CHAPTER III

METHODOLOGY

I. MATERIALS

1. Plant materials

Rice (*Oryza sativa* L.) cultivar 'Leung Pra Tew 123' (LPT123) obtained from the Agriculture Department, Ministry of Agriculture and Cooperation, Thailand

Arabidopsis thaliana ecotype Columbia-0 (Col)

Arabidopsis thaliana ecotype Landsberg *erecta* (Ler)

Arabidopsis thaliana SALK T-DNA insertion mutants ordered from Arabidopsis Biological Resource Center (ABRC)

2. Instruments

2.1 Equipment for rice growing

Grass bottle 50 mL
Grass bottle 100 mL
Plastic tray 8 x 12 x 4 $inch^2$
Sand

2.2 Equipment for Arabidopsis growing

3M™ micropore™ medical tape (3M, USA)
Dessicator jar
Fisherbrand™ Square Disposable Petri dish with grid (Fisher Scientific, USA)
Microcentrifuge tube 1.5 mL
Micropipette (Gilson, USA)
Plastic pot
Plastic tray
Soil (Sunshine Professional Peat-Lite mix 4; SunGro Horticulture, Vancouver, BC, Canada)

2.3 Equipment for collecting plant sample

Aluminum foil
Balance (Sartorius CP423s; Scientific Promotion Co., USA)
Deep freezer -80°C
Forceps
Scissors

2.4 Equipment for proteomic analysis

Digital camera
ESI ion trap MS (HCT ultra PTM Discovery system; Bruker, Germany)
Incubator (Gemmyco, USA)
Mortar and pestle
Nano column (Monolithic Nano Column 100 μm i.d. x 5 cm)
Orbital shaker (Biosan, USA)
Pre-column (Monolithic Trap Column 200 μm i.d. x 5 cm)
Protein electrophoresis (Bio-Rad, USA)
Spatula
Spectrophotometer (Agilent Technology, USA)
Surgical scalpel
Ultimate 3000 LC system (Dionex, USA)

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2.5 Equipment for quantitative real-time polymerase chain reaction (qPCR)

CFX96™ Real-Time PCR Detection System (Bio-Rad, USA)
Gel Doc™ 2000 and UV transilluminator (Bio-Rad, USA)
Horizontal gel electrophoresis (MiniRun GE-100; Hangzhou BIOER Technology Co., Ltd., China)
Low-profile 8-tube strips 0.2 ml (Bio-Rad, USA)
Microcentrifuge (Sorvall Biofuge Pico; Germany)
Microcentrifuge tubes 1.5 mL
Micropipette (Gilson, USA)
Mortar and pestle
Optical flat 8-cap strips (Bio-Rad, USA)
PTC-100™ Peltier Thermal Cycler (MJ Research, USA)
Refrigerated centrifuge (Universal 32R; Hettich, Germany)
Spectrophotometer (Agilent Technology, USA)

2.6 Equipment for chitosan-responsive gene identification in *Arabidopsis*

2.6.1 EMS-mutagenized seed preparation

Microcentrifuge tube 1.5 mL
Micropipette (Rainin, USA)
Pasteur pipette
Scintillation grass vial with screw cap 20 mL

2.6.2 Library preparation for high-throughput sequencing

Bioanalyzer (Agilent Technology, USA)
Centrifuge 5424/5424 R (Eppendorf, USA)
DynaMag™-2 Magnet (Invitrogen, USA)
Genome Analyzer II (Illumina Inc., USA)
Micropipette (Rainin, USA)
NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA)
PCR machine (Thermal Cycler; Bio-Rad, USA)
Pellet pestle
The Qubit® 2.0 Fluorometer (Invitrogen, USA)
Thermomixer® R (Eppendorf, USA)
UVP imaging (Labworks software)

2.6.3 ABRC mutant confirmation

Gel Doc™ 2000 and UV transilluminator (Bio-Rad, USA)
Horizontal gel electrophoresis (MiniRun GE-100; Hangzhou BIOER Technology Co., Ltd., China)
Microcentrifuge (Sorvall Biofuge Pico; Germany)
Microcentrifuge tubes 1.5 mL
Micropipette (Gilson, USA)
Microwave oven
Pellet pestle
PCR tubes 0.2 mL (Axygen, USA)
PTC-100™ Peltier Thermal Cycler (MJ Research, USA)
Refrigerated centrifuge (Universal 32R; Hettich, Germany)
Spectrophotometer (Agilent Technology, USA)

