# Phosphoproteome analysis reveals chitosan-induced resistance to osmotic stress in rice (*Oryza sativa* L.) seedlings

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**ABSTRACT**

This study sought to identify the mechanism underlying the response to chitosan at the posttranslational level. Khao Dawk Mali 105 seeds were soaked in 40 mg l$^{-1}$ of chitosan, and leaves of 2- and 4-week-old seedlings were sprayed with chitosan before starting osmotic stress conditions. Chitosan induced resistance to osmotic stress by enhancing shoot fresh and dry weights and maintained increased photosynthetic pigments. Leaf phosphoproteomes were examined using gel-free LC-MS/MS. Of the 60 phosphoproteins showed a significant difference in protein expressions under osmotically-stressed plants treated with chitosan. More than 40% of the phosphoproteins involved in signaling pathways, including OsCML12 calmodulin-related calcium sensor protein, ubiquitin carboxyl-terminal hydrolase 15, U-box domain-containing protein 45, HEAT repeat family protein, BRCA1 C terminus domain-containing protein, pectinesterase, protein kinase domain-containing protein, and receptor-like protein kinase. Chitosan enhanced rice seedling growth and drought resistance via multiple complex networks, including metabolism, transport, transcription, and signaling under osmotic stress.

## 1. Background

Drought is a major abiotic stress that affects plant growth and, depending on the developmental timing, can significantly reduce crop production (Kanya et al. 2019). Notably, the ongoing changes in the global climate may potentially increase osmotic deficits (Lamaoui et al. 2018). Rice (*Oryza sativa* L.), the most important crop worldwide, is the main staple food source of more than half of the global population (Wu et al. 2004). Areas in Asia dedicated to rice production (approximately 130 million ha) are under constant threat from abiotic stresses, especially drought. These areas, consisting of irrigated and rainfed lowlands, account for more than 85% of total rice production globally (Ali et al. 2006). Therefore, improved drought resistance against temperature fluctuations is crucial to the production of high-quality rice. The development of conventional breeding of drought-tolerant rice cultivars is a feasible strategy to avoid drought stress-induced yield losses. However, the lack of genetic data and manipulation methods remains problematic. Exogenous compounds, such as putrescine, spermine, spermidine (Ndayiragije and Lutts 2006), and glucose (Cha-um et al. 2007), have the potential to acclimatize plants under osmotic stress. In addition, chitosan can purportedly stimulate plant growth and tolerance to abiotic stress. It has been also reported that large-scale chitosan commercialization originates from the chemical alkaline hydrolysis of shrimp chitin, with a cost of nearly USD 10/g (Sigma Chemical Con., St. Louis, MO 63118, USA) (Batista et al. 2020). In agriculture, chitosan is applied under field conditions in plants, including turmeric (*Curcuma longa* L.), oregano (*Origanum*

*vulgare* ssp. *hirtum*), and sage (*Salvia officinalis* L.) (Stasińska-Jakubas and Hawrylak-Nowak 2022). Moreover, under drought stress, chitosan is reported to stimulate the growth of several plant species, including wild apples (*Malus sieversii* (Lebed.) Roem.) native to the mountains of Central Asia (Yang et al. 2009), coffee (*Coffea canephora* var. *robusta*) (Dzung et al. 2011), wheat (*Triticum aestivum* L.) (Zeng et al. 2012), and the common bean (*Phaseolus vulgaris* L.) (Abu-Muriefah 2017). During osmotic stress, soaking the seeds and foliar spraying of rice with chitosan was reported to significantly increase shoot growth (Pongprayoon et al. 2013) and the yield (Boonlertnirun et al. 2007). However, the effects of chitosan on plants reportedly vary according to the chitosan structure and concentration, as well as the plant species, developmental stage, and genotype (Pichyangkura and Chadchawan 2015).

Therefore, the mechanism underlying the ability of chitosan to regulate the growth and stress response of plants should be elucidated. Numerous studies have reported that chitosan can enhance the defense responses of plants by activation of hydrogen peroxide ($H_2O_2$) through an octadecanoid pathway and nitric oxide (NO) in the chloroplast, MAP kinase activation, oxidative burst, and hypersensitive responses (Rakwal et al. 2002), as well as activation of defense-related genes via chromatin alterations (Hadwiger et al. 2015). Chitosan can also act as a trigger of plant defense mechanisms against pathogen attacks by inducing the activation of transcription factors (TFs) related to defense-responsive genes and hormone signaling (Povero et al. 2011), and has been reported to elicit signal transduction

via the generation of $H_2O_2$ as a second messenger in abiotic stress responses, leading to increased drought resistance in rice seedlings (Pongprayoon et al. 2013). Gel-based proteomics and coexpression network analysis have shown that chitosan induced changes in several related proteins localized in the chloroplasts (Chamnanmanoontham et al. 2015). Consequently, to clarify the crucial role of chitosan, particularly modulation of proteins involved in signal transduction pathways, a quantitative phosphoproteomics method was employed to identify phosphorylated proteins in rice leaves. Phosphorylation of proteins is central to several metabolic, hormonal, developmental, and stress responses and is extensively employed in signal transduction, frequently involving cascades of protein kinases and phosphatases (Mayya and Han 2009). Protein phosphorylation seems to be regulated by the coordinated actions of protein kinases and phosphatases, which account for about one-third of all proteins in eukaryote cells (Qeli et al. 2010). In eukaryotic cells, the most common sites of protein phosphorylation are serine, threonine, and tyrosine residues (Pearlman et al. 2011). Large-scale phosphoproteomics analysis has been conducted to assess the responses of crops to drought stress. For example, in maize (*Zea mays* L.), highly significant changes were identified in 138 phosphopeptides involved in epigenetic control, gene expression, cell cycle-dependent processes, and phytohormone-mediated responses (Bonhomme et al. 2012). In addition, phosphoproteins related to drought tolerance and osmotic regulation have been identified in different cultivars of wheat (*Triticum* spp.) (Lv et al. 2014; Zhang et al. 2014). Therefore, an investigation of phosphorylated proteins in rice plants would be not only relevant for a more in-depth understanding of the molecular mechanisms of signal transduction pathways involved in defense responses, but also crucial to reveal the coexpression gene interaction networks involved in responses to chitosan.

Therefore, the present study aimed to investigate the growth induction events in rice potentially triggered by chitosan in response to osmotic stress with the use of a gel-free quantitative phosphoproteomics approach by liquid chromatography-electrospray ionization tandem mass spectrometry (ESI-LC-MS/MS). This assumption reflects significant phosphoprotein changes at the posttranslational level in response to chitosan under osmotic stress. The novel findings of this study provide new insights into the understanding of the regulatory mechanisms underlying the action of chitosan-induced resistance to osmotic stress in rice.

## 2. Materials and methods

### 2.1. Plant material, growth conditions, chitosan and osmotic stress treatments

The rice cultivar Khao Dawk Mali 105 (KDML105; *Oryza sativa* L. ssp. *indica* cv. KDML105), a drought-sensitive cultivar (Pamuta et al. 2020), was provided by the Agriculture Department Ministry of Agriculture and Cooperation, Thailand. Rice germination and growth conditions were similar to those described previously (Pongprayoon et al. 2013; Chintakovid et al. 2017). Briefly, rice seeds were soaked in distilled water for 48 h, then germinated on sterilized sand flooded with distilled water under natural light. After 2

weeks of germination, a modified WP medium (Vajrabhaya and Vajrabhaya 1991) was added, and seedlings were grown in the greenhouse under natural light ($37 \pm 2°C$, $74 \pm 5\%$ relative humidity, and $93 \pm 5$ µmol m$^{-2}$ s$^{-1}$ photosynthetic photon flux). The nutrient solution was refreshed every 7 days. After 4 weeks, the rice seedlings were divided into two groups. Plants in one group continued to grow in freshly prepared nutrient solutions as a standard condition. In contrast, plants in the other group were transferred to a nutrient solution containing 10% (w/v) polyethylene glycol 6000 (PEG6000) to simulate osmotic stress conditions for 7 days.

For chitosan treatment (Figure 1), rice seeds were first soaked in a 40 mg l$^{-1}$ solution of oligomeric chitosan with an 80% degree of deacetylation solution, prepared as described (and called O80) in Limpanavech et al. 2008, whereas for the control groups, rice seeds were soaked with distilled water for 48 h (Figure 1, step 1). The seeds were then germinated on sand supplemented with WP nutrient solution for 2 weeks before transferring to WP nutrient solution (Figure 1, step 2). Seedlings (2- and 4-week-old) were sprayed twice with a solution of chitosan at the same concentration containing 0.01% (v/v) Triton X-100 until thoroughly soaked. A control treatment was performed by spraying as above except with 0.01% (v/v) Triton X-100 but no chitosan (Figure 1, step 3). After 2 days of the last chitosan treatment, 10% (w/v) PEG6000 was added to the nutrient solution as a surrogate model of osmotic stress conditions (Figure 1, step 4). Then, the rice seedlings were transferred to a freshly prepared nutrient solution without PEG6000 as osmotic stress-free ('re-watering') conditions (Figure 1, step 5). Four replicates for each treatment were arranged in a completely randomized design. Leaf samples were collected during 7 days of osmotic stress and consequently re-watered from four independent replicates to determine growth and photosynthetic pigments. Leaf tissues were harvested during 3 days of osmotic stress from three independent replicates for phosphoproteome, phenylalanine, and amino acid analyses in each treatment.

