan increase Os04g50204 protein level in 'LPT123-TC171' after 48 h of drought stress (Figure 4.49).

The above five genes were selected for TILLING as the representative of the gene that responded differently in 'LPT123' and 'LPT123-TC171' under drought stress with/without chitosan application

7.2 Comparing the expression pattern of three chitosan-responsive proteins/genes by qPCR

Gene expression at transcription level of chitosan-responsive proteins/genes selected for TILLING study were performed in 'LPT123' and 'LPT123-TC171' lines. The similar level of the transcript *Os01g42260*, during drought stress was found in 'LPT123' with or without chitosan response (Figure 4.50 B). This was quite different from the pattern obtained by proteomics approach (Figure 4.50 A). In 'LPT123-TC171', chitosan treatment lowered the transcript level after 24 h of drought stress. The lower level of this gene at transcriptional level in 'LPT123-TC171' was found (Figure 4.51 B), which was similar to the expression pattern generated from proteomics data (Figure 4.51 A).

For *Os05g40220*, when it was detected by qPCR, it had lower expression level in 'LPT123' with/without chitosan (Figure 4.52 B). Although, slightly higher level of this gene expression was found in 'LPT123-TC171' without chitosan treatment, when the plants treated with chitosan, the expression was decreased (Figure 4.53 B).

For the last regulated gene, *Os04g50204*, it was found that this gene was up-regulated by drought stress in ‘LPT123’, but not in ‘LPT123-TC171’ when plants were not treated with chitosan, but after chitosan application, the expression of this gene was lowered in ‘LPT123’ and higher in ‘LPT123-TC171’ (Figure 4.54-4.55)

(A) **(B)**

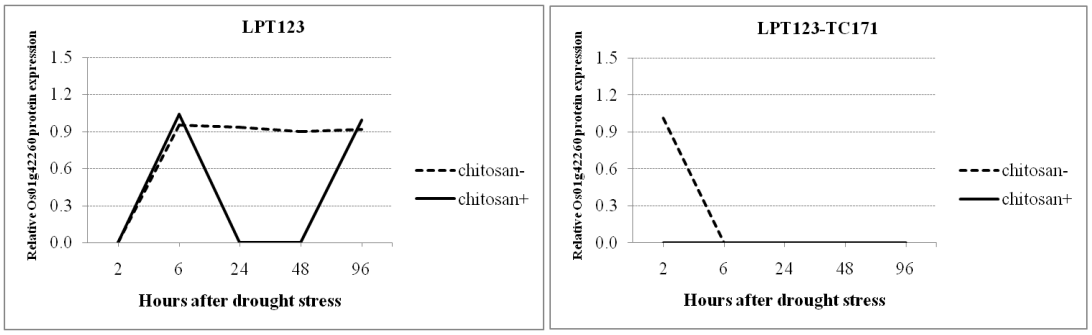


Figure 4.45 Os01g42260 protein during drought stress in 'LPT123'(A) and 'LPT123-TC171'(B).

(A) **(B)**

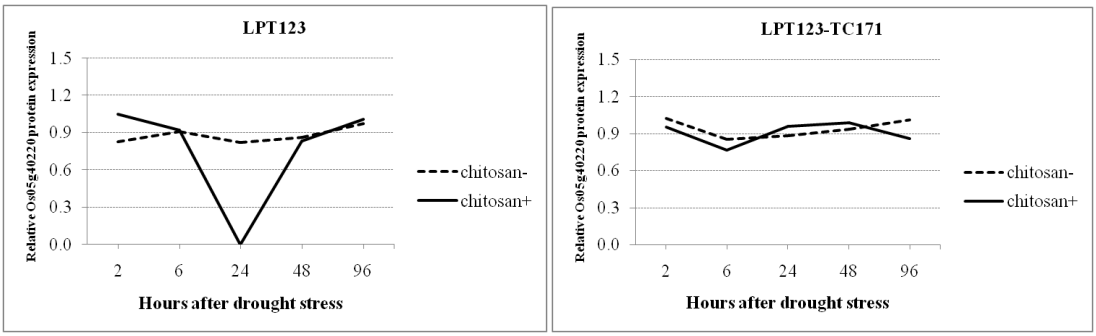


Figure 4.46 Os05g40220 protein during drought stress in 'LPT123'(A) and 'LPT123-TC171'(B).

(A) **(B)**

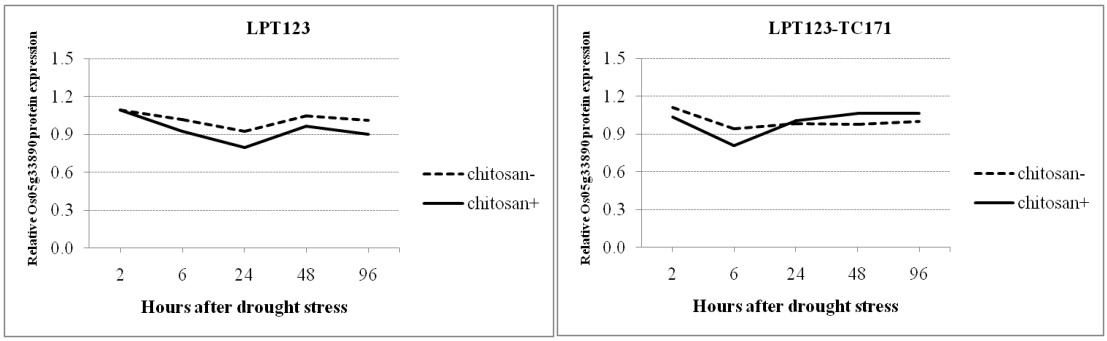


Figure 4.47 Os05g33890 protein during drought stress in 'LPT123'(A) and 'LPT123-TC171'(B).

(A) **(B)**

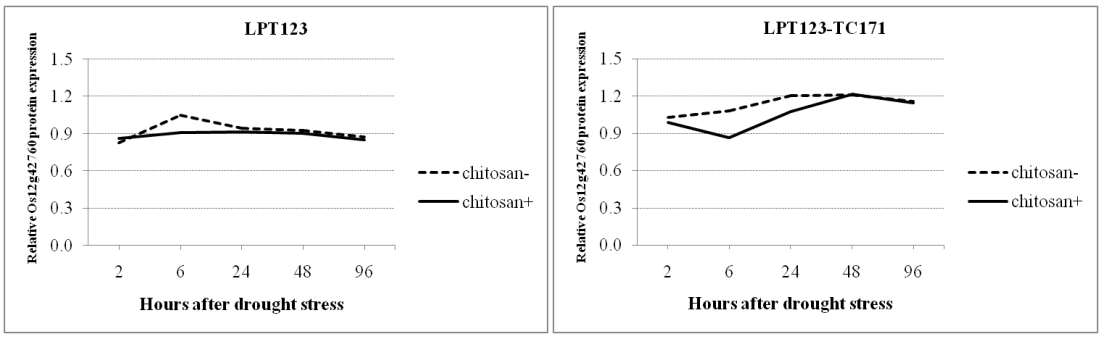


Figure 4.48 Os12g42760 protein during drought stress in 'LPT123'(A) and 'LPT123-TC171'(B).

(A) **(B)**

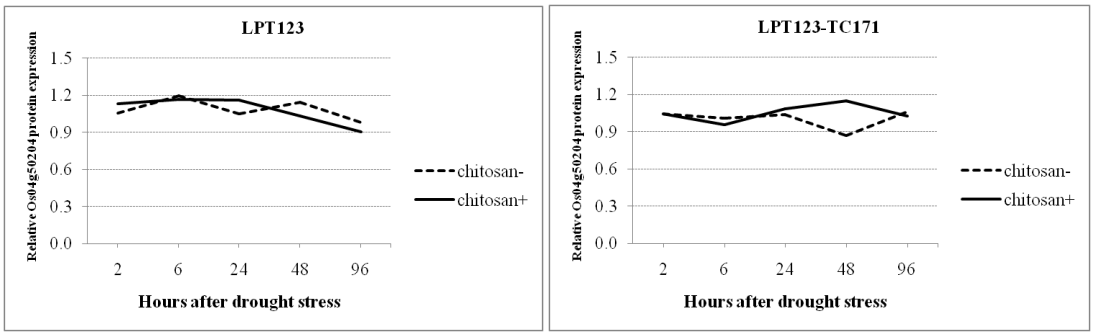


Figure 4.49 Os04g50204 protein during drought stress in 'LPT123'(A) and 'LPT123-TC171'(B).

(A) **(B)**

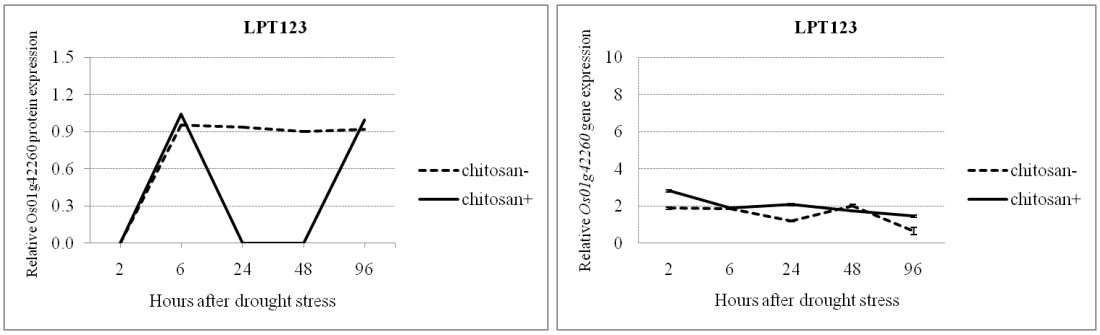


Figure 4.50 The relative Os01g42260 protein expression (A) and *Os01g2260* gene expression (B) in 'LPT123' leaves at 2, 6, 24, 48 and 96 h during drought stress. Bar is a standard error.

(A) **(B)**

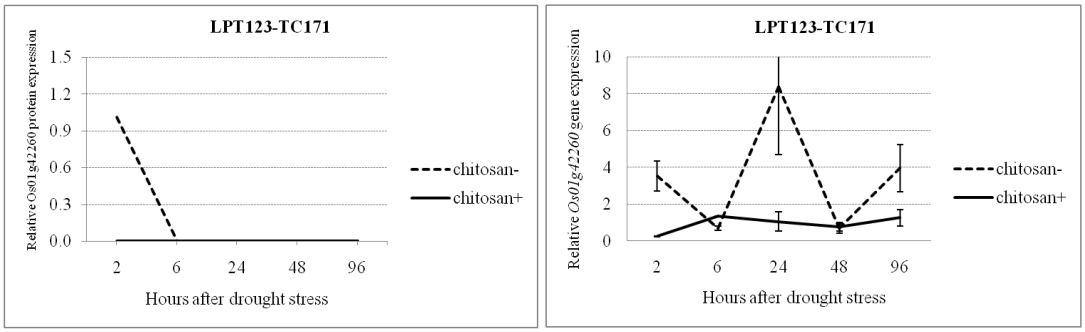


Figure 4.51 The relative Os01g42260 chitosan-responsive protein (A) and *Os01g42260* chitosan-responsive gene (B) in 'LPT123-TC171' leaves at 2, 6, 24, 48 and 96 h during drought stress. Bar is a standard error.

(A) **(B)**

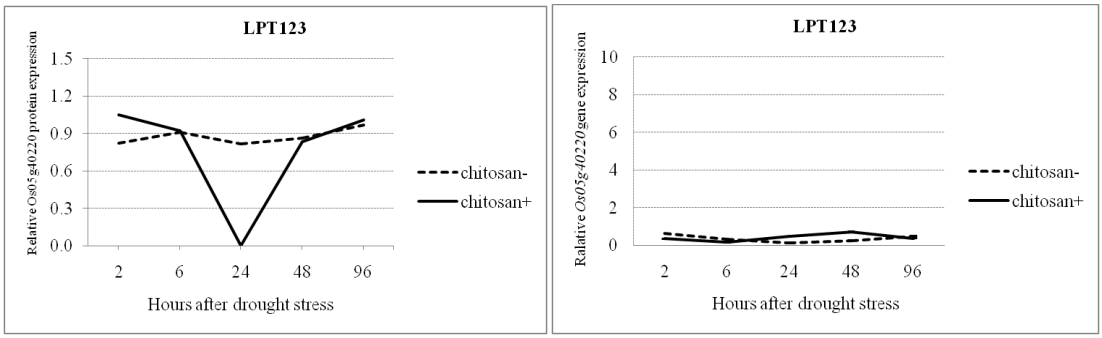


Figure 4.52 The relative Os05g40220 chitosan-responsive protein (A) and *Os05g40220* chitosan-responsive gene (B) in 'LPT123' leaves at 2, 6, 24, 48 and 96 h during drought stress. Bar is a standard error.