3. Chemicals and reagents

3.1 Chemicals for rice growing

Distilled water
Modified WP No.2 nutrient solution (see in Appendix A)
Oligomeric chitosan with deacethylation percentage of 80 (O80) (M_W 20,000) provided by Assistant Professor Dr. Rath Pichyangkura
Triton X-100 (Merck, Germany)

3.2 Chemicals for *Arabidopsis* growing

Distilled water
Hydrochloric acid (HCl) concentrate
Murashige and Skoog medium (MS medium; MP biomedicals, USA)
Oligomeric chitosan with deacethylation percentage of 80 (O80) (M_W 20,000) provided by Assistant Professor Dr. Rath Pichyangkura
Sodium hypochlorite (Clorox®, USA)

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3.3 Chemicals for proteomic analysis

3.3.1 Protein extraction and precipitation

0.1% sodium dodecyl sulfate (SDS)
0.15% deoxycholic acid (DOC)
72% trichloroacetic acid (TCA)
Liquid nitrogen

3.3.2 Protein determination by Lowry method

0.2% copper sulfate ($CuSO_4$)
0.4% tartaric acid ($C_4H_6O_6$)
0.8 N sodium hydroxide (NaOH)
20% sodium carbonate ($NaCO_3$)
5% sodium dodecyl sulfate (SDS)
Bovine serum albumin (BSA) (2 µg/µL)
Folin-Ciocalteu phenol: H_2O (1:5) (v/v)

3.3.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

3.3.3.1 Separating and Stacking gel preparation (see in Appendix A)

0.5 M Tris.HCl pH 6.8
1.5 M Tris.HCl pH 8.8
10% ammonium persulfate (APS)
10% sodium dodecyl sulfate (SDS)
40% (w/v) acrylamide/bis-acrylamide solution (29:1)
Distilled water
Tetramethylethylenediamine (TEMED)

3.3.3.2 Protein electrophoresis

Protein Ladder 10-250 kDa (New England Biolabs, USA)
Protein loading buffer (see in Appendix A)
Tris-glycine running buffer (see in Appendix A)

3.3.4 Coomassie staining and destaining

Coomassie Brilliant Blue R-250
Acetic acid
Methanol
Distilled water

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

0.1% trifluoroacetic acid (TFA)
10 mM ammonium bicarbonate
10 mM dithiothreitol (DTT)
10 ng/mL trypsin (Promega, USA)
100 mM iodoacetamide (IAM)
100% acetonitrile (ACN)
Sterile milli Q water

3.4 Chemicals for quantitative real-time polymerase chain reaction (qPCR)

3.4.1 RNA extraction and cDNA preparation

0.5x TBE (see in Appendix A)
3M sodium acetate (CH_3COONa)
5M sodium chloride (NaCl)
Absolute ethanol (Merck, Germany)
Agarose (Research Organics, USA)
Chloroform (Merck, Germany)
Cloned DNase I (RNase-free) (Takara Bio Inc., Japan)
Concert™ Plant RNA Reagent (Invitrogen, USA)
DEPC-treated distilled water
Elution buffer (Qiagen, Germany)
Ethidium bromide (Promega, USA)
iScript™ Reverse Transcription Supermix (Bio-Rad, USA)
Isopropanol (Merck, Germany)
Liquid nitrogen
Phenol:chloroform:isoamyl alcohol (25:24:1) (v/v)
RNA loading dye (see in Appendix A)

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3.4.2 qPCR

SsoFast™ EvaGreen® Supermix (Bio-Rad, USA)
Distilled water

3.5 Chemicals for chitosan-responsive gene identification in *Arabidopsis*

3.5.1 EMS-mutagenized seed preparation

Ethyl methanesulfonate (EMS) (Sigma-Aldrich, USA)
Sodium hydroxide (NaOH)
Tween-20

3.5.2 Library preparation for high-throughput sequencing

0.5x TBE (see in Appendix A)
100 mM dATP (New England Biolabs, USA)
3M sodium acetate (CH_3COONa)

