CHAPTER III
MATERIALS AND METHODS

Plant materials

Two Thai indica rice lines, Leung Pratew123 rice and Leung Pratew123-TC171 rice line, were used in this study.

Seeds of the LPT123 rice were kind gifts of Department of Rice, Ministry of Agriculture and Cooperative, Thailand.

The LPT123-TC171 rice is a salt resistant mutant line derived from somaclonal variation of the LPT123 rice under salt stress condition (Sripinyowanich, 2010; Udomchalotorn et al., 2009; Vajrabhaya and Vajrabhaya, 1991). Therefore, the genetic background of these two rice lines was similar. It is also considered to be a drought resistant line (Klomsakul, 2004; Thikart et al., 2005). Seeds of the LPT123-TC171 rice having been selected under salt stress and self-fertilized for nine generations were provided by the Center of Excellence in Environment and Plant Physiology, Department of Botany, Faculty of Science, Chulalongkorn University.

Instruments

1. Equipments for rice planting
   - cylinder
   - glass bottle 150 ml
   - plastic tray
   - sand
   - sprayer

2. Equipments for sample collection
   - aluminum foil
   - forceps
   - liquid nitrogen container
   - scissors
   - balances (Mettler Toledo AG285, Mettler Toledo, Switzerland)
   - -80 °C deep freezer (Thermo-Scientific, USA)

3. Equipments for Identification of chitosan responsive proteins in LPT123 and LPT123-TC171 rice during drought stress

3.1 Protein extraction and protein concentration measurement

- ice box
- liquid nitrogen container
- microcentrifuge tube 1.5 ml
- micropipette tip 10, 200 and 1,000 µl
- mortar and pestle
- spatula
- autoclave (Taichung, Taiwan)
- micropipette 10, 20, 200 and 1,000 µl (Gilson, France)
- refrigerated centrifuge (Universal 32R, Hettich, Germany)
- spectrophotometer (Agilent Technology, USA) and cuvette
- vortex mixer (Labnet, USA)
- water bath (LabTech, USA)

3.2 Protein separation (SDS-polyacrylamide gel electrophoresis)

- beaker 100 ml
- filter paper (Whatman®, GE healthcare, USA)
- ice box
- micropipette tip 10, 200 and 1,000 µl
- plastic tray
- micropipette 10, 20, 200 and 1,000 µl (Gilson, France)
- shaker (Biosan, USA)
- vertical gel electrophoresis unit (Bio-Rad, USA)

3.3 Protein digestion and peptide analysis (ESI-MS/MS)

- forceps
- glass
- microcentrifuge tube 1.5 ml
- micropipette tip 200 µl
- petri dish (Pyrex, USA)
- scalpel
- vial and insert tube
- wrap

- 96 well microplate
- ESI ion Trap MS (HCT ultra PTM Discovery System, Bruker Daltonik, Germany)
- hot air oven
- microcentrifuge
- micropipette 20 µl
- multi-channel micropipette 200 µl
- shaker (Biosan, USA)
- ultimate 3000 LC system (Dionex, USA)
- -80 °C deep freezer (Thermo-Scientific, USA)

4. Equipments for expression analysis of a chitosan-responsive gene during drought stress, *transcriptional repressor*

4.1 Total RNA extraction

- ice box
- liquid nitrogen container
- microcentrifuge tube 1.5 ml
- micropipette tip 10, 200 and 1,000 µl
- mortar and pestle
- parafilm (Whatman®, GE healthcare, USA)
- spatula
- autoclave (Taichung, Taiwan)
- dry bath incubator (MD-01N model, Major Science, Taiwan)
- gel documentation system (Gel DOC™2000, Bio-Rad, USA)
- horizontal gel electrophoresis system (MiniRun GE-100, Hangzhou Bioer Technology, China)
- micropipette 10, 20, 200 and 1,000 µl (Gilson, France)
- microwave oven (Toshiba, Thailand)
- refrigerated centrifuge (Universal 32R, Hettich, Germany)
- spectrophotometer (Agilent Technology, USA) and cuvette
- vortex mixer (Labnet, USA)
- -20 °C freezer (SANYO biomedical freezer, Japan)