(A) **(B)**

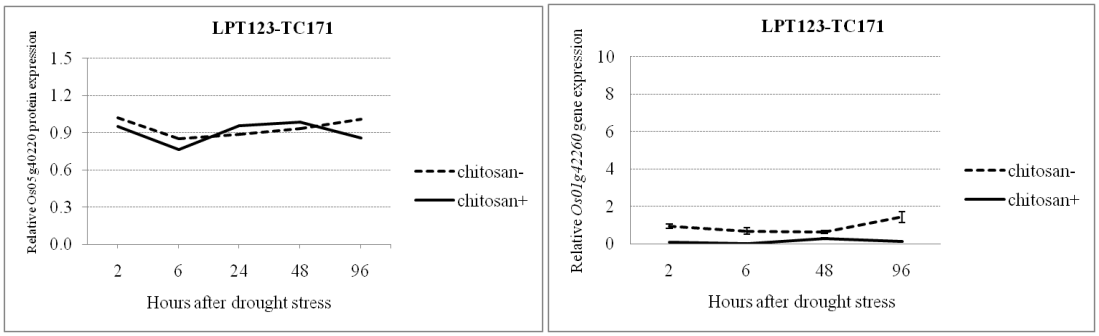


Figure 4.53 The relative Os05g40220 chitosan-responsive protein (A) and *Os05g40220* chitosan-responsive gene (B) in 'LPT123-TC171' leaves at 2, 6, 24, 48 and 96 h during drought stress. Bar is a standard error.

(A) **(B)**

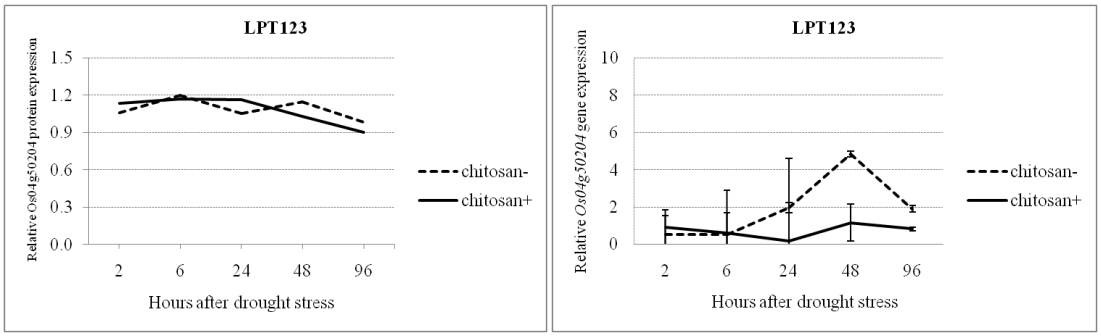


Figure 4.54 The relative Os04g50204 chitosan-responsive protein (A) and *Os04g50204* chitosan-responsive gene (B) in 'LPT123' leaves at 2, 6, 24, 48 and 96 h during drought stress. Bar is a standard error.

(A) **(B)**

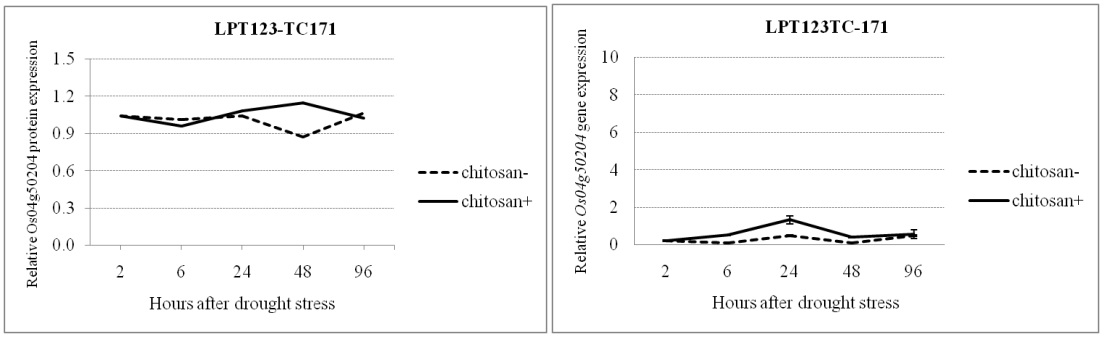


Figure 4.55 The relative Os04g50204 chitosan-responsive protein (A) and *Os04g50204* chitosan-responsive gene (B) in 'LPT123-TC171' leaves at 2, 6, 24, 48 and 96 h during drought stress. Bar is a standard error.

7.3 Nucleotide sequences variation (changes) of 5 genes by TILLING study

The process of TILLING was started when protein sequences were aligned to obtain the genomic sequence encoding the selected proteins. Five target genes consisted of *Os01g42260*, *Os05g40220*, *Os05g33890*, *Os12g42760* and *Os04g50204* were selected from chitosan-responsive proteins in 'LPT123' and 'LPT123-TC171' lines (Figure 4.46-4.50).

Sixteen mutation candidates were identified in *Os01g42260* which showed the severe mutated changing from T to C or G to A on 1350 and 440 TILLed regions, respectively. These mutations caused the possibly-severe mutation (PSM). Other mutations were found to be nonsense mutations (nonsense and silent mutations) (Table 4.2).

Os05g40220 target gene was mutated showing the silent, splicing mutation and PSM including 13 mutation candidates. The severe-mutated changing from A to G at 171 on TILLed fragment was considered to be the splicing mutation (Table 4.3).

Os05g33890 mutated gene was observed that the base change from G to A at 657 regions caused the splicing mutation. Thirteen mutations consisted of silent, intron and non- possibly severe mutations (NSM) (Table 4.4).

Os12g42760 gene, the changing from C to T at 1142 position causing caused the possibly-severe mutation (PSM), while the changing of base from C to T at 913 on TILL fragments caused truncation mutation. Eight candidate mutations consisted of PSM, NSM, silent and truncation mutations (Table 4.5).

Finally, sixteen candidate mutations were found in *Os04g50204* target gene, the changing of A to G and C to T at 583 and 1255 positions caused possibly-

severe mutations. Other mutations involved in NSM and silent mutations (Figure 4.6).

The 66 nucleotide changes were identified in the Az-MNU-treated 'Nipponbare' population. Nucleotide changes were classified into 4 groups; silent and non-severe mutations (NSM) (51.5%), possibly-severe mutation (PSM) and truncation mutation (19.7%), splicing mutation (3.0%) and mutation within intron (25.8%). The main of these mutants were transitions consisting of G to A (34.8%) or A to G (7.6%) and C to T (36.3%) or T to C (15.2%) changes. Moreover, the mutations were identified as transversions changing from A to T (3.1%), T to A (1.5%) and C to A (1.5%). Furthermore, fifty-three nonsense or splice site changes caused truncation and 15 missense mutations were found, eight nucleotide changes affected the damage of protein function are highlight in bold (Table 4.2-4.6). These severe mutations of genes on the TILLed regions were selected to place an order the M3 seeds for genotyping or phenotyping studies in further work.

Table 4.2 Mutations discovered in *Os01g42260* genes on 'Nipponbare' population mutagenized with Az-MNU

Gene	**Nucleotide Change**	**Amplicon Position**	**Effect** [a]	**Type**
Os01g42260	C->T	1337	intron	intron
	C->T	697	intron	intron
	T->C	1274	intron	intron
	G->A	1016	intron	intron
	T->C	**1350**	**I232T**	**PSM**
	G->A	384	Q131=	silent
	A->T	626	intron	intron
	G->A	954	intron	intron
	G->A	762	L220=	silent
	G->A	498	R169=	silent
	C->T	654	intron	intron
	C->T	1066	intron	intron
	G->A	390	Q133=	silent
	G->A	**440**	**G150D**	**PSM**
	C->T	960	intron	intron
	G->A	85	intron	intron

[a] Synonymous (=). Predicted damaging mutations are highlighted in bold. PSM is a possibly-severe mutation.

Table 4.3 Mutations discovered in *Os05g40220* genes on 'Nipponbare' population mutagenized with Az-MNU

Gene	**Nucleotide Change**	**Amplicon Position**	**Effect** [a]	**Type**
Os05g40220	T->A	1269	V423D	PSM
	C->T	117	intron	intron
	T->C	138	intron	intron
	C->T	1154	P385S	PSM
	C->T	429	T143I	PSM
	C->T	1158	A386V	NSM
	A->G	**171**	**splice**	**splice**
	C->T	735	S245F	PSM
	T->C	115	intron	intron
	G->A	346	R115=	silent
	C->T	415	S138=	silent
	T->C	692	S231P	PSM
	T->C	646	S215=	silent

[a] Synonymous (=). Predicted damaging mutation is highlighted in bold. NSM and PSM are non severe and possibly-severe mutations, respectively.

Table 4.4 Mutations discovered in *Os05g33890* genes on mutant 'Nipponbare' population treated with Az-MNU

Gene	Nucleotide Change	Amplicon Position	Effect [a]	Type
Os05g33890	G->A	156	E30K	NSM
	C->T	1071	intron	intron
	C->T	456	L105=	silent
	A->T	1010	intron	intron
	C->T	339	intron	intron
	G->A	**657**	**splice**	**splice**
	G->A	153	A29T	NSM
	C->T	807	L196F	NSM
	G->A	608	R155=	silent
	G->A	851	L210=	silent
	T->C	773	C184=	silent
	T->C	174	L36=	silent
	C->T	1132	intron	intron

[a] Synonymous (=). Predicted damaging mutation is highlighted in bold. NSM is a possibly-severe mutation.

Table 4.5 Mutations discovered in *Os12g42760* genes on 'Nipponbare' population with Az-MNU

Gene	Nucleotide Change	Amplicon Position	Effect [a]	Type
Os12g42760	**C->T**	**1142**	**S408F**	**PSM**
	C->A	1212	I431=	silent
	G->A	523	A202T	NSM
	G->A	601	A228T	NSM
	C->T	1058	S380F	PSM
	G->A	424	A169T	NSM
	C->T	**913**	**Q332***	**truncation**
	T->C	887	L323P	PSM

[a] Synonymous (=), nonsense (*). Predicted damaging mutations are highlighted in bold. NSM and PSM are non severe and possibly-severe mutations, respectively.

Table 4.6 Mutations discovered in *Os12g42760* genes on 'Nipponbare' population mutagenized with Az-MNU

Gene	Nucleotide Change	Amplicon Position	Effect [a]	Type
Os04g50204	C->A	1115	N632K	NSM
	G->A	1188	D657N	NSM
	G->A	759	V514I	NSM
	C->T	1055	F612=	silent
	C->T	1137	L640F	NSM
	A->G	**583**	**E455G**	**PSM**
	G->A	868	S550N	NSM
	T->C	356	L379=	silent
	A->G	543	K442E	NSM
	G->A	480	A421T	NSM
	G->A	1034	E605=	silent
	G->A	83	E288=	silent
	A->G	1225	N669S	NSM
	A->G	1197	T660A	NSM
	C->T	**1255**	**P679L**	**PSM**
	C->T	1418	G733=	silent

[a] Synonymous (=). Predicted damaging mutations are highlighted in bold. NSM and PSM are non severe and possibly-severe mutations, respectively.

II. Discussion

1. Determination of the appropriate chitosan types and concentrations for drought resistance induction in rice (*Oryza sativa* L.)