5M sodium chloride (NaCl)
5x Second strand synthesis buffer (see in Appendix A)
Absolute ethanol (Merck, Germany)
AMPure® XP Beads (Beckman Coulter Inc., USA)
Chloroform (Merck, Germany)
Concert™ Plant RNA Reagent (Invitrogen, USA)
DNA polymerase I (New England Biolabs, USA)
Dynabeads® mRNA Purification Kit (Invitrogen, USA)
Elution buffer (Qiagen, Germany)
End repair module (New England Biolabs, USA)
Ethidium bromide (Promega, USA)
Fragmentase enzyme (New England Biolabs, USA)
GeneRuler™ 1kb DNA ladder plus (Fermentas, USA)
Genomic DNA extraction buffer (see in Appendix A)
Hypure™ molecular biology grade water (HyClone, USA)
Isopropanol (Merck, Germany)
Klenow fragment (New England Biolabs, USA)
Phenol:chloroform:isoamyl alcohol (25:24:1) (v/v)
Phusion DNA polymerase (New England Biolabs, USA)
Quick ligation kit (New England Biolabs, USA)
RNA loading dye
RNase A (Sigma-Aldrich, USA)
SuperScript™ III First-Stand Synthesis System (Invitrogen, USA)
TURBO™ DNase (Ambion, USA)

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3.5.3 ABRC mutant confirmation

0.5x TBE (see in Appendix A)
3M sodium acetate (CH_3COONa)
5M sodium chloride (NaCl)
Absolute ethanol (Merck, Germany)
Agarose (Research Organics, USA)
Chloroform (Merck, Germany)
Cloned DNase I (RNase-free) (Takara Bio Inc., Japan)
Concert™ Plant RNA Reagent (Invitrogen, USA)
Sterile distilled water
dNTP (Roche, Switzerland)

Elution buffer (Qiagen, Germany)
Ethidium bromide (Promega, USA)
Gene-specific primers (see in Appendix D)
GeneRuler™ 1kb DNA ladder plus (Fermentas, USA)
Genomic DNA extraction buffer (see in Appendix A)
iScript™ Reverse Transcription Supermix (Bio-Rad, USA)
Isopropanol (Merck, Germany)
Liquid nitrogen
Phenol:chloroform:isoamyl alcohol (25:24:1) (v/v)
RNA loading dye (see in Appendix A)
Taq DNA polymerase (RBC Bioscience, Taiwan)

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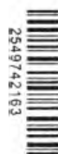
II. METHODS

1. Determination of chitosan effects on protein profiles of rice seedlings after chitosan application

1.1 Plant growth condition

Rice seeds were soaked with oligomeric chitosan with deacetylation percentage of 80 (O80) at concentration of 40 mg/L for 24 hours. Seeds were germinated on sterile sand for 2 weeks; one-week-old seedlings were irrigated once with modified WP No.2 nutrient solution (Vajrabhaya and Vajrabhaya, 1991). After that, seedlings were transferred to grass bottle filled with modified WP No.2 nutrient solution. Rice seedlings were sprayed with 40 mg/L O80 chitosan supplemented with 0.01% Triton X-100 when they were 2-week-old and 4-week-old. The control treatment was sprayed with distilled water containing 0.01% Triton X-100 at the same time period. Plants were grown in a green house under natural light and the temperature was 32°C during the day and 29°C during the night. The nutrient solution was refreshed every 7 days for the whole experiment. The level of solution was maintained every day by addition of water to the same initial solution level. Three biological replicates were used for proteomic analysis. Each biological replicate consisted of three individual seedlings.



1.2 Plant tissues collection

Leaf tissues were separately collected from 4-week-old plants at 0 and 24 hours after the last chitosan treatment and then transfer to new freshly prepared nutrient solution. Plant samples were immediately frozen in liquid nitrogen and stored at -80°C prior to protein extraction.

1.3 Protein extraction and separation by one-dimensional SDS-PAGE

For protein extraction, leaf tissues (300 mg) were ground in liquid nitrogen with mortar and pestle to a fine powder. The powder were homogenized with 0.1% SDS and incubated at 37°C for 3 hours followed by centrifugation at 13,000 rpm for 15 min. The supernatant was collected and used as the total protein extract. The protein contents were determined according to Peterson (1982 using BSA as a standard protein.