4.2 DNA-free RNA preparation

- ice box
- microcentrifuge tube 1.5 ml
- micropipette tip 10, 200 µl
- parafilm (Whatman®, GE healthcare, USA)
- dry bath incubator (MD-01N model, Major Science, Taiwan)
- gel documentation system (Gel DOC™2000, Bio-Rad, USA)
- horizontal gel electrophoresis system (MiniRun GE-100, Hangzhou BIOER Technology, China)
- micropipette 10, 20, 200 µl (Gilson, France)
- microwave oven (Toshiba, Thailand)
- refrigerated centrifuge (Universal 32R, Hettich, Germany)
- spectrophotometer (Agilent Technology, USA) and cuvette
- -20 °C freezer (SANYO biomedical freezer, Japan)

4.3 First-strand cDNA synthesis

- ice box
- micropipette tip 10 and 200 µl
- PCR tube (Axygen Inc., USA)
- microcentrifuge (Sorvall® Biofuge Pico, Germany)
- micropipette 10, 20 and 200 µl (Gilson, France)
- PCR thermal cycler (PTC-100™, Peltier Thermal Cycler, MJ Research, USA)
- -80 °C deep freezer (Thermo-Scientific, USA)

4.4 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

- ice box
- micropipette tip 10 and 200 µl
- 8 tube strip and flat cap (Bio-Rad, USA)
- CFX96™ Real-Time PCR Detection System (Bio-Rad, USA)
- microcentrifuge (Sorvall® Biofuge Pico, Germany)
- micropipette 10, 20 and 200 µl (Rainin, USA)

Chemicals and reagents

1. Reagents for rice planting
   - distilled water
   - filtered water
   - modified WP nutrient solution (appendix A)
   - 0.01% Triton-x-100
   - 10% Polyethylene glycol (PEG) 6000
   - 80% deacetylated oligomeric chitosan solution at a concentration of 40 mg/L

2. Reagents for sample collection
   - liquid nitrogen (Linde, Thailand)

3. Reagents for identification of chitosan-responsive proteins in LPT123 and LPT123-TC171 rice during drought stress

   3.1 Protein extraction and protein concentration measurement
   - bovine serum albumin (BSA) (2µg/µl)
   - liquid nitrogen (Linde, Thailand)
   - reagent A (alkaline copper reagent; appendix A)
   - reagent B (diluted Folin-Ciocalteu's phenol reagent; appendix A)
   - 0.1% sodium dodecyl sulfate (SDS)
   - 0.15% deoxycholic acid (DOC)
   - 72% trichloroacetic acid (TCA)

   3.2 Protein separation (SDS-polyacrylamide gel electrophoresis)
   - destaining solution (appendix A)
   - distilled water
   - protein loading dye (appendix A)
   - protein ladder 10-250 kDa (New England Biolabs, USA)
   - staining solution (appendix A)
   - tetramethylethylenediamine (TEMED)
   - Tris-glycine electrophoresis buffer (appendix A)
   - 0.5 M Tris HCl pH 6.8
   - 1.5 M Tris HCl pH 8.8
   - 0.1% acetic acid
   - 0.5% sodium dodecyl sulfate (SDS)

- 10% sodium dodecyl sulfate (SDS)
- 10% ammonium persulfate (APS)
- 40% acrylamide

3.3 Protein digestion and peptide analysis (ESI-MS/MS)

- bovine serum albumin (BSA)
- destaining solution (appendix A)
- methanol (Merck , Germany)
- steriled milli Q water
- 10 mM dithiothreitol/10 mM ammonium bicarbonate
- 10 ng trypsin/50% acetronitrile/10 mM ammonium bicarbonate
- 100 mM iodoacetamide/10 mM ammonium bicarbonate
- 0.1% trifluoroacetic acid
- 30% acetronitrile (ACN)
- 50% acetronitrile/0.1% trifluoroacetic acid
- 100% acetronitrile (ACN)