In normal condition, chitosan had a positive effect on the growth of roots, shoots and leaves of various plants including Gerbera (Wanichpongpan et al., 2001) and other crop plants (Chibu and Shibayama, 2001). Chitosan spraying on orchid roots showed the induction of flower production, growth, and increased the resistance against virus and fungi (Chandrkrachang, Sompongchaikul and Teuntai, 2003). In rice, Boonlertnirun, Sarobol and Sooksathan (2006) reported that four times of foliar sprays to 'Suphanburi 1' cultivar significant affected yield, panicle per plant and dry matter accumulation. The applying polymeric chiotosan at 80 mg/L by seed soaking and soil treatment for four times at rice seedling stage tended to stimulate growth and significantly increased rice yield (Boonlertnirun et al., 2008). Contrastingly, in the present study, the oligomeric chitosan at 40 mg/L application indicated the best treatment for induction the growth of shoot in original 'LPT123' rice under drought stress and re-watering conditions (Figure 4.1-4.6).

Chitosan acted as drought resistance inducer in some plant species. It was shown to induce drought tolerance in apple (Yang et al., 2009) and coffee (Dzung, Khanh and Dzung, 2011). Chitosan could improve efficiency of water use in pepper (Bittelli et al., 2001), chitosan can induce stomatal closure through the ROS signal (Lee et al., 1999; Srivastava et al., 2009). Chitosan-treated rice before drought stress exhibited the highest yield components and showed good recovery, including the less of damaged percentage in leaves (Boonlertnirun et al., 2007). Similar to the present study, we applied chitosan four times by seed soaking and foliar spraying before applying drought stress. The data showed that chitosan could maintain 'LPT123' shoot growth under drought stress (Figure 4.2).

The effects of chitosan were reported to be species and genotype dependent responses (Jamal Uddin et al., 2004; Kananont et al., 2010; Korsangruang et al., 2010). As 'LPT123' and 'LPT123-TC171' have the similar genetic background, the difference in chitosan response will provide a good model to identify the required mechanism for chitosan induced growth enhancement during drought stress.

2. Investigation of chitosan effects on physiological changes during drought stress

Chitosan induced the significant drought resistance showing by shoot growth enhancement during drought stress in 'LPT123', but not in 'LPT123-TC171' (Figure 4.1-4.3). This was also supported by the photosynthetic pigment contents after drought stress. Without drought stress at day 0, the increase in photosynthetic pigments after spraying oligomeric chitosan was found in both rice lines (Figure 4.7). The same results were also found on the other plants. It was shown that 2 kDa oligomeric chitosan increased chlorophyll content in soybean (Dzung and Thang, 2004) and peanut (Dzung, 2005) by 17.9% and 23.0%, respectively in the field experiment. The application of 30 mg/L oligomeric chitosan was suitable for increasing chlorophyll contents of soybean and peanut as same as plant growth. Moreover, application of oligomeric chitosan of 60 mg/L in the field condition increased total chlorophyll content of coffee seedlings up to 15.36%, comparing to the control (Dzung et al., 2011). This is consistent with the results found in oligomeric chitosan application to both rice lines, before drought stress. However, after 7 days of drought stress, chitosan application only enhanced plant growth and accumulation ofphotosynthetic pigments of 'LPT123', but not in 'LPT123-TC171' (Figure 4.7). Normally, drought stress caused a decrease in chlorophyll concentrations in apple tree (*Malus domestica* Borkh.) (Sircelj et al., 2005) *Pyracantha fortuneana* and *Pteroceltis tatarinowii* (Liu et al., 2011), whereas chitosan-treated 'LPT123' under drought stress could maintain the photosynthetic pigments as the same level with non stress plants (Figure 4.7). The result demonstrated that this oligomerlic chitosan (O-80) at 40 mg/L is the best type and concentration to increase drought resistance in 'LPT123'.

The different responses in ‘LPT123’ and ‘LPT123-TC171’ suggested the genetic regulation in chitosan response.

H_2O_2 was proposed as the signal molecule in chitosan response in plants. Singha et al. (2008) reported that chitosan caused the increase in H_2O_2 in cultures of *Sphaeropsis sapinea*, and decreased in superoxide. Moreover, in cultured cells, it was shown that chitosan treatment could trigger the production H_2O_2 and defense responses such as the increasing of the activities of phenylalanine ammonialyase, the transcription of defense-related genes *β-1,3-glucanase* (*glu*), chitinase (*chi*) and pathogen-related protein (PR1) (Lin et al., 2005). However, in this study, drought induced H_2O_2 production was detected in both rice lines, but chitosan treatment clearly showed the reduction of H_2O_2 content only in ‘LPT123-TC171’ rice during drought stress (Figure 4.9 B). It was found that chitosan application inhibited H_2O_2 production scavenging the significant lower level up to 56 % reduction of H_2O_2 in ‘LPT123-TC171’ plants on day 2, 4 and 7 during drought stress (Figure 4.9 B). It suggested that the lower H_2O_2 production during drought stress in ‘LPT123-TC171’ resulted in less growth and drought resistant enhancement by chitosan, when compared to ‘LPT123’.

3. Determination of chitosan effects on antioxidative systems during drought stress

Reactive oxygen species (ROS) caused the damage to plant cells under drought stress. Mechanisms to detoxify ROS in plant cell are divided into two groups; the enzymatic components which are catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APx), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR) and non-enzymatic components, which are ascorbic acid (AA), carotenoids, flavanones and anthocyanins. The levels of the activities and amount of antioxidants enzymes will be variable among plant species under drought stress (Reddy et al., 2004).

3.1 Determination of non-enzymatic antioxidants

The chitosan-treated plants produce larger amount of free phenolic contents than those of controls in 'LPT123' rice line, reaching the maximum levels on day 2 after drought stress (Figure 4.9 A). Khan (2003) proposed that total phenolic contents and enzymatic activities (tyrosine ammonia-lyase and phenylalanine ammonia-lyase) increased after chitosan and oligomeric chitin applications in soybean leaves. The key enzymes of the phenylpropanoid pathway were inducible in response to abiotic and biotic stresses. Phenolic compounds act as scavengers of activated free radicals and showed the antioxidant properties (Bonoli et al., 2004).

Ascorbic acid (AA) is one of the antioxidants reacting with ROS such as H_2O_2, $O_2^{-\cdot}$ and OH^- as well as lipid hydroperoxidases. The functions of AA are involved in biological activities such as an enzyme co-factor, antioxidant and a donor/acceptor in electron transport at the plasma membrane or in the chloroplasts (Conklin et al., 2001). In contrast, from the results, ascorbic acid content under drought stress did not significantly change both 'LPT123' and 'LPT123-TC171' (Figure 4.10). It is possible that plants may use other non-enzymatic antioxidants to combat with drought stress such as xanthophylls pigments, zeaxanthin and antheraxanthin (Alonso et al., 2001). Similar to chitosan application, ozone stress did not affect the ascorbic acid content, when compared to the control in soybean (Zhao et al., 2010). Sreenivasulu et al. (2000) revealed that total ascorbate did not change in sensitive seedling of foxtail millet (*Setaria italoca*) under salt stress. In chloroplasts, ''Halliwell-Asada'' pathway indicated that AA was used for ascorbate peroxidase (APx) and oxidized it to monodehydroascorbate (MDA) (Reddy et al., 2004).

The glutathione is the most abundant non-proteinic. It works in intracellular to protect the cells from ROS (Mosleh et al., 2007). The glutathione is used in metabolism of phase II by GST activities including glutathione peroxidase (GPx), and glutathione reductase (GR) (Suntres, 2002; Lei et al., 2003). Moreover,

the glutathione acts as a source of amino acid for the synthesis of the proteins (Meister and Anderson, 1983). From the result, chitosan application did not increase GSH content during drought stress (Figure 4.11 A), but chitosan could reduce the GSH content (Figure 4.11 B). The reduction of GSH in chitosan treatment led to the accumulation of GSSG of glutathione-ascorbate cycle in the system. This could support the lower level of H_2O_2 in 'LPT123-TC171' in chitosan-treated plants. The GSH and GSSG should be balance to maintain of cellular redox state (Foyer and Nocter, 2005). GSH was reduced state to maintain the normal in cells encountering with ROS during oxidative stress (Meyer, 2008).

On the other hand, the increasing of GSSG when subjected to drought stress acts as a good indicator and the level of GSSG, lipid peroxidation, solute leakage, glutathione peroxidase and glutathione-*S*-transferase, is showed negative correlation with protein synthesis rate (Dhindsa, 1991). Similar to the result, in chitosan-treated 'LPT123-TC171', the significantly increase of GSSG was detected on day 7 after drought stress (Figure 4.12 B) correlated to the higher level of GSH (Figure 4.11 B). It is suggested that GSH acted a substrate for multiple cellular reactions and yielded to GSSG (Gill and Tuteja, 2010). The level of GSH in 'LPT123' is much lower than in 'LPT123-TC171'. This result indicated that the limiting of GSH in 'LPT123' led to the accumulation of H_2O_2 during drought stress. In 'LPT123-TC171', the lower level of H_2O_2 during drought stress correlating to the higher level of GSH to scavenge H_2O_2 via glutathione-ascorbate cycle.

3.2 Determination of enzymatic antioxidants

Acidic/neutral/basidic GPx, APx, and GST were the important enzymes in antioxidative system. The results indicated that chitosan and non chitosan treatments did not significantly affect the increasing of acidic GPx activity in roots and leaves of both rice lines under drought stress (Figure 4.13-4.14). In contrast, Screenivasulu et al. (1999) reported that the increasing of total peroxidase activity and acidic GPx activity in foxtail millet (*Setaria italic*). The opposite responses in neutral GPx activity were found in 'LPT123' and 'LPT123-TC171'. Chitosan affected only

the neutral GPx activity in 'LPT123-TC171' leaves but not in roots of both lines (Figure 4.15-4.16). Only the basidic GPx activity was found in roots of both lines, especially in 'LPT123' (Figure 4.18). The chitosan induced neutral GPx activity of the same species but can be different in cultivar as shown in maize cultivar 'Mol7' and 'HuangC'. When maize, cultivar 'Mol7' was treated with 0.25% chitosan, peroxide activity was increased, but in 'HuangC' cultivar, chitosan did not significantly change the activity of the enzyme (Guan et al., 2009). Furthermore, chitosan treatment effectively enhanced the activities of neutral peroxidase in orange (*Citrus sinensis* L. cv. 'Osbeck') (Zeng et al., 2010). Chitosan affected peroxidase activity in cell wall-bound proteins (Flocco and Giulietti, 2003).

APx located in different part of cellular components such as cytosolic, mitochondria, chloroplast (Amako, Chen and Asada, 1994; Hong et al., 2007). Ascorbate used as the specific electron donor and it played an important role to scavenge and protect the plant cells from H_2O_2 toxicity (Shigeoka, Nakano and Kitaoka, 1980; Asada, 1999). In the present study, before drought stress, APx activity in 'LPT123-TC171' was about 3 times higher than the activity in 'LPT123' in leaves (Figure 4.19 A). Under drought stress condition, chitosan application did not show the effects on APx activity in leaves of 'LPT123-TC171' and in roots of both lines (Figure 4.20 B and 4.21). However, chitosan treatment induced the APx induction after a day of drought stress in 'LPT123' (Figure 4.2 A), correlating with the ascorbic acid contents which was found on day 2 after drought stress (Figure 4.19 A). Chitosan could induce APx in rice (*Oryza sativa* L.) and oranges (*Citrus sinensis* L. cv. 'Osbeck') (Agrawal et al., 2002; Zeng et al., 2010). These results suggest that 'LPT123' could maintain growth under drought stress resulting from chitosan caused the increase in APx (AA is the substrate). However, chitosan did not induce APx activity in 'LPT123-TC171' via glutathione-ascorbate cycle to eliminate H_2O_2 during drought stress.