The extracted proteins were fractionated on 12.5% polyacrylamide gels one-dimensional SDS-PAGE (Laemmli, 1970) and visualized by Coomassie brilliant blue staining (Meyer and Lamberts, 1965; see in Appendix B). The stained gels were excised according to molecular weight range of protein ladder (see in Appendix B).

1.4 In-gel digestion

The gel fragments in each marker (marker A to marker F) were subjected to in-gel digestion using an in-house method developed by Jaresitthikunchai *et al.* (2009. Briefly, the gel plugs were washed twice with sterile milli Q water, dehydrated with 100% ACN and dried at room temperature. Then, dried gel plugs were reduced with 10 mM DTT/10 mM ammonium bicarbonate at room temperature for an hour and alkylated with 100 mM iodoacetamide (IAM)/10 mM ammonium bicarbonate at room temperature for an hour in a dark container. After that, the gel plugs were dehydrated for three times with 100% ACN for 5 min, then 40 µL of trypsin solution (10 ng/µL trypsin in 50% ACN/10 mM ammonium bicarbonate) was added to the gel plugs and incubated at room temperature for 20 min, then 30% ACN was added and followed by incubation overnight at room temperature. After digestion, the digested peptides were extracted from gel plugs with 30 µL of 50% ACN/0.1% TFA at room temperature for 10 min in an orbital shaker. The extracted peptides were collected, pooled and overnight dried by hot air oven at 40°C. The dried peptides were kept at -80°C for further mass spectrometric analysis.

1.5 Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS/MS)

The digested peptides were analyzed by Ultimate 3000 LC system (Dionex) coupled with ESI-Ion Trap MS (HCT ultra PTM Discovery System; Bruker) with electrospray at a flow rate of 20 µL/min to µ-precolumn (Monolithic Trap Column, 200 µm i.d. x 5 cm). The peptides were separated on a nano column (Monolithic Column, 100 µm i.d. x 5 cm) at a flow rate of 1.0 µL/min. A solvent gradient (Solvent A: H_2O, 0.1% FA; solvent B: 20% H_2O, 80% ACN, 0.1% FA) was started as being 10% - 70%, solvent B at 0-13 min, 90% solvent B at 13-15 min and 10% solvent B at 15-20 min.

1.6 Protein quantitation and identification

The acquired LC-MS/MS data from the different samples were displayed using DeCyderMS Differential Analaysis software (DeCyderMS, GE Healthcare; Johansson *et al.*, 2006; Thorsell *et al.*, 2007). To achieve peptide detection, acquired LC-MS/MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignment, and quantitation based on the peptide ions signal intensities. MS/MS data corresponding to the detected peptides was exported and searched in the National Center for Biotechnology Information (NCBI) non redundant database with Mascot software (Matrix Science, London, UK; Perkins *et al.*, 1999). Search parameters were set as follows: peptide tolerance (±1.2 Da), NCBInr database, *Oryza sativa* L. (taxonomy), carbamidomethylation of cysteine (fixed modification), and methionine oxidation (variable modification). Protein scores were derived from ion scores as a non-probabilistic basis for ranking protein hits, and derived as the sum of a series of peptide scores. To achieve $p<0.05$, the score threshold was set by Mascot algorithm based on the size of the database used in the search. The identified protein data were imported back into DeCyder MS.

1.7 Gene ontology and expression clustering

The identified proteins were used for gene ontology (GO) using rice genome annotation project (http://rice.plantbiology.msu.edu). The significantly expressed protein 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.

1.8 Gene co-expression network construction

The locus numbers of significantly expressed proteins were subjected to construct the gene co-expression network in Rice Oligonucleotide Array Database (http://www.ricearray.org/coexpression/network.shtml; Cao *et al.*, 2012). The predicted localization of the gene co-expression network and GO were retrieved from Rice Genome Annotation Project (http://rice.plantbiology.msu.edu; Ouyang *et al.*, 2007).

1.9 Determination of proteomic-based chitosan-responsive gene expression by quantitative real-time polymerase chain reaction (qPCR)

1.9.1 Total RNA extraction and cDNA preparation

Frozen tissue was ground in liquid nitrogen with pellet pestle in a microcentrifuge tube to a fine powder. Total RNA was isolated using Concert™ Plant RNA reagent (Invitrogen) according to manufacturer's protocol. Contaminated DNA was eliminated by DNAse I digestion at 37°C for an hour according to manufacturer's protocol. Treated RNA was eluted in 20 µL elution buffer and the concentration was determined by spectrophotometry.