### 2.2. Photosynthetic pigments analysis

Six randomly selected plants from each replicate were used to determine the fresh weight (FW), dry weight (DW), and photosynthetic pigments. The Chl *a*, Chl *b*, and carotenoid contents were measured following the methods of Shabala et al. (1998) and Lichtenthaler (1987). Briefly, 100–200 mg of fresh leaves were homogenized with 5 ml of 80% (v/v) acetone, then wrapped in aluminum foil and placed in a refrigerator for 48 h. The Chl *a* and Chl *b* were quantified at wavelengths of 662 and 644 nm, whereas carotenoids were determined at 470 nm on a microplate spectrophotometer (Multiscan GO; Thermo Fisher Scientific). We used 80% (v/v) acetone solution as a blank control. Statistical analysis of the data was performed using analysis of variance (ANOVA) and the mean comparison was performed with Tukey's test, establishing statistical significance at $P$-values of $p < 0.05$. The bars in all figures represent the standard deviation of the mean.

### 2.3. Phosphoproteomics analysis

Leaf samples from ten seedlings were pooled from each replicate treated with or without chitosan application.
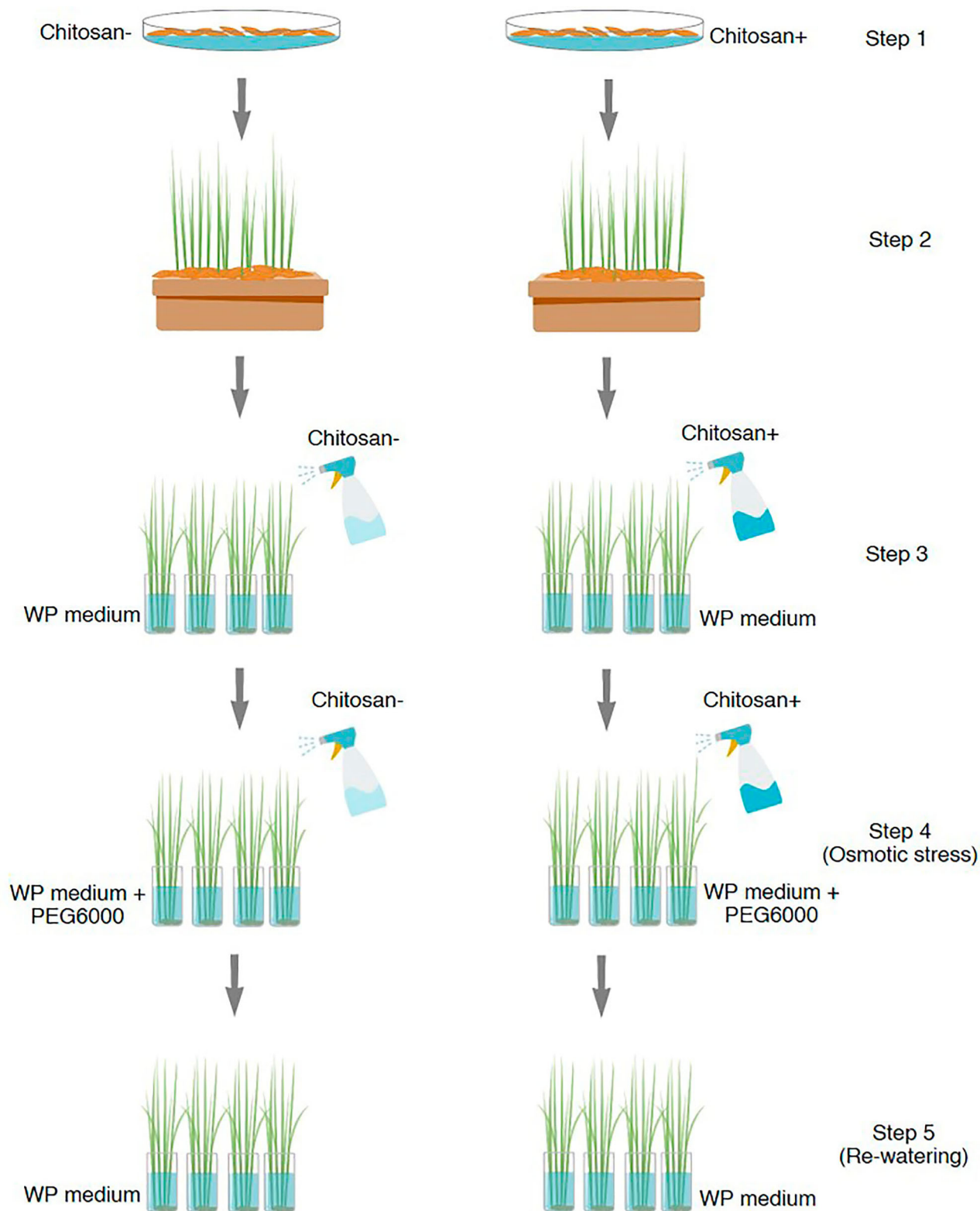
**Figure 1.** Scheme of the methodology for chitosan treatment.

Phosphoproteins were extracted from 0.5 g of leaf samples after being ground in liquid nitrogen and precipitated with acetone, according to Aroonluk et al. (2020). The protein concentrations were determined as described by Lowry et al. (1951).

Phosphoproteomic analysis was performed according to the phosphoproteome workflow procedure described by Nakagami et al. (2012). One hundred micrograms of the protein were used for phosphoproteins enrichment using the Pierce® Phosphoprotein Enrichment Kit according to the manufacturer's instructions and desalted by the HiTrap® Desalting Columns (Merck KGaA, Darmstadt, Germany). The phosphoproteins were reduced with 10 mM dithiothreitol (DTT), alkylated with 30 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate, and then digested with sequencing-grade trypsin (Promega Corporation, Madison, WI, USA) for 16 h at 37°C. Tryptic peptides were then concentrated by a SpeedVac™ Vacuum Concentrator (Thermo Fisher Scientific) and dissolved in 0.1% formic acid (FA) for MS analysis.

Phosphopeptide samples were assessed by liquid chromatography (LC) using an Ultimate 3000 LC System (Dionex Corporation, Sunnyvale, CA, USA) coupled to an ESI ion trap mass spectrometer (HCT Ultra PTM Discovery System; Bruker Daltonik GmbH, Bremen, Germany) and equipped with a monolithic nanocolumn (100-µm i.d. × 5 cm; Thermo Fisher Scientific) at an electrospray flow rate of 20 µl/min and a mobile phase flow rate of 0.3 µl/min. The mobile phase consisted of a nonlinear gradient of solvent A (0.1% (v/v) FA in $H_2O$) and solvent B (20% (v/v) $H_2O$, 80% (v/v) acetonitrile, 0.1% (v/v) FA) changing from 9:1 (v/v) A:B to 3:7 (v/v) A:B from 0 to 13 min, then 1:9 (v/v) A:B from 13 to 15 min and 9:1 (v/v) A:B from 15 to 20 min. Electrospray ionization was carried out at 1.6 kV using the CaptiveSpray. Nitrogen was used as a drying gas (flow rate about 50 l/h). Collision-induced-dissociation product ion mass spectra were obtained using nitrogen gas as the collision gas. Mass spectra (MS) and MS/MS spectra were obtained in the positive-ion mode at 2 $Hz$ over the range (m/z) 150-2200. The collision energy was adjusted to 10 eV as a function of the $m/z$ value.

## 2.4. Protein quantitation and identification

The Decyder MS 2.0 analysis software (GE Health-care, Chicago, IL, USA) was used to measure the relative protein abundance based on peptide MS signal intensities of the individual LC-MS analyzed data. An average abundance ratio of more than two-fold was determined as an over-expressed protein with a significant standard $t$-test and one-way ANOVA $p < 0.05$. All MS/MS spectra from the Decyder MS analysis were performed by applying the global variable mode of carbamidomethyl, variable mode of oxidation (M), phospho (ST) and phospho (Y), peptide charge state (1+, 2 + and 3 +), and m/z tolerance 0.1 u. These spectra were searched against NCBI protein databases (https://www.ncbi.nlm.nih.gov/) with an *Oryza sativa* L. proteome (downloaded March 4th, 2019) to identify matching peptides by using the Mascot software search engine (Matrix Science, London, UK). Identified proteins were filtered with a one-way ANOVA $p < 0.05$. In this experiment, 200 fg of BSA was used as an internal standard to normalize protein intensities from each set of data.

The identified proteins were used for GO analysis to identify the biological processes according to the Protein ANalysis THrough Evolutionary Relationships (PANTHER; http://www.pantherdb.org/) classification system (Thomas et al. 2003) and the Rice Genome Annotation Project (Ouyang et al. 2007). The levels of significantly expressed proteins in the hierarchical clustering were determined with MultiExperiment Viewer software (MEV) (Saeed et al. 2003) and analyzed using the $t$-test ($p < 0.05$) and the Pearson correlation coefficient. Coexpression network analysis was conducted to identify significantly upregulated proteins following chitosan treatment under osmotic stress relative to the control plants without chitosan application using a guide gene approach by Rice Functionally Related gene Expression Network Database (RiceFREND; https://ricefrend.dna.affrc.go.jp/) with the multiple gene approach with a hierarchy of 1 and mutual rank of 5 (Sato et al. 2013).