The functions of GST are reported as detoxification, the regulation of apoptosis and hormone homeostasis as well as responses to abiotic and biotic stresses (Dixon, Skipsey and Edwards, 2010). From the result, chitosan could not induce the GST activity in leaves, but induced the GST activity in roots of both lines under drought stress (Figure 4.21-4.22). Similarly, Dhindsa (1991) indicated that activities of glutathione peroxidase (GP), glutathione reductase (GR), glutathione-*S*-tranferase (GST) did not change, but showed GSSG level changed under drought stress. The GSSG content increased after drought stress (Figure 4.12). Therefore, chitosan could not induce the GST activity to scavenge H_2O_2 via glutathione-ascorbate cycle. Taken together, all of the results showed that chitosan had no significant effects on the enzymes in glutathione-ascorbate cycle, APx and GST activities (Figure 4.19 and 4.21), suggesting that the enzyme activity may not have the direct effects on H_2O_2 scavenging system via glutathione-ascorbate cycle in leaves.

4. Determination in chitosan effects on ATPase activity during drought stress

In membrane system or membrane proteins have the important role for ion selectively to regulate ion transport on the proton gradients by proton pumps in salt/drought tolerance in plants (Ashraf and Harris, 2004). Three types of distinct proton pumps consist of the vacuolar H^+-ATPase (*V-type* ATPase), *P-type* ATPase which H^+ pump across the plasma membrane into the extracellular space, and the vacuolar H^+-pyrophosphatase, which acidifies in vacuolar lumen and other intracellular compartments (Gaxiola et al., 2001). In the present study, activity of *P-type* ATPase and *V-type* ATPase were determined. The result showed that chitosan treatment did not affect the increasing of ATPase activities in leaves and roots of both lines under drought stress. Without chitosan application, drought stress increased both ATPase activities in 'LPT123' and 'LPT123-TC171' (Figure 4.23-4.26). It indicated that chitosan did suppress both *P-type* and *V-type* ATPases activity in leaves and roots of 'LPT123' and 'LPT123-TC171' lines. Similarly, PEG-treated roots showed the increase of *P-type* ATPase activity in salt-tolerant wheat (*Triticum*

aestivum L.) (Liu et al., 2005). Gong et al. (2003) indicated that drought stress stimulated p-nitrophenyl phosphate (PNPP) hydrolysis activity of *P-type* ATPase. Conversely, water stress inhibited the *P-type* ATPase activity in root of wheat (*Triticum aestivum* L.) and while spruce needles (Qiu, 1999). Moreover, Barkla et al. (1999) reported that the *V-type* ATPase was regulated through ABA-independent pathways in *Mesembryanthemum crystallinum*.

5. Proteomic analysis of chitosan responsive proteins in 'LPT123' and 'LPT123-TC171' rice lines

The present study indicates that chitosan-responsive proteins can be divided into 7 categories according to their functions which are metabolic process, signal transduction, transcription, transport, disease resistance/defense, growth and protein degradation (Figure 4.56).

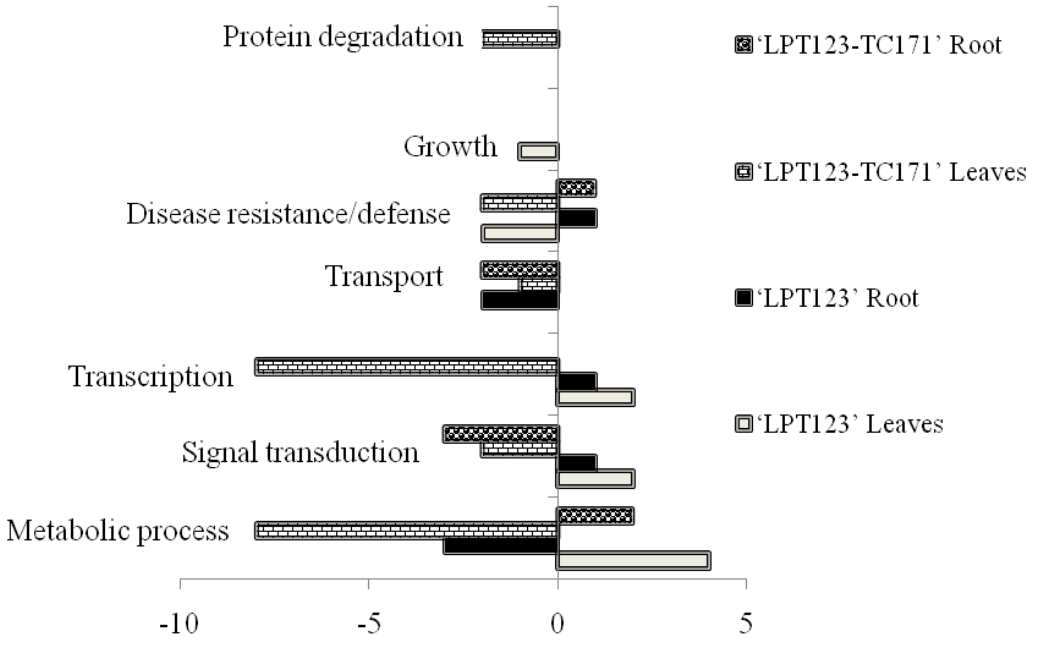


Figure 4.56 Summary functional categories of protein in leaves and roots of 'LPT123' and 'LPT123- TC171'

Mostly chitosan-responsive proteins under drought stress related to metabolic process, signal transduction and transcription regulations in leaves and roots of 'LPT123' and 'LPT123-TC171' (Figure 4.27-4.42). Similarly, Gorantia et al. (2007) reported that genes involved in cellular metabolism, signal transduction and transcription were found to be expressed in leaves and roots of 'Nagina 22' during drought stress.

Proteins related to metabolic process involving aspartic proteinase, 5-formyltetrahydrofolate cyclo-ligase, cytochrome c, cytochrome P450, chalcone synthase, alpha-amylase were found to be down regulated in 'LPT123-TC171'. Cytochrome P450 is the drought-responsive protein involving in photosynthetic process (Gorantia et al., 2007). With this study, the chitosan application to 'LPT123-TC171' led to the reduction of this protein during drought stress. Consistent with the reduction of photosynthetic pigments, it can be implied that chitosan application to 'LPT123-TC171' interface to photosynthetic system, leading to the degradation of both protein and pigments.

Proteins related to metabolic process were found in root such as GDSL-like lipase, jjH-GPD, glucan endo-1, 3-ß -glucosidase, lactose permeases and peroxidase. Glucan endo-1, 3-ß-glucosidase is the enzyme in carbohydrate metabolic process (Opassiri et al., 2010). In the result, peroxidase protein was down regulated in root of 'LPT123'. This protein might be a basidic GPx because it showed the lower level of enzymatic activity in chitosan application under drought stress comparing to the control (Figure 4.18 A).

Proteins involving in signal transduction in this study such as jasmonate O-methytransferase, protein kinase, cyclin-dependent kinase, polygalacturonase inhibitor 2 precursor were observed. Most of them were up-regulated in 'LPT123' leaves and roots (Figure 4.32 and 4.35), while these proteins were down-regulated in

‘LPT123-TC171’ (Figure 4.38). The result indicated that signal transduction may play a role in chitosan-responsive protein under drought stress in ‘LPT123’. Moreover, the cyclin-dependent kinase (Os12g23700) responded to chitosan in the early phase (Figure 4.43). Similarly, chitosan application induced the expression *of* Cyp71A13 and Cyp71B15 in both in *cerk1* mutant and wild type *Arabidopsis* (Povero et al., 2011). Moreover, chitosan application induced mitogen activated protein kinase (MAPK) activity in *Rubia tinctorum* (Vasconsuelo, Giulietti, and Boland, 2004).

Transcriptional proteins were identified including zinc finger, C_3HC_4 type, zinc finger C-x8-C-x5-C-X3-H type, DNA- directed RNA polymerase III which were up-regulated proteins in ‘LPT123’. While, ribosomal L18p/L5e family protein, leucine rich repeat family protein, OsFBX306-F-box domain containing protein, zinc finger protein-related, DNA-directed RNA polymerase III subunit RPC1 were mostly down-regulated proteins in ‘LPT123-TC171’ leaves. This protein group may act as chitosan-responsive protein under drought stress in ‘LPT123’. Moreover, in the result, zinc finger C-x8-C-x5-C-x3-H type (Os02g58440) was up-regulated in ‘LPT123’. Chitosan treatment induced the Os02g58440 gene to the highest expression level at 2 h which was higher than the control (Figure 4.44). The results of proteins/genes in ‘LPT123’ were supported by Povero et al. (2011). They indicated that 49 transcription factors genes were up-regulated after chitosan treatment. Moreover, some transcription factors are responsive to both chitin and chitosan such as *WRKY46, WRKY18*, *ERF2* and *WRKY40* (Gorantla et al., 2007).

Proteins involved in disease resistance/ defense were identified in this study including chitinase, RPM1, multidrug resistance, NBS-LRR. These proteins were down- regulated in leaves of both lines. Whereas, power mildew PM3b and strip rust Yr10 showed up-regulation of the proteins. In other works, chitosan stimulated the production of anti-fungal phytoalexins, the diterpenoid lactone momilactone A, flavonoid sakuranetin in rice leaves (Agrawal et al., 2002). Povero et al. (2011)

showed a large number of PR-proteins after chitosan application. Moreover, Doares et al. (1995) proposed that chitosan induced plant defensive genes via the octadecanoid pathway. However, there are only a few reports on chitosan-responsive proteins under abiotic stresses.

Moreover, proteins associated with growth and protein degradation were found in 'LPT123' and 'LPT123-TC171' leaves. Expansin was down-regulated the growth protein. STIP1 and proteasome are down-regulated the protein degradation. While, mitochondrial carrier, transmembrane amino acid transporter, auxin efflux carrier, transporter proteins were down-regulated in roots of both rice lines. Expansins are the key proteins in wall extension during plant growth. Over-expression of the *TaEXPB23* gene (expansin gene) could enhance the drought resistance in tobacco plants (Li et al., 2011). However, there were no reports on the responsiveness of expansin to chitosan.

6. Targeting Induced Local Lesions IN Genomes (TILLING)

6.1 Selection the chitosan-responsive proteins/genes for TILLING approach

The five chitosan-responsive proteins were selected for TILLING study consists of Os01g42260, Os05g40220, Os05g33890, Os12g42760 and Os04g50204. Three chitosan-responsive genes; Os01g42260, Os05g40220 and Os04g50204 were detected by qPCR technique. The detail of proteins obtained from rice database (http://rice.plantbiology.msu.edu/) as shown in the Table 4.8

Table 4.7 Summary the chitosan-responsive proteins in TILLING

Locus	**Acc number (gi #)**	**Putative function**	**Functional group**
Os01g42260	gi|53792190	LEUNIG Transcription	Transcription
Os05g40220	gi|87009959	Transposon protein	Transposon
Os05g33890	gi|115463857	Microtubule associated protein	Transcription
Os12g42760	gi|115489640	Type IIB DNA topoisomerase	Translation
Os04g50204	gi|115460228	Protein transport	Transport

Interestingly, these chitosan-responsive proteins/genes in 'LPT123' and 'LPT123-TC171'were selected to discover the point mutation or nucleotide change in TILLING approach of 'Nipponbare' rice. Moreover, these chitosan-responsive proteins did not present in the proteomics result (*Experiment 4*). These proteins were analyzed a little differently a bit from the present proteomics study in terms of protein identification and classification (see in method 4.5). These 5 proteins were identified in MASCOT MS/MS Ions search, but did not through DeCyder MS 2.0 program. The obtained number of protein by this method was less than the proteomics study. Thirteen and three of chitosan-responsive proteins were identified in leaves and roots, respectively. Furthermore, the significant proteins in present study were generated by Multi experiment Viewer (MeV) software using t-test p value < 0.05. It is possible that these 5 proteins were detectable, but were classified in non significant protein. From the result of proteomics study, the significant proteins were found only 20% and 30% of total proteins in leaves and roots, respectively.

