CHAPTER IV
RESULTS

Identification of chitosan-responsive proteins in LPT123 and LPT123-TC171 rice during drought stress

The proteomic analysis showed 3,327 proteins found in leaves and 1,068 proteins found in roots of LPT123 and LPT123-TC171 rice. Venn diagram gave an overview of proteins appearing in each treatment. Most of the proteins were common in all treatments, 2,140 proteins (64%) in leaves and 792 proteins (74%) in roots (Figure 4.1).

Chitosan-responsive proteins during drought stress in LPT123 rice

Abundance of total proteins was compared between chitosan and non-chitosan treatments with *t*-test ($P < 0.05$) in order to identify chitosan-responsive proteins in each rice line. In LPT123 rice, significantly differentially expressed proteins in leaves were 401 proteins: 295 proteins were down-regulated while 106 proteins were up-regulated (Figure 4.2). Their identities are given in table D.1. Interestingly, the characteristics of this response were also found in root proteome. Among 115 significantly differentially expressed proteins in roots, 72 proteins were down-regulated. The others were up-regulated (Figure 4.3 and table D.2).

The chitosan-responsive proteins were classified into several groups as shown in Figure 4.4 based on their involvement in biological process, a term in gene ontology. Proteins with unknown function and transposable elements were 2 major groups of them found in both leaf and root parts. For the known function proteins, those related to metabolic process were the predominant group.

The majority of the proteins that were up-regulated by chitosan under drought stress involved in metabolic process (18%) such as cytochrome P450, ribose-5-phosphate isomerase A, prenyltransferase and transketolase. The others were proteins involving in stress response/defense (8%; e.g. NBS-LRR disease resistance protein and disease resistance protein RPM1), nucleic acid metabolic process (6%; e.g. MYB family transcription factor and RNA dependent RNA polymerase), transport (4%; e.g. cyclic nucleotide-gated ion channel 2 and nucleotide-sugar transporter family protein), signal transduction (4%; e.g. ras-related protein), protein modification process (4%; e.g. OsWAK55, OsWAK71 and OsWAK81), cellular homeostasis (1%; thioredoxin) and growth and development (1%) (Figure 4.4A and table D.1).

Down-regulated proteins in leaf tissues included proteins involving in metabolic process (15%; e.g. dihydrolipoyl dehydrogenase 1, AAA-type ATPase family protein, NADH dehydrogenase I subunit N, NADPH reductase, aconitase hydratase and GDSL-like lipase/acylhydrolase), transport (8%; e.g. ABC transporter family protein, mitochondrial carrier protein, amino acid/polyamine transporter II and CorA-like magnesium transporter protein), nucleic acid metabolic process (7%; e.g. transcriptional repressor, AP2 domain containing protein and pre-mRNA-splicing factor SLU7), stress response/defense (5%; e.g. NBS-LRR disease resistance protein and monodehydroascorbate reductase), signal transduction (3%; e.g. receptor-like kinase), protein modification process (2%; e.g. ubiquitin conjugating enzyme), growth and development (2%), cellular process and cellular component organization (Figure 4.4A and table D.1).

Moreover, among proteins that were up-regulated in root tissues, those involving in metabolic process (16%; e.g. ferredoxin-nitrite reductase, polygalacturonase and tetrahose-6-phosphate synthase) and protein modification process (11%; e.g. protein kinase domain containing protein and protein phosphatase 2C) were 2 main groups. Proteins related to nucleic acid metabolic process (5%), signal transduction (5%), transport (5%) and stress response (5%) were also found (Figure 4.4B and table D.2).

Within 43 down-regulated proteins, proteins belonging to metabolic process were the largest group (13%; e.g. cytochrome P450, hydrolase, alpha/beta fold family domain containing protein and aldehyde dehydrogenase). The others were proteins involving in nucleic acid metabolic process (11%; e.g. high mobility group), protein modification process (6%; e.g. OsWAK13), stress response/defense (6%; e.g. peroxidase precursor), transport (4%; e.g. clathrin assembly protein), cellular process (1%; myosin), cellular component organization (1%; SET domain containing protein) and growth and development (1%; PINHEAD) (Figure 4.4B and table D.2).

Mapping all of these proteins into biological pathway by using MapMan software facilitated the visualization of the proteins on them. An overview of RNA-protein synthetic and metabolic pathways were shown in Figure 4.5. The 58 and 46 proteins were mapped into RNA-protein synthetic and metabolic pathways, respectively (table D.3 and D.4). Chitosan treatment decreased abundance of proteins assigned to amino acid activation. For proteins assigned to transcription, RNA processing, protein synthesis, posttranslational modification, protein targeting and protein degradation, some of them were chitosan down-regulated proteins while the others were chitosan up-regulated proteins (Figure 4.5A and table D.3).

Chitosan application prior to drought stress led to decreased abundance of many proteins assigned to photosynthesis, tetrapyrrole synthesis, cellular respiration, redox, nucleotide metabolism, amino acid metabolism (both synthesis and degradation), secondary metabolism, lipid metabolism (degradation), cell wall metabolism, major carbohydrate metabolism (degradation) and fermentation (Figure 4.5B and table D.4). On the other hand, abundance of many proteins belonging to nitrogen metabolism, amino acid metabolism (synthesis), secondary metabolism, lipid metabolism, cell wall metabolism (degradation), minor carbohydrate metabolism and pentose phosphate pathway were induced (Figure 4.5B and table D.4).

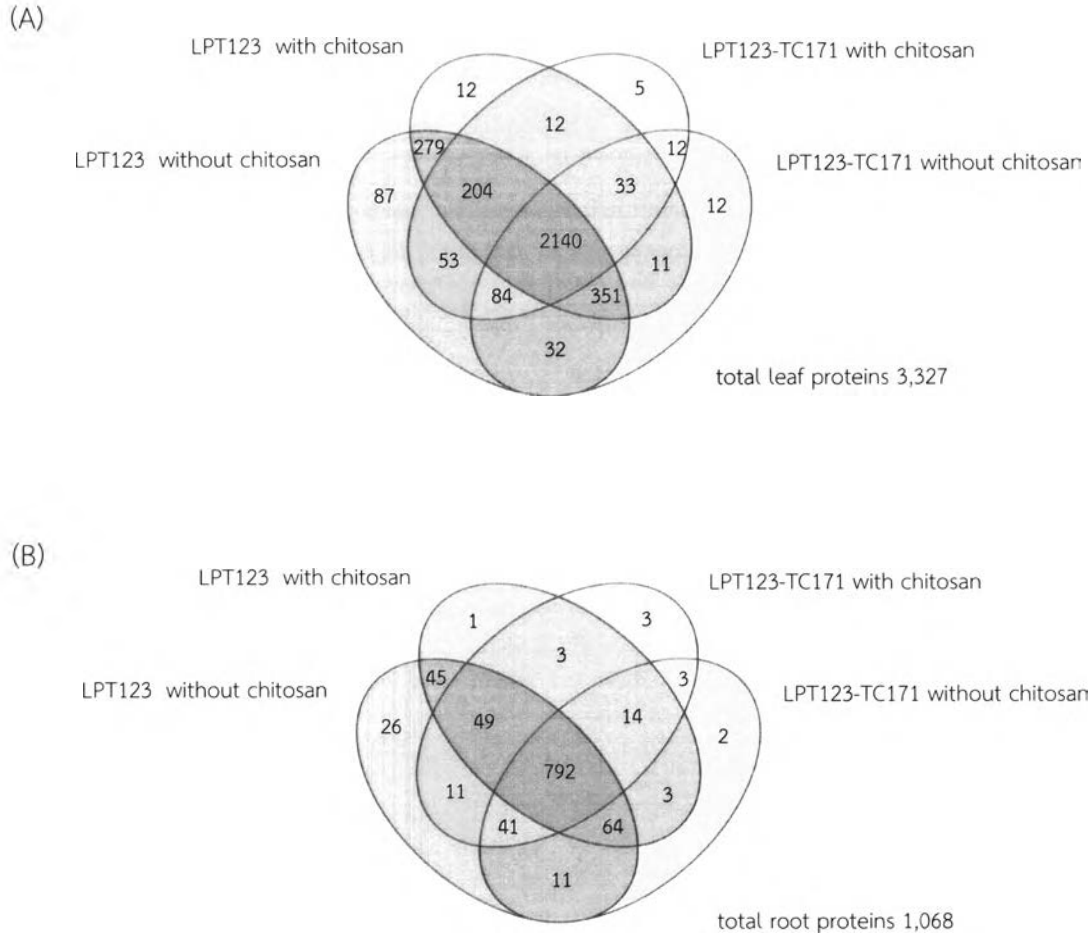


Figure 4.1 Venn diagram analysis of proteins found in (A) leaf tissue and (B) root tissue of LPT123 and LPT123-TC171 rice treated with and without chitosan during drought stress