One microgram of the DNase-treated RNA was added to the iScript™ Reverse Transcription Supermix according to manufacturer's instruction (Bio-Rad, USA). The reaction was incubated in PCR machine at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min.

1.9.2 qPCR amplification

The qPCR amplification by SsoFast™ EvaGreen® Supermix according to manufacturer's instruction (Bio-Rad, USA) was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) in a final volume of 20 µl using 1 µl of cDNA, 10 µl of 2x SsoFast™ EvaGreen® Supermix, 250 nM gene-specific primers and 7 µl sterile water. The amplification cycling condition were as follows: enzyme activation at 95°C for 30 second, followed by 40 cycles of denaturation at 95°C for 5 second, annealing and extension at 55°C (for *OsBRL1* and *OsPSKR*) or 58°C (for *OsEF1α*) °C for 5 min.

The specific primers for qPCR amplification were designed manually and confirmed with the OligoAnalyzer 3.1 software (www.idtdna.com) and NCBI Primer-BLAST tool. The *OsEF1α*, the housekeeping gene, were used as an endogenous reference gene.

2. Identification and characterization of some chitosan-responsive genes in *Arabidopsis*

2.1 Determination of chitosan concentration for chitosan insensitive screening

To find the appropriate chitosan concentration for mutant screening, wild-type *Arabidopsis* (Col) were tested on ½ MS medium (pH 5.8) with chitosan O80 at concentration of 0, 20, 40, 60 and 80 mg/L. Phenotypes of *Arabidopsis* responding to chitosan application were observed to select the easiest visual method the evaluation for mutant phenotype.

2.2 Transcriptomic analysis of chitosan-responsive genes in *Arabidopsis*

2.2.1 Plant materials

Wild-type Col seeds were sterilized and grown on ½ MS medium (pH 5.8) with or without 80 mg/L O80 chitosan for 10 days in a controlled growth room at 22 °C ± 2 °C. Whole plant tissues were collected, then immediately frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. The experiment was performed with completely randomized design (CRD) with two independent biological replicates. Each replicate consisted of a pool of 10 plants.

2.2.2 Total RNA extraction

Frozen tissue was ground in liquid nitrogen with pellet pestle in a microcentrifuge tube to fine powder. Total RNA was isolated using Concert™ Plant RNA reagent (Invitrogen) according to manufacturer's protocol. Contaminated DNA was eliminated by DNAse I digestion at 37°C for an hour according to manufacturer's protocol. Treated RNA was eluted in 20 µL elution buffer and the concentration was determined by NanoDrop 1000 Spectrophotometer.

2.2.3 Library preparation and Illumina sequencing

All libraries were prepared according to Burkart-Waco *et al.* (2013). In short, 10 μg of total RNA was subjected to purify mRNA using Dynabeads (Invitrogen). First-strand cDNA was synthesized with random primer using SuperScript® III Reverse Transcriptase (Invitrogen) followed by second-strand cDNA synthesis. The cDNA was cleaned using AMPure XP (Beckman Coulter Genomics) with a 1.8:1 ratio (AMPure:sample reaction) (v/v). Then, each library was fragmented by dsDNA fragmentase (New England Biolabs; NEB) and cleaned with AMPure. The fragment was transformed into blunt end by End Repair Module Enzyme Mix (NEB) and added 'A' base to 3'-end by Klenow fragment (NEB). Each step was followed by AMPure cleanup. Bioo Scientific's NEXTflex™ DNA barcode adapters were ligated to the end of DNA fragments by Quick ligase (NEB). The final product was purified using AMPure with ratio 0.7:1 (v/v), then 0.8:1 (v/v) to get the fragments between 300-350 bp and eluted in 20 μL elution buffer (Qiagen).

For enrichment of each library, 10 μL was amplified with the following reaction: 15 μL Phusion 2x HF master mix (NEB), 3 μL water, and 2 μL 5 μM premixed PE primers. The reaction condition was 98°C for 30 s; 14 cycles of 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s; final extension at 72°C for 5 min. The amplicon was cleaned with AMPure with 0.8:1 ratio (v/v) and qualified and quantified using the Agilent BioAnalyzer. All libraries were sequenced by Illumina's Genome Analyzer II (UC Davis Genome Center DNA Technologies Core Facility) (50-bp single-end reads) according to manufacturer's instructions.