6.2 Nucleotide sequences variation (changes) of 5 genes by TILLING study

Till et al. (2007) applied the TILLING method in rice and used two different mutagens in rice. Within 10 target genes showed 57 nucleotide changes. Twenty-nine missense and a nonsense mutations were identified, six nucleotide changes showed the damaging to protein function. In this study, 5 target genes showed 66 nucleotide changes of Az-Mnu-treated 'Nipponbare' population which consisted of 1 nonsence of truncation, 18 silent mutations and 13 missense mutations as well as 2 splicing mutations (Table 4.2-4.6). Eight candidate mutations revealed to be damaged in protein function (Table 4.2-4.6 in bold). The results of this study differ from other works (Till et al. 2004; 2007) which showed the higher number of nucleotide changes. It is possible that the difference of nucleotide changes resulting from the response to chemical mutagen types depend on plant species. The combination of sodium azide plus methyl-nitrosourea (Az-MNU) and EMS were applied in rice population (Till et al. 2007). The data showed that 10 target genes contained 27 nucleotide changes in the EMS-treated population and 30 nucleotide changes in the Az-MNU population. EMS concentration at 0.8% and 1% showed the low number of mutation in indica rice 'IR64' (Wu et al., 2005). EMS application was not appropriate in seed germination of japonica 'Nipponbare' due to toxicity (Till et al., 2007). In this study, mixing of sodium azide and methyl nitrosourea (Az-MNU) used for forward genetic screening in barley (Szarejko et al., 1999) and provided a higher number of mutations and less toxicity to 'Nipponbare' (Till et al., 2007).

Moreover, the higher number of nucleotide changes in this study might occur by discovering of rare mutation by sequencing (Tsai et al., 2011). The mutations discovering were detected on PCR products and digested at mismatched sites in heteroduplexes with CELI endonuclease and enzymatic mixes from celery (*Apium graveolens*) extracts (Oleykowski et al., 1998; Till et al., 2004). In this study, the candidate mutations were detected by sequencing following by comparing of new mutations with a reference sequence and bioinformatics were used to find the sequencing errors from real changes. Additionally, pooling strategy must be

represented at an equivalent concentration to the members in a pool (Tsai et al., 2011). In this study, total of 768 rice individuals were designed in term of a tridimension pooling, the same method was reported by Tsai et al. (2011).

CHAPTER V

CONCLUSION

1. Determination of the appropriate chitosan type and concentration for drought resistance induction in rice (*Oryza sativa* L.)

During drought stress and re-watering conditions, O-80 chitosan at the concentration of 40 mg/L significantly increased SFW and SDW in 'LPT123', while SWC tended to increase but chitosan did not affect 'LPT123-TC171' growth under drought stress.

2. Investigation of chitosan effects on physiological changes during drought stress

Oligomeric chitosan application could increase photosynthetic pigments in both rice lines. After drought stress for 7 days, 'LPT123' could maintain the pigment contents at the same level on control without chitosan application, while it showed the negative effects on 'LPT123-TC171'. Drought induced H_2O_2 production was detected in both rice lines, but chitosan treatment clearly showed the reduction of H_2O_2 content only in 'LPT123-TC171' rice during drought stress.

3. Determination of chitosan effects on antioxidative systems

3.1 Determination of non-enzymatic antioxidants

The ascorbic acid, GSH and GSSG did not seem to be involved in drought resistance, induced by chitosan in 'LPT123'. The results did not correlate well with drought resistant phenotype.

3.2 Determination of enzymatic antioxidants

Summary of the enzymatic antioxidant system was shown in Table 5.1

Table 5.1 Summary the effects of chitosan treatment on enzymatic antioxidants during drought stress

Enzymatic activity	**'LPT123'**		**'LPT123-TC171'**	
	Leaves	**Roots**	**Leaves**	**Roots**
1. GPx				
1.1 Acidic GPx	NS	NS	NS	NS
1.2 Neutral GPx	NS	↑(0h)	↑	↓(0h)
1.3 Basidic GPx	NS	↑	NS	↓
2. APx	↑	NS	↑(0h)	NS
3. GST	NS	↑	NS	↓

NS= no significant difference of enzymatic activity between chitosan treatment and non chitosan treatment; ↑= presence chitosan showed the higher of enzymatic activity than absence chitosan, conversely, ↓= presence chitosan showed the lower of enzymatic activity than absence chitosan.

In 'LPT123', higher antioxidant enzyme activities were found in roots than shoots, which is opposite to what found in 'LPT123-TC171, this supports lower H_2O_2 production during drought stress in 'LPT123-TC171' resulted in less growth and

drought resistant enhancement by chitosan, when compared to 'LPT123'. This suggested that H_2O_2 might be the required signal for chitosan responses in rice.

4. Determination of chitosan effects on ATPase activity during drought stress

Chitosan treatment did not affect the increasing of ATPase activities in leaves and roots of 'LPT123' and 'LPT123-TC171' under drought stress. Without chitosan application, drought stress increased both ATPase activities in 'LPT123' and 'LPT123-TC171'. It indicated that chitosan did suppress both *V-type* and *P-type* ATPase activities in leaves and roots of both lines.

5. Proteomic analysis of chitosan responsive proteins in 'LPT123' and 'LPT123-TC171' lines

By LC-MS analysis, 2768 and 1525 proteins were found in leaves and roots, of both lines, respectively. The total of 168 and 92 proteins found in leaves and roots showed homology to the previous known studies. The responsive proteins can be divided into 7 categories according to their functions. Moreover, the chitosan-responsive genes in leaves, *Os12g23700* and *Os02g58440* were detected at early phase of drought stress involving in signal transduction and transcription, respectively.

6. Targeting Induced Local Lesions IN Genomes (TILLING)

The five genes; *Os01g42260, Os05g40220, Os05g33890, Os12g42760* and *Os04g50204* were selected for TILLING as the representative of the gene that responded differently in 'LPT123' and 'LPT123-TC171' under drought stress with/without chitosan application. Three genes; *Os01g42260*, *Os05g40220* and *Os04g50204*, were detected by qPCR. The Az-MNU-treated 'Nipponbare'

population found total 66 nucleotide changes. Among nucleotide changes consisted of silent mutation, non-severe mutation (NSM), possibly-severe mutation (PSM), truncation mutation, splicing mutation and mutation within intron. The majority of these mutants were transitions. Eight severe mutations of genes on the TILLed regions were selected to place an order the M3 seeds for genotyping or phenotyping studies in further work.

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APPENDICES

APPENDIX A

REAGENT RECIPES

1. Proteomic study

1.1 Protein extraction

0.1% SDS

1.2 Deoxycholate-trichloroacetic acid (DOC-TCA) precipitation

0.15% (w/v) deoxycholate (DOC)	950 µl
72% (w/v) trichloroacetic acid (TCA)	100 µl

1.3 Lowry protein assay

1.3.1 Reagent A

(0.2% $CuSO_4$ + 0.4% Tartaric acid)	5 ml
20% Na_2CO_3	5 ml
0.8 N NaOH	10 ml
5% SDS	20 ml

1.3.2 Reagent B

Folin-Ciocalteu phenol	10 ml
Distilled water	50 ml

1.4 SDS-PAGE analysis

1.4.1 Separating gel preparation (10 ml)

40% Acrylamide	3125 µl
1.5 M Tris.HCl pH 8.8	2500 µl
10% SDS	125 µl

dH_2O	4200 µl
10% APS	50 µl
TEMED	6 µl

1.4.2 Stacking gel preparation (3 ml)

40% Acrylamide	300 µl
0.5 M Tris.HCl pH 6.8	742 µl
10% SDS	30 µl
dH_2O	1900 µl
10% APS	23 µl
TEMED	3.5 µl

1.5 Silver staining

1.5.1 Fixing solution

(50% methanol, 12% acetic acid0.05% of 37% formaldehyde)

Methanol	50 ml
48% acetic acid	25 ml
37% formaldehyde	50 µl
Distilled water	25 ml
Total	100 ml

1.5.2 Washing solution

35% ethanol

1.5.3 Sensitizing solution

0.02% sodium thiosulfate ($Na_2S_2O_3$)

1.5.4 Staining solution

0.2% $AgNO_3$

1.5.5 Developing solution

(6% Na_2CO_3, 0.0004% $Na_2S_2O_3$, 0.05% of 37% formaldehyde)

Na_2CO_3	6 g
0.02% $Na_2S_2O_3$	2 ml
Adjust volume with dH_2O	100 ml
37% formaldehyde	50 µl

1.5.6 Stopping solution

EDTA	14.6 g
Adjust volume with dH_2O	1000 ml

1.5.7 Gel storage solution

0.1% acetic acid

2. Reagents for RNA extraction

2.1 RNA extraction buffer

100 mM Tris pH 9.0

100 mM NaCl

20 mM EDTA pH 8.0

1% lauryl sarcosinate

0.1% β-mercaptoethanol

0.1% diethylpyrocarbonate (DEPC)

2.2 RNA loading dye and DNA loading dye

50% glycerol

0.25% bromophenol blue

0.25% xylene cyanol FF

2.2.1 5X TBE

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA pH 8.0	20 ml

Adjust volume to 1000 ml with dH_2O

2.2.2 DEPC-treated H_2O

H_2O	1000 ml

DEPC	1 ml

Shake vigorously; incubate at least 1 h at 37 °C and then autoclave

2.2.3 DEPC-treated TE buffer

10 mM Tris pH 8.0

1 mM EDTA

0.1% DEPC

2.2.4 Tris HCl (pH 8.0)

Tris base	121.1 g
dH_2O	800 ml
Conc. HCl	42 ml

Adjust volume to 1000 ml and pH to 8.0

2.2.5 EDTA (pH 8.0)

EDTA	186.1 g
dH_2O	800 ml
NaOH	20 g

Adjust volume to 1000 ml and pH to 8.0

10 M LiCl

3. Potassium phosphate buffer, 0.1 M

Solution A: 27.2 g KH_2PO_4 per liter (0.2 M final) in water.

Solution B: 34.8 g K_2HPO_4 per liter (0.2 M final) in water.

Referring to Table A.1 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 200 ml.

Table A.1 Preparation of 0.1 M potassium phosphate buffer

Desired pH	Solution A (ml)	Solution B (ml)	Desired pH	Solution A (ml)	Solution B (ml)
5.7	93.5	6.5	6.9	45.0	55.0
5.8	92.0	8.0	7.0	39.0	61.0
5.9	90.0	10.0	7.1	33.0	67.0
6.0	87.7	12.3	7.2	28.0	72.0
6.1	85.0	15.0	7.3	23.0	77.0
6.2	81.5	18.5	7.4	19.0	81.0
6.3	77.5	22.5	7.5	16.0	84.0
6.4	73.5	26.5	7.6	13.0	87.0
6.5	68.5	31.5	7.7	10.5	90.5
6.6	62.5	37.5	7.8	8.5	91.5
6.7	56.5	43.5	7.9	7.0	93.0
6.8	51.0	49.0	8.0	5.3	94.7

3. Modified WP nutrient solution (WP No.2) (Vajrabhaya and Vajrabhaya, 1991)

Table A.2 Modified WP nutrient solution

Chemicals	**Content (mg/l)**
Macroelements:	
Potassium nitrate (KNO_3)	580
Calcium sulfate($CaSO_4$)	500
Magnesium sulfate ($MgSO_4.7H_2O$)	450
Triple superphosphate	250
Ammonium sulfate ($(NH_4)_2SO_4$)	100
Microelements:	
Di-sodium ethylene diamine tetraacetate (Na_2EDTA) [a]	160
Ferrous sulfate ($FeSO_4.7H_2O$)[a]	120
Manganese sulfate ($MnSO_4.H_2O$)	15
Boric acid (H_3BO_3)	5
Zinc sulfate ($ZnSO_4.7H_2O$)	1.5
Potassium iodide (KI)	1.0
Sodium molybdate ($Na_2MoO_4.\ 2H_2O$)	0.1
Copper sulfate($CuSO_4.\ 5H_2O$)	0.05
Cobalt chloride ($CoCl_2.\ 6H_2O$)	0.05

[a] Preparation of FeSO4 stock concentration 30 g/l

1. Weight Na_2EDTA 40 g and $FeSO_4.7H_2O$ 30 g.

2. Dissolve Na_2EDTA 40 g in distilled water 500 ml and $FeSO_4.7H_2O$ 30 g in distilled water 500 ml at 70-90 ºC, separately.

3. Add the Na_2EDTA solution into $FeSO_4.7H_2O$ solution, mix and then prepare this solution under the O_2 at the room temperature for 3-4 h until getting the clear solution.