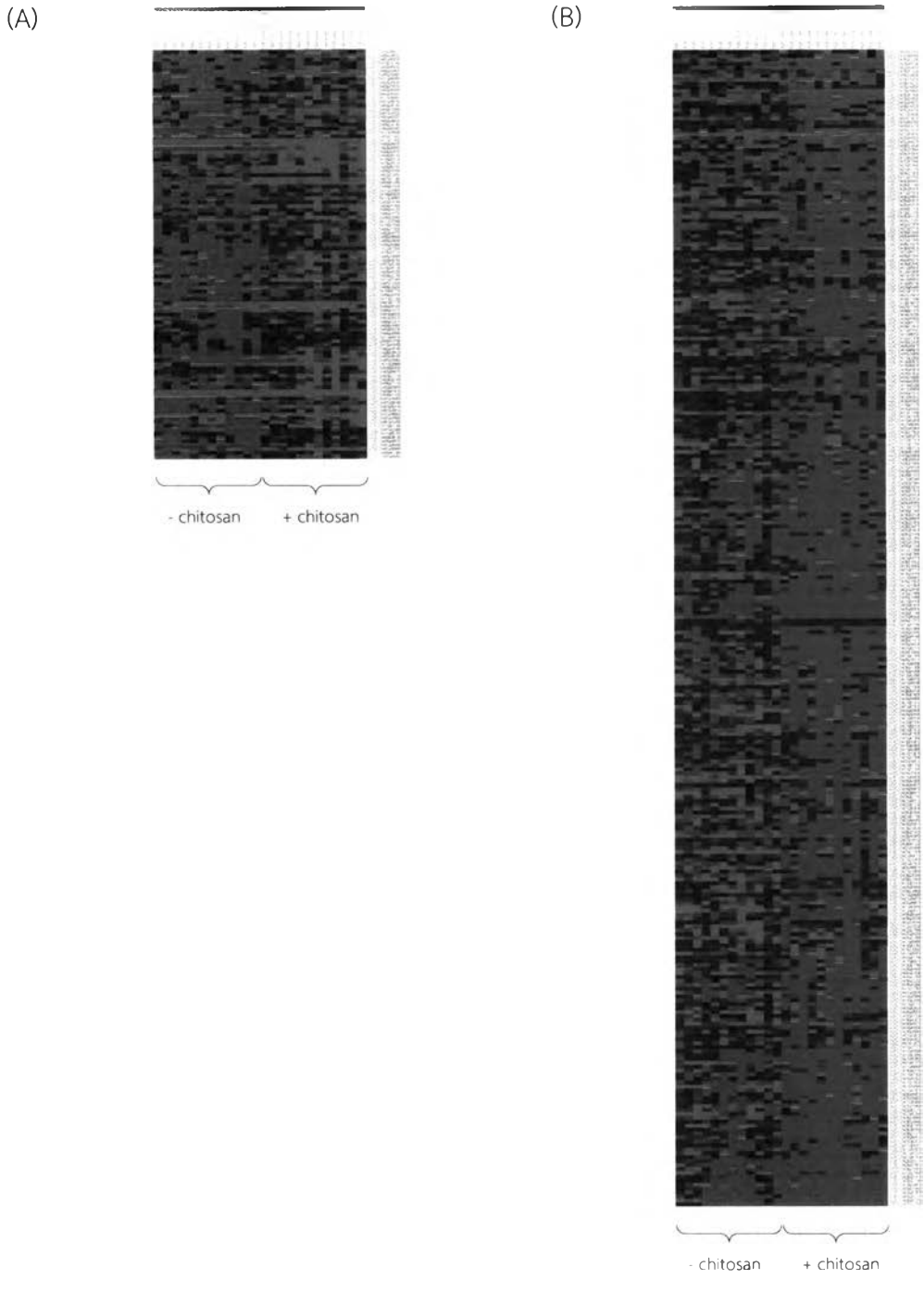


Figure 4.2 Expression patterns of (A) significantly chitosan up-regulated proteins and (B) significantly chitosan down-regulated proteins during drought stress found in leaf tissue of LPT123 rice at 0, 2, 6 and 24 hours after drought treatment. Heat map were created using MultiExperiment Viewer (MeV) software. Each row shows an individual protein. Light green to light red color bar represents low to high protein abundance.

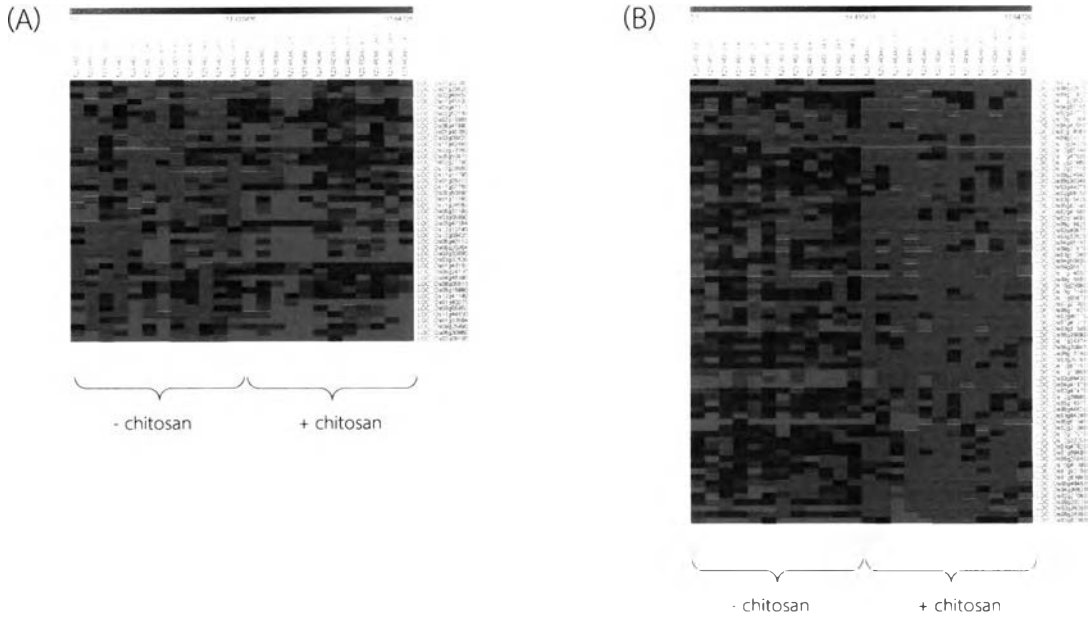
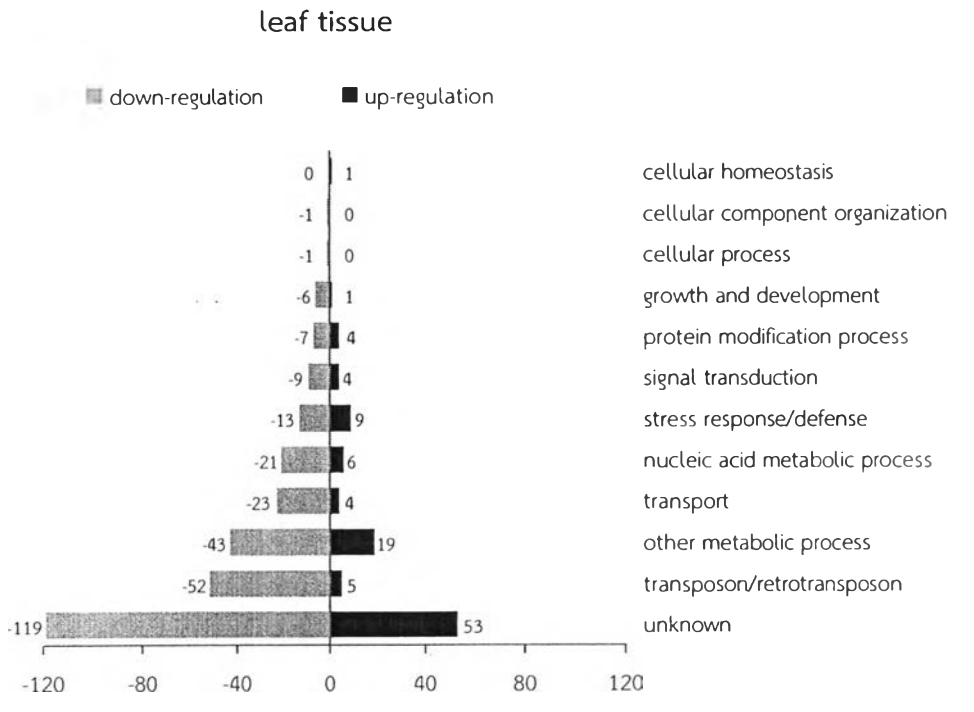