2.2.4 Computational analysis, sequence processing and expression analysis

All sequences were processed according to Burkart-Waco *et al.* (2013). Each library was aligned to TAIR10 cDNA database. Gene expression was evaluated by using the R package DESeq (version 1.6.1; http://bioconductor.org/packages/release/bioc/html/DESeq.html) (Anders and Huber, 2010). All read counts were accounted to each library and normalized between all libraries by scaled read counts up or down. To retrieve differentially expressed genes, the threshold was set at adjusted $p<0.05$ (Benjamini and Yekutieli, 2005). Gene descriptions and GO were retrieved from The Arabidopsis Information Resource (TAIR) and MapCape (Thimm *et al.*, 2004).

2.2.5 Gene co-expression network construction

The locus number of significantly differentially expressed genes were subjected to construct the gene co-expression network in Arabidopsis Interactions Viewer in Bio-Analytic Resource database (the BAR; version 13-06; Toufighi *et al.*, 2005) and visualized by Cytoscape 3.0.2 (Saito *et al.*, 2012). Gene group analysis was explored by retrieving gene list from CoexSearch and gene ontology in ATTED-II (version 7.1; Obayashi *et al.*, 2011)

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2.3 EMS-mutagenesis *Arabidopsis* seed preparation

Arabidopsis thaliana ecotype Columbia-0 (Col) seeds were mutagenized by treated with ethyl methanesulfonate (EMS) according to Comai laboratory's protocol. In short, seeds were washed with 4 mL distilled water with 0.1% Tween 20 in scintillation vial. The vial was agitated at 180 rpm for 15 min, then water was discarded. Seeds were washed four times with 4 mL distilled water and agitated at 180 rpm for 5 min. After washing, seeds were treated with 25 mM EMS and agitated at 180 rpm for 17 h. Treated seeds were washed five times with 4 mL distilled water at 180 rpm for 5 min and stored at 4°C for 2-3 days before sowing. All solution and disposable equipment contaminated with EMS were disposed into 1 M NaOH. Mutagenized seeds (M_1) were planted on soil in a controlled growth room at 22 °C ± 2 °C for 2 months to obtain M_2 seeds. After that, M_2 seeds of 5-10 M_1 plants per pool were collected and used as a population for chitosan insensitive phenotype screening.

2.4 Screening of mutagenized *Arabidopsis* with chitosan-insensitive phenotype

The pools of M_2 seed were screened on 80 mg/L O80 chitosan supplemented medium with the same growth condition as described in step 2.1. The selected plants were transferred and grown individually on soil for 2 months, then the M_3 seeds from individual mutant were separately collected for the second round of screening.

To select chitosan-insensitive mutant plants, the M_3 seeds were surface-sterilized and grown on ½ MS medium with or without chitosan for 10 days. To confirm the existence of the insensitive phenotypes, the third and fourth screening with the same condition were performed. The positive candidate M_3 plants were crossed to commonly used background line, Landsberg *erecta* (L*er*) and Col to check their genetic characteristics i.e. phenotypes and mode of inheritance. The cross seeds (F_1) were planted on soil to generate F_2 progeny. The pools of F_2 progeny of each candidate mutant were selected on the selective medium as the same growth condition as described above. Recessive pool of F_2 progeny was sequenced in the following steps.

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2.5 Genomic DNA extraction

Genomic DNA of recessive pool of F_2 progeny was isolated using plant DNA extraction buffer (see in Appendix A) according to laboratory's protocol. In short, 50 mg of plant tissues was ground in liquid nitrogen to a fine powder with pellet pestle in microcentrifuge tube. The 500 µL DNA extraction buffer was added and thoroughly mixed. The mixture was separated by centrifugation at 13,000 rpm for 10 min. Supernatant was transferred to a new microcentrifuge tube, added 0.7 mL isopropanol and mixed by inverting followed by centrifugation at 13,000 rpm for 5 min. Pellet was resuspended in 100 µL TE. To remove contaminated RNA, the reaction was incubated with 25 µg RNase A at 37 °C for an hour followed by phenol extraction and ethanol precipitation. The DNA was resuspended in 50 µL TE. DNA quantity and quality were determined using spectrophotometry and the visualization on the 1% agarose in 0.5x TBE, respectively.