4. Reagents for expression analysis of a chitosan-responsive gene during drought stress, *transcriptional repressor*

4.1 Total RNA extraction

- absolute ethanol (Merck, Germany)
- agarose (USB Corporation, Ohio, USA)
- ethidium bromide (Gibco BRL, USA)
- DEPC-treated RNA loading dye (appendix A)
- DEPC-treated TE buffer (appendix A)
- liquid nitrogen (Linde, Thailand)
- phenol:chloroform:isoamyl alcohol (25:24:1) (v/v)
- RNA extraction buffer (appendix A)
- 5x TBE buffer (appendix A)
- 10 M lithium chloride ($LiCl_2$)
- 3% hydrogen peroxide (H_2O_2)
- 80% ethanol

4.2 DNA-free RNA preparation

- agarose (USB Corporation, Ohio, USA)
- ethidium bromide (Gibco BRL, USA)

- DEPC-treated RNA loading dye (appendix A)
- DEPC-treated TE buffer (appendix A)
- DEPC-treated water (appendix A)
- isopropanol (UNIVAR, Australia)
- phenol:chloroform:isoamyl alcohol (25:24:1) (v/v)
- recombinant DNase I (RNase-free) (Takara Bio Inc., Japan)
- 3 M sodium acetate (CH_3COONa)
- 5x TBE buffer (appendix A)
- 3% hydrogen peroxide (H_2O_2)
- 80% ethanol

4.3 First-strand cDNA synthesis

- M-MLV reverse transcriptase (Promega, USA)
- nuclease-free water
- Oligo (dT) 15 primer (Promega, USA)
- 100 mM dATP, dCTP, dGTP, dTTP (Promega, USA)

4.4 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

- forward primer
- reverse primer
- SsoFast™ EvaGreen® Supermix (Bio-Rad, USA)
- ultrapure water

Methods

1. **Plant growing condition, chitosan application and drought stress treatment**

The experiment was performed as described previously (Pongprayoon et al., 2013). Rice seedlings were treated with chitosan solution three times before subjected to drought stress. Seeds of the LPT123 and the LPT123-TC171 rice were soaked in 80% deacetylated oligomeric chitosan (O80) solution at a concentration of 40 mg/L (chitosan treatment) and distilled water (control treatment) for 24 hours before planted on moist sand in a greenhouse under natural light. Seven days after sowing, the sand was supplemented with modified WP nutrient solution (Vajrabhaya and Vajrabhaya, 1991) to prevent the plants from nutritional deficiency. Fifteen-day-

old plants were sprayed with 40 mg/L O80 solution supplemented with 0.01% Triton-x-100 until fully soaked. The plants sprayed with the distilled water supplemented with the 0.01% Triton-x-100 were used as a control group. Then, seedlings of comparable growth were rigorously transferred to the modified WP nutrient solution and allowed to grow until subjected to drought stress. During this period, nutrient solution level was maintained at the same level every day by addition of filtered water and the plants were supplied with the fresh nutrient solution once a week. The chitosan solution was applied again when the plants were thirty-day-old. Two days after the last chitosan application, the plants were subjected to drought stress by transferring them to the nutrient solution containing 10% polyethylene glycol (PEG) 6000. The plants grown in the nutrient solution without the 10% PEG 6000 were used as a control group.

Leaf and root samples were collected and immediately frozen in liquid nitrogen after 0, 2, 6 and 24 hours of the treatment. The samples were stored at -80 °C prior to further studies.

The experiment was carried out with a completely randomized design (CRD) with three biological replicates. Each of which was a collection of three individual seedlings.

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

2.1 Protein extraction and protein concentration measurement

Total protein was extracted from plant tissues, 300 mg of the leaf tissues or 200 mg of the root tissues, with 0.1% sodium dodecyl sulfate (SDS). The tissues were ground in liquid nitrogen into fine powder using a chilled mortar and pestle and immediately mixed homogeneously with 900 µl of 0.1% SDS for the leaf tissue or 300 µl of 0.1% SDS for the root tissue in microcentrifuge tube. The mixture was incubated at 37 °C for 3 hours before centrifuged at 13,000 rpm for 15 minutes to collect supernatant.

