Plant Physiology and Biochemistry 149 (2020) 301–312

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

# Proteomics analysis reveals the defense priming effect of chitosan oligosaccharides in *Arabidopsis-Pst* DC3000 interaction

Xiaochen Jia[a,1], Hongqiang Qin[b,1], Santosh Kumar Bose[a], Tongmei Liu[a], Jinxia He[a], Shangqiang Xie[a], Mingliang Ye[b], Heng Yin[a,*]

[a] Dalian Engineering Research Center for Carbohydrate Agricultural Preparations, Liaoning Provincial Key Laboratory of Carbohydrates, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, 116023, China
[b] Key Laboratory of Separation Science for Analytical Chemistry, National Chromatographic R & A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, 116023, China

ARTICLE INFO

*Keywords:*
*Pst* DC3000
*Arabidopsis*
Chitosan oligosaccharides
Plant defense
Proteomics
Defense priming

ABSTRACT

Chitosan oligosaccharides (COS) worked effectively in multiple plant-pathogen interactions as plant immunity regulator, however, due to the complexity of the COS-induced immune signaling network, the topic requires further investigation. In the present study, quantitative analysis of proteins was performed to investigate the underlying mechanism of COS induced resistance to *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) in *Arabidopsis thaliana*. 4303 proteins were successfully quantified, 186, 217 and 207 proteins were differently regulated in *mock* + *Pst*, COS, and *COS* + *Pst* treated plants, respectively, compared with mock plants. From detailed functional and hierarchical clustering analysis, a priming effect of COS on plant immune system by pre-regulated the key proteins related to signaling transduction, defense response, cell wall biosynthesis and modification, plant growth and development, gene transcription and translation, which confers enhanced resistance when *Pst* DC3000 infection in *Arabidopsis*. Moreover, RACK1B which has the potential to be the key kinase receptor for COS signals was found out by protein-protein interaction network analysis of COS responsive proteins. In conclusion, COS treatment enable plant to fine-tuning its defense mechanisms for a more rapid and stronger response to future pathogen attacks, which obviously enhances plants defensive capacity that makes COS worked effectively in multiple plant-pathogen interactions.

## 1. Introduction

Plant disease caused by pathogens is a fatal threat to field crops growth and yield (Strange and Scott, 2005). During the long evolutionary process, plants have developed sophisticated defense mechanisms to cope with multiple pathogens, including pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Berg, 2009; Silva et al., 2018; Spence et al., 2015). As the first layer of plant immune system, PTI can prevent the pathogen from infecting and colonizing host tissues, which confers resistance to broad-spectrum pathogens in plants (de Vega et al., 2018; Ramirez-Prado et al., 2018).

PAMPs such as flg22, elf18, lipopolysaccharide and oligosaccharides are potent plant immunity elicitors to initiate PTI and enhance plant innate resistance (Kutschera and Ranf, 2019; Seo et al., 2019). Elicitor-induced resistance is the fine regulation of multiple biological processes including transcriptional, post-translational, metabolic, physiological and epigenetic reprogramming (Hake and Romeis, 2019; Martinez-Medina et al., 2016). Immune system in the plants pretreated with elicitors can be temporarily and even trans-generationally adjusted to a 'primed state' (Aranega-Bou et al., 2014; Martinez-Medina et al., 2017; Xu et al., 2019). These 'post-primed' plants enable activating defense response in a more rapid and stronger manner when pathogens attack than untreated plants (de Vega et al., 2018).

Chitosan oligosaccharides (COS) which obtained by enzymatic degradation of chitosan are well-known plant immunity elicitors that have been widely used to enhancing plant resistance to various pathogens for many years (Jia et al., 2018; Yin et al., 2010b, 2016). As elicitors, the perception of COS by the host cell triggers typical PTI responses, including the transient accumulation of signal molecules such as $Ca^{2+}$, NO, and $H_2O_2$, the up-regulation of the MAPK signaling cascade pathway, the transcription and translation of defense-related proteins,

* Corresponding author.
*E-mail addresses:* mingliang@dicp.ac.cn (M. Ye), yinheng@dicp.ac.cn (H. Yin).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.plaphy.2020.01.037
Received 18 August 2019; Received in revised form 29 January 2020; Accepted 30 January 2020
Available online 03 February 2020

and activation of the SA and JA-mediated signaling pathways (Jia et al., 2016, 2018; Poncini et al., 2017; Yang et al., 2017; Yin et al., 2013). However, overall understanding of the signaling networks that involved in COS induction mechanism and the regulation pattern of the biological processes in COS induced 'primed state' plants is still remains unknown.

Proteomics is a powerful tool for revealing the proteins involved in complex biological processes and exploring the molecular regulatory pathways (Fabre et al., 2019; Lakra et al., 2018; Wu et al., 2019). Several proteomics studies have been carried out in recent years to investigate the induced resistance of many elicitors, such as $H_2O_2$, flg22, MSP1, SA, chitin, etc (Chen et al., 2018; Liu et al., 2015; Sun et al., 2018). These studies provided global insights into the complex induction mechanisms of elicitors, and some new clues of components that involved in PTI signaling and elicitor induced 'primed state' plants were also found out.

