CHAPTER V
DISCUSSION

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

Mechanism of chitosan response during drought stress in LPT123 rice

Triple chitosan application prior to drought stress not only enhanced shoot growth but also influenced protein expression changes in LPT123 rice more than that of LPT123-TC171 rice. Most of changed proteins were down-regulated in chitosan treatment. Almost half of the changed proteins were proteins with unknown function and transposable elements.

For known function proteins, proteins involved in metabolic process were the predominant group. AAA-type ATPase family protein which is similar to Rubisco activase was down-regulated. The Rubisco activase regulates Rubisco activity by removing the inhibitors. Antisense of this gene in rice resulted in decreasing of initial activity of Rubisco and net photosynthetic rate (Ji et al., 2012). It can be implied that CO_2 assimilation in LPT123 rice may be suppressed because of chitosan application prior to drought stress even the chitosan can increase content of photosynthetic pigments in LPT123 rice and its mutant line (Pongprayoon et al., 2013). Increasing of photosynthetic pigments and chloroplast size by chitosan has been reported (Dzung et al., 2011; Limpanavech et al., 2008). In light reaction of photosynthesis, NADH dehydrogenase I subunit N (Ndhn) was decreased. It has been proposed that it functions in stabilization of plastid Ndh complex (Rumeau et al., 2005) which plays role in cyclic electron transport around PSI. The non-photochemical quenching (NPQ), process for dissipation of excessive light energy, was increased in the *ndhn-1* mutant (Rumeau et al., 2005). These indicate that chitosan application affects photosynthesis both light reaction and CO_2 fixation.

Cellular respiration in LPT123 rice seems to be impaired in chitosan treatment. There were down-regulation of aconitase hydratase which catalyzes citrate to isocitrate, dihydolipoyl dehydrogenase which is component of pyruvate dehydrogenase complex, and dihydolipoyllysine residue succinyl transferase component of 2-oxoglutarate dehydrogenase complex which catalyzes α – keto glutarate to succinyl CoA. These proteins function in TCA cycle. Furthermore, lactate/malate dehydrogenase was also down-regulated. Two proteins involved in mitochondrial electron transport chain, ATP synthase subunit beta and NADH-

ubiquinone oxidoreductase, were decreased. The reduction of proteins involved in cellular respiration suggests that may be to balance the carbon sink-source that correlated with reduction of photosynthesis. Depletion of proteins involved in cellular respiration under drought stress has been reported in 'Penncross' and 'Penn-A4' bentgrass (Xu and Huang, 2010).

Chitosan has been reported that it induced several proteins involved in secondary metabolism including phenylalanine ammonia-lyase (Ferri et al., 2009; Khan et al., 2003; Lin et al., 2005), chalcone flavanone isomerase (Ferri et al., 2009). That is consistent with this study. Several proteins were induced by chitosan during drought stress including terpene synthase 8, transketolase, chalcone synthase, leucoanthocyanidin reductase, transferase family domain containing protein and prenyltransferase. This prenyltransferase is similar to homogentisate prenyltransferase in Arabidopsis (ATHST or PDS2) which involved in plastoquinone-9-biosynthesis and localized to chloroplast membrane. The plastoquinone plays important role as electron carrier in carotenoid biosynthesis, chlororespiration pathway and photosynthetic electron transport (Tian et al., 2007). This may contribute to maintenance of photosynthetic pigments found in chitosan-treated LPT123 rice after 7 days of drought stress (Pongprayoon et al., 2013).

Osmoregulation is common plant adaptation to drought stress. The plant accumulates osmolytes to reduce cellular water potential and consequently can take up water from the low water potential media. It was found that chitosan application could stimulate osmolyte biosynthesis in root tissue of LPT123 rice as trehalose-6-phosphate synthase increased in chitosan treatment. The trehalose-6-phosphate synthase catalyzes glucose-6-phosphate to trehalose-6-phosphate (Iordachescu and Imai, 2008). Overexpression of *OsTPS1* enhanced salt, drought, cold and high temperature stress tolerance in rice (Li et al., 2011). This action of chitosan may result in more drought tolerance and higher plant growth than non-chitosan treatment. Moreover, Increasing of ferredoxin-nitrite reductase in root tissue was found in both LPT123 and LPT123-TC171 rice in chitosan treatment, indicating that chitosan could enhance nitrogen assimilation. There have been reported that exogenous application of nitrogen (soil application) to drought-treated plant can increase nutrient contents, leaf turgor pressure, cell membrane stability (Saneoka et al., 2004), and improve transpiration, photosynthesis and plant growth (Ashraf et al., 2011).