Before protein concentration was determined, the supernatant was aliquoted to purify protein by using deoxycholate - trichloroacetic acid precipitation method (Peterson, 1983) with some modifications. 950 µl of 0.15% deoxycholic acid (DOC) was added to 50 µl of the supernatant, mixed well before incubated at room temperature for 10 minutes. After incubation, 100 µl of 72% trichloroacetic acid (TCA) was added and subsequently incubated once at 4 °C overnight. Protein was harvested by centrifugation at 13,000 rpm for 15 minutes at 4 °C. Upper, aqueous

phase was discarded. Protein pellet was air dried approximately 5 minutes at room temperature before dissolved in 50 µl of 0.15% DOC or 30 µl of 0.15% DOC (for root tissue).

Protein concentration was evaluated using the Lowry's method (Lowry et al., 1951) which is based on the reduction of phosphomolybdotungstate by copper oxidized peptide bond producing hetero-polymolibdenum blue. Reaction between 200 µl of reagent A (alkaline copper reagent; appendix A) and 5 µl of the purified protein sample was performed at room temperature for 30 minutes. 10 µl of the purified protein sample was used for root tissue. Then 50 µl of reagent B (diluted Folin-Ciocalteu's phenol reagent; appendix A) was added and incubated at room temperature for 30 minutes. The absorbance was recorded at 750 nm using spectrophotometer and bovine serum albumin (BSA) as a standard. The concentration of protein was calculated according to the following equation:

Protein concentration (µg/µl)

= average OD750 of sample/m x dilution factor/testing volume

m is slope of standard curve.

2.2 Protein separation (SDS-polyacrylamide gel electrophoresis, SDS-PAGE)

To prepare protein saparation, 15 µg of leaf protein or 20 µg of root protein extracted with 0.1% SDS was incubated at 37 °C overnight or until it dried. Then it was dissolved in 10 µl of 0.5% SDS, mixed well with 20 µl of protein loading dye and boiled for 5 minutes before loaded into a well. The protein sample was separated on SDS-PAGE with 4% stacking gel and 12.5% separating gel (Laemmli, 1970). The electrophoresis was done with constant current at 15 mA. Separated protein was detected with Coomassie Brilliant Blue staining (Sambrook et al., 1989). The gel was immersed in staining solution and shaken gently at room temperature until the protein bands appeared. The solution was removed before the gel was soaked in destaining solution and agitated slowly for an hour. During this period, the destaining solution was changed 3-4 times. After that the gel was destained overnight or until the background was clear and stored in 0.1% acetic acid.

2.3 Protein digestion and peptide analysis (ESI-MS/MS)

To reduce protein complexity in the peptide analysis, gel of each sample was segmented into six parts according to protein molecular weight; 10-20, 20-30, 30-50, 50-80, 80-150 and more than 150 kDa (Figure C.2). Each of which was cut into small pieces about 1 mm^3 and destained. These gel plugs were aliquoted

into 96-well microplate for in-gel digestion which was performed as described by Jaresitthikunchai et al. (2009). The gel plugs were washed by shaking in 200 µl of sterile water for 5 minutes before dehydrated twice by shaking in 200 µl of 100% acetronitrile for 5 minutes. Carbamidomethyl reaction was conducted by incubating the dried gel plugs with 50 µl of 10 mM dithiothreitol/10 mM ammonium bicarbonate for an hour before incubating the gel plugs with 50 µl of 100 mM iodoacetamide/10 mM ammonium bicarbonate in the dark for an hour. After that the gel pieces were dehydrated three times. All of these processes, the former solution in the plate was always taken away before new solution was added. Proteins were digested with 40 µl of trypsin solution (10 ng trypsin in 50% acetronitrile/10 mM ammonium bicarbonate) at room temperature for 20 minutes, subsequently immersed in 30 µl of 30% acetronitrile and incubated overnight. The digested peptide solution was carefully transferred to a new plate (avoiding any of gel pieces) and the residues in the gel pieces were extracted twice by adding 30 µl of 50% acetronitrile/0.1% trifluoroacetic acid and agitating for 10 minutes. All of the procedures were carried out at room temperature. The extracted peptide solution was dried at 40 °C overnight and stored at -80 °C for further analysis.