However, proteomics analysis on COS induced resistance is still lacking. A label-free proteomics was used to analyze differentially expressed proteins in rice after COS treatment in 2017, however, no significantly rules were find out due to the limited proteins numbers that quantified (Yang et al., 2017). Thus, more accurate and sensitive proteomic approach, together with multiple treatment groups' comparison are necessary for COS induction mechanism investigation. Fortunately, proteomics approach was developing rapidly in recent years, such as isobaric tags for relative and absolute quantification (iTRAQ) which has been proven to be a highly sensitive quantitative proteomics approach (Evans et al., 2012). The iTRAQ-based proteomics analysis enables quantitatively analyzing protein abundance in eight samples simultaneously with high confidence and repeatability, and has been widely used to investigating the proteomics variation in multiple treatment samples (Li et al., 2018; Ma et al., 2019; Wu et al., 2018).

The *Arabidopsis-Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) interaction has been used as a typical plant-pathogen system to study plant defense response for many years. And in previous studies, we verified that COS worked effectively in inducing resistance to *Pst* DC3000 in *Arabidopsis* by activating both SA- and JA-mediated pathways (Jia et al., 2018). However, the detailed induction mechanism of COS, important nodes and components of the involved biological processes in *Arabidopsis-Pst* DC3000 interaction remains unclear. Thus, to investigate the underlying mechanism of COS induced resistance to *Pst* DC3000 in *Arabidopsis*, an iTRAQ-based proteomic quantification method was carried out in this study.

## 2. Materials and methods

### 2.1. Plant materials and treatment

COS was obtained from Dalian GlycoBio Co. Ltd. (Dalian, China), with a degree of polymerization from 2 to 10 and a degree of deacetylation of 95%. *Arabidopsis* ecotype Columbia (Col-0, WT) plants were cultivated as described previously (Jia et al., 2016). More than 240 WT plants that grown in soil for 30 days, were evenly assigned into either the mock group (spraying water 3 days before inoculating with 10 mM $MgSO_4$), COS group (spraying 50 mg/L COS 3 days before inoculating with 10 mM $MgSO_4$), the *mock* + *Pst* group (spraying water 3 days before inoculating with *Pst* DC3000), or the *COS* + *Pst* group (spraying 50 mg/L COS 3 days before inoculating with *Pst* DC3000). Each treatment group contained three biological replicates, and each replicate included at least 20 plants. The inoculation of *Pst* DC3000 was carried out by using the same method which described in our previous study (Jia et al., 2018). By a high bacterial titer inoculation under laboratory conditions, *Pst* DC3000 could invades *Arabidopsis* leaf tissue and caused water-soaked patches which eventually become necrotic on infected leaves (Xin and He, 2013). After 4 days, the disease symptoms of infected leaves were fully emerged in mock-treated plants, while much lighter in COS pre-treated plants (Jia et al., 2018). Therefore, to revealing the protein regulation pattern in *Arabidopsis* during COS induced resistance and plant innate defense to *Pst* DC3000, the infected leaves which collected at 4 days after *Pst* DC3000 inoculations were used for proteomics analysis.

### 2.2. Protein extraction and digestion

Plant materials were ground to a fine powder in liquid nitrogen. One-gram powder was suspended in 20 mL of 65 mM DTT and 4% SDS in an ice bath. The suspension was extracted via sonication for 14 min (80 cycles of 8 s sonication/5 s rest). After centrifugation (20,000 g × 10 min), the proteins in the supernatant were precipitated with acetone/ethanol/acetic acid (50/50/0.1) reagent overnight at −20 °C in a volume ratio of 1:4. The protein sediment was collected by centrifugation as before, and washed twice with 1 mL of 100% ice-cold acetone. Sediment was dissolved in 100 mM Tris-HCl supplemented with 6 M guanidine hydrochloride (pH 8.0). Five hundred microgram of protein from each biological replicate were mixed together, and then reduced by adding DTT to a final concentration of 10 mM and incubating for 2 h at 37 °C. Iodoacetamide (IAA) was then added to a final concentration of 20 mM, and the mixture was incubated for 30 min in the dark. The proteins were then diluted 8-folds using 100 mM Tris-HCl (pH 8.0) and digested with trypsin (20:1) for 20 h at 37 °C. Peptides were acidified with 10% TFA (adjust pH 2–3) and desalted on an HLB SPE cartridge (Waters, 60 mg). The desalted peptides were collected and dried by using speed vacuum dryer (Thermo Fisher).

### 2.3. iTRAQ labelling and RP-HPLC separation

Two hundred microgram of dried peptides were dissolved in 50 μL 100 mM HEPS buffer (pH8.0), then labelled using iTRAQ 8-plex kits (AB Sciex Inc., Framingham, MA, USA) according to the manufacturer's protocol. The mock, COS, *mock* + *Pst* and *COS* + *Pst* treated samples were labelled with iTRAQ tags 114, 115, 116 and 117 respectively. These labelled samples were mixed together, divided into 10 tubes and dried by using speed vacuum dryer (Thermo Fisher). One tube of the iTRAQ-labelled samples mixtures were dissolved by using buffer A (10 mM ammonium formate, pH 9.5), then loaded onto a 2.1 × 150 mm column packed with C18 particles 5 μm, 150 Å (Agela). The peptides were eluted at a flow rate of 0.2 ml/min with a gradient of 0–5% buffer B (10 mM ammonium formate in 80% acetonitrile, pH 9.5) for 2 min, 5–38% buffer B for 46 min, 38–90% buffer B for 5 min, and 90% buffer B for 10 min. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled into 12 fractions and vacuum dried, the workflow details were shown in Fig. 1.