Figure 4.3 Expression patterns of (A) significantly chitosan up-regulated proteins and (B) significantly chitosan down-regulated proteins during drought stress found in root tissue of LPT123 rice at 0, 2, 6 and 24 hours after drought treatment. Heat map were created using MultiExperiment Viewer (MeV) software. Each row shows an individual protein. Light green to light red color bar represents low to high protein abundance.

(A)



(B)

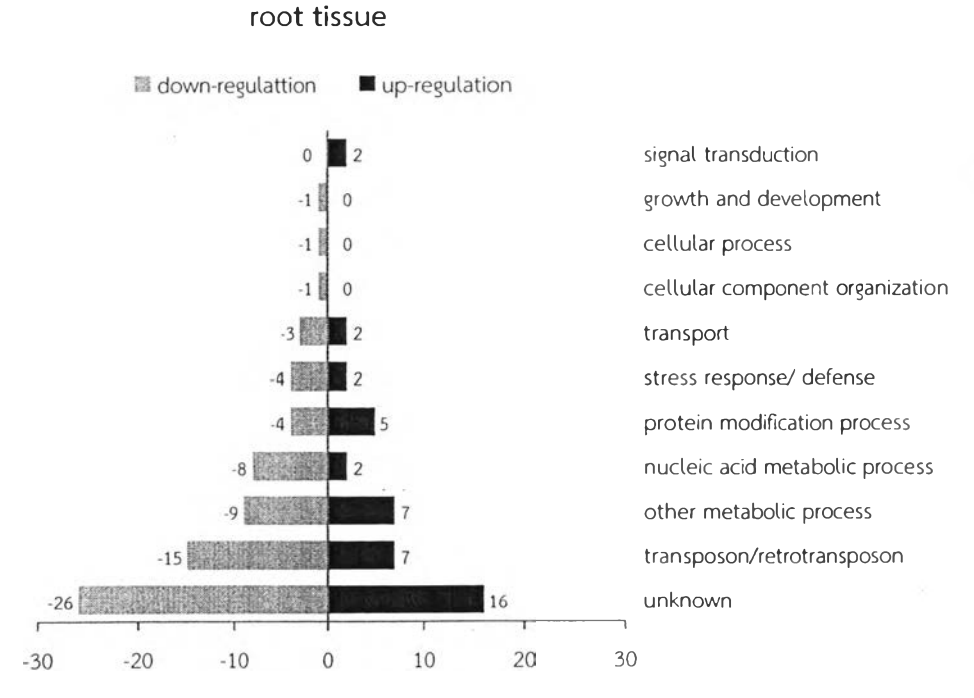


Figure 4.4 Functional classification of chitosan responsive proteins in (A) leaf tissue and (B) root tissue of drought-treated LPT123 rice. Negative sign indicates down-regulation.

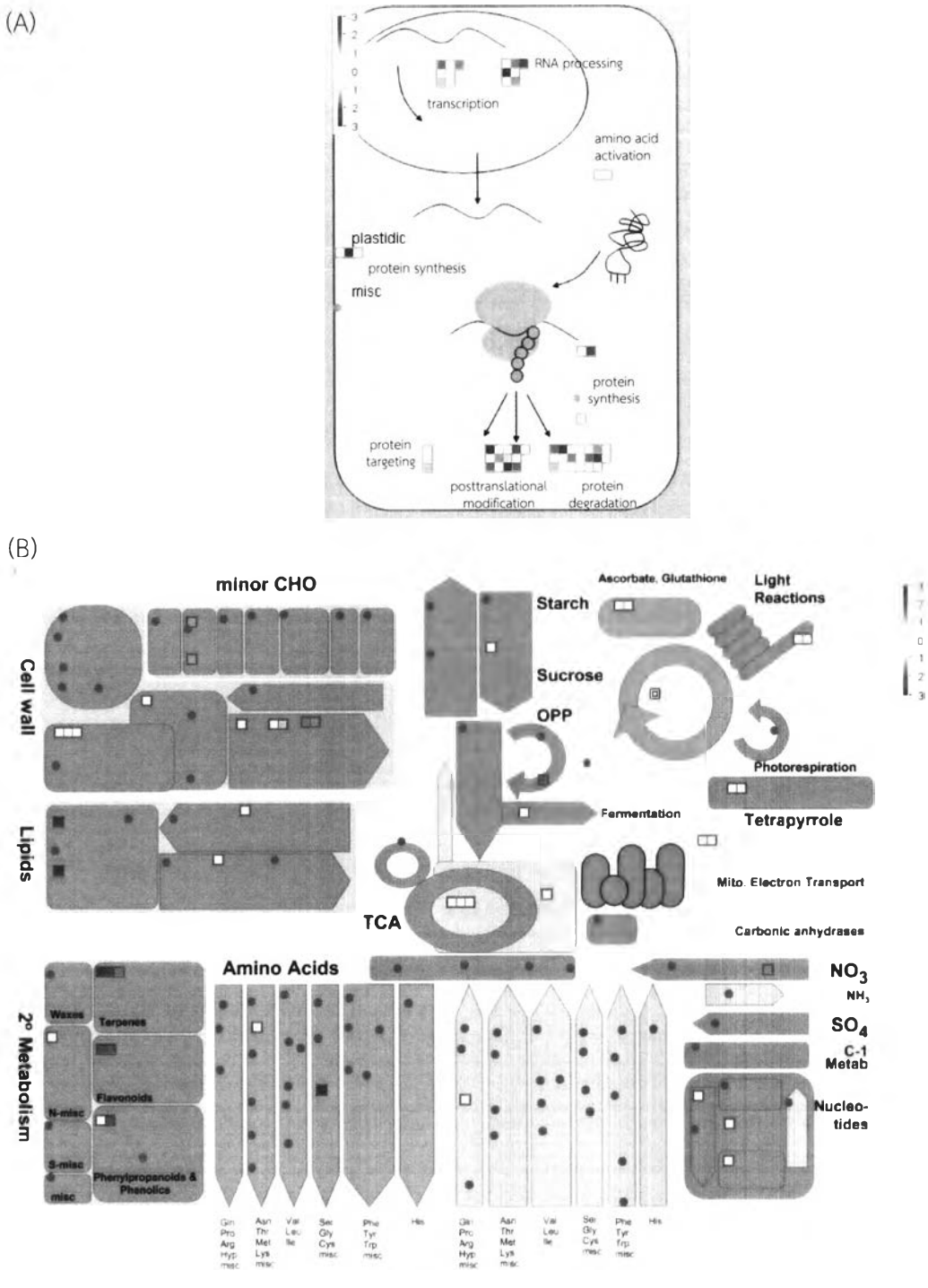


Figure 4.5 Schematic representation of chitosan-responsive proteins in drought-treated LPT123 rice in (A) RNA-protein synthesis and (B) an overview of metabolic pathway created by using MapMan software. Each box represents each protein that is mapped into the pathway whereas gray circles are data bins which no proteins are mapped into. Light green to light red color represents low to high protein abundance.