The reduction of CO_2 assimilation in drought stress may result in imbalance between light capture by photosynthetic pigments and utilization in Calvin cycle and

consequently lead to reactive oxygen species (ROS) generation which can damage cellular components (Miller et al., 2010). Plants cope with that situation by activation of antioxidant system to eliminate the ROS (Ji et al., 2012; Miller et al., 2010; Xu and Huang, 2010). Effect of chitosan on antioxidant system has been reported (Yang et al., 2009; Yin et al., 2006). Under drought stress, chitosan application could enhance superoxide dismutase and catalase activity in apple seedling (Yang et al., 2009). In this study, reduction of monodehydroascorbate reductase, which converts monodehydroascorbate into ascorbate (a H_2O_2 dissipating enzyme), and peroxidase precursor was observed in leaf and root tissues, respectively, in chitosan-treated plant under drought stress. Thioredoxin, a redox carrier, was up-regulated in leaf tissue. Previous study showed that chitosan had minor effect on antioxidant enzyme activity in LPT123 rice (Pongprayoon et al., 2013). H_2O_2 accumulation pattern was not drastically changed by chitosan application and the level of H_2O_2 in LPT123 rice was higher than that of its mutant line in both chitosan and non-chitosan treatment (Pongprayoon et al., 2013).

The other proteins involved in stress response/defense were disease resistance proteins. There have been reported that chitosan acts as elicitor and can activate plant defense responses (Povero et al., 2011; Yin et al., 2006). Annexin protein abundance was also changed with chitosan treatment. It is Ca^{2+} dependent phospholipid binding protein. It responded to chitosan differently in LPT123 rice and its mutant line. It was up-regulated in LPT123 rice but down-regulated in LPT123-TC171 rice. Jami et al. (2012) showed that expression of this gene was down-regulated under salt and cold stresses. The function of Arabidopsis annexin have been investigated. Loss of function *annAt1* and *annAt4* mutants resulted in the increased salt and drought tolerance. *AnnAt4* overexpression caused plant more sensitive to stress (Huh et al., 2010). In this study, the down-regulation of annexin in LPT123-TC171 rice did not lead to more drought tolerant ability.

Several proteins belonging to nucleic acid metabolic process responded to chitosan during drought stress such as transcriptional repressor, AP2 domain containing protein, pre-mRNA-splicing factor SLU7, MYB family transcription factor and RNA dependent RNA polymerase. The transcriptional repressor is similar to AtSin3-like3 which acted as a negative regulator of ABA responses. It was down-regulated in chitosan treatment. Its RNA interference lines were hypersensitive to ABA during germination. ABA delayed seed germination, growth and development of the RNAi transgenic plants (Song et al., 2005). MYB transcription factors have been found in abiotic stress responses (Abe et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2006;

Yanhui et al., 2006). Changes of protein abundance in this group may be important to drought tolerant improvement by chitosan application since their role in gene expression regulation.

Chitosan application affected proteins belonging to protein modification process. Most of these proteins were protein kinases and proteins involved in protein degradation such as OsWAK55, OsWAK71, OsWAK81, OsWAK13 and ubiquitin conjugating enzyme. It can be implied that cell requires both protein modification and protein degradation to respond to chitosan during drought stress.

To respond to environmental stimuli, proteins involved in signal transduction pathway are important as they likely regulate cellular responses. Ras-related protein was up-regulated in chitosan treatment. This protein is similar to AtRabA1d which is involved in vasicular trafficking (Takáč et al., 2012). Moreover, there has been reported that chitosan affected proteins in signal transduction pathway. MPK9 and MPK12 involved in chitosan induced stomatal closure (Salam et al., 2012).

Mechanisms of chitosan response during drought stress in LPT123-TC171 rice

Similarly to the significantly changed proteins found in LPT123 rice, most of chitosan-responsive proteins during drought stress in LPT123-TC171 rice were down-regulated and proteins with unknown functions and transposable elements were the majority. However, fewer number of changed proteins due to chitosan application under drought stress were found in this rice line than that of LPT123 rice.

Proteins involved in metabolic process were the main group. In this study, ribulose bisphosphate carboxylase small chain was up-regulated, but AAA-type ATPase family protein, which is similar to Rubisco activase, and transketolase were down-regulated. All of these enzymes function in Calvin cycle. Rubisco activase had higher impact on CO_2 fixation than Rubisco (Fukayama et al., 2012). Moreover, ribulose-1,5-bisphosphate (RuBP) regeneration and photosynthesis were decreased in *transketolase* antisense plant (Henkes et al., 2001). Therefore, it can be inferred from the reduction of these enzymes that CO_2 assimilation in LPT123-TC171 rice may decline in chitosan treatment as found in LPT123 rice. In light reaction, chlorophyll A/B-binding protein (Psbs1) which plays role in photoprotection through non-photochemical quenching (NPQ) was decreased in chitosan treatment. Increasing of *OsPsbs1* expression increased NPQ (Kasajima et al., 2011). Loss of function *psbs* mutant rice was highly sensitive to photoinhibition under high light condition (Koo et al., 2005) and had higher cyclic electron transfer than that of wild type plant

(Zulfugarov et al., 2010). However, recent study in Arabidopsis revealed that NPQ could occur in *psbs* antisense plant, suggesting that Psbs acted as a modulator of NPQ (Johnson and Ruban, 2010).