### 2.4. Liquid Chromatography − Mass spectrometry (LC-MS/MS) analysis

LC-MS/MS analysis of peptides fractions were performed on a Q-Exactive mass spectrometer (Thermo, San Jose, CA) equipped with an Ultimate 3000 system (Thermo, San Jose, CA). The LC-MS/MS system contained a capillary trap column (200 μm i.d., 4 cm length, C18 AQ beads (5 μm, 120 Å)) and a 15-cm capillary separation column (150 μm i.d., C18 AQ beads (1.9 μm, 120 Å)). The fractions were loaded at the flow rate of 4.0 μL/min using 0.1% FA in water for 10 min. The nano-LC gradient was set as followed at the flow rate of 550 nL/min: from 3 to 6% Buffer B (98% ACN/0.1% FA) in 5 min, from 6 to 35% Buffer B in 100 min, from 35% to 45% Buffer B in 10 min, and from 45% to 90% Buffer B in 5 min. The separation system was equilibrated by Buffer A (98% $H_2O$/0.1% FA) for 10 min. The temperature of the ion transfer capillary was set as 275 °C and the S-lens RF was set as 60. The normalized collision energy was set as 30 ± 3 for the analysis of iTRAQ labelled peptides (HCD mode). The resolution of full mass was set to 70,000 and the resolution of MS/MS was set to 17,500. Survey full scan MS was acquired from m/z 350 to 1800 with AGC of $3 \times 10^6$ and IT of

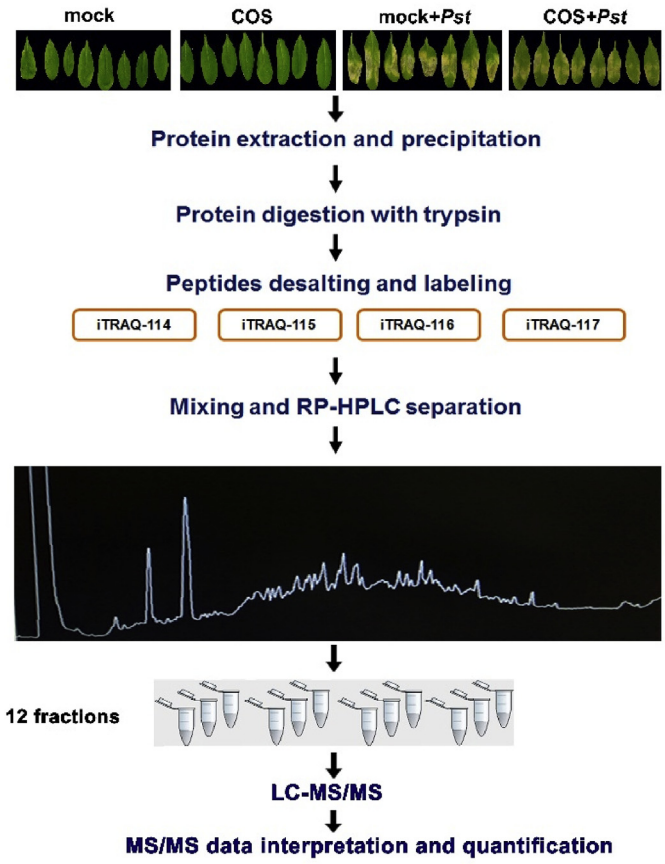


**Fig. 1.** Workflow for the proteomic analysis of COS induced resistance to *Pst* DC3000 in *Arabidopsis*. The "Mock" or "COS" group denoted as pretreated *Arabidopsis* with water or 50 mg/L COS, 3 d before inoculated with 10 mM MgSO4. The "Mock + *Pst*" or "COS + *Pst*" group indicated as pretreated *Arabidopsis* with water or 50 mg/L COS, 3d before inoculated with *Pst* DC3000. Each group contained more than sixty *Arabidopsis* plants.

36 ms, and 15 most intense ions (charge 2–7) with the intensity threshold of $5 \times 10^3$ were selected for MS/MS with maximum IT of 120 ms.

### 2.5. Data analysis

The raw data were searched against UniProtKB *Arabidopsis* database with 20,193 proteins by using iTRAQ labeling in Maxquant (1.5.8.3) (Cox et al., 2014). The parameters used for searching were: mass tolerances were 10 ppm and 50 ppm for the precursor and fragments, respectively; for trypsin digested samples, enzyme specificity was set to KR/P with up to 2 missed cleavage sites; cysteine residue was set as a static modification of 57.0215 Da; and iTRAQ-4 plex was utilized to the quantitative analysis of proteins. The quantitative protein ratios were weighed and normalized by the median ratio in Maxquant, and the ratios with p values $< 0.05$ were considered significant.