Chitosan-responsive proteins during drought stress in LPT123-TC171 rice

Proteomic analysis demonstrated that chitosan affected LPT123 and LPT123-TC171 protein patterns during drought stress differently. Interestingly, chitosan-responsive proteins during drought stress found in LPT123-TC171 rice were less than that found in LPT123 rice, but they responded as the same trend as they did in LPT123 rice. Most of them were down-regulated. Within 314 significantly differentially expressed proteins found in leaves, 213 proteins were down-regulated. The others were up-regulated (Figure 4.6 and table D.5). In root part, 88 proteins responded to chitosan during drought stress significantly. Among these proteins, 65 proteins were down-regulated whereas 23 proteins were up-regulated (Figure 4.7 and table D.6).

The proteins were categorized into several groups as detailed in Figure 4.8. Proteins with unknown function and transposable elements were predominant in both leaf and root parts.

In leaf tissues, chitosan up-regulated proteins in drought treated plant included proteins involved in metabolic process as the predominant group, (19%; e.g. glycosyltransferase, acyltransferase and ribulose bisphosphate decarboxylase small chain), nucleic acid metabolic process (10%; e.g. WRKY27, WRKY94 and MYB family transcription factor), stress response/defense (5%; e.g. NBS-LRR disease resistance protein and NB-ARC domain containing protein), transport (4%; e.g. CASP), protein modification process (4%; e.g. CAMK_CAMK_like.24), cellular component organization (2%) and growth and development (2%) (Figure 4.8A and table D.5).

The main group of chitosan down-regulated proteins in leaves of drought treated plant was metabolic process (16%) such as hydroxyacid oxidase 1, amino acid kinase, transketolase, AAA type ATPase family protein and phospholipase D1. The others were proteins belonging to nucleic acid metabolic process (9%; e.g. transcription elongation factor, bZIP transcription factor and homeobox associated leucine zipper), stress response/defense (6%; e.g. superoxide dismutase, copper/zinc superoxide dismutase and NBS-LRR disease resistance protein), transport (3%; e.g. ABC transporter family protein and phosphate carrier protein), protein modification process (3%; e.g. OsWAK81 and tyrosine protein kinase domain containing protein), growth and development (3%; e.g. late embryogenesis abundant domain containing protein and auxin responsive factor 14), cellular process (1%; e.g. kinesin domain containing protein) and cellular component organization (1%; e.g. chloroplast unusual positioning protein) (Figure 4.8A and table D.5).

In root tissues, 23 proteins that were up-regulated in drought treated plant consisted of those related to metabolic process (13%; e.g. ferredoxin-nitrite reductase and jasmonate O-methyltransferase), signal transduction (9%; e.g. receptor protein kinase-like), stress response/defense (9%; e.g. NB-ARC domain containing protein) and cellular component organization (4%) (Figure 4.8B and table D.6).

Furthermore, down-regulated protein during drought stress were classified into metabolic process (12%; e.g. gibberellins 20 oxidase 2 and lipoxygenase), nucleic acid metabolic process (11%; e.g. bZIP transcription factor and splicing factor 3B subunit 1), stress response response/defense (6%; e.g. peroxidase precursor), protein modification process (3%; e.g. OsWAK103) and transport (1%) (Figure 4.8B and table D.6).

All of these proteins were mapped into an overview of RNA-protein synthetic and metabolic pathways by using MapMan software to facilitate the visualization of the proteins on them (Figure 4.9). The 39 and 29 proteins were mapped into RNA-protein synthetic and metabolic pathways, respectively (table D.7 and D.8). Abundance of proteins assigned to amino acid activation reduced under chitosan treatment. Most of proteins assigned to transcription, RNA processing, protein synthesis, posttranslational modification and protein degradation were chitosan down-regulated proteins (Figure 4.9A and table D.7).

Among 29 proteins mapped into an overview of metabolism, most of them were down-regulated by chitosan treatment. It reduced abundance of many proteins involved in photosynthesis (except ribulose bisphosphate carboxylase small chain), photorespiration, sulfur assimilation, nucleotide metabolism, amino acid metabolism, secondary metabolism, lipid metabolism, cell wall metabolism (degradation), minor carbohydrate metabolism and major carbohydrate metabolism (degradation). Conversely, chitosan increased abundance of proteins assigned to pentose phosphate pathway, cellular respiration, nitrogen metabolism, lipid metabolism and cell wall metabolism (Figure 4.9B and table D.8).

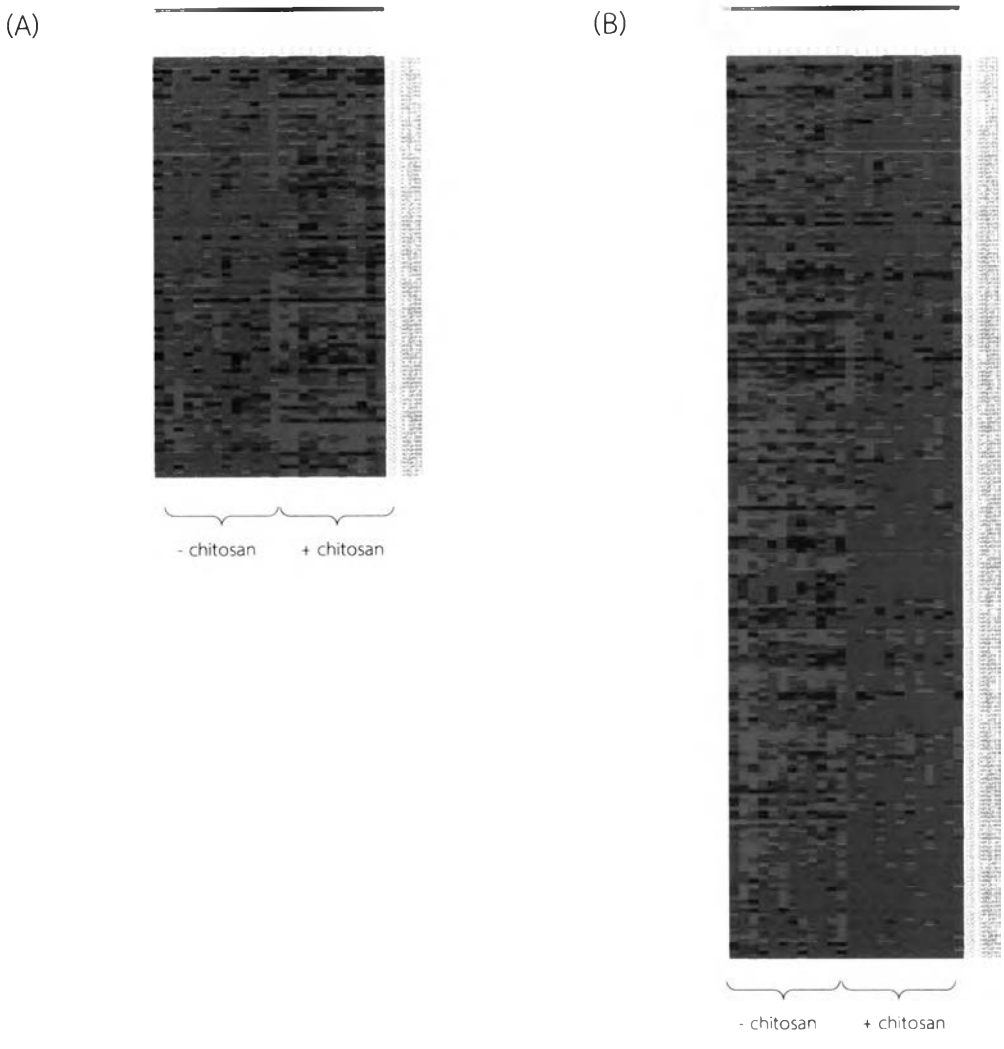


Figure 4.6 Expression patterns of (A) significantly chitosan up-regulated proteins and (B) significantly chitosan down-regulated proteins during drought stress found in leaf tissue of LPT123-TC171 rice at 0, 2, 6 and 24 hours after drought treatment. Heat map were created using MultiExperiment Viewer (MeV) software. Each row shows an individual protein. Light green to light red color bar represents low to high protein abundance.