2.6 Library preparation and genome sequencing of mutants

500 ng of genomic DNA was fragmented by dsDNA fragmentase. The reaction was incubated at 37 °C for 45 min and stopped by adding 5 µL of 0.5 M EDTA to total reaction. The fragment was purified using Ampure according to laboratory's protocol. The fragment was transformed into blunt end and added 'A' base to 3'-end by polymerase enzyme mix and Klenow fragment, respectively. Bioo's adapters were ligated to the end of DNA fragments. The product was purified using Ampure by 300 to 350 bp size-selection.

To amplify the amount of DNA in the library, the PCR was performed with Bioo's primer. The protocol was 98 °C for 30 sec, 14 cycles of 98 °C for 10 sec, 65 °C for 30 sec, and 72 °C for 30 sec followed by 72 °C for 5 min, and hold at 10 °C. The amplicon was quantified SYBR Green I by running on 1.5% agarose gel with low mass ladder. Enrichment reaction were purified with AMPure (0.8:1) and quantified with The Qubit® 2.0 Fluorometer. The libraries were sequenced on one lane using Illumina's Genome Analyzer II (50-bp single-end reads) according to manufacturer's instructions.

2.7 Data processing and gene mutation prediction

Reads from Illumina sequencing were processed in a bioinformatic pipeline for sequence alignment with BWA (version 0.7.3a) and SAMtools (version github-1.18). The sequences were mapped to *Arabidopsis* reference genome TAIR10. After analysis, the list of candidate genome positions with associated mutational effects was obtained. The result was analyzed by SnpEff program (Cingolani *et al.*, 2012) to predict the effects of variants on genes such as single-nucleotide polymorphisms (SNPs), multiple-nucleotide polymorphisms (MNPs) and insertion-deletion (In-del) in the whole genome sequences. Annotated genes can be classified into synonymous or non-synonymous SNPs, stop codon gains or losses.

To determine which mutation actually caused the damage, the probability of an amino acid substitution that affects protein function was evaluated using SIFT (Kumar *et al.*, 2009). The prediction based on the degree of amino acid residues conservation in sequence alignments from closely related sequences through PSI-BLAST in NCBI.

2.8 Mutant ordering and confirmation

2.8.1 Mutant ordering from ABRC

The predicted mutation from step 2.7 was searched for T-DNA insertion mutant from Arabidopsis Biological Resource Center (ABRC) seed stock.

2.8.2 Mutant confirmation

Genomic DNA was extracted from ABRC mutants as previously described in step 2.5. 100 ng of genomic DNA was used as template for PCR amplification with specific primers (see in Appendix D) designed by searching SALK T-DNA lines in SALK T-DNA verification primer design. The protocol was 94 °C for 5 min, 30 cycles of 94 °C for 30 sec, 54 °C for 30 sec, and 72 °C for 1 min followed by 72 °C for 7 min, and hold at 10 °C. The amplicon was qualified by running on 1% agarose gel with GeneRuler^{TM} 1kb DNA ladder plus.

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To detect the level of RNA in ABRC mutants, total RNA was extracted as previously described in step 2.2.2. DNase-treated RNA was eluted in 20 µL elution buffer and the concentration was determined by spectrophotometer. One microgram of the DNase-treated RNA was added to the iScript™ Reverse Transcription Supermix according to manufacturer's instruction.

First-strand cDNA was subjected to reverse transcription PCR (RT-PCR) reaction with gene-specific primers (see in Appendix D). The specific primer pairs for amplification were designed manually and confirmed with the OligoAnalyzer 3.1 software (www.idtdna.com) and NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). *AtEF1α*, a housekeeping gene, were used as an endogenous reference gene. The protocol was 94 °C for 5 min, 28 or 32 (for line 1 and 8) cycles of 94 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 30 sec followed by 72 °C for 7 min, and hold at 10 °C. The amplicon was qualified by running on 1.5% agarose gel with GeneRuler^{TM} 1kb DNA ladder plus.

3. Identification of ortholog gene(s) involving in chitosan responses in rice

Locus number (TAIR AGIs) of RNA-seq data and predicted mutated genes were subjected to RICE DB 0.6 (Narsai *et al.*, 2013) to identify ortholog genes in rice. The results were compared with locus number of significantly expressed proteins from proteomic analysis.

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