Preparing peptide solution for ESI-MS/MS analysis, all of the dried tryptic peptides derived from the same mass interval of the same sample were resuspended in 20 µl of 0.1% trifluoroacetic acid, pooled together in a new microcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes at room temperature to eliminate any of contaminants. The bovine serum albumin (BSA) serving as internal standard to normalize protein intensity was added into tryptic peptide solution. The peptides were analyzed by ESI-MS/MS using an Ultimate 3000 LC system (Dionex, USA) coupled with an ESI-Ion Trap MS, HCT ultra PTM Discovery System (Bruker Daltonik, Germany).

2.4 Protein quantification and identification

The raw data from LC-MS/MS analysis were converted into mzXML format with CompassXport 1.3.10 program (Bruker Daltonik GmbH). Proteins were quantified with DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare) (Johansson et al., 2006; Thorsell et al., 2007) and identified with MASCOT software (Matrix Science, London, UK) (Perkins et al., 1999) by searching against non-redundant database of National Center for Biotechnology Information (NCBInr) 20130206 with the following parameters, taxonomy: *Oryza sativa* (rice), enzyme: trypsin, allow up to: 1 missed cleavage, fixed modifications: carbamidomethyl (C), variable

modifications: oxidation (M), peptide tolerance: ± 1.2 Da, MS/MS tolerance: ± 0.6 Da, peptide charge: 1+, 2+ and 3+ (monoisotopic) and instrument: ESI-TRAP. Protein loci and functions in biological process were assigned by using blastp and gene ontology (GO) browsers in rice genome annotation project (http://rice.plantbiology.msu.edu) (Ouyang et al., 2007), respectively. For the proteins assigned to the same locus, a protein having the highest Mascot score was selected. In the case of equal Mascot score, ANOVA *P*-value derived from analysis with DeCyder MS Differential Analysis software would be considered. Protein having the lowest *P*-value was chose.

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

There are four treatments in this experiment, drought-treated LPT123 rice with and without chitosan applications and drought-treated LPT123-TC171 rice with and without chitosan applications. Venn diagrams were created to show proteins found in each treatment in leaf and root parts. The protein missing at least 2 of 3 replicates was assigned as absent. In each treatment, protein appearing in at least one of four time points was designated as present in the treatment.

Total proteins which were found in LPT123 or LPT123-TC171 rice were subjected to statistical analysis. T-test ($P < 0.05$) was adopted to identify chitosan responsive proteins during drought stress in each rice line. Hierarchical clustering analysis grouped the significant expressed proteins into two main groups, down-regulation and up-regulation. Heat map was also generated by using MultiExperiment Viewer (MeV) software (Saeed et al., 2003). MapMan software v. 3.5.1R2 (http://mapman.gabipd.org) was employed to visualize the proteins in biological pathway. For each treatment, protein intensities of four time points were averaged. The data were represented as fold change of average protein intensity in chitosan treatment compared with non-chitosan treatment under drought stress.

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

From proteomic data, a gene significantly responding to chitosan treatment during drought stress was selected to study its expression at transcriptional level by using qRT-PCR.

3.1 Bioinformatics

Co-expression network of total chitosan responsive proteins in leaf and root tissues of LPT123 rice were predicted with rice interactions viewer in the Bio-Analytic Resource for Plant Biology (http://bar.utoronto.ca) (Ho et al., 2012). Transcriptional repressor (LOC_Os01g01960) which was down-regulated in leaf tissues of chitosan-treated LPT123 rice under drought stress was the largest node. Therefore, it was selected to study its expression at transcriptional level. To verify this gene responds to stress, *in silico* analysis of *cis*-acting elements in promoter region and expression level under abiotic stress were performed. Putative stress-responsive *cis*-acting elements in 2 kb upstream region of *transcriptional repressor* gene were annotated by using Rice Stress-Responsive Transcription Factor Database (http://www.nipgr.res.in/RiceSRTFDB.html) (Priya and Jain, 2013). Its expression level under abiotic stresses were evaluated from microarray experiments (GSE6901, GSE24048, E-MEXP-2401 and GSE26280) in Rice Oligonucleotide Array Database (http://www.ricearray.org) (Jung et al., 2008). The microarray experiment details are in table E.2-E.5.