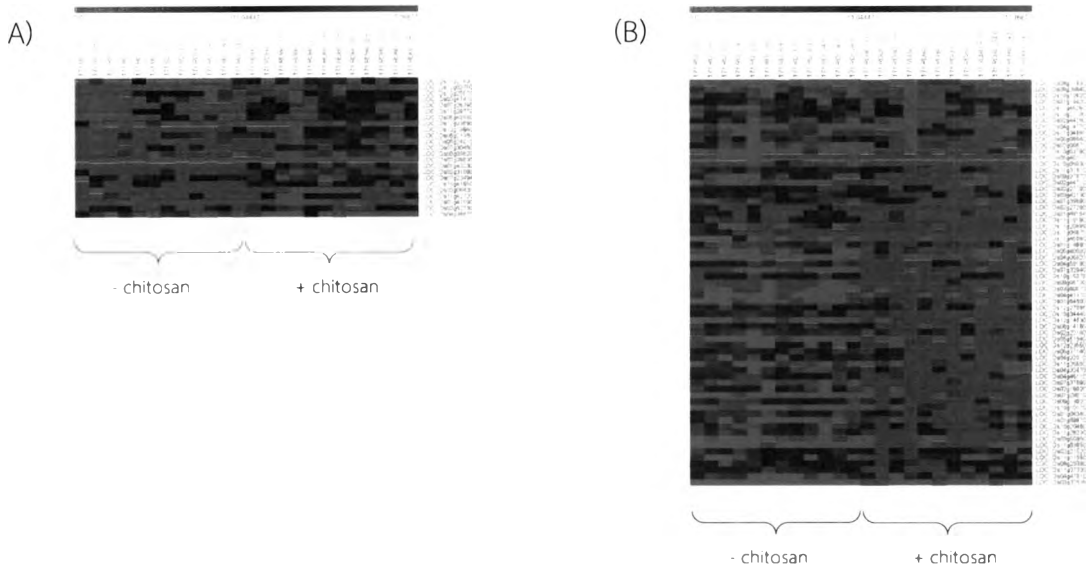
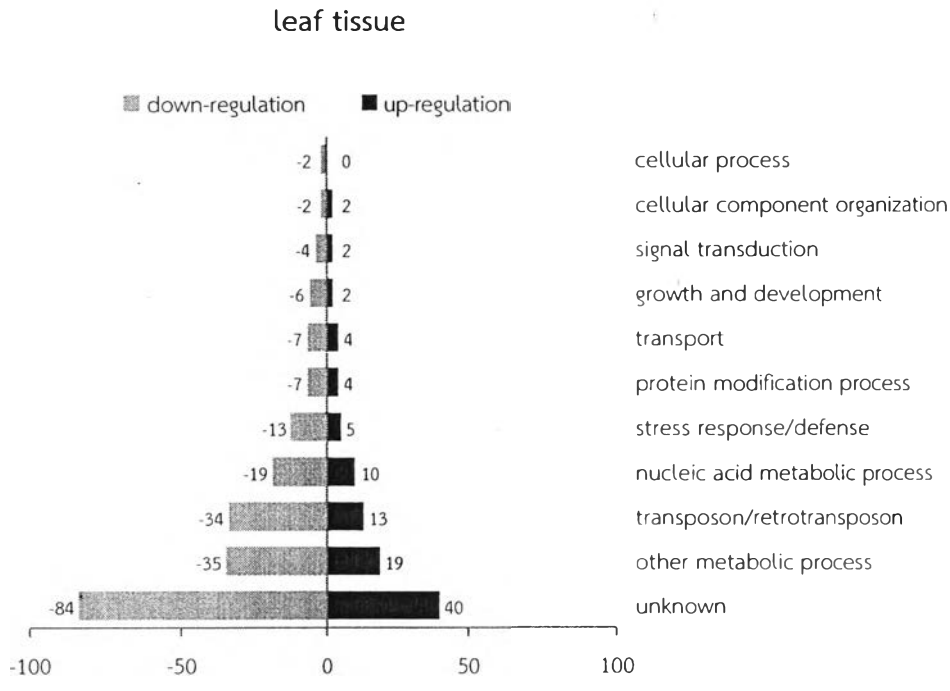


Figure 4.7 Expression patterns of (A) significantly chitosan up-regulated proteins and (B) significantly chitosan down-regulated proteins during drought stress found in root tissue of LPT123-TC171 rice at 0, 2, 6 and 24 hours after drought treatment. Heat map were created using MultiExperiment Viewer (MeV) software. Each row shows an individual protein. Light green to light red color bar represents low to high protein abundance.

(A)



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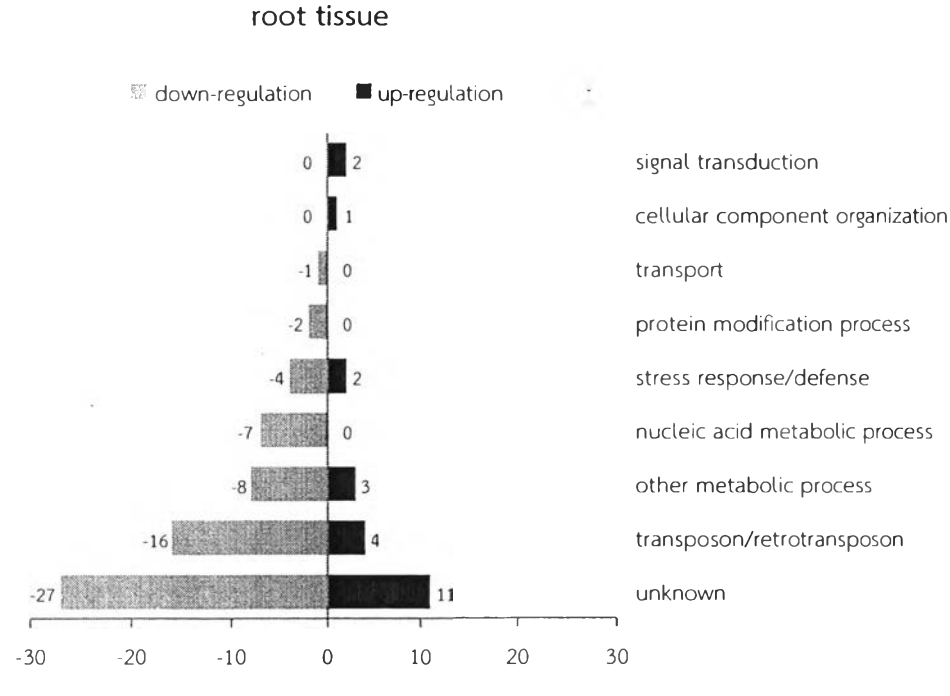


Figure 4.8 Functional classification of chitosan responsive proteins in (A) leaf tissue and (B) root tissue of drought-treated LPT123-TC171 rice. Negative sign indicates down-regulation.