## 2.5. Measurement of phenylalanine content

The phenylalanine content of rice leaves was extracted using ice-cold methanol: osmotic as described by Uawisetwathana

et al. (2015) with some modifications. Briefly, plant sample powder (100 mg) was mixed with 2 ml of cold extraction solvent, shaken at 2,400 rpm for 5 min using a multi-tube vortexer (MTV-100; Hangzhou Allsheng, Inc., Hangzhou, Zhejiang, China) and then sonicated using an ultrasonic cleaner (CP2600D; Crest Ultrasonics, Penang, Malaysia) at 4°C for 15 min. The supernatant was subsequently separated from debris by centrifugation at 4°C and 3,500 rpm for 5 min (Allegra X-22R Centrifuge; Beckman Colter, Inc., Brea, CA, USA) and then vacuum dried at 40°C for 3 h to remove the solvents. The dried crude samples were redissolved in 150 µl of osmotic and filtered through a 0.22-µm cellulose acetate membrane (Costar® Spin-X® centrifuge tube filter; Corning Incorporated, Corning, NY, USA) at 4°C and 3,500 rpm for 5 min. Then, 100 µl of the flow-through samples were derivatized and quantified by gas chromatography (GC)-MS. Pretreatment and derivatization of phenylalanine were performed using a Phenomenex EZ: faast™ kit (Phenomenex, Inc., Torrance, CA, USA) with 0.2 mM norvaline in 10% n-propanol (100 µl) added to the crude extract (100 µl) as an internal standard. The mixture was aspirated through the sorbent tip and then washed with 200 µl of absolute n-propanol. Phenylalanine adsorbed onto the sorbent particles was dispensed using 200 µl of 3-picoline in NaOH as an eluting solution into a flat vial. Then, 25 µl of 20% (v/v) propyl chloroformate in 60% (v/v) chloroform and 20% (v/v) iso-octane were added to the vial and mixed for 10 s. The reaction was neutralized by the addition of 50 µl of iso-octane. After vortexing for 10 s, phase separation was allowed to proceed for about 1 min. Then, 50 µl of the upper layer were collected and dried under a stream of $N_2$ gas for 5 min. The derivatized form of phenylalanine was dissolved in 50 µl of iso-octane: chloroform (80:20, v/v) and then transferred to a GC vial.

A gas chromatograph (7890B; Agilent Technologies, Inc., Santa Clara, CA, USA) coupled with an EI source (Agilent Technologies, Inc.) and a triple quadrupole mass spectrometer (7000D; Agilent Technologies, Inc.) was employed to separate and quantify the targeted ions of phytohormones. Two microliters of the derivatized samples were injected at 250°C with a split mode of 1:5 into a polysiloxane (phenyl 50% and dimethyl 50%) or ZB-AAA column (10 m x 0.25 mm; Phenomenex, Ez: faast kit). The separation was performed under a flow of helium gas at 1.1 ml/min and an initial temperature of 110°C, which was increased to 170°C at 20°C /min and then further increased to 320°C at 30°C /min. Electron ionization in the positive mode (EI+) was performed at 70 eV and a vaporizer temperature of 240°C. Qualitative and quantitative analyses of the ions were performed in the selected ion monitoring mode. Statistical analysis of the data was performed using ANOVA and the mean comparison was performed with Tukey's test, accepting significance at the $p < 0.05$ level.

## 2.6. Analysis of free physiological amino acids by GC-TQ/MS

Free physiological amino acids were analyzed according to the method described by Jimenez-Martín et al. (2012) with some modifications. To extract free amino acids, 100 mg of ground leaf tissues were homogenized with 4 ml of 25% acetonitrile in 0.1 M HCl for 2 min and incubated at room temperature for 20 min. The homogenate was centrifuged

at 10,000 rpm for 10 min. Aliquots of the supernatant (50 μl) were transferred to GC glass vials containing 50 μl of norleucine (200 nmol/ml) as internal standards. The samples were dried at 60°C for 2 h, then mixed with 50 μl of dichloromethane and incubated at room temperature for 30 min. Afterward, the dried samples were mixed with 50 μl of a derivatizing agent (N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane) and 50 μl of acetonitrile. Then, 2 μl of each sample were incubated at 100°C for 4 h prior to triple quadrupole GC/MS with a gas chromatograph (7890B; Agilent Technologies, Inc.) equipped with a mass spectrometer (7000D; Agilent Technologies, Inc.) and a PAL autosampler system (CTC Analytics AG, Zwingen, Switzerland). Aliquots of the derivatized amino acids (2 μl) were injected using the pulsed split mode at a 1:5 split ratio and 280°C into a HP-5MS column (30, 0.25 mm i.d.; J&W GC column; Agilent Technologies, Inc.). Helium was used as the carrier gas at a constant flow rate of 1.4 ml/min. The temperature of the GC oven was increased from 130°C to 190°C at 6°C /min, to 230°C at 30°C /min for 5 min, and then to 325°C for 6 min. The transfer line, ion source (EI), and quadrupole temperatures were set at 325°C, 240°C, and 180°C, respectively. The mass spectrometer was operated in selected ion monitoring mode. Statistical analysis of the data was performed using a $t$-test, accepting significance at the $p < 0.05$ level.

# 3. Results

## 3.1. Osmotic stress response of the KDML105 in terms of shoot growth and photosynthetic pigment contents

Under standard conditions without PEG6000-treated KDML105 rice cultivar showed a significantly, 2.0 and 1.4 times higher shoot fresh weight (SFW) and shoot dry weight (SDW), respectively, after plant culturing for 7 days. In contrast, PEG6000 affected shoot growth (SFW and SDW) during osmotic stress conditions in the KDML105 rice cultivar. In particular, the KDML105 rice plants under osmotic stress for 7 days exhibited reduced SFW and SDW by approximately 47.6% and 15.2%, respectively, compared to non-PEG6000-treated plants (Figure 2a and b). In addition, the photosynthetic pigment levels in rice under standard conditions for 7 days showed an increase in chlorophyll $a$ (Chl $a$), chlorophyll $b$ (Chl $b$), and carotenoids by 2.0, 2.5, 1.9 times, respectively. At the same time, the effect of osmotic stress treatment caused a numerical reduction in Chl and carotenoids. After 7 days of osmotic stress, a significant decrease could be observed in the Chl $a$ (29.6%) and carotenoid (38.3%) content in KDML105 rice leaves, while the level of Chl $b$ content did not change compared to the control plants (Figure 2c–e).

## 3.2. Chitosan improved growth enhancement and maintained photosynthetic pigments during osmotic stress and re-watering conditions

Chitosan at 40 mg l$^{-1}$ by seed soaking and foliar spraying could enhance the shoot growth and the level of photosynthetic contents after 7 days of osmotic stress and nonosmotic stress (re-watering) conditions. The chitosan-treated KDML105 rice seedlings promoting SFW were 1.3- and 2.0-fold higher than nonchitosan-treated plants under osmotic stress and re-watering conditions, respectively (Figure 3a). In addition, the SDW of chitosan-treated plants were significantly higher by 1.2- and 1.1-fold than untreated control plants under osmotic stress and re-watering conditions, respectively (Figure 3b). In addition, the chitosan-treated KDML105 cultivar maintained increased photosynthetic pigment levels during osmotic stress. After removing osmotic stress (re-watering), foliar spray chitosan increased the content of Chl $a$ (1.3-fold) and Chl $b$ (1.2-fold), but not the content of carotenoids (Figure 3c–e).

## 3.3. Phosphoproteome profile and coexpression network of rice leaves induced by chitosan during osmotic stress

Phosphoproteomics analysis using gel-free LC-MS/MS identified a total of 2,398 proteins in rice leaves under osmotic stress (Table S1). In total, 324 and 755 responsive proteins were detected in rice leaves with and without chitosan treatment, respectively. Of the 2,398 proteins, 1,319, as shown in the Venn diagram in Figure 4a, were assigned to the following 11 GO functional categories: cellular process (33.9%), metabolic process (25.8%), biological regulation (10.9%), cellular component organization (10.7%) response to stimulus (6.9%), localization (6.7%), signaling (1.7%), developmental process (0.9%), multi-organism process (0.9%), reproduction (0.4%), and others (1.20%; Figure 4b).

Hierarchical clustering analysis was performed with a total of 2,398 proteins from the leaves of rice plants treated with or without chitosan under osmotic stress and then categorized based on expression patterns. The 60 most significantly expressed proteins are shown in Figure 5a as a heat map based on expression levels in each replication. Putative functions were assigned to only 32 phosphoproteins, as the remaining 28 were either hypothetical or unknown (Table S2). The significantly expressed proteins were classified into the six following groups based on GO functional annotation in reference to the Rice Genome Annotation Project: signal transduction (41%), metabolic process (34%), transport (13%), transcription factor (6%), defense response (3%), and other (3%; Figure 5b). Besides, the expression levels of 19 proteins were upregulated, including metabolic processes (GDSL-like lipase/acylhydrolase, dirigent protein 16, and potassium efflux antiporter protein), transport (aminotransferase, IAA-amino acid hydrolase, transmembrane amino acid transporter protein, integral membrane protein, and piezo-type mechanosensitive ion channel), TFs (transcriptional factor B3 family protein and SC35-like splicing factor SCL30), and mainly in signal transduction (OsCML12-calmodulin-related calcium sensor protein, ubiquitin carboxyl-terminal hydrolase 15, U-box domain-containing protein 45, HEAT repeat family protein, BRCA1 C terminus domain-containing protein, pectinesterase, protein kinase domain-containing protein, and receptor-like protein kinase; Table 1).