3.2 Expression analysis of *transcriptional repressor* gene at transcriptional level

Both LPT123 and LPT123-TC171 rice were grown and treated with chitosan with the same condition as that of proteomic study. Samples were collected and immediately frozen in liquid nitrogen after 0, 2, 6 and 24 hours of drought treatment. The experiment was carried out with a completely randomized design (CRD) with three biological replicates. Each of which was a collection of three individual seedlings.

3.2.1 Total RNA extraction

Plant total RNA was extracted from leaf sample that was treated and harvested with the same condition as that of proteomic study by hot phenol method (Thikart et al., 2005) with some adjustments. Leaf tissue was ground in liquid nitrogen into fine powder using a cooled mortar and pestle and immediately homogenized in an equal volume of RNA extraction buffer and phenol:chloroform: isoamyl alcohol (25:24:1, v/v) which was preheated at 80 °C. The homogenate was centrifuged at 13,000 rpm for 15 minutes at 4 °C. Supernatant was thoroughly transferred into a new microcentrifuge tube, mixed well with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) before re-centrifuged at 13,000 rpm for 15 minutes at 4 °C. The upper, aqueous phase was carefully transferred into a

fresh microcentrifuge tube, mixed well with two volumes of ice cold absolute ethanol and incubated at -20 °C for 30 minutes. The pellet was harvested by centrifugation at 13,000 rpm for 15 minutes at 4 °C. The pellet was washed with ice cold 80% ethanol and air dried at room temperature before resuspended in 160 µl of ice cold DEPC-treated TE buffer. RNA was precipitated by incubation with 40 µl of ice cold 10 M lithium chloride at -20 °C overnight. The RNA was thawed on ice before pelleted by centrifugation at 13,000 rpm for 15 minutes at 4 °C. After the centrifugation, the RNA pellet was washed with ice cold 80% ethanol and air dried at room temperature. Ultimately, the RNA pellet was dissolved in 20 µl of ice cold DEPC-treated TE buffer and stored at -20 °C until use.

The quality of RNA was checked by running the RNA sample on 0.8% agarose gel electrophoresis. Agarose solution was prepared by melting agarose powder in 0.5x TBE buffer. 1 µl of RNA sample was mixed with 1 µl of 6x RNA loading dye and 4 µl of DEPC-treated water and then loaded into a well. The electrophoresis was carried out at 100 Volt. The RNA was stained with 0.5 µg/ml of ethidium bromide for 15 minutes and visualized under UV light by using gel documentation system. RNA concentration and purity were determined by measuring absorbance at 260 and 280 nm using spectrophotometer and calculated according to the following equations:

RNA concentration (µg/ml) = A_{260} x dilution factor x 40

RNA purity = A_{260}/ A_{280}

* The concentration of RNA is around 40 µg/ml when A_{260} is 1 (Sambrook et al., 1989).

3.2.2 DNA-free RNA preparation

To prepare DNA-free RNA, recombinant DNase I (RNase-free) was used to eliminate contaminated DNA from RNA samples, which based on manufacturer's instruction. The 10 µg of total RNA was incubated with DNase I reaction mixture containing 5 µl of 10x DNase I buffer and 2 µl of DNase I (10 units) at 37 °C for an hour. Total volume of this reaction was adjusted with DEPC-treated water to 50 µl. After incubation, 100 µl of DEPC-treated water and 150 µl of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) were added, mixed gently and then centrifuged at 12,000 rpm for 5 minutes at 4 °C to collect supernatant. RNA was precipitated by incubation with 0.1 volumes of ice cold 3 M sodium acetate and 0.6 volumes of ice cold isopropanol at -20 °C for 30 minutes. The DNA-free RNA pellet was harvested by centrifugation at 12,000 rpm for 10 minutes at 4 °C. The pellet was

washed with ice cold 80% ethanol, air dried at room temperature and resuspended in 10 µl of DEPC-treated TE buffer.