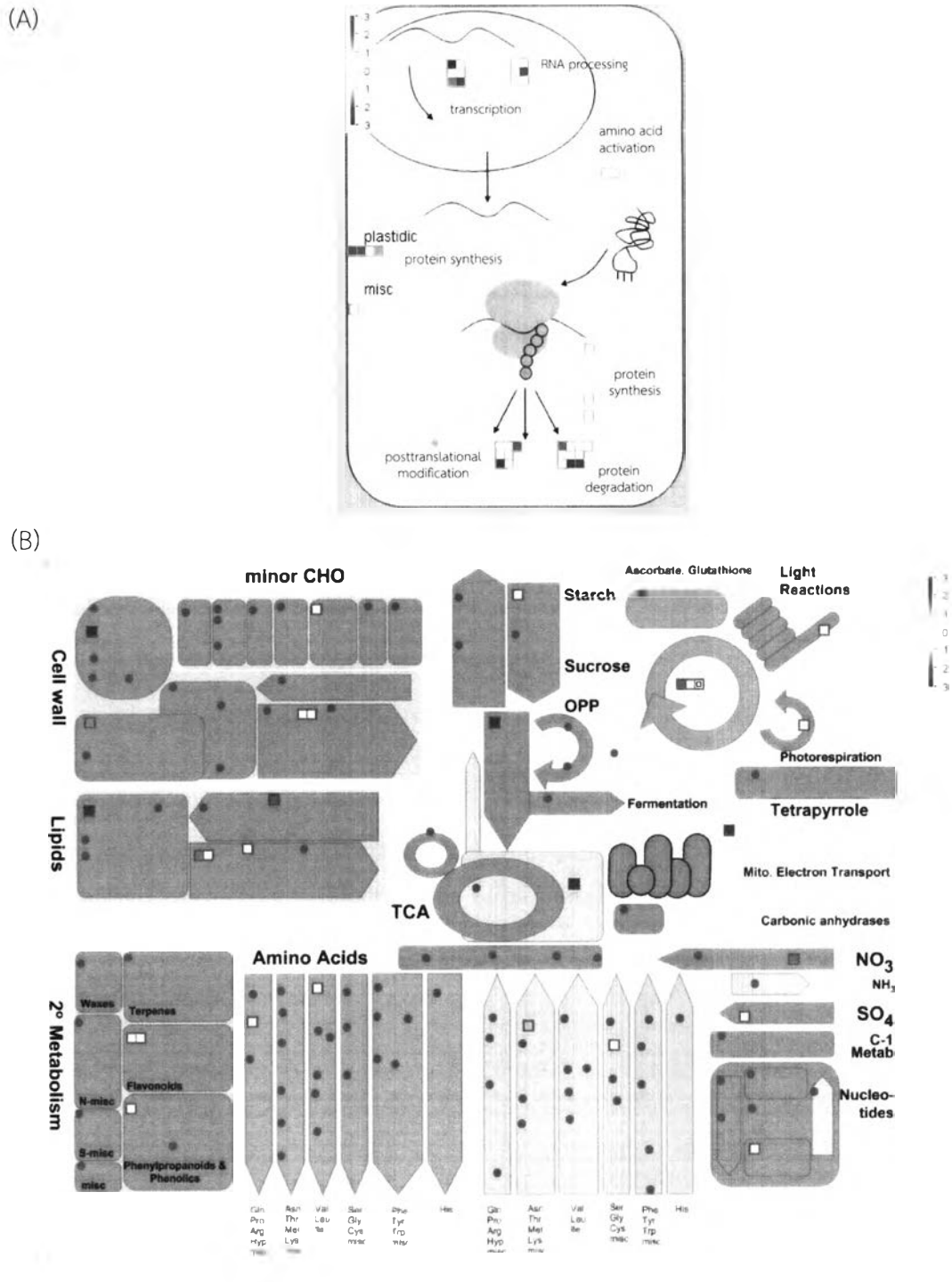


Figure 4.9 Schematic representation of chitosan-responsive proteins in drought-treated LPT123-TC171 rice in (A) RNA-protein synthesis and (B) an overview of metabolic pathway created by using MapMan software. Each box represents each protein that is mapped into the pathway whereas gray circles are data bins which no proteins are mapped into. Light green to light red color represents low to high protein abundance.

Moreover, not only chitosan-responsive proteins under drought stress specific to LPT123 or LPT123-TC171 rice but also similar proteins in both rice lines were found. There were 37 and 13 similar proteins in leaf and root, respectively (Figure 4.10 and table D.1, D.2, D.5 and D.6). Some of these proteins responded in the same manner, up-regulation or down-regulation in both rice lines, for example, ferredoxin-nitrite reductase, an up-regulated protein, and phospholipase D p1 and AAA-type ATPase family protein, down-regulated proteins. The others responded to chitosan during drought stress in the opposite manner in LPT123 and LPT123-TC171 rice such as annexin which was up-regulated in LPT123 rice but it was down-regulated in LPT123-TC171 rice.

According to functional classification, the majority of chitosan responsive proteins found in both LPT123 and LPT123-TC171 rice involved in metabolic process. Based on these data and an overview of metabolism created by MapMan software, proteins involving in predominant metabolic processes in both rice lines were summarized in table 4.1. Chitosan reduced abundance of proteins belonging to photosynthesis in both rice lines (e.g. AAA-type ATPase family protein and NADH dehydrogenase I subunit N in LPT123 rice; AAA-type ATPase family protein, transketolase and chlorophyll A-B binding protein in LPT123-TC171 rice) except ribulose bisphosphate carboxylase small chain which was up-regulated in LPT123-TC171 rice. Abundance of proteins in cellular respiration decreased in LPT123 rice (ATP synthase subunit . beta, NADH-ubiquinone oxidoreductase 49kDa subunit, lactate/malate dehydrogenase, dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, dihydrolipoyl dehydrogenase 1 and aconitate hydratase protein), but increased in LPT123-TC171 rice (ATP synthase subunit beta and lactate/malate dehydrogenase) in chitosan treatment. Proteins involving in osmolyte synthesis in both rice lines responded to chitosan in different manner. In LPT123 rice, trehalose-6-phosphate synthase was up-regulated whereas amino acid kinase and late embryogenesis abundant domain containing protein were down-regulated in LPT123-TC171 rice. Furthermore, chitosan changed abundance of proteins functioning in antioxidant system. Only one protein was up-regulated in each rice line: thioredoxin in LPT123 rice and glutathione-s-transferase in the other. Meanwhile, chitosan treatment led to decreased abundance of two antioxidant enzymes, monodehydroascorbate reductase and peroxidase precursor, in LPT123 rice, but five enzymes, superoxide dismutase, copper/zinc superoxide dismutase, hydroxyacid oxidase 1 and two of peroxidase precursors, in LPT123-TC171 rice.

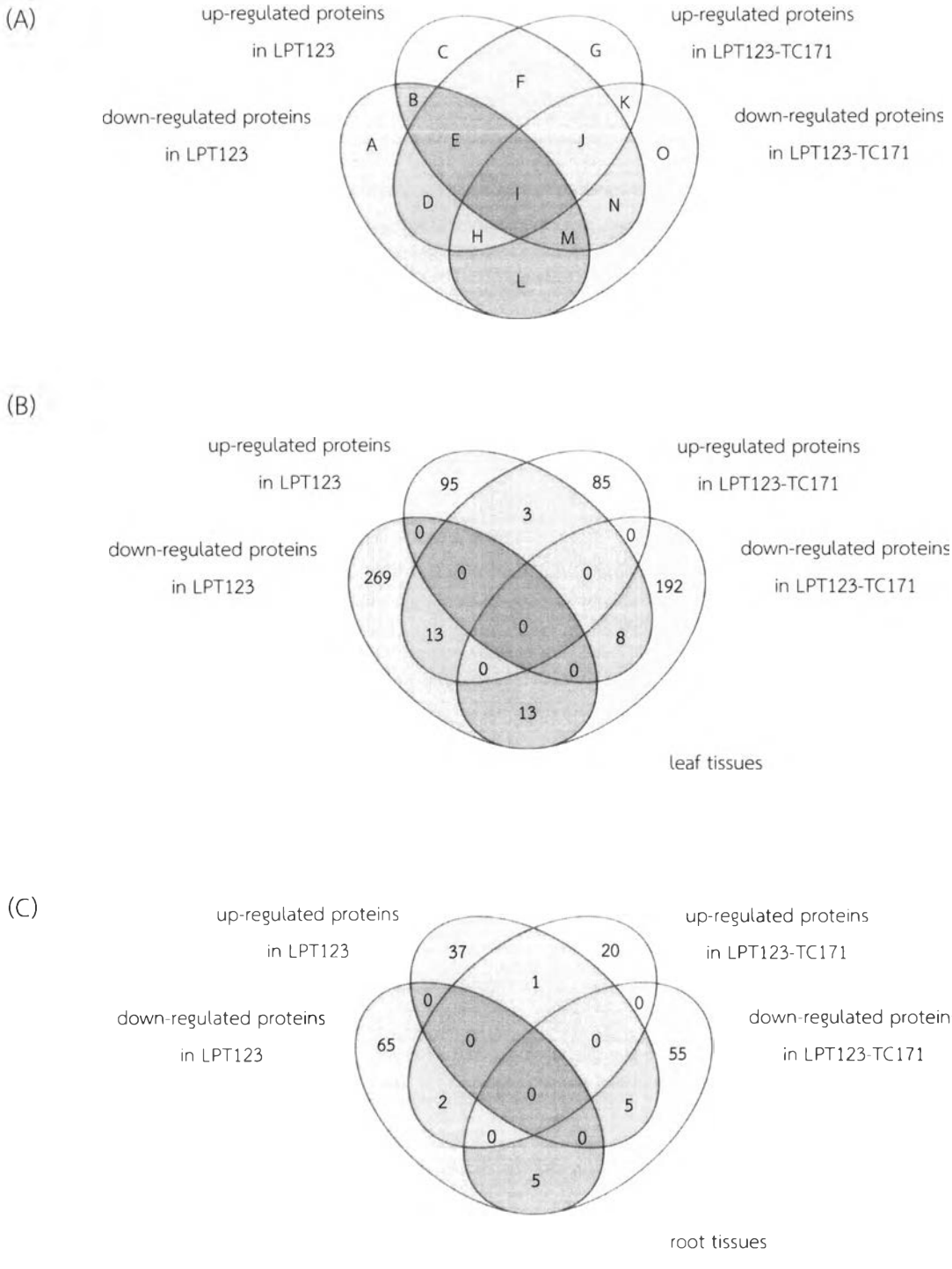


Figure 4.10 Venn diagrams showing significantly chitosan responsive proteins during drought stress. (A) represents Venn diagram pattern. The others are significantly chitosan responsive proteins during drought stress found in (B) leaf tissues and (C) root tissues of LPT123 and LPT123-TC171 rice.