To investigate the main chitosan-induced metabolic processes, 19 upregulated proteins were subjected to coexpression network analysis using the RiceFREND database. Of these 19 chitosan-responsive phosphoproteins, 13 (68.4%) were found to form a positive coexpression network with other genes represented as nodes a-m, as shown in Figure 6 (Table S3). The thirteen phosphoproteins with the
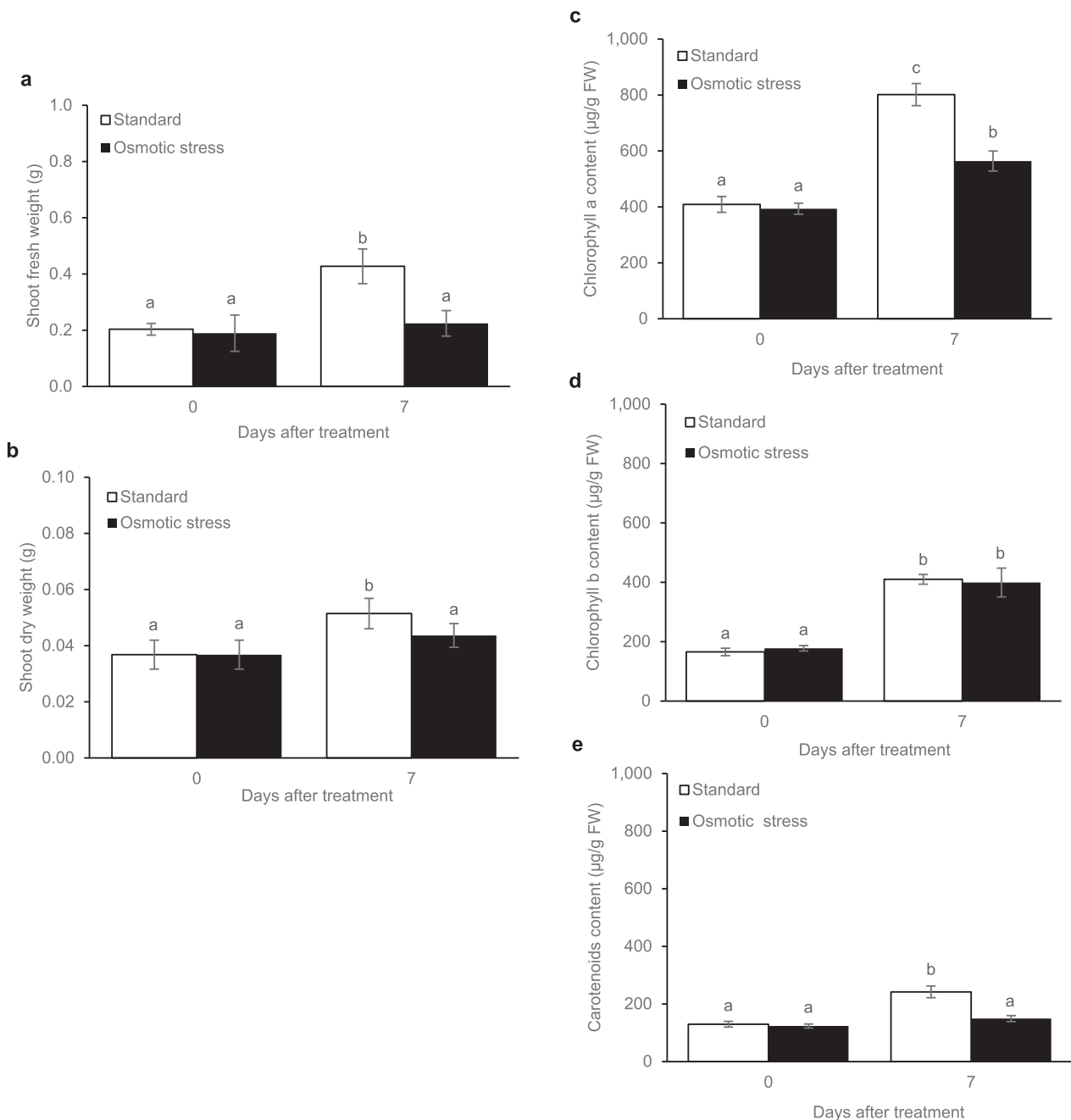
**a**



**b**



**c**



**d**



**e**



**Figure 2.** Growth parameters and photosynthetic pigments of KDML105 rice cultivar under standard and osmotic stress conditions (10% PEG6000) for 7 days in the shoot fresh weight (**a**), shoot dry weight (**b**), Chl *a* (**c**), Chl *b* (**d**), and carotenoids (**e**). Data are represented as mean ± SD, derived from 4 independent repeats. Different lowercase letters indicate significant differences, $p < 0.05$, ANOVA followed by Tukey's test.

coexpression were GDSL-like lipase/acylhydrolase (Os07g0668300; LOC_Os07g47210; node a), potassium efflux antiporter protein (Os12g0617800; LOC_Os12g42300; node b), transmembrane amino acid transporter protein (Os01856500; LOC_Os01g63770; node c), integral membrane protein (Os06g0103800; LOC_Os06g01440; node d), transcriptional factor B3 family protein (Os02g0598200; LOC_Os02g38470; node e), SC35-like splicing factor SCL30 (Os12g0572400; LOC_Os12g38420; node f), ubiquitin carboxyl-terminal hydrolase 15 (Os02g0244300; LOC_Os02g14730; node g), U-box domain-containing protein 45 (Os02g0539200; LOC_Os02g33590; node h), HEAT repeat family protein (Os03g0721200; LOC_Os03g51140; node i), BRCA1 C terminus domain-containing protein (Os06g0144000; LOC_Os06g05190; node j), pectinesterase (Os07g0675100; LOC_Os07g47830; node k), protein kinase domain-containing protein (Os03g0745700;

LOC_Os03g53410; node l), and receptor-like protein kinase (Os11g0208700; LOC_Os11g10280; node m).

## 3.4. The effects of chitosan application and osmotic stress on the phenylalanine (PHE) and amino acids profile

Without the addition of chitosan, osmotic stress increased leaf PHE levels the first day, then they returned to normal levels 2 days after the osmotic stress in the KDML105 cultivar. In contrast, chitosan-treated KDML105 did not alter PHE content levels the first 2 days. Then, it showed the highest PHE content levels (50.1 mg/100 g of DW) on day 3 of osmotic stress by approximately 8.7-fold compared to non-chitosan-treated plants (Figure 7a).

Furthermore, amino acid contents were quantified in rice leaves with or without chitosan application before exposure
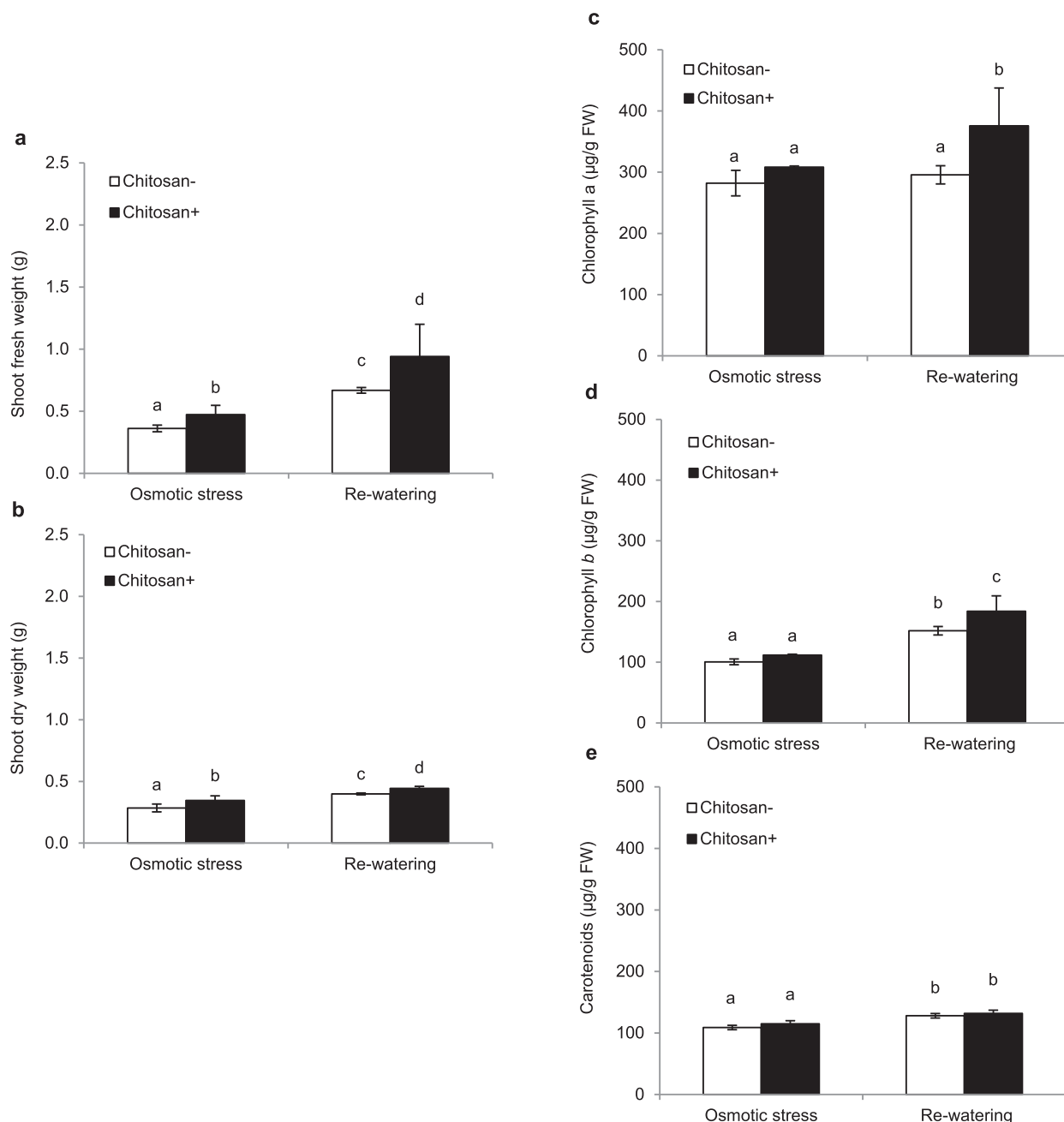
**Figure 3.** Growth parameters and photosynthetic pigments of KDML105 rice treated with and without chitosan under osmotic stress and re-watering, indicated by shoot fresh weight (**a**) and shoot dry weight (**b**), Chl *a* (**c**), Chl *b* (**d**), and carotenoids (**e**). Data are presented as mean ± SD, derived from 4 independent repeats. Different lowercase letters indicate significant differences ($p < 0.05$).

to osmotic stress. After 3 days of osmotic stress, 22 amino acids were detected, including alanine (ALA), glycine (GLY), α-aminobutyric acid (ABA), valine (VAL), leucine (LEU), isoleucine (IEU), threonine (THR), serine (SER), proline (PRO), asparagine (ASN), aspartic acid (ASP), glutamic acid (GLU), phenylalanine (PHE), α-aminoadipic acid (AAA), glutamine (GLN), ornithine (ORN), glycine-proline (GPR), lysine (LYS), histidine (HIS), tyrosine (TYR), tryptophan (TRP), and γ- aminobutyric acid (GABA). Chitosan-treated plants exhibited higher amino acid contents than untreated control plants, ranging between 1.11 and 688.42 mg/100 g of DW. The amino acids with the highest contents (per 100 g DW) were glutamine (688.42 mg), glutamic acid (334.73 mg), histidine (231.44 mg), alanine (182.89 mg), and proline (163.60 mg; Figure 7b).