Unlike in LPT123 rice, proteins involved in cellular respiration, lactate/malate dehydrogenase and ATP synthase subunit beta in LPT123-TC171 rice were up-regulated. Increasing of proteins involved in respiration under drought stress has been reported in grapevine (Cramer et al., 2013). Meanwhile, chitosan-treated LPT123 rice increased proteins involved in secondary metabolism. Chitosan application in LPT123-TC171 rice decreased proteins involved in that process such as leucoanthocyanidin reductase, transferase and chalcone isomerase-3.

Chitosan increased osmolyte biosynthetic enzyme in LPT123 rice but it decreased such protein in LPT123-TC171 rice. Amino acid kinase which is similar to Δ^1-pyrroline-5-carboxylate synthetase (P5CS1) in Arabidopsis, a key enzyme in proline biosynthetic pathway, was down-regulated in chitosan treatment. Abiotic stress induced expression of proline-related genes and proline accumulation in plants (Hien et al., 2003; Igarashi et al., 1997; Yooyongwech et al., 2012). Moreover, it has been found that proline accumulation was higher in stress tolerant plants than that of stress sensitive ones (Choudhary et al., 2005; Hien et al., 2003; Igarashi et al., 1997). Loss of function *OsP5CS2* mutants were more sensitive to abiotic stress than wild-type plants (Hur et al., 2004). Late embryogenesis abundant domain containing protein (OsLEA15) was also down-regulated in chitosan treatment under drought stress. Its expression from semiquantitative RT-PCR analysis was not detected under stress and normal condition, which was consistent with microarray data (Wang et al., 2007). That chitosan down-regulated proteins involving in this osmolyte biosynthesis may result in the improvement of drought tolerance by chitosan in LPT123-TC171 rice not being as well as that in LPT123 rice which the osmolyte biosynthesis was stimulated. Ferredoxin-nitrite reductase in root tissue was also increased as found in LPT123 rice.

Chitosan also changed abundance of antioxidant enzymes in LPT123-TC171 rice. These effects were lesser in LPT123 rice. In LPT123-TC171 rice, the increasing of glutathione-s-transferase was found in leaf tissue. On the contrary, decreased abundance of superoxide dismutase, copper/zinc superoxide dismutase and hydroxyacid oxidase 1 in leaf tissue and two of peroxidase precursors in root tissue was found in chitosan-treated plant. The superoxide dismutase, copper/zinc superoxide dismutase and hydroxyacid oxidase 1 involved in H_2O_2 production. The superoxide dismutase and copper/zinc superoxide dismutase dissipate superoxide by

converting into H_2O_2. Hydroxyacid oxidase 1 or glycolate oxidase functions in photorespiration. It oxidizes glycolate to glyoxylate, which leads to the production of H_2O_2 in peroxisome. Acceleration of glutathione-s-transferase couple with reduction of H_2O_2 production by superoxide dismutase, copper/zinc superoxide dismutase and glycolate oxidase may lead to lower level of H_2O_2 in chitosan treatment compared with control as found in previous study (Pongprayoon et al., 2013). In LPT123-TC171 rice, chitosan decreased H_2O_2 content significantly on day 2 after drought stress (Pongprayoon et al., 2013). The researchers suggested that under such condition, LPT123-TC171 rice did not derive benefit from growth improvement by chitosan. H_2O_2 was proposed as a signaling molecule in biotic and abiotic stress responses. Exogenous application of H_2O_2 couple with chitosan treatment could enhance plant growth in drought-treated LPT123-TC171 rice (Pongprayoon et al., 2013). Several studies showed that H_2O_2 was required for chitosan induced stomatal closure (Iriti et al., 2009; Lee et al., 1999; Srivastava et al., 2009). Furthermore, abundance of disease resistance proteins were changed with chitosan treatment as found in LPT123 rice.

It was found that chitosan application changed abundance of proteins belonging to nucleic acid metabolic process and protein modification process. For example, WRKY27, WRKY94, MYB family transcription factor, bZIP transcription factor, homeobox associated leucine zipper, OsWAK81, OsWAK103, and tyrosine protein kinase domain containing protein. WRKY and MYB transcription factors have been reported to respond to biotic and abiotic stresses (Abe et al., 2003; Povero et al., 2011; Ross et al., 2007; Yamaguchi-Shinozaki and Shinozaki, 2006; Yanhui et al., 2006). This bZIP transcripton factor is OsABI5. It was down-regulated by chitosan treatment. It acted as a negative regulator of ABA. Mutant plant was more tolerance to drought and salt stresses (Zou et al., 2008). As discussed earlier, changes of protein abundance in these groups may be important to drought tolerant improvement by chitosan application since their role in gene expression and protein functional control.