Table 4.1 Up-regulated and down-regulated proteins in predominant metabolic pathways

	LPT123		LPT123-TC171	
	up-regulation	down-regulation	up-regulation	down-regulation
photosynthesis	-	2	1	3
cellular respiration	-	6	2	-
osmolyte accumulation	1	-	-	2
antioxidant system	1	2	1	5

Expression analysis of a chitosan-responsive gene during drought stress, *transcriptional repressor*

Proteomic analysis revealed that protein patterns in LPT123 rice were affected by chitosan application under drought stress more than that in LPT123-TC171 rice. Co-expression network analysis of total chitosan responsive proteins in leaf and root tissues of LPT123 rice showed that transcriptional repressor (LOC_Os01g01960) which was down-regulated in leaf tissues of chitosan-treated LPT123 rice under drought stress was the largest node. A lot of proteins were predicted as partner of this gene, including proteins involved in cell cycle, protein modification process and gene expression (Figure 4.11 and table E.1). Therefore, it was selected to study its expression at transcriptional level.

According to analysis of nucleotide sequence on promoter of the *transcriptional repressor*, a large number of stress-responsive *cis*-acting elements were found (table 4.2 and E.2). *Cis*-acting elements that respond to drought stress, including ABRELATERD1, ACGTABREMOTIFA2OSEM, ACGTATERD1, DRE2COREZMRAB17, DRECRTCOREAT, MYB2AT, MYBCORE, MYCATERD1 and MYCATRD22, were detected. Moreover, some pathogen-associated elements, namely GT1GMSCAM4 and WBOXATNPR1, were found as well. These suggested that this gene can respond to stress. Microarray experiment data showed that it was up-regulated under drought stress (Figure 4.12).

The effect of chitosan on its expression during drought stress at transcription level was monitored with qRT-PCR. Interestingly, it responded to drought stress and chitosan in LPT123 and LPT123-TC171 rice in opposite manner. In LPT123 rice, there was no significant difference in *transcriptional repressor* gene expression due to chitosan application under drought stress condition at any time points in this study. However, its expression level tended to increase in non-chitosan treated plant under drought stress and be higher than that of chitosan-treated plant (Figure 4.13A). These responses were in accordance with those found in microarray experiment and proteomic data, showing the down-regulation of these protein when plants were treated with chitosan. In LPT123-TC171 rice, *transcriptional repressor* transcript was likely to decline during drought stress in non-chitosan treatment, especially at 6 hours after drought stress which showed significantly decrease. On the other hand, its expression was liable to increase under drought condition in chitosan-treated plant except at 6 hours after drought stress which significantly declined (Figure 4.13B). This

result suggested that the *transcriptional repressor* may play a role in different response to chitosan during drought stress in LPT123 and LPT123-TC171 rice.

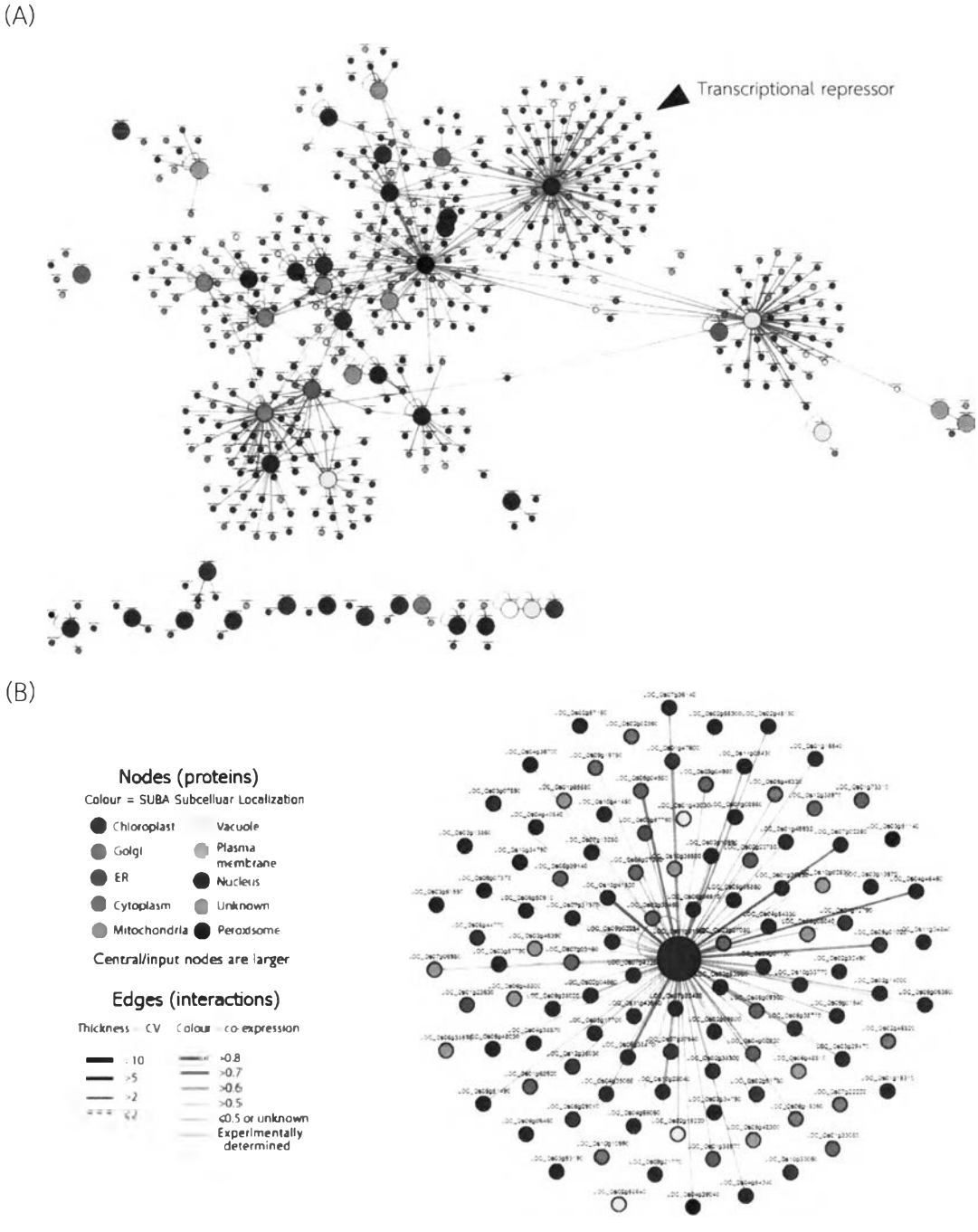


Figure 4.11 Predicted co-expression network of total chitosan responsive proteins in LPT123 rice (A) and the enlargement of predicted co-expression network of transcriptional repressor (B) analyzed with rice interaction viewer in the Bio-Analytic Resource for Plant Biology (http://bar.utoronto.ca). The circles were colored based on their subcellular localization. The color and thickness of edge line indicate Pearson correlation coefficient and interolog confidence value of interaction, respectively.