### 2.6. Bioinformatic analysis

Functional annotations of the proteins were conducted using the string database and proteins were categorized according to their biological process, molecular function, and cellular localization (https://string-db.org). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) and the Clusters of Orthologous Groups of Proteins (COG) database (http://www.ncbi.nlm.nih.gov/COG/) were used to classify and group these identified proteins. GO and pathway enrichment analysis were performed to determine the functional subcategories and metabolic pathways that the differentially accumulated proteins were significantly enriched in. Hierarchical clustering of the protein profiles was performed using R pheatmap package.

## 3. Results

### 3.1. Proteomics analysis of COS induced resistance to Pst DC3000 in Arabidopsis

To reveal the underlying mechanism of COS induced resistance in *Arabidopsis-Pst* DC3000 interaction, proteomic variations under *mock*, *mock + Pst*, *COS*, and *COS + Pst* treatments were investigated. By using an iTRAQ method (Fig. 1), 4312 proteins were totally identified from four treatment groups, and among them 4303 proteins were successfully quantified. The protein number that detected in this paper were much higher than most of the previously proteomics studies on plant immunity (Cui et al., 2018; Sun et al., 2018; Zhang et al., 2018a), and 2-fold higher than COS-treated rice (Yang et al., 2017), which provide more abundant data for COS induction mechanism investigation.

Protein abundance with significant change in different treatments was selected using a method described by Abdallah et al. (2012). Protein ratios outside this range were defined as being significantly different at $P = 0.05$. The cutoff value for the down-regulated proteins were 0.64, 0.56, 0.58-fold, and for the up-regulated proteins were 1.24, 1.46, 1.47-fold in *COS*, *mock + Pst*, *COS + Pst* treatment groups, respectively. Based on this criterion, 137, 117 and 167 proteins were up-regulated, and 49, 100 and 40 proteins were down-regulated in *mock + Pst*, COS, and *COS + Pst* treated plants, respectively, compared with mock-treated plants.

The coverage of significantly changed proteins in different treatment groups were shown in Fig. 2A. For COS-pretreated plants, 18.8% of up-regulated proteins and 31% of down-regulated proteins were also significantly changed in *Pst* DC3000-infected plants, suggested that COS pretreatment pre-changed the expression of some key functional proteins that functioned in *Arabidopsis-Pst* DC3000 interaction, which may benefit for defense activating when *Pst* DC3000 infection. Although the significantly changed proteins shared a high similarity between *mock + Pst* and *COS + Pst* groups (57.8%), however, there are 54 up-regulated proteins and 9 down-regulated proteins were only differently expressed in *COS + Pst* groups. Moreover, besides the effect of COS pretreatment on *Arabidopsis, Pst* DC3000 invasion also caused an obviously proteomic variation in COS pretreatment groups, up-regulating 149 proteins and down-regulating 19 proteins in *COS + Pst* groups. These results suggested that COS pretreatment or/and *Pst* DC3000 invasion lead to a significantly proteomic variation, which will facilitate revealing the induction mechanism of COS in *Arabidopsis-Pst* DC3000 interaction.

### 3.2. GO and KEGG enrichment analysis of COS pretreatment and Pst DC3000 infection responsive proteins

To gain more knowledge of differentially expressed proteins in COS pretreatment or/and *Pst* DC3000 infected plants, GO enrichment and KEGG analysis were used to annotate the target proteins (Fig. 2B and C, Fig. 3). According to the GO enrichment results, 117 up-regulated and 100 down-regulated proteins in COS pretreated plants could be localized to major cellular compartments such as cytoplasm, vacuole and membrane. The up-regulated proteins cover a diverse range of biological processes, including the process related to stimulus response, metabolism, gene expression and photosynthesis (Fig. 2B). These results are in agreement with previous reports showing that gene reprograming and plant growth promoting effects of COS pretreatment on different plants, including rice (Yang et al., 2017) and wheat (Wang et al., 2015). The down-regulated proteins mainly enriched to the process related to transport, metabolism, and modification (Fig. 2C).