APPENDIX B

PROTOCOLS

1. Silver staining

After electrophoresis, the gel was fixed in fixing solution by slightly shaking for 30 min, then washed with washing solution for 5 min followed by sensitizing solution for a minute. After that, the gel was soaked in dH_2O for 5 min and stained with staining solution for 10 min. The stained gel was rinsed twice with dH_2O. The gel was transferred to the developing solution until a brown or grey precipitate normally develops within a few seconds. When the adequate degree of staining has been achieved, transfer the gel to the stopping solution for at least 10 min. The gel was rinsed with dH_2O and stored in storage solution.

2. RNA extraction

Total RNA was extracted by hot phenol as described by Thikart et al. (2005). Plant tissues were ground to fine powder in liquid nitrogen using chilled mortar and pestle, and then hot RNA extraction buffer (80 °C) was added to the powder. The mixtures were homogenized by vigorous shaking for 30 seconds. Then, it was centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was transferred to a fresh microcentrifuge tube and reprecipitated with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (v/v). The mixtures were centrifuged at 14,000 rpm for 5 min at 4 °C and carefully transferred aqueous phase (avoiding any of the lower phase) to a fresh microcentrifuge tube. After that, two volume of ice cold absolute ethanol was added to the supernatant, mixed well and incubated at -20 °C for 30 min. The pellet was separated by centrifugation at 10,000 rpm for 10 min at 4 °C and air dried at room temperature for 5-10 min. The dried pellet was resuspended in

DEPC-treated TE buffer. RNA was precipitated by addition of 0.2 volumes of 10 M LiCl and stored overnight at -20 °C. The mixture was left at room temperature for 5 minutes and centrifuged at 10,000 rpm for 10 min at 4 °C. After centrifugation, the pellet was washed by 80% ethanol, air dried and dissolved in 20 µl DEPC-treated TE buffer.

3. *DNase* I treatment

Ten micrograms of total RNA sample were added to the *DNase*I treatment mixture (1X *DNase* I buffer, 10 units of *DNase* I) according to manufacturer's protocol. The reaction was incubated at 37°C for 1 h. After incubation, the mixture was added 100 µl of DEPC-treated water and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), then it was centrifuged at 14,000 rpm for 5 min at 4°C. The upper aqueous phase was transferred to a fresh microcentrifuge tube. RNA was precipitated by the addition of 0.1 volumes of 3M NaOAc (pH 5.2) and 0.6 volumes of ice cold isopropanol and stored at -20°C for 30 min. After centrifugation, the pellet was washed with 80% ethanol and air dried at room temperature for 5-10 min. The DNA-free RNA pellet was resuspended in 20 µl DEPC-treated water.

4. Reverse transcription

Two micrograms of the DNA-free RNA was added to the reverse transcription mixture (1X M-MLV Reverse Transcriptase buffer, 100 ng of oligo$(dT)_{15}$ primer, 100 ng of the dNTP mix and 200 units of M-MLV Reverse Transcriptase). The reaction was incubated at 42°C for 1 h. The first strand cDNA was stored at - 20°C.

5. Real-time PCR

Real-time PCR amplification was performed with the specific primer sequences for each gene. The parallel amplication of *OsEF1α*, the housekeeping

control gene, was used to normalize gene expression. The quantitative real-time PCR was conducted on CFX96 Real-time system (Bio-Rad, USA).

5.1 Calculation of relative gene expression level

The level of gene expression was determined in comparison with the housekeeping gene (reference gene) expression in reference to the expression on day 0 of the treatment (control). The relative expression ratio of target gene was calculated based on PCR efficiency (E) and the CT deviations. CT was defined as the point at which the florescence rises appreciably above the background fluorescence (Pfaffl, 2001).

$$\text{Relative gene expression level} = \frac{(E_{target})^{\Delta CTtarget}}{(E_{ref})^{\Delta CTref}}$$

$E_{target} = 10^{(-1/slope)}$ of interested gene

$E_{ref} = 10^{(-1/slope)}$ of *OsEF1α*

$\Delta CT_{target} = CT_{control\ at\ 0h} - CT_{sample\ at\ any\ time\ point\ of\ interested\ gene}$

$\Delta CT_{ref} = CT_{control\ at\ 0h} - CT_{sample\ at\ any\ time\ point\ of\ } OsEF1\alpha$

APPENDIX C

STANDARD CURVES

1. H_2O_2 standard

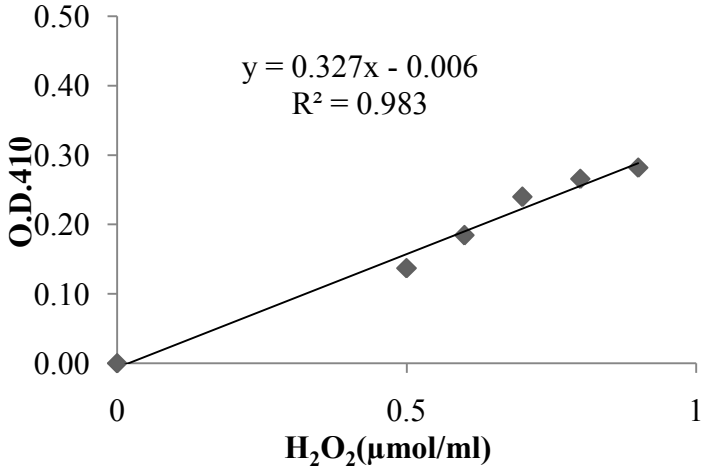

Figure C.1 Stand curve of H_2O_2

2. Phenolic standard

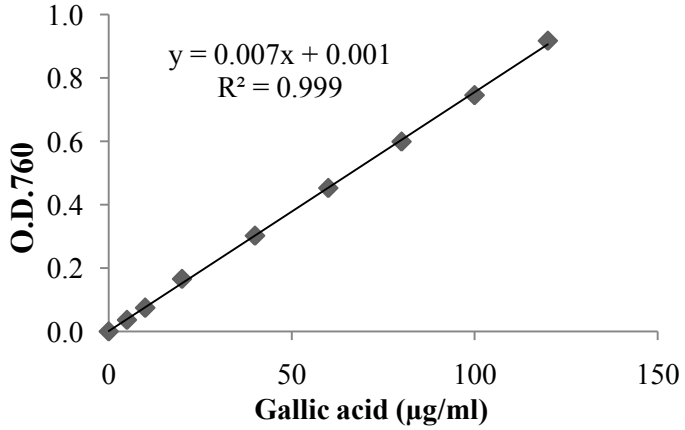

Figure C.2 Stand curve of phenolic compound

3. Ascorbic acid (AA) standard

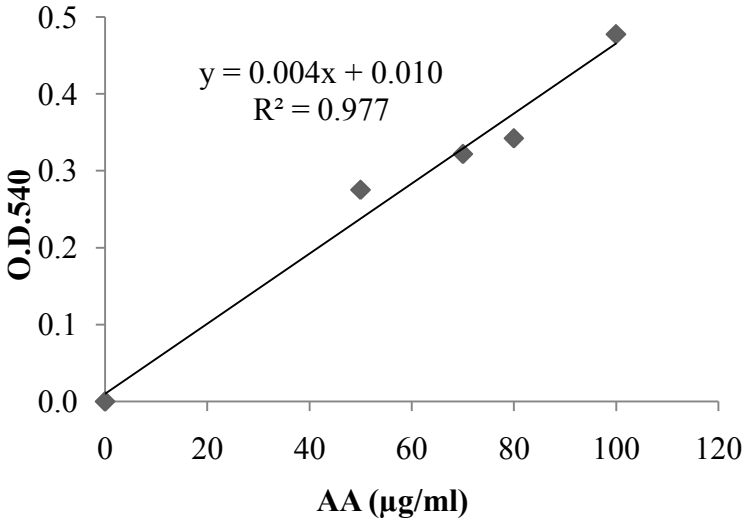

Figure C.3 Stand curve of AA

4. GSH standard

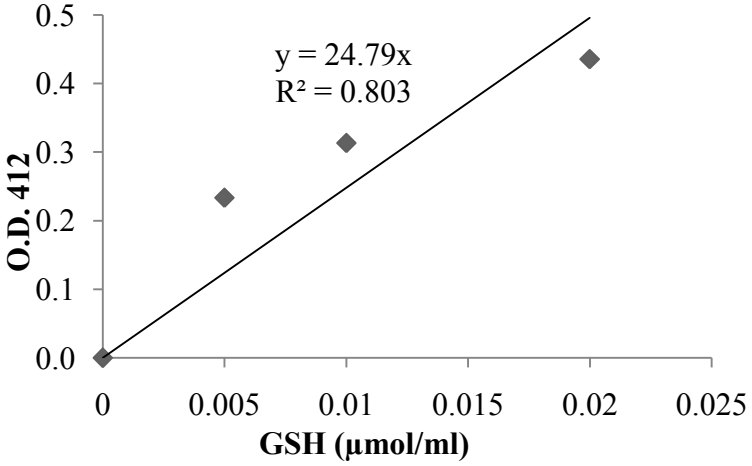

Figure C.4 Stand curve of GSH

5. Protein standard

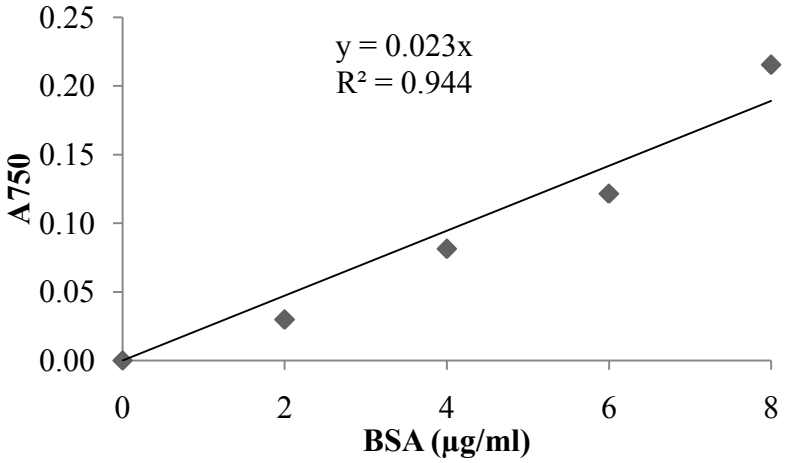

Figure C.5 Stand curve of protein (BSA)

6. ATPase standard

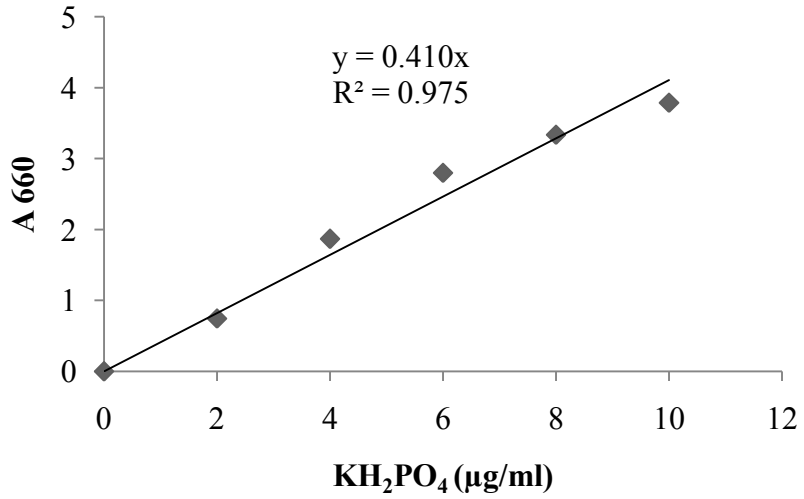

Figure C.6 Stand curve of APTase

APPENDIX D

PROTEINS

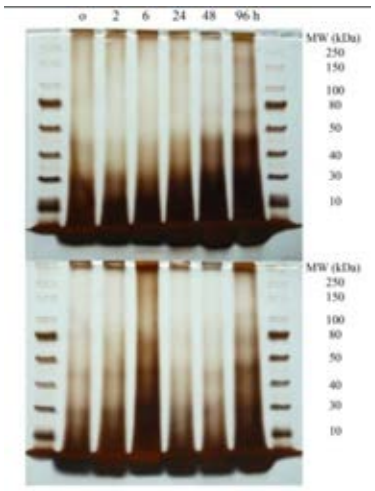

Figure D.1 The isolated proteins from ‘LPT123’ leaves treated with water (upper) or chitosan (lower) at 0, 2, 6, 24, 48 and 96 h were separated on 12.5% SDS-PAGE and visualized by silver stain.