Table 4.2 *In silico* characterization of stress-responsive *cis*-elements in 2 kb upstream region *of transcriptional repressor*

Site Name	Signal Sequence	Stress Response
ABRELATERD1	ACGTG	drought stress
ACGTABREMOTIFA2OSEM	ACGTGKC	drought stress | salinity stress
ACGTATERD1	ACGT	drought stress
AGCBOXNPGLB	AGCCGCC	salinity stress
ASF1MOTIFCAMV	TGACG	abiotic and biotic stress | xenobiotic stress
DRE2COREZMRAB17	ACCGAC	drought stress | salinity stress | cold stress
DRECRTCOREAT	RCCGAC	drought stress | salinity stress | cold stress | heat stress
GT1GMSCAM4	GAAAAA	pathogen-induced stress | salinity stress
MYB1AT	WAACCA	stress responsive
MYB2AT	TAACTG	drought stress
MYB2CONSENSUSAT	YAACKG	stress responsive
MYBCORE	CNGTTR	drought stress
MYCATERD1	CATGTG	drought stress
MYCATRD22	CACATG	drought stress
MYCCONSENSUSAT	CANNTG	abiotic stress
WBOXATNPR1	TTGAC	pathogen-induced stress response

R=A/G, Y=C/T, K=G/T, S=C/G, W=A/T, B=C/G/T, H=A/C/T, V=A/C/G, N=A/C/G/T

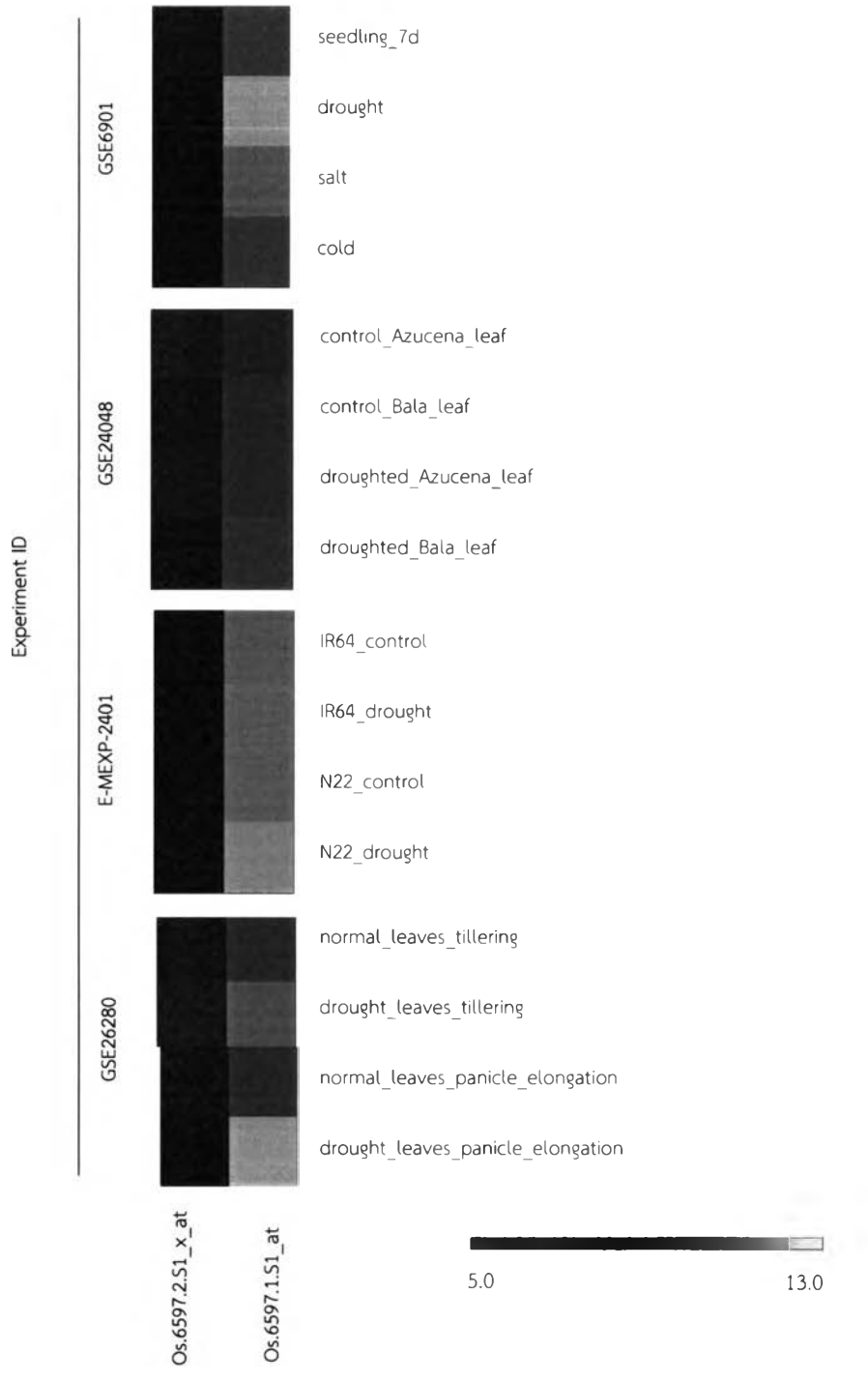
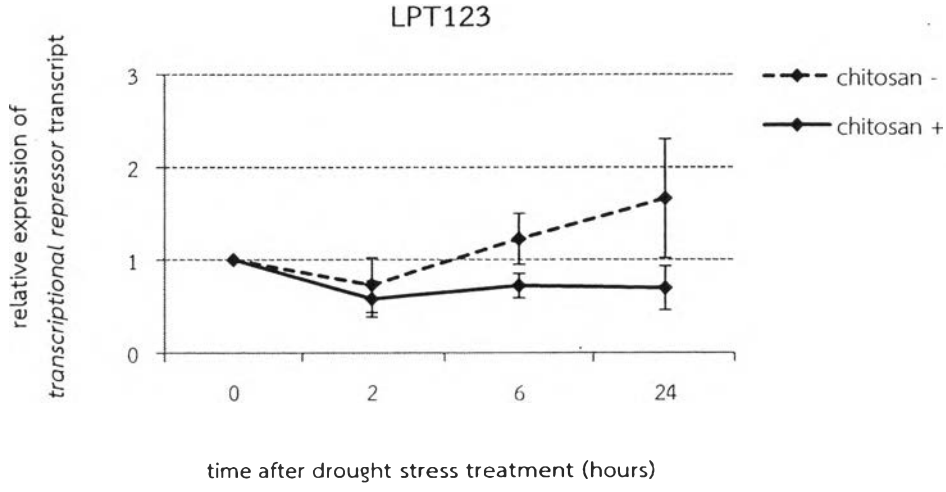


Figure 4.12 Heat map showing expression level of *transcriptional repressor* under abiotic stresses downloaded from Rice Oligonucleotide Array Database (http://www.ricearray.org)

(A)



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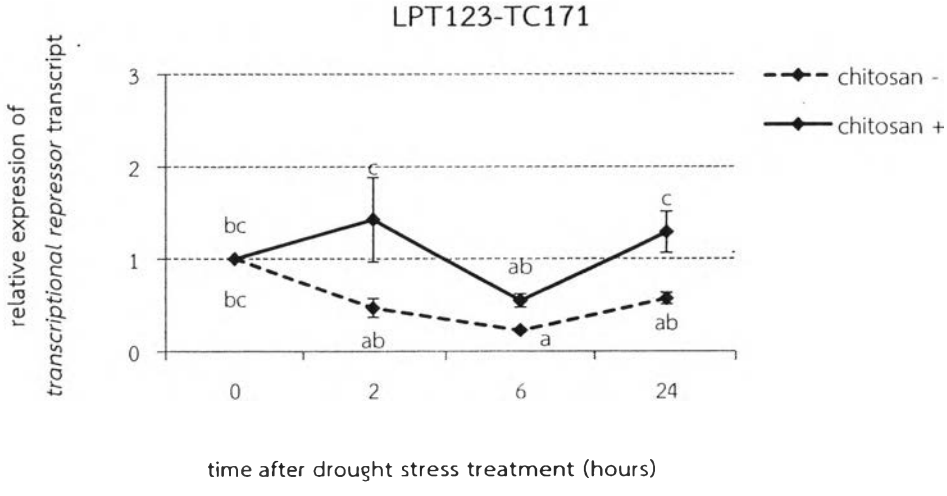

Figure 4.13 Expression patterns of chitosan responsive gene, *transcriptional repressor*, in leaf tissue of (A) LPT123 and (B) LPT123-TC171 rice during drought stress. The data are means of three biological replications with standard error. The lowercase letters indicate significant difference ($P < 0.05$; DMRT).