Despite their small number, proteins involving in signal transduction pathway, for example, receptor protein kinase-like and phytosulfokine receptor precursor, play important role to respond to environmental stimuli. These proteins were up-regulated in chitosan-treated LPT123-TC171 rice. Phytosulfokine receptor precursor is similar to PSY1 in Arabidopsis which involved in plant immunity (Mosher and Kemmerling, 2013).

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

LPT123 rice is more responsive to chitosan during drought stress than LPT123-TC171 rice in both growth (Pongprayoon et al., 2013) and protein changes. Co-expression analysis of total chitosan-responsive proteins in LPT123 rice revealed that transcriptional repressor, which was down-regulated by chitosan application under drought stress, was the largest node that correlated with a great number of proteins. These demonstrated its crucial role in cell function.

Bioinformatic analyses show *transcriptional repressor* has the capability to respond to stresses. It contains a large number of stress-responsive *cis*-acting elements in promoter region, for example, ABRE (ACGTATERD1), DRE (DRE2COREZMRAB17 and DRECRTCOREAT), MYB (MYB2AT and MYBCORE) and MYC (MYCATERD1 and MYCATRD22) recognition sites. This suggests that its expression under drought stress may be regulated by basic domain leucine zipper (bZIP) transcription factor, dehydration responsive element binding protein (DREB), MYB transcription factor or basic helix-loop-helix (bHLH) transcription factor, which recognize and bind to those elements. Some of which have been characterized. *OsDREB1A*, *OsDREB2A* and *OsDREB2B* encoded proteins interacting specifically with DRE. *OsDREB1A* was up-regulated under cold stress while both of *OsDREB2* were up-regulated under dehydration and salt stresses (Dubouzet et al., 2003; Matsukura et al., 2010). Overexpression of *OsDREB1* and *OsDREB2B* could improve drought tolerance in transgenic plants (Ito et al., 2006; Matsukura et al., 2010). AtMYC2 and AtMYB2 acted as trans-acting elements binding to MYC and MYB recognition sites in promoter region of *RD22*, a drought and ABA responsive gene, respectively. Transgenic plants overexpressing the *AtMYC2* and *AtMYB2* were drought tolerance (Abe et al., 2003). Microarray experiment data supported drought stress response of the *transcriptional repressor*- it was up-regulated under drought stress, which was the same responsive pattern as it did in LPT123 rice.

According to qRT-PCR analysis, the *transcriptional repressor* tended to express in opposite manner in LPT123 and LPT123-TC171 rice in both drought stress and chitosan application under drought stress. It was likely to up-regulate in LPT123 rice but down-regulate in LPT123-TC171 rice under drought stress. Its expression was liable to decrease in LPT123 rice but increase in the other due to chitosan application under drought stress. In addition, chitosan treatment tended to decrease its transcript in drought-treated LPT123 rice but increase in drought-treated LPT123-

TC171 rice compared with non-chitosan treatment. It encodes protein similar to AtSin3-like3 (Figure E.1). The AtSin3-like3 is a component of transcriptional repressor complex. It represses gene expression through recruitment of DNA binding proteins to form complex at promoter region of target genes. It could interact with AtERF7, an ABA-responsive transcriptional repressor, and HDA19. Moreover, the AtSin3-like3 and HDA19 could trigger transcriptional repression activity of AtERF7. The *AtSin3-like3* acted as a negative regulator of ABA responses. Its RNA interference lines were hypersensitive to ABA during germination. ABA delayed not only the germination but also growth and development in the RNAi plants (Song et al., 2005). Even though the qRT-PCR and proteomic study did not exactly show the same results, which may arise from post-transcriptional control (Mazzucotelli et al., 2008), tendency of responses could be seen. It could be inferred from those study that trend of the reduction in *transcriptional repressor* transcript due to chitosan application under drought stress and maintenance of lower level compared with non-chitosan treated LPT123 rice may make the plant more responsive to ABA than non-chitosan treated one and consequently lead to more drought tolerance. On the other hand, the trend of higher level of its expression in chitosan treated LPT123-TC171 rice may result in less responsive to ABA than non-chitosan treated plants and hence less drought tolerance. These trends correspond with the study of Iriti et al. (2009) showing that chitosan could reduce transpiration by inducing ABA-dependent stomatal closure via H_2O_2 mediated process. That study correlate with the study of Pongprayoon et al. (2013) which showed that chitosan-treated LPT123 rice under drought stress had higher level of H_2O_2 than LPT123-TC171 rice. This study points to the important role of *transcriptional repressor* that may contribute to different responses to chitosan during drought stress in LPT123 and LPT123-TC171 rice.