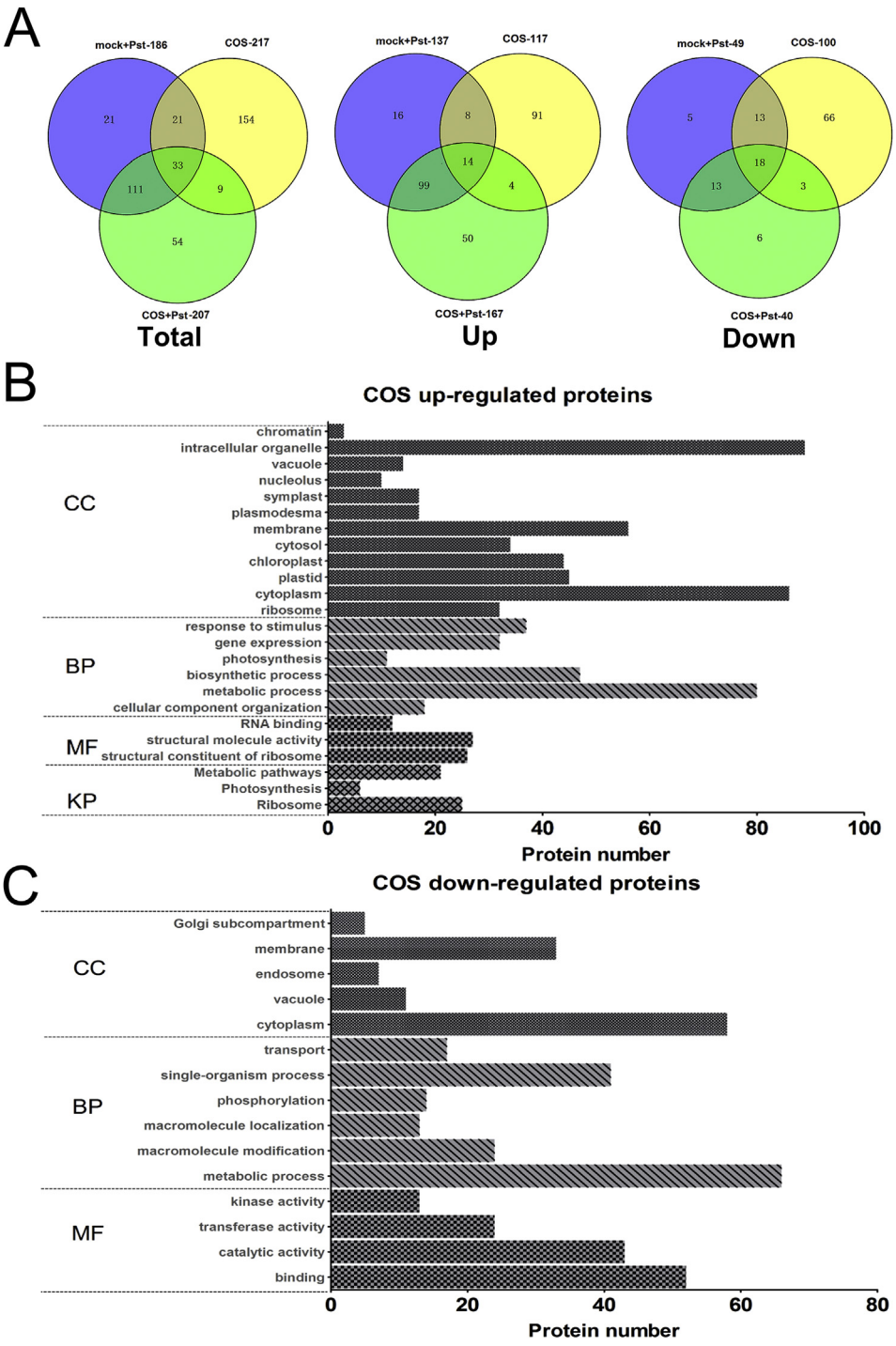


**Fig. 2.** Venn diagram and functional analysis of the significantly changed proteins in *Pst* DC3000 infected and/or pretreated *Arabidopsis*. (A) Venn diagram analysis of the differentially changed proteins identified in *Arabidopsis* leaves from three groups, including COS, *mock* + *Pst* and *COS* + *Pst*, compared with mock group. (B, C) Classification of the up-regulated or down-regulated proteins after COS pretreatment according to its enriched GO terms, including cellular component (CC), biological process (BP), molecular function (MF) and KEGG pathway (KP).

The proteins up-regulated due to *Pst* DC3000 infection, including *mock* + *Pst* and *COS* + *Pst* groups, are mainly locates in cytoplasm, vacuole, membrane, cytosol and cell wall (Fig. 3). The enriched biological processes and molecular functions were similar between *mock* + *Pst* and *COS* + *Pst* groups. The process related to stimulus response, defense response, catabolic process and oxidation-reduction process, and the proteins possessed catalytic activity, binding, oxidoreductase activity, hydrolase activity were all up-regulated in both *Pst* DC3000 infected plants and COS + *Pst* treated plants. Certainly, COS pretreatment caused some difference compared with *mock* + *Pst* groups, proteins involved in protein transport process, and proteins possessed antioxidant activity, dioxygenase activity and glucose transmembrane transporter activity were only up-regulated in *COS* + *Pst* groups (Fig. 3B).