To confirm the genomic DNA was removed completely, agarose gel electrophoresis was performed. RNA concentration and purity were evaluated at 260 and 280 nm.

3.2.3 First-strand cDNA synthesis

First strand cDNA was synthesized by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) according to manufacturer's protocol. 2 µg of DNA-free RNA was incubated with 0.5 µg of oligo$(dT)_{15}$ primer at 70 °C for 5 minutes and cooled on ice immediately. The mixture containing 5 µl of 5x M-MLV reaction buffer, 5 µl of 10 mM dNTP and 200 units of M-MLV RT was subsequently added and mixed gently. The reaction was incubated at 37 °C for an hour. The first strand cDNA was stored at -80 °C.

3.2.4 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The quantitative expression of *transcriptional repressor* transcript was monitored with CFX96™ Real-Time PCR Detection System (Bio-Rad, USA) by using specific primers; a forward primer: 5'-CTGG AAAGGAATAAGCGGAAG-3' and a reverse primer: 5'-GCAGCTTCTCAGGCACATTT-3' to amplify a PCR product of 211 bps. The 10 µl qRT-PCR solution contained 0.5 µl of 5 µM forward primer, 0.5 µl of 5 µM reverse primer, 5 µl of 1x SsoFast™Evagreen® Supermix, 3 µl of sterile water and 1 µl of 1:2 dilution of first strand cDNA. The qRT-PCR conditions was set as followed: enzyme activation at 95 °C for 30 seconds, 40 cycles of PCR (denaturation at 95 °C for 5 seconds and annealing/extension at 56.4 °C for 30 seconds) and finally, melting curve analysis at 70-90 °C for 5 seconds. The qRT-PCR of each sample was done with three technical replicates and three biological replicates.

The expression of a housekeeping gene, *OsEF-1α* detected with specific primers; a forward primer: 5'-ATGGTTGTGGAGACCTTC-3' and a reverse primer: 5'-TCACCTTGGCACCGGTTG-3' (Saeng-ngam et al., 2012) was used to normalize *transcriptional repressor* gene expression. The 10 µl qRT-PCR solution contained 0.5 µl of 5 µM forward primer, 0.5 µl of 5 µM reverse primer, 5 µl of 1x SsoFast™Evagreen® Supermix, 3 µl of sterile water and 1 µl of 1:5 dilution of first strand cDNA. The reaction was carried out under the same condition as that of *transcriptional repressor* gene.

Relative expression level of *transcriptional repressor* gene was calculated according to ΔΔCT method which is based on real-time PCR efficiency (*E*) and CP deviation (Pfaffl, 2001). The CP is defined as the point at which the fluorescence signal rises appreciably above the background fluorescence. The expression of *transcriptional repressor* and *EF-1α* in each treatment at 0 hour (without drought stress) were set as controls of interested gene and reference gene, respectively.

$$R = \frac{(E_{target})^{\Delta CP\ target(control-sample)}}{(E_{ref})^{\Delta CP\ ref(control-sample)}}$$

R = relative expression ratio of target gene

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

E_{ref} = $10^{-1/slope}$ of reference gene

ΔCP_{target}(control-sample) = $CP_{0\ hour}$ – $CP_{any\ time\ point\ of\ interested\ gene}$

ΔCP_{ref}(control-sample) = $CP_{0\ hour}$ – $CP_{any\ time\ point\ of\ reference\ gene}$

3.2.5 Statistical analysis

The effect of chitosan on the relative expression level of *transcriptional repressor* transcript was tested by analysis of variance (ANOVA) at $P < 0.05$ with SPSS Statistics 17.0 software (IBM SPSS Modeler). Where this effect was significant, means were compared by Duncan's multiple range test (DMRT). The significant difference was accepted at $P < 0.05$. The data were shown as mean ± S.E.