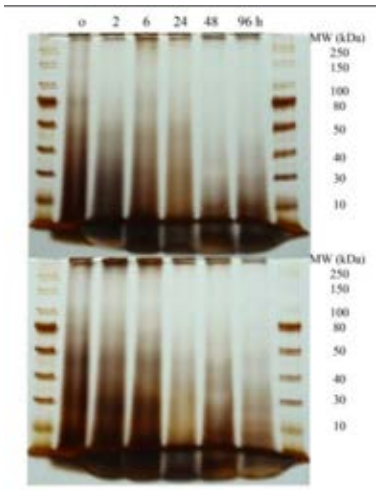

Figure D.2 The isolated proteins from ‘LPT123’ roots treated with water (upper) or chitosan (lower) at 0, 2, 6, 24, 48 and 96 h were separated on 12.5% SDS-PAGE and visualized by silver stain.

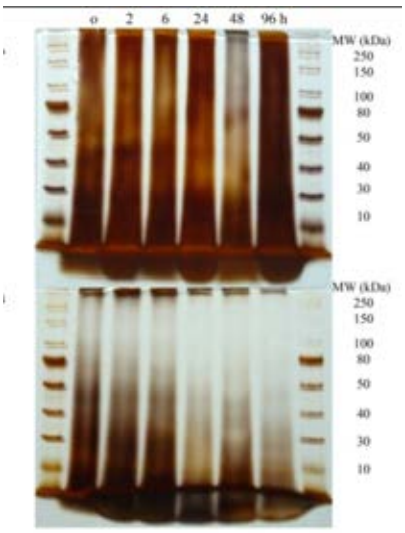

Figure D.3 The isolated proteins from ‘LPT123-TC171’ leaves treatedwith water (upper) or chitosan (lower) at 0, 2, 6, 24, 48 and 96 h were separated on 12.5% SDS-PAGE and visualized by silver stain.

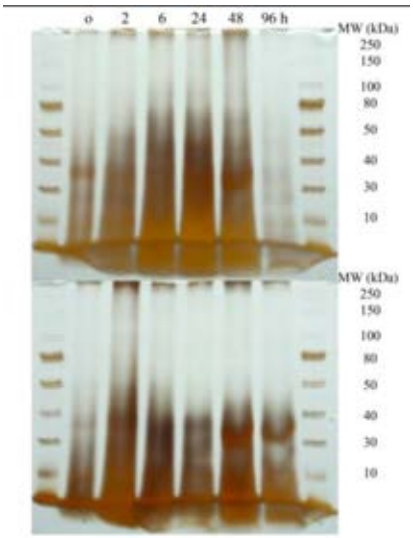

Figure D.4 The isolated proteins from ‘LPT123-TC171’ roots treated with water (upper) or chitosan (lower) at 0, 2, 6, 24, 48 and 96 h were separated on 12.5% SDS-PAGE and visualized by silver stain.

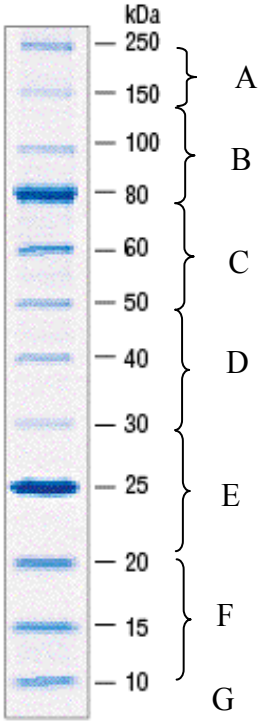

Figure D.5 Protein bands were excised according to protein ladder (10-250 kDa, New England Biolabs); A (>150 kDa), B (80-150 KDa, C (50-80 KDa), D (30-50 kDa), E (20-30 kDa), F (10-20 kDa) and G (<10 kDa).

Table D.1 List of similar proteins in leaves and roots of both rice lines

LOC	**Putative functions**	**Functional groups**
Os06g24050	transposon protein	transposon protein
Os09g31458	expressed protein	unknown
Os07g09990	retrotransposon protein	retrotransposon protein
Os11g06720	abscisic stress-ripening	unknown
Os04g51940	YT521-B	unknown
Os04g41490	DNA-directed RNA polymerase III subunit RPC1	transcription
Os07g44780	GDSL-like lipase/acylhydrolase	metabolic process
Os07g30150	phosphoribosyl transferase	metabolic process
Os12g36720	RGH1A	unknown

Table D.2 List of down-regulated proteins in ‘LPT123’ leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1233.716966	NMGAVEKVQSR	gi|51038233	LOC_Os05g34210	expressed protein	unknown
1183.833941	MTTTTTTTRR	gi|115483208	LOC_Os10g39700	CHIT15 - Chitinase family protein precursor	disease resistant protein
1117.26661	CFGYMVSKK	gi|3646373	LOC_Os03g40270	alpha-1,4-glucan-protein synthase	other
1129.82536	LVKDTINLSQ	gi|115435146	LOC_Os01g10630	expressed protein	unknown
1211.629551	LTGECISQFR	gi|297728483	LOC_Os11g35210	NB-ARC domain containing protein	other
1196.930164	LPHRLGAPPLK	gi|297721843	LOC_Os03g08114	expressed protein	unknown
1168.986156	MTLQGSSTSSR	gi|115473865	LOC_Os07g46600	RNA pseudouridine synthase	other
903.0103083	TMAMASMK	gi|115461150	LOC_Os04g56950	jasmonate O-methyltransferase,	signal transduction
1156.461251	GMRMVDGSMR	gi|108706557	LOC_Os03g01060	retrotransposon protein	retrotransposon

Table D.2 (Cont.) List of down-regulated proteins in ‘LPT123’ leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1398.959448	GGACGYGDLDIFR	gi|16517043	LOC_Os06g41700	expansin precursor	growth
896.7628055	VFDEMEK	gi|115483719	LOC_Os11g01210	HCF152	other
960.9536559	EPPRAAGLR	gi|38345347	LOC_Os07g12520	zinc ion binding protein	other
982.7998352	SKGNEFMR	gi|38345522	LOC_Os04g59394	expressed protein	unknown
941.7099009	SLGAWRLSG	gi|51536065	LOC_Os06g12050	mTERF family protein	other
778.4803441	RMSAGSR	gi|297603899	LOC_Os04g51940	YT521-B	other
2242.186821	EVIALHGMGGLGKT ALAANVYR	gi|18057109	LOC_Os10g21400	disease resistance protein RPM1	disease resistant protein

Table D.2 (Cont.) List of down-regulated proteins in ‘LPT123’ leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1121.342831	FQSLHFVSR	CA759966	-	unknown	unknown
1110.760081	GDGGGGYRR	041020	-	unknown	unknown
1187.805757	AALSSSGLVGAPTG	gi|14718312	LOC_Os07g09990	retrotransposon protein	retrotransposon protein

Table D.3 List of up-regulated proteins in ‘LPT123’ leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1492.531666	IEEELGAAAVYAGAK	gi|780372	LOC_Os10g08550	enolase	carbohydrate metabolic process
783.9367424	NYHLIK	gi|125552173	LOC_Os09g32450	DNA topoisomerase 3 protein	metabolic process
1150.380189	VGCTAKVTSTK	gi|154550669	LOC_Os01g01080	decarboxylase	metabolic process
2270.993056	ARAEGLPEGA	gi|125541277	LOC_Os10g01060	protein kinase family protein	signal transduction
2209.977492	YRTAATAMLARP	gi|42407554	LOC_Os08g01040	zinc finger, C3HC4 type domain	transcription
795.5402326	LGHDLLK	gi|115472161	LOC_Os07g30940	expressed protein	unknown
698.2886737	GKPHTR	gi|218192868	LOC_Os04g22400	retrotransposon protein	retrotransposon protein

Table D.3 (Cont.) List of up-regulated proteins in 'LPT123' leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
698.2886737	GKPHTR	gi|218192868	LOC_Os04g22400	retrotransposon protein	retrotransposon protein
930.3610012	ANPGPISGGR	gi|15128439	LOC_Os12g23700	cyclin-dependent kinase G-2	signal transduction
850.5132787	ANATEKGR	gi|28209459	LOC_Os03g34070	retrotransposon protein	retrotransposon protein
949.7885814	LKTAQNSQK	gi|38345968	LOC_Os04g27490	retrotransposon protein	retrotransposon protein
947.7076751	TMRVGVDR	gi|115481010	LOC_Os10g04074	retrotransposon protein	retrotransposon protein
844.1657844	ANAGVRTR	gi|125544376	LOC_Os07g20544	aspartokinase, chloroplast precursor	metabolic process
844.1657844	ANAGVRTR	gi|125544376	LOC_Os07g20544	aspartokinase, chloroplast precursor	metabolic process
991.6285176	MVVVTLAMK	gi|46805523	LOC_Os02g58440	zinc finger C-x8-C-x5-C-x3-H type family protein	transcription

Table D.3 (Cont.) List of up-regulated proteins in ‘LPT123’ leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
930.3610012	ANPGPISGGR	gi|15128439	LOC_Os12g23700	cyclin-dependent kinase G-2	signal transduction
850.5132787	ANATEKGR	gi|28209459	LOC_Os03g34070	retrotransposon protein	retrotransposon protein
949.7885814	LKTAQNSQK	gi|38345968	LOC_Os04g27490	retrotransposon protein	retrotransposon protein
947.7076751	TMRVGVDR	gi|115481010	LOC_Os10g04074	retrotransposon protein	retrotransposon protein

Table D.4 List of down-regulated proteins in ‘LPT123’ roots

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1141.028524	IYICLDGCK	gi|38346848	LOC_Os04g17190	transposon protein, putative, Mutator sub-class	transposon protein
1229.164616	LEIKNCSANGK	gi|62733801	LOC_Os11g15670	NBS-LRR disease resistance protein	other/unknown
1237.380616	FPSAPLLLLLR	gi|115474549	LOC_Os08g02580	conserved hypothetical protein	unknown
915.1008651	GCDGSVLIDTVPGSTTR	gi|55701011	LOC_Os05g06970	peroxidase precursor	metabolic process
1393.752257	DVPGIIEGK	gi|77551929	LOC_Os11g39470	retrotransposon protein	retrotransposon protein
1237.37294	MTRWYGGAGLK	gi|77555031	LOC_Os12g20260	hyprothetical protein	unknown
736.3736122	AMLDIR	gi|115460434	LOC_Os04g51940	YT521-B	other/unknown

Table D.4 (Cont.) List of down-regulated proteins in ‘LPT123’ roots

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1070.01439	LAVVFSSGHR	gi|218191060	LOC_Os02g01010	OsPDIL1-4 protein disulfide isomerase PDIL1-4	other/unknown
1050.158968	SGDGSMQLLE	gi|32488046	LOC_Os04g12490	retrotransposon protein	retrotransposon protein
1245.626551	SDRQFK	gi|222624670	LOC_Os07g20510	auxin efflux carrier	transport
829.7625205	TLQTIQK	gi|115454487	LOC_Os03g06510	KIP1	transcription factor
827.7462847	VMPPLTR	gi|27764656	LOC_Os03g32600	retrotransposon protein	retrotransposon protein
797.2960696	GMVHAIR	gi|115452405	LOC_Os03g18160	mitochondrial carrier protein	transport
840.3591903	VQSGKYR	gi|34395178	LOC_Os07g28930	express protein	unknown
1671.416784	FVGVEEAERVSSMGR	gi|115455553	LOC_Os03g55704	express protein	unknown