## 4. Discussion

### 4.1. Osmotic stress induced by PEG6000 and chitosan affected growth enhancement and photosynthetic pigments in the osmotic stress condition

Khao Dawk Mali 105 or KDML105, known in the world market as Thai Hom Mali or Thai jasmine rice, has a unique fragrance and good eating/cooking quality (Pamuta et al. 2020). KDML105 local landrace varieties were widely distributed throughout Thailand and mainly cultivated in northeast Thailand (Vanavichit et al. 2018). However, its growth and yield are threatened by drought stress (Kanjoo et al. 2012). Previous studies on rice drought stress responses have been performed by transferring rice seedlings into nutrient solutions containing the osmotic agents PEG6000 (Xiong et al. 2010; Maksup et al. 2014) to create osmotic stress. Drought stress reportedly damages the photosynthetic
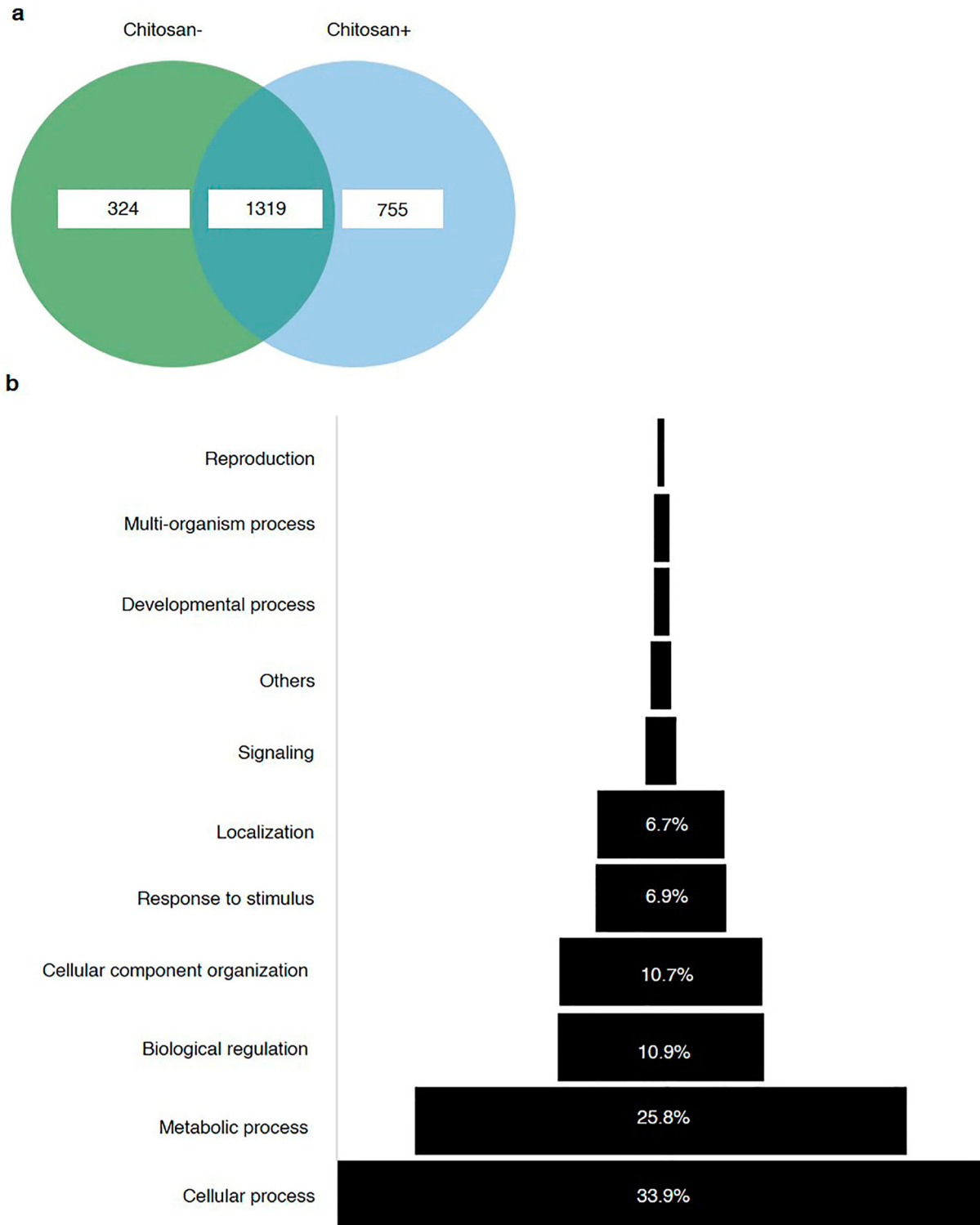
**Figure 4.** In total, 324 and 755 phosphoproteins detected with and without chitosan treatment. The 1,319 proteins were found upon both treatments in rice leaves (**a**). Functional classification of the total proteins were constructed based on gene ontology (GO) annotated assignments of proteins in the Protein ANalysis THrough Evolutionary Relationships (PANTHER) (**b**).

system by severely damaging the chloroplast envelope (Yamane et al. 2003; Vassileva et al. 2012) and the PSII complex. A consequence of this damage is a reduced chlorophyll content level (Maksup et al. 2014). In the present study, we observed that the Chl *a* and carotenoid contents were remarkable reduced after 7 days of osmotic stress (Figure 2c–e). This suggests that chlorophyll degradation occurs under osmotic stress.

Applying exogenous chitosan to osmotic stress resulted in marked drought resistance and improved shoot growth (SFW and SDW) during osmotic stress and after osmotic stress removal (re-watering) (Figure 3a and b), and also supported by the photosynthetic pigment contents (Chl *a* and Chl *b*) after osmotic stress (re-watering) (Figure 3c and d). These results were consistent with previous reports of the ability of chitosan to stimulate growth and drought resistance in LPT123 rice cultivar (Pongprayoon et al. 2013) and induce drought tolerance in white clover (*Trifolium repens* L.) (Li et al. 2017), sweet basil (*Ocimum ciliatum* L. and *O. basilicum* L.) (Ghasemi et al. 2017), cowpea (*Vigna unguiculata* (L.) Waip) (Farouk et al. 2012), and creeping bentgrass (*Agrostis stolonifera* L.) (Liu et al. 2020).
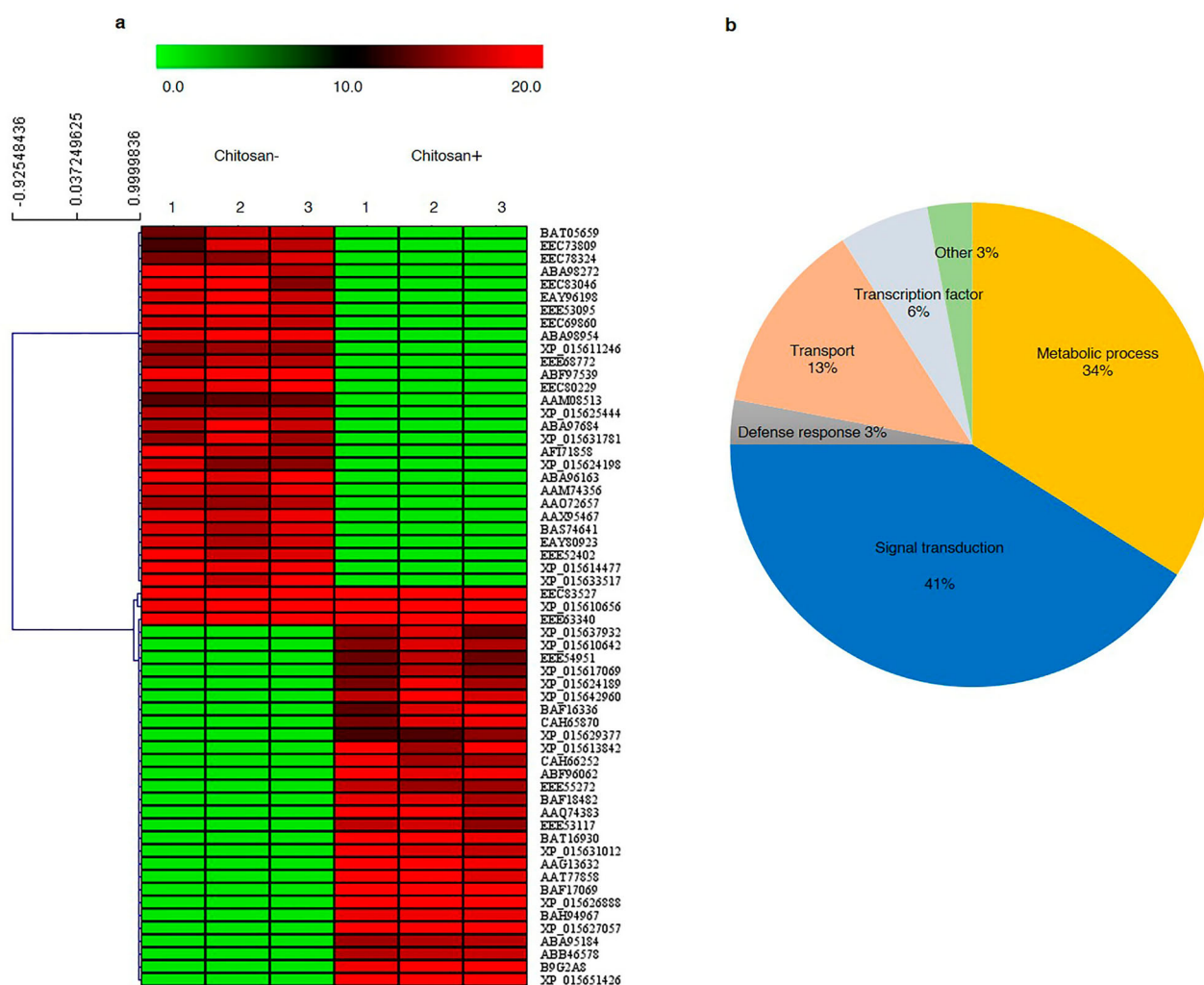
**Figure 5.** Heat map of significantly changed proteins in KDML105 rice cultivar under osmotic stress for 3 days with and without chitosan treatment. The heat map was created using the MultiExperiment Viewer (**a**). Each row in the hierarchical clustering analysis represents an individual protein. The color scale (green to red) indicates low to high protein expressions. Numbers above the column refer to the number of replications. The pie chart was constructed based on the gene ontology annotated assignments of proteins in Rice Genome Annotation Project (**b**).

## 4.2. Phosphoproteome profiles of rice leave after O80 chitosan application prior to osmotic stress

In contrast to the study of plant growth responses, phosphoproteomics was used to investigate protein phosphorylation, one of the most common posttranslational modification in proteins, controlling nearly all intracellular biological events, such as signal transduction, protein–protein interactions, protein stability, protein localization, apoptosis, and cell cycle control. Protein phosphorylation plays an important role in the regulation of cellular signaling pathways. Detecting changes in protein phosphorylation can be a difficult task because of the transient labile state of the phosphate group. Furthermore, low phosphoprotein abundance and poorly developed phospho-specific antibodies also contribute to difficulties in phosphoprotein detection. As a result, phosphoproteome analysis necessitates highly sensitive and specific methods. Currently, the majority of phosphoproteomic studies are performed by mass spectrometric approaches combined with phospho-specific enrichment methods (Thingholm et al. 2009) Using quantitative proteomics approaches, these variations at the protein level can be detected and measured, providing valuable information about the understanding of molecular mechanisms (Gondkar et al. 2021). In the present study, we

conducted a gel-free-based quantitative phosphoproteomics analysis of the chitosan response to short-term osmotic stress in rice leaves. A large number of phosphoproteins were identified. Indeed, the upregulated phosphoproteins were coexpressed with other genes that might be involved in chitosan-induced resistance to osmotic stress. These chitosan-responsive phosphoproteins and the related signaling and metabolic pathways might play important roles in chitosan signaling and response to osmotic (drought) stress in rice leaves.

## 4.3. Chitosan-induced phosphoproteins involved in secondary metabolic processes under osmotic stress

Coexpression network analysis showed that GDSL-like lipase/acylhydrolase (Os07g0668300) interacted with other genes, especially peroxidase 11 precursors (Os06g0274800), which is involved in secondary metabolic pathways, such as phenylalanine and phenylpropanoid biosynthesis (Figure 6, node a). Peroxidases act as antioxidant enzymes and contribute to the removal of hydrogen peroxide (Singh et al. 2013). Interestingly, the peroxidase and dirigent (Os08g349100) proteins have been implicated in the modulation of lignification levels upon exposure to abiotic stress (Paniagua et al. 2017). Dirigent-like genes are responsive to
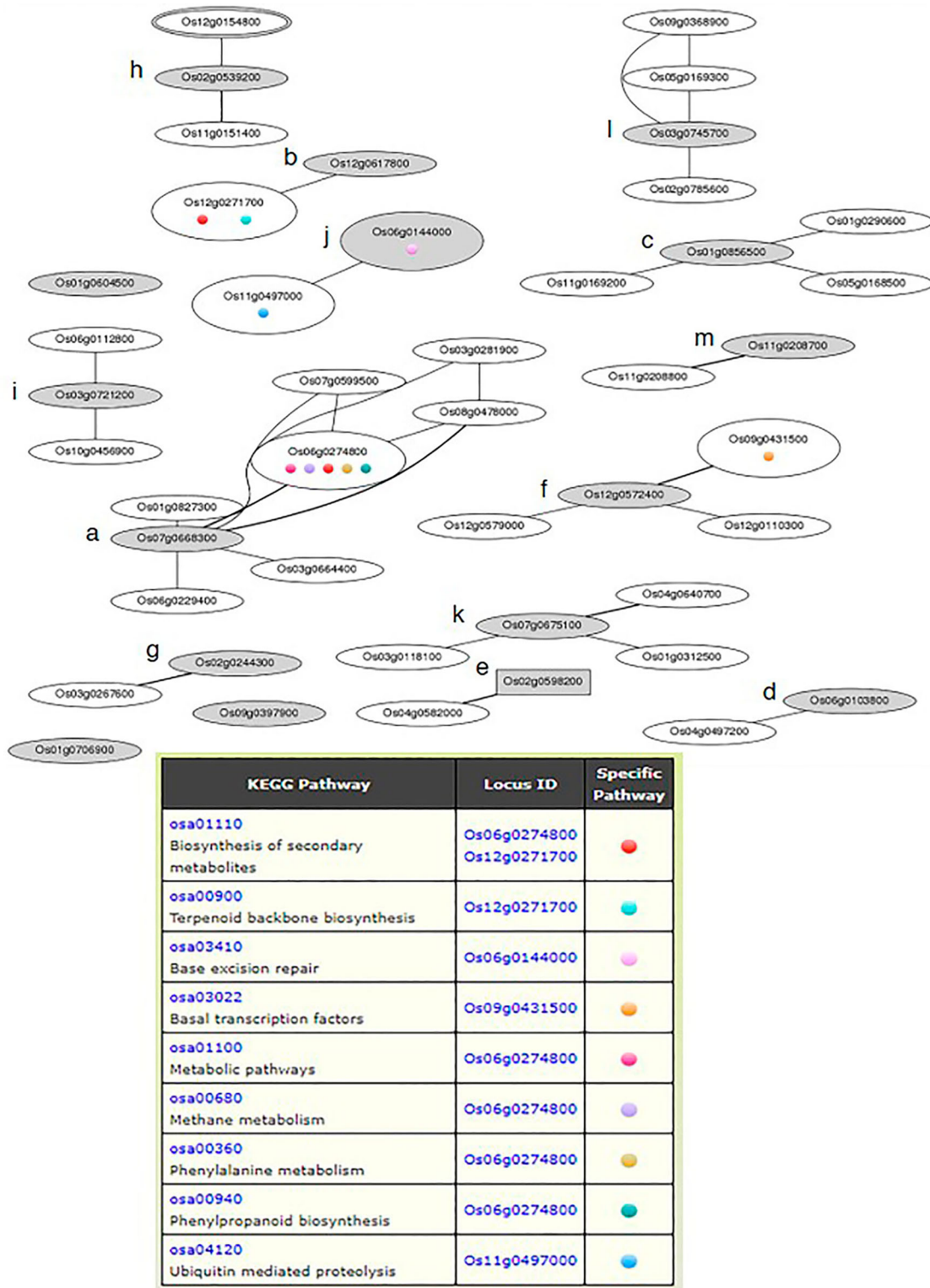
| KEGG Pathway | Locus ID | Specific Pathway |
|---|---|---|
| osa01110 Biosynthesis of secondary metabolites | Os06g0274800 Os12g0271700 | 🔴 |
| osa00900 Terpenoid backbone biosynthesis | Os12g0271700 | 🔵 |
| osa03410 Base excision repair | Os06g0144000 | 🩷 |
| osa03022 Basal transcription factors | Os09g0431500 | 🟠 |
| osa01100 Metabolic pathways | Os06g0274800 | 🔴 |
| osa00680 Methane metabolism | Os06g0274800 | 🟣 |
| osa00360 Phenylalanine metabolism | Os06g0274800 | 🟡 |
| osa00940 Phenylpropanoid biosynthesis | Os06g0274800 | 🟢 |
| osa04120 Ubiquitin mediated proteolysis | Os11g0497000 | 🔵 |

**Figure 6.** Coexpressed networks of upregulated proteins with significant expression level changes induced by chitosan treatment in rice leaves under osmotic stress, generated by the RiceFREND. Gray and white ellipses indicate query proteins (node a-m) and related genes in nodes, respectively. The square represents the transcription factor. The colored circles in the ellipses represent the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the node gene shown in the table.

osmotic, leading to increased lignification and phenylpropanoid biosynthesis as plant defense mechanisms (Caño-Delgado et al. 2003; Miedes et al. 2014). These data correlate with previous reports that chitosan stimulated an increase in peroxidase activity under stress conditions in plants (El Hadrami et al. 2010). Chitosan treatment-induced resistance to osmotic stress via $H_2O_2$ production and increased peroxidase levels in rice seedlings (Pongprayoon et al. 2013). Also, chitosan was shown to induce resistance against *Blumeria graminis* f. sp. *hordei* in barley plants (*Hordeum vulgare* L.) by oxidative burst induction and phenolic compound deposition (Faoro et al. 2008), and significantly improved phenol accumulation and flavonoid metabolism in white clover (*Trifolium repens* L. cv. Ladino) under dehydration stress (Li et al. 2017). Flavonoids participate in the production of phenylalanine and phenylpropanoid secondary metabolites,

**Table 1.** Upregulated phosphoproteins in rice in response to chitosan treatment under osmotic stress.

| NCBI accession number | Protein identification | Locus ID[a] | MSU ID[b] | Functional category | Peptide[c] | ID score[d] | Node[e] |
|---|---|---|---|---|---|---|---|
| ABA95184 | GDSL-like lipase/acylhydrolase | Os07g0668300 | LOC_Os07g47210 | Secondary metabolic processes | DVLTLVTK | 12.9 | a |
| XP_015631012 | Dirigent protein 16 | Os08g0349100 | LOC_Os07g44260 | Secondary metabolic processes | VGSTMCK | 17.5 | – |
| AAQ74383 | Potassium efflux antiporter | Os12g0617800 | LOC_Os12g42300 | Secondary metabolic processes | SLHSPLLTR | 14.5 | b |
| ABF96062 | Aminotransferase | Os08g0245400 | LOC_Os03g24460 | Transport | RNSPNSIDSK | 6.8 | – |
| EEE55272 | IAA-amino acid hydrolase | Os01g0706900 | LOC_Os01g51060 | Transport | GRLAPSR | 11.3 | – |
| B9G2A8 | Transmembrane amino acid transporter protein | Os01g856500 | LOC_Os01g63770 | Transport | KLGSSILSSR | 3.1 | c |
| XP_015642960 | Integral membrane protein | Os06g0103800 | LOC_Os06g01440 | Transport | AGLKVITIIDK | 2.9 | d |
| XP_015610642 | Piezo-type mechanosensitive ion channel | Os01g0388566 | LOC_Os01g29230 | Transport | FLWVYR | 8.7 | – |
| XP_015624189 | Transcriptional factor B3 family protein | Os02g0598200 | LOC_Os02g38470 | Transcription factor | TSNQNGEKNMK | 15.4 | e |
| XP_015626888 | SC35-like splicing factor SCL30 | Os12g0572400 | LOC_Os12g38430 | Transcription factor | EHEVDK | 9.4 | f |
| EEE54951 | OsCML12-calmodulin-related calcium sensor protein | Os01g0604500 | LOC_Os01g41990 | Signal transduction | RNGCISIER | 3.4 | – |
| XP_015627057 | Ubiquitin carboxyl-terminal hydrolase 15 | Os02g0244300 | LOC_Os02g14730 | Signal transduction | VEALKKPSK | 30.2 | g |
| XP_015613842 | U-box domain-containing protein | Os02g0539200 | LOC_Os02g33590 | Signal transduction | GSSCK | 2.9 | h |
| AAT77858 | HEAT repeat family protein | Os03g0721200 | LOC_Os03g51140 | Signal transduction | AILGGSEGK | 9.2 | i |
| AAG13632 | BRCA1 C terminus domain | Os06g0144000 | LOC_Os06g05190 | Signal transduction | MTAMAAARR | 22.7 | j |
| XP_015610656 | Pectinesterase | Os07g0675100 | LOC_Os07g47830 | Signal transduction | GGGFK | 1.2 | k |
| EEE63340 | Protein kinase domain-containing protein | Os03g0745700 | LOC_Os03g53410 | Signal transduction | MYVRADK | 9.1 | l |
| XP_015617069 | Receptor-like protein kinase | Os11g0208700 | LOC_Os11g10280 | Signal transduction | TAQAK | 5.0 | m |
| XP_015651426 | Metallo-beta-lactamase | Os09g0397900 | LOC_Os09g23380 | Other | HLGAAQIDR | 8.0 | – |

[a]Locus names were retrieved from the RiceFREND database
[b]Locus number of the matched protein from the rice genome annotation project
[c]Predicted peptide obtained from Mascot™
[d]The score obtained from Mascot™ for each match
[e]Node genes were subjected to coexpression network analysis using the RiceFREND database shown in Figure 6

which function as scavengers of radicals (Fini et al. 2011) and are associated with enhanced drought tolerance in plants (Nakabayashi et al. 2014). Indeed, the results of the present study revealed that chitosan treatment induced the highest levels of phenylalanine (50.1 mg/100 g of DW) on day 3 of osmotic stress, and chitosan-treated plants had higher phenylalanine contents than untreated plants by approximately 8.7-fold (Figure 7a). The phosphoproteomics data demonstrated that chitosan induced significant upregulation of many phosphoproteins and genes associated with secondary metabolism, which potentially contributed to coping with osmotic stress. Furthermore, a putative potassium efflux antiporter protein (Os12g0617800) was found to regulate the efflux of monovalent cations to maintain ion homeostasis in plant cells. This protein is localized in chloroplasts and associated with chlorophyll biosynthesis, development, and photosynthesis under abiotic conditions (Luo et al. 2018). It is involved in a coexpression network with solanesyl diphosphate synthase 1 (Os12g0271700) in pathways associated with the production of secondary metabolites and terpenoid biosynthesis (Figure 6, node b). The terpenoid metabolism plays a role in various functions of plants as hormones (gibberellins, abscisic acid), photosynthetic pigments (phytol, carotenoids), and electron carriers (ubiquinone, plastoquinone) (McGarvey et al. 1995). In this study, the chitosan-treated KDML105 cultivar maintained high photosynthetic pigments (Chl *a*, Chl *b* and carotenoids) during osmotic stress (Figure 3c–e). These findings imply that chitosan increased the expression of phosphoproteins involved in secondary metabolisms and could help rice seedlings maintain photosynthetic pigments during osmotic stress by affecting the potassium efflux antiporter protein.

## 4.4. Chitosan elevated the amino acid metabolites and transporter proteins in response to osmotic stress

Amino acids played an important compatible osmolyte and provided available precursors for nitrogen, carbon (Ali et al. 2019), and protein synthesis in plants (Burg et al. 2008). It is known that during drought stress, free amino acids are a critical adaptive response in plants (Good et al. 1994). Proline has been reported to osmotic adjustment and acts as an effective osmotic protector and scavengers of reactive oxygen species when plants are subjected to various environmental stresses (Yamada et al. 2005). Glutamine is converted to glutamic acid via the catabolic pathway. It serves as a source of nitrogen and a transport substance. In addition, histidine is metabolized to glutamic acid by four enzymatic steps in animals. However, this pathway has not yet been investigated in plants (Hildebrandt et al. 2015). The glutamic acid can serve as the precursor of proline and is involved in Chl biosynthesis in plants (Burg et al. 2008). In the present study, chitosan treatment promoted glutamic acid and proline accumulation, and it may maintain the Chl content in rice leaves under osmotic stress. The result was also validated by phosphoproteomics study. During osmotic stress, chitosan-treated KDML105 induced aminotransferase (Os08g0245400) and IAA-amino acid hydrolase (Os01g0706900), and also are reportedly associated with amino acids and nitrogen metabolisms (Schultz et al. 1995; Schultz et al. 1998). Furthermore, phosphoproteomics study revealed that in rice leaves, chitosan-responsive transport proteins were upregulated, including transmembrane amino acid transporter protein (Os01g856500), integral membrane protein (Os03g0721200), and piezo-type mechanosensitive ion channel (Os01g0388566).
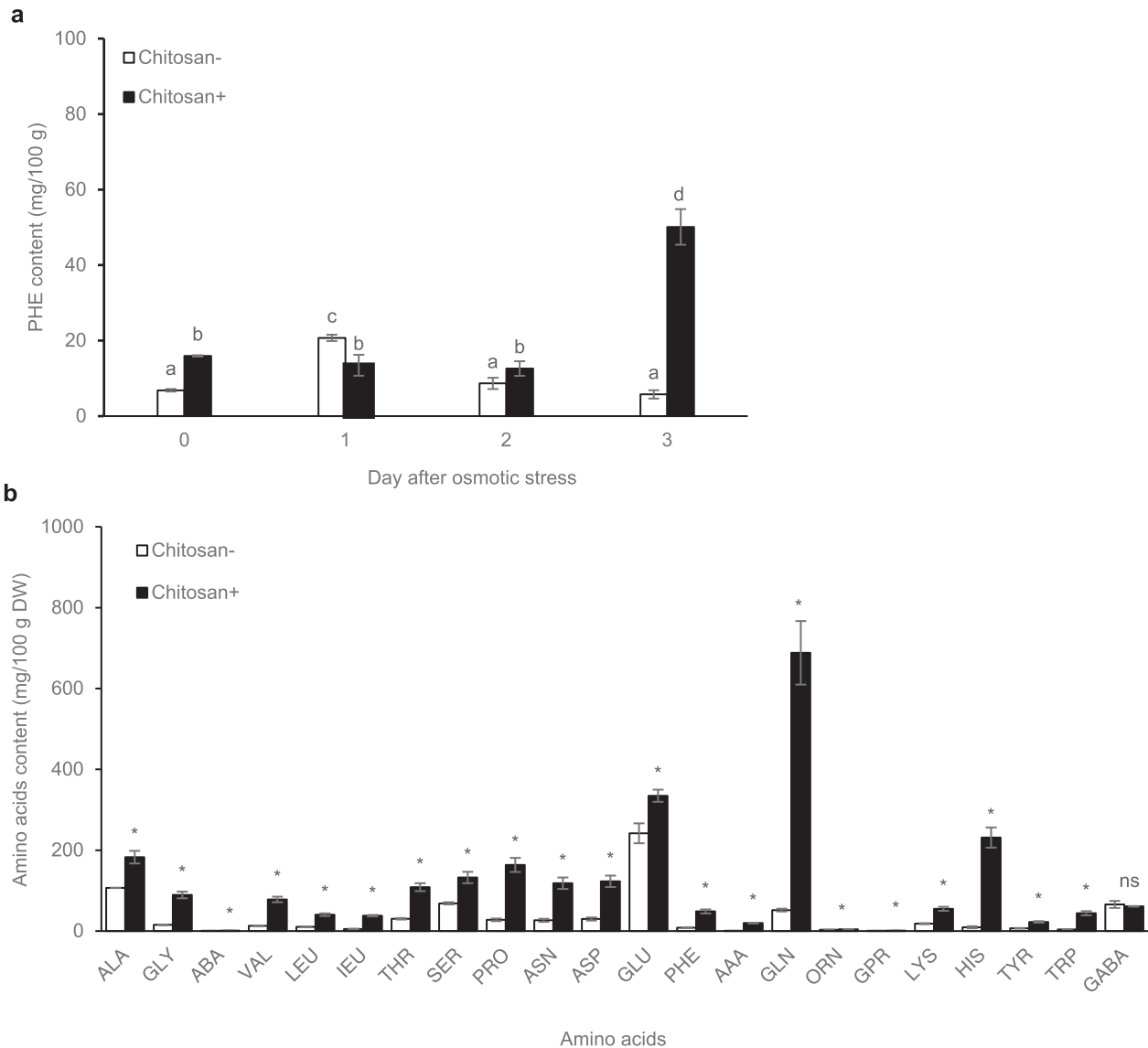
**Figure 7.** PHE in rice leaves on days 1, 2, and 3 (**a**) and amino acid compositions on day 3 with and without chitosan treatment under osmotic stress (**b**). Data are presented as mean ± SD derived from 3 independent repeats. Different lowercase letters indicate significant differences ($p < 0.05$).

Coexpression gene networks, the transmembrane amino acid transporter interacted with aminotransferase, class V family protein (Os01g0290600), amino acid/polyamine transporter II family protein (Os11g0169200), and nonaspanin (TM9SF) family protein (Os05g0168500) (Figure 6, node c), whereas the integral membrane transport protein showed an interaction with glycoside hydrolase, family 9 protein (Os04g0497200) (Figure 6, node d). Most amino acid transporters are considered to be proton-amino acid symporters coupled to amino acid uptake across the plasma membrane of plant cells (Ortiz-Lopez et al. 2000). Recently, it was discovered that the piezo-type mechanosensitive protein responsible for Ca$^{2+}$ transduction and involved in the abscisic acid signaling pathway in rice. It is located on the plasma and vacuole membranes (Heng et al. 2021).

## 4.5. Chitosan induced phosphoproteins associated with TFs and signal transduction during osmotic stress

TFs act as upstream triggers in a variety of signaling pathways. Chitosan stimulated the expression of transcriptional factor B3 family protein (Os02g0598200) (Figure 6, node

e), which participates in TF-mediated embryo axis formation and vascular development similar to the TF auxin response factor 1 (Möller et al. 2017). Typically, auxin plays a critical role in meristematic cell differentiation in the shoot apical meristem and is a necessary signal responding to abiotic stresses (Zhang et al. 2012). The serine/arginine-rich SC35-like splicing factor SCL30 (Os12g0572400) was identified as an essential splicing factor in spliceosome assembly and splicing regulation, either directly or indirectly, to maintain gene expression and development (Yan et al. 2000). This phosphoprotein interacted with TATA box-binding protein-associated factor 10 (Os09g0431500), a transcription factor involved in basal transcription (Figure 6, node f).

Another noteworthy, chitosan treatment increased the expression of eight signal transduction phosphoproteins in rice leaves under osmotic stress, including OsCML12 calmodulin-related calcium sensor protein (Os01g0604500), ubiquitin carboxyl-terminal hydrolase 15 (Os02g0244300), U-box domain-containing protein 45 (Os02g053200), HEAT repeat family protein (Os06g0103800), BRCA1 C terminus domain-containing protein (Os06g0144000), pectinesterase (Os07g0675100), protein kinase domain-containing protein (Os03g0745700), and receptor-like protein kinase

(Os11g0208700). The calmodulin family is a vital class of $Ca^{2+}$ sensor proteins that play crucial roles in cellular signaling cascades through the regulation of numerous target proteins (Ranty et al. 2006). $Ca^{2+}$ is a signaling molecule for chitosan induction. Oligomeric chitosan is reported to induce cytosolic accumulation of $Ca^{2+}$ in guard cells as a signal for stomatal closure. Changes in intracellular $Ca^{2+}$ levels have been reported in the early response to various abiotic signals, including mechanical stimuli, osmotic stress, cold and heat shocks (Klsuener et al. 2002). Low concentrations (50 μg/ml) of chitosan can induce an increase in the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyt}$) and the accumulation of $H_2O_2$ (Zuppini et al. 2004). However, previous studies reported chitosan-induced drought resistance in the LPT123 rice cultivar but not in the mutated LPT123-TC171 line via $H_2O_2$ signaling (Pongprayoon et al. 2013), suggesting that the response to chitosan might vary depending on the genotype or cultivar. Alternatively, $Ca^{2+}$ may act as a signaling component to elevate calmodulin-related calcium sensor proteins in response to osmotic stress, enhancing drought resistance and growth improvement in KDML105 rice cultivar through chitosan priming.

The results of phosphoproteomics analysis identified four phosphoproteins involved in the ubiquitin pathway, including U-box domain-containing protein, HEAT repeat family protein, BRCA1 C terminus domain-containing protein, and ubiquitin carboxyl (C)-terminal hydrolase 15 protein linking to proteolysis of the signaling pathways involved in hormone responses, environmental adaptation, and development in higher plants (Callis and Vierstra 2000). The ubiquitin carboxyl (C)-terminal hydrolase 15 protein hydrolyzes a variety of ubiquitin linkages, either before or after proteolysis, and plays a role in recycling ubiquitin and reversing ubiquitin conjugation in DNA repair, chromatin modification, and signal transduction (Yang et al. 2007). U-box domain-containing protein acts as a single peptide E3 ligase (Azevedo et al. 2001), while HEAT repeat family protein has been implicated in protein degradation (Cheng et al. 2004). These proteins are also related to hormone signaling. In addition, BRCA1 C terminus domain-containing protein is known to participate in base excision repair, as this domain is an integral signaling module in the DNA damage response and has been established as a phosphor-peptide binding module (Leung et al. 2011). The coexpression network analysis showed that the BRCA protein interacted with ubiquitin-activating enzyme-like protein (SUMO activating enzyme 1a) associated with the ubiquitin-mediated proteolytic pathway (Figure 6, node j). SUMO participates in regulated protein degradation and is involved in plant growth and the response to drought stress (Catala et al. 2007). Our results revealed that chitosan-responsive proteins were upregulated in ubiquitination and deubiquitination pathways in response to osmotic stress, leading to enhanced drought resistance and growth in rice.

Furthermore, pectinesterase (Os07g0675100) was detected in the enzyme-catalyzed first essential step in bacterial invasion of plant tissues (Fries et al. 2007) and acts in defense mechanisms in plants against pathogens by releasing pectin fragments that function as messenger molecules (Collmer and Keen 1986). Chitosan was shown to elicit a response to biotic stress and induces an increase in defense-related compounds (Katiyar et al. 2014). Generally, plants respond to biotic stress by producing phytoalexin,

pathogenesis-related proteins, such as chitinase, β-glucanase, and proteinase inhibitors, and inducing stress-responsive genes (Hidangmayum et al. 2019). Chitosan can also induce activation of the chitinase and glucanase enzymes associated with resistance to pathogens in various plants, including the peach (*Prunus persica* L. Batsch) (Ma et al. 2013), tomato (*Solanum lycopersicum* L.) (Sathiyabama et al. 2014) and dragon-fruit (*Hylocereus undatus* (Haworth) Britton & Rose) (Ali et al. 2014). However, in rice species, the difference of pathogenesis-related proteins is dependent on the molecular weight of chitosan (Lin et al. 2005) and the cultivar genotype.

Moreover, protein kinase domain-containing protein (Os03g0745700) and receptor-like protein kinase (Os11g0208700) are involved in the regulation of the stress signal transduction pathways. It has been reported that chitosan application induces a receptor-like kinase gene, MAP kinase pathway, and lysin motif receptor-like kinase, chitin elicitor receptor kinase 1 (*CERK1*) binding with chitin and chitosan (Petutschnig et al. 2010). In contrast, chitosan signaling did not activate the *CERK1*-independent pathway in *Arabidopsis thaliana* seedlings (Povero et al. 2011). Although the binding receptors remain unclear, chitosan might elicit effects in plant cells through charge–charge interactions owning to the positively charged amine groups that could bind to the negatively charged membrane phospholipids and thus, might not be involved with a specific receptor (Kauss et al. 1989; Shibuya and Minami 2001). However, the results of the present study indicate that chitosan can induce the phosphorylation of proteins in response to osmotic stress by acting as a receptor in a signaling pathway to increase drought resistance in rice.

## 5. Conclusions

Drought stress is one of the major abiotic stresses, causing a severe reduction in plant growth and crop production. Chitosan has been proposed as a chemical with the potential to induce significant drought resistance and improve growth in various plant species. Oligomeric chitosan with a degree of deacetylation of 80% (O80) at 40 mg $l^{-1}$ can stimulate SFW and SDW at the seedling stage. Quantitative phosphoproteomics and coexpression network analyses revealed that chitosan-induced expression of phosphoproteins involved in several processes, including secondary metabolism, transport, transcription, and signaling, in response to osmotic stress to enhance drought resistance and growth in rice cultivar.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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