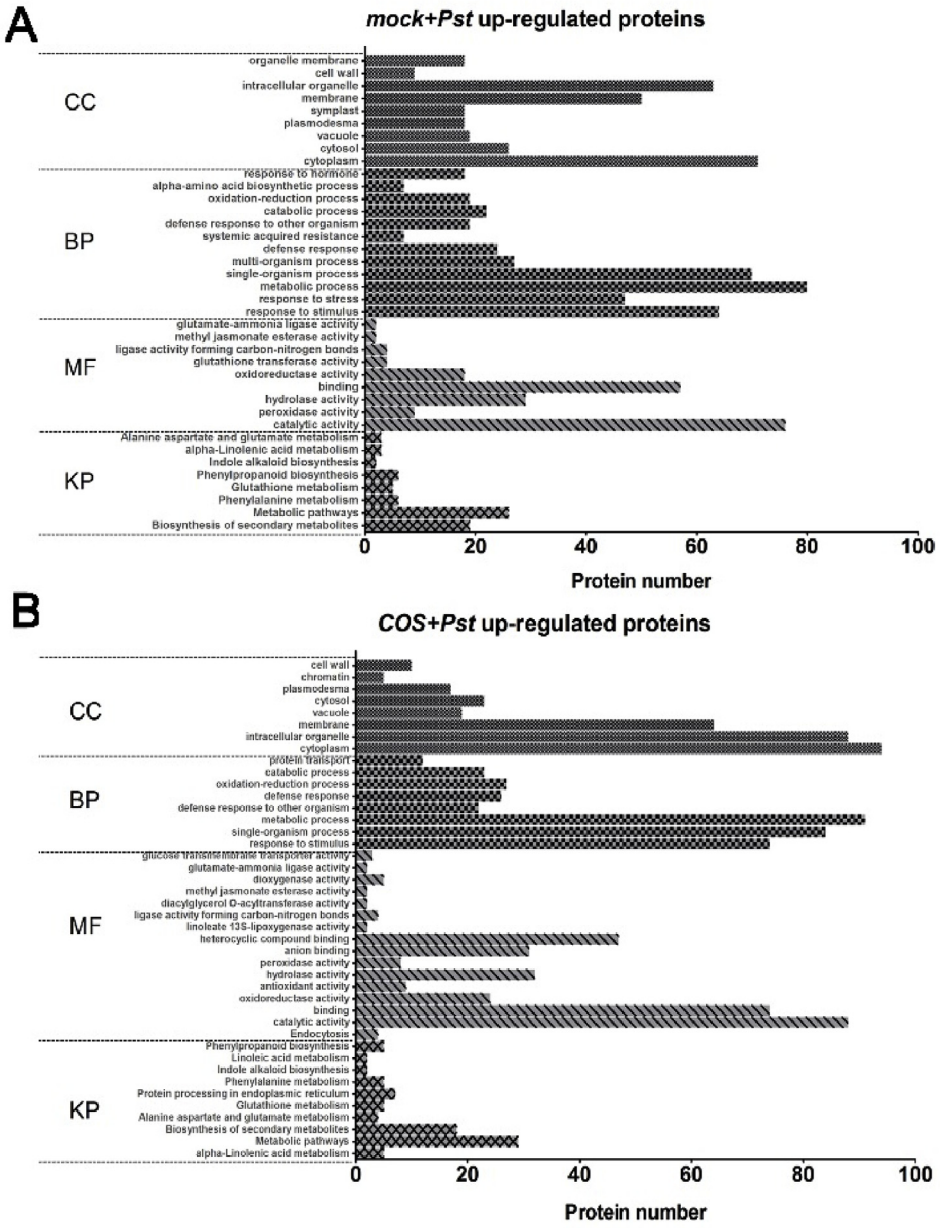


**Fig. 3.** Functional analysis of the significantly changed proteins in *Pst* DC3000 infected *Arabidopsis*. Classification of the up-regulated proteins in *mock + Pst* (A) and *COS + Pst* (B) treatment groups according to its involved enriched GO terms, including cellular component (CC), biological process (BP), molecular function (MF) and KEGG pathway (KP).

### *3.3. Hierarchical clustering of the proteins response to COS pretreatment and Pst DC3000 infection*

To reveal the expression pattern of the proteins responding to COS pretreatment and *Pst* DC3000 infection, the significantly changed proteins were analyzed by hierarchical clustering using the average fold change of intensity ratios (Fig. 4A). 403 significantly changed proteins were divided into three big clusters according to the hierarchical clustering analysis results. The regulation pattern of most proteins from cluster II and III are shown high similarity in different treatment groups, while proteins in cluster I are shown obviously difference between COS pretreated plants and *Pst* DC3000 infected plants. To gain more information from the regulation pattern, the proteins in each cluster were divided by the biological processes they enriched in (Fig. 4B).

Defense response related proteins are mainly enriched in cluster I, such as the proteins involved in response to bacterium, multi-organism process, defense response to bacterium, systemic acquired resistance and oxidation-reduction process. The distinct regulation of defense response related proteins, which are obviously down-regulated in COS pretreated plants while highly up-regulated in *Pst* DC3000 infected plants, may due to the difference between pre-activated and fully activated plant immune response. The proteins enriched in cluster II are mainly related to biological regulation, localization, transport, phosphorylation and system development, which all obviously decreased in COS pretreated or/and *Pst* DC3000 infected plants. Furthermore, the processes related to protein metabolic process and protein modification process were also down-regulated in each treatment groups.

Most proteins in cluster III are involved in biosynthetic process, gene expression, translation, photosynthesis, cellular component organization and cellular component assembly, which all up-regulated in COS pretreated or/and *Pst* DC3000 infected plants. The similar regulation pattern of the proteins in cluster II and III suggesting the pre-reprogramming effect of COS on some processes are consistent with *Pst*

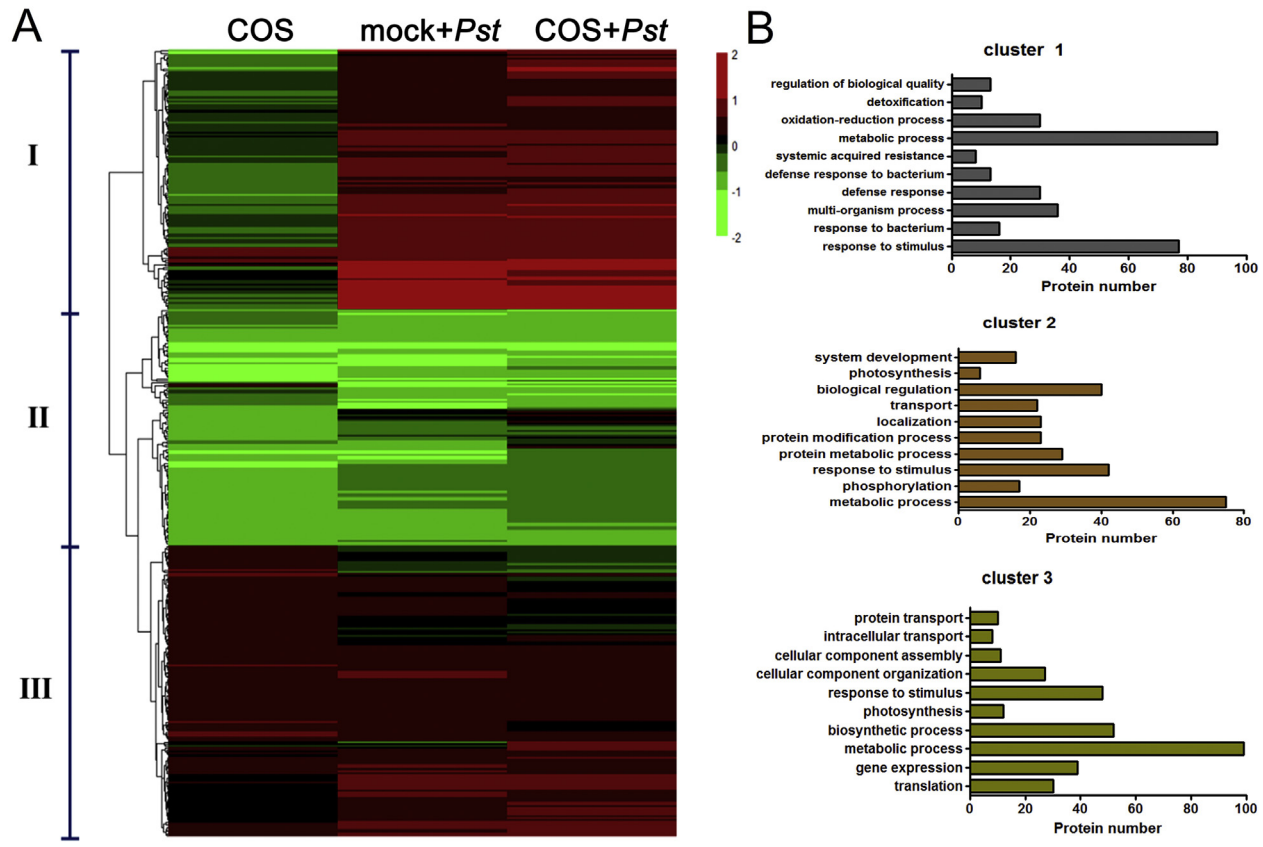


**Fig. 4.** Hierarchical cluster analysis (A) and biological process classification (B) of the significantly changed proteins in COS, *mock* + *Pst* and *COS* + *Pst* groups, compared with *mock* treated *Arabidopsis*. The "COS" group means pretreated *Arabidopsis* with 50 mg/L COS 3 d before inoculated with 10 mM $MgSO_4$. The "Mock + *Pst*" or "COS + *Pst*" group means pretreated *Arabidopsis* with water or 50 mg/L COS 3d before inoculated with *Pst* DC3000. Each group contained more than sixty *Arabidopsis* plants.

DC3000 infection, which benefit for enhancing *Arabidopsis* defense resistance to *Pst* DC3000 in advance. Unexpectedly, the proteins related to metabolic process and response to stimulus are enriched in all three clusters, suggesting that the key proteins involved in these processes are distinct regulated in the COS pretreated or/and *Pst* DC3000 infected plants.

## 4. Discussions

The proteomics analysis carried out in this study was aimed to revealing the distinct regulation pattern of biological processes that involved in COS induced resistance and plant innate immune response to *Pst* DC3000 infection. Therefore, to gain more information from the abundant proteomics data and further uncovering the defense priming effect of COS on plant, we described and discussed a series of distinct regulated proteins in different treatment groups in details as below.

### 4.1. Proteins related to signaling transduction

Chitosan's function mechanism in pea tissue is clear by inserting itself into the minor groove of DNA and activates DNA damage responses to potentiate plant immunity (Hadwiger, 2015; Hadwiger and Tanaka, 2017). However, COS signals recognition in *Arabidopsis* may also via PRRs-mediated recognition pattern like other PAMPs, since several candidates who have the potential to be COS receptors are found out (Liu et al., 2018). However, the special receptor for COS perception in *Arabidopsis* remains unclear thus far. In COS pretreated plants, some receptor-like proteins which may have potential roles in COS perception, such as MTH12.12 (AT5G59670), NAK (AT5G02290), MAPR4 (AT4G14965), F3O9.6 (AT1G16260) and T7N9.25 (AT1G27190) were significantly changed. As PAMPs, the perception of COS by the host cell leads to the activation of a complicated signaling network. And according to our proteomic analysis, the key proteins involved signal transduction, such as CBL3 (AT4G26570), ROC5 (AT4G34870), CPK7 (AT5G12480), RACK1B (AT1G48630) and MPK16 (AT5G19010), were significantly up-regulated after COS pretreatment (Table S1).

When *Pst* DC3000 invasion, plant activates many immune responses, including hypersensitive response (HR) at the infection site, callose deposition to fortify cell walls, antibacterial phytoalexins, reactive oxygen species (ROS), pH changes, possibly restriction of nutrient release, accumulation of SA and JA, and etc (Xin and He, 2013). Signal transduction also plays key role in these processes. Thus, in *Pst* DC3000 infected plants, proteins related to signaling transduction were also significantly up-regulated, such as F28J7.16 (AT3G01830), CPK26 (AT4G38230), Hsp70-2 (AT5G02490), CPK7 (AT5G12480) and GAD (AT1G65960) (Table S1). And there are no significantly difference between *mock* + *Pst* and *COS* + *Pst* treated plants. CPK26 which play a role in signal transduction pathways that involve calcium as a second messenger, were down-regulated in COS pretreated plants while up-regulated after *Pst* DC3000, which indicating the different regulation pattern of some key component involved in signaling transduction between COS induced resistance and plant innate immunity.

### 4.2. Proteins related to ROS homeostasis

ROS burst is one of the earliest hallmarks of the plant defense response, it transmit signals to induce defense pathways in the host plants (Carr et al., 2010). ROS is quickly accumulating in either *Pst* DC3000 infected or COS treated plants (Li and Zhu, 2013; Xin and He, 2013).

However, the highly accumulated ROS is harmful for many cellular molecules such as proteins, nucleic acids and lipids (Ali et al., 2018; Vellosillo et al., 2010). In response to the ROS burst during PAMP treatment or pathogen infection, many key enzymes serve as ROS scavengers will be enhanced in host plants to protect themselves from oxidative damage, which also appeared in our detection (Zhong et al., 2017).

In COS pretreated plants, HIRD11 (AT1G54410), CYP71B26 (AT3G26290), CP12-2 (AT3G62410), ACHT1 (AT4G26160), GPX7 (AT4G31870) and COX6A (AT4G37830), which plays key roles in oxidation reduction, were significantly up-regulated (Table S2). Moreover, 20 and 24 proteins which plays a key role in hydrogen peroxide removal and oxidation reduction were also enhanced obviously in *mock + Pst* and *COS + Pst* treated plants, respectively (Table S2). Unexpectedly, except COX6A, other oxidation reduction related proteins which up-regulated in COS groups were not involved in plant resistance to *Pst* DC3000. Thus, the ROS scavenge mechanism may different in COS induced resistance and *Pst* DC3000 infection activated plant defense responses.

### 4.3. Proteins related to defense response

Once plants recognize the PAMP signals, plant immune responses were activated. In our proteomic analysis, 79 proteins related to plant defense response were significantly changed in COS pretreatment and/or *Pst* DC3000 infection (Table S3). In COS pretreated plants, LTPG1 (AT1G27950), MLP43 (AT1G70890), TIR (AT1G72930), GPX7 (AT4G31870) and T20D1.30 (AT5G19510), which plays pivotal positive roles in plant defense response were up-regulated. And GRP-3, which encoding interactors of WAK1, negatively affect defense responses induced by oligogalacturonides were down-regulated after COS pretreatment. All these differently expressed proteins suggesting the pre-activated plant defense response in COS pretreated plants, which will easily be triggered a stronger defense resistance when *Pst* DC3000 invasion than mock treated plants.

SA- and JA-mediated signaling pathway are essential for plant defense response, SA mediates systemic acquired resistance (SAR), while JA mediates induced systemic resistance (ISR) (Janda and Ruelland, 2015; Koornneef and Pieterse, 2008; Loake and Grant, 2007). COS induction effect on SA and JA signaling pathway without bacteria or virus infection were already confirmed in many plants, including *tobacco* (Chen et al., 2009), *Arabidopsis* (Jia et al., 2016), rice (Yang et al., 2017) and *Brassica napus* (Yin et al., 2006; Yin et al., 2010a). After COS pretreatment, WAT1 (AT1G75500) which prevent salicylic-acid (SA) accumulation were down-regulated, suggesting the activating SA pathway in COS pretreated plants (Table S3).

Both the SA and JA pathways are required for *Arabidopsis* response to *Pst* DC3000 (Betsuyaku et al., 2017; Jia et al., 2018). In *Pst* DC3000 infected plants, 8 proteins involved in SA biosynthesis and response pathway, such as EDS16 (AT1G74710), ALD1 (AT2G13810) and PR1 (AT2G14610) were enhanced and 5 proteins which related to JA-mediated pathway, including TAT3 (AT2G24850), NATA1 (AT2G39030) and MES16 (AT4G16690) were highly up-regulated (Table S3). All these increased proteins suggesting the activated SA and JA pathway in *Pst* DC3000