Table D.4 (Cont.) List of down-regulated proteins in ‘LPT123’ roots

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1105.504255	LIVDFIVER	gi|115473671	LOC_Os07g44780	GDSL-like lipase/acylhydrolase	metabolic process
1132.728629	GCDLAPSNLDA	gi|115450929	LOC_Os03g06910	PPR repeat	other/unknown
1550.598181	QENSVSLQFSPEPS	gi|115475115	LOC_Os08g08860	CRS2-associated factor 2, mitochondrial precursor	other/unknown
1415.779064	VDLSLSK	gi|115448361	LOC_Os02g54790	hyprothetical protein	unknown
1371.901033	AEIGGNR	gi|297727099	LOC_Os09g31458	express protein	unknown

Table D.5 List of up-regulated proteins in ‘LPT123’ roots

Mass	peptide	protein	LOC	Putative function	Functional groups
1113.722911	EVKLIDLER	gi|115481008	LOC_Os10g04060	powdery mildew resistance protein	disease resistant protein
1037.161787	KPPSKDIVR	gi|115483917	LOC_Os12g02950	EMB2748, (pentatricopeptide)	other
742.2035034	LTGSIPR	gi|115461607	LOC_Os05g01430	polygalacturonase inhibitor 2 precursor	signal tranduction
933.9651462	LALIDMGAK	gi|34015136	LOC_Os05g10710	retrotransposon protein	retrotransposon protein
1025.851885	MAFADLDTK	gi|108708879	LOC_Os03g01060	retrotransposon protein	retrotransposon protein
1025.851885	MAFADLDTK	gi|50872417	LOC_Os03g30810	retrotransposon protein	retrotransposon protein
754.8767598	EIINAAK	gi|115459150	LOC_Os04g41490	DNA-directed RNA polymerase III	transcription

Table D.6 List of down-regulated proteins in ‘LPT123-TC171’ leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
2209.977492	YRTAATAMLAR PGGGGGGAADFK	gi|42407554	LOC_Os08g43530.1	OsFBX306 - F-box domain containing protein	transcription
999.0329634	GDIDIIDLK	gi|27368877	LOC_Os01g25386.1	multidrug resistance-associated protein	disease resistant protein
1174.79175	GAFDGAMGALPR	gi|90399033	LOC_Os04g58070.1	aspartic proteinase nepenthesin precursor	metabolic process
1150.133139	MVRLLHLPR	gi|34393338	LOC_Os07g39070.1	5-formyltetrahydrofolate cyclo-ligase	metabolic process
969.5293722	AHVLEHHK	gi|54287472	LOC_Os07g39070.1	5-formyltetrahydrofolate cyclo-ligase	metabolic process
1231.423526	QSQAPTQKTSR	gi|115478857	LOC_Os09g20550.1	zinc finger protein	transcription

Table D.6 (Cont.) List of down-regulated proteins in 'LPT123-TC171' leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1133.300088	MGPNDAGAVRK	gi|222637521	LOC_Os07g43900.1	protein kinase domain containing protein	signal transduction
1110.945317	GVISLRNDIK	gi|77554434	LOC_Os12g39610.1	retrotransposon protein,putative, Ty3-gypsy subclass	retrotransposon
998.101384	ATAADGVAVAR	gi|218195927	LOC_Os05g01460.1	STIP1 homology and U box-containing protein 1	protein degradation
990.5328538	DIMVANRR	gi|297603248	LOC_Os04g49430.1	ZR1 protein	unknown
754.9051407	EIINAAK	gi|115459150	LOC_Os04g41490.1	DNA-directed RNA polymerase III subunit RPC1	transcription
1071.090307	AAAAALARAGAR	gi|218186357	LOC_Os12g03250.1	retrotransposon	retrotransposon

Table D.6 (Cont.) List of down-regulated proteins in 'LPT123-TC171' leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1091.115251	HEVVNLQPR	gi|108707760	LOC_Os05g34770.1	cytochrome c	metabolic process
1071.304954	ELKGNPLAAR	gi|218189931	LOC_Os02g02640.1	NBS-LRR disease resistance protein	disease resistant protein
760.4764567	DGDGGGGAR	gi|42409402	LOC_Os03g04195.1	expressed protein	unknown
2235.692271	IMLHDADAYVAEIDGKVVMK	gi|169769	LOC_Os06g49970.2	alpha-amylase precursor	metabolic process
1253.206913	ACLNAGR	gi|115486413	LOC_Os11g42100.1	leucine rich repeat family protein	transcription
924.2601962	WSDDEGSK	gi|51091043	LOC_Os06g41930.2	zinc-binding protein	transcription

Table D.6 (Cont.) List of down-regulated proteins in 'LPT123-TC171' leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
778.4803441	RMSAGSR	gi|297603899	LOC_Os05g07240.1	expressed protein	unknown
1398.959448	GGACGYGDLDIFR	gi|16517043	LOC_Os03g49600.1	Os3bglu7 - beta-glucosidase, exo-beta-glucanse	unknown
1168.986156	MTLQGSSTSSR	gi|115473865	LOC_Os07g46600.1	RNA pseudouridine synthase	unknown
628.194867	GAAGPEK	gi|297607977	LOC_Os08g05540.1	expressed protein	unknown
812.1313269	HSANGKAK	gi|20330742	LOC_Os07g17010.1	chalcone synthase	metabolic process
1101.754019	GIPAVSVSMSR	gi|115454743	LOC_Os03g48840.1	ribosomal L18p/L5e	transcription
1233.716966	NMGAVEKVQSR	gi|51038233	LOC_Os05g34210.1	expressed protein	unknown
554.639358	EMMDAYR	gi|56783671	LOC_Os01g17180.1	proteasome subunit	protein degradation
785.2223537	RGGGGGGGGR	gi|8096645	LOC_Os10g22200.1	-	unknown

Table D.6 (Cont.) List of down-regulated proteins in ‘LPT123-TC171’ leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1576.560242	GYPDIR	gi|77553263	LOC_Os12g09089.1	protein kinase PKN/PRK1, effector	signal transduction
820.4464492	AGVTHVLK	gi|115435496	LOC_Os01g13190.1	histidinol dehydrogenase, chloroplast precursor	other
720.1539878	SNMVSLG	gi|38568038	LOC_Os04g22050.1	retrotransposon protein	transposon
848.1301234	DDAHTYK	gi|55168112	LOC_Os05g15750.1	transposon protein, putative, CACTA, En/Spm	transposon
760.1448678	SSGGGGAAAK	gi|34394087	LOC_Os07g09990.1	retrotransposon	retrotransposon

Table D.6 (Cont.) List of down-regulated proteins in 'LPT123-TC171' leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
981.6315192	XPGVYHGQL	AA752797	-	unknown	unknown
962.0917058	GGVRVEPPR	gi|125603593	LOC_Os08g34740.1	SGT1 protein	unknown
954.9734434	HKGEGGGTGR	gi|77555414	-	unknown	unknown
1103.644649	LIVDFIVER	gi|115473671	LOC_Os07g44780.1	GDSL-like lipase/acylhydrolase,	metabolic process
778.4452219	DFLNNR	gi|115489000	LOC_Os12g36720.1	RGH1A	unknown
702.0265051	AAAGETGK	gi|222630385	LOC_Os05g08420.1	expressed protein	unknown
817.5273844	DAVMGLGR	gi|38423993	-	-	unknown

Table D.6 (Cont.) List of down-regulated proteins in ‘LPT123-TC171’ leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
783.9367424	NYHLIK	gi|125552173	LOC_Os05g32130.1	expressed protein	unknown
922.5258193	LREMFPK	gi|115488352	LOC_Os12g23700.1	cyclin-dependent kinase G-2	signal transduction
1331.705696	GGSVRPSRNSSTK	gi|115471239	LOC_Os07g12520.1	zinc ion binding protein	other
935.3248229	STGTIAYPK	gi|108862071	LOC_Os12g01320.1	retrotransposon protein, putative, Ty3-gypsy	retrotransposon
880.8911148	DGDATMTR	gi|38605892	LOC_Os04g45310.1	retrotransposon protein, putative, Ty3-gypsy subclass	retrotransposon

Table D.7 List of up-regulated proteins in ‘LPT123-TC171’ leaves

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1135.143348	SDVDIISFLK	gi|115446575	LOC_Os02g33750.1	transposon protein	transposon
738.9603564	KHHLFG	gi|218194846	LOC_Os04g34600.1	abscisic stress-ripening	other
1162.339394	MVATIAPDISK	gi|77552388	LOC_Os11g43770.1	leucine rich repeat family protein	transcription
735.7433019	QCNLFG	CA754864		unknown	unknown

Table D.8 List of down-regulated protein in ‘LPT123-TC171’roots

Mass	Peptide	Protein	LOC	Putative function	Functional groups
742.2035034	LTGSIPR	gi|115461607	LOC_Os05g01430.1	polygalacturonase inhibitor 2 precursor	signal transduction
797.2960696	GMVHAIR	gi|115452405	LOC_Os03g18160.1	mitochondrial carrier protein	transport
1056.501678	EEHRLGGGGGC GGGGGRPPIPG	gi|115468776	LOC_Os06g39600.1	protein phosphatase 2C	signal transduction
1060.452703	ELLGMKAER	gi|78708074	LOC_Os10g14850.1	retrotransposon	retrotransposon
840.3591903	VQSGKYR	gi|34395178	LOC_Os09g37949.1	serine/threonine-protein kinase SRPK1	unknown
1145.990807	EGSTSLSVDVR	gi|115455147	LOC_Os03g52320.1	GRF-interacting factor 1	other
1034.895038	KVSSGSGNGSR	gi|110289319	LOC_Os10g35040.1	receptor kinase	signal transduction

Table D.8 (Cont.) List of down-regulated protein in ‘LPT123-TC171’roots

Mass	Peptide	Protein	LOC	Putative function	Functional groups
916.2188962	AGALGLAWR	gi|34394079	LOC_Os07g09920.1	-	unknown
1162.630887	ATLSDSAGGG(GGR	gi|297721463	LOC_Os02g43040.1	expressed protein	unknown
1115.70127	KLVLDAGQFK	gi|115438645	LOC_Os01g43320.1	transmembrane amino acid transporter protein	transport
1025.851885	MAFADLDTK	gi|108708879	LOC_Os03g30810.1	retrotransposon protein	retrotransposon
1025.851885	MAFADLDTK	gi|50872417	LOC_Os03g30810.1	retrotransposon	retrotransposon

Table D.9 List of up-regulated protein in ‘LPT123-TC171’roots

Mass	Peptide	Protein	LOC	Putative function	Functional groups
1037.161787	KPPSKDIVR	gi|115483917	LOC_Os11g03200.1	EMB2748	other
1161.368258	DLIPRGADFR	gi|28812148	LOC_Os03g41419.1	serpin domain containing protein	unknown
1191.234717	IYDTNIDVIK	gi|115439837	LOC_Os01g53750.1	glucan endo-1,3-beta-glucosidase precursor	metabolic process
828.1445014	ITEGLGLK	gi|115450973	LOC_Os03g07170.1	lactose permease-related	metabolic process
793.8647954	SMQATNK	gi|115445033	LOC_Os02g12490.1	expressed protein	unknown
2263.159149	HVGLGNLLQLNTVTIDLECR	gi|218186046	LOC_Os11g37870.1	stripe rust resistance protein Yr10	defense
1237.37294	MTRWYGGAGLK	gi|77555031	LOC_Os12g20260.1	expressed protein	unknown
1146.835985	NFKVMEHDK	gi|77552440	LOC_Os11g44840.1	expressed protein	unknown

BIOGRAPHY

Ms. Wasinee Pongprayoon was born on June 13, 1981 in Suphanburi Province. She got the scholarship from the Development and Promoting Sciences and Technology Talents Project (DPST) and finished the secondary school from Phrapathom Witthalai School in 2000. Continually, she was supported by scholarship from DPST and enrolled in Bachelor's degree, majored in Biology, Faculty of Science, at Silpakorn University from 2000 to 2004. After that she continued on Master degree in Plant Science, Faculty of Science, Mahidol University from 2004 to 2007. She has been supported by DPST to study and doing her research for the degree of Doctor of Philosophy in Biological Science program, Faculty of Science, Chulalongkorn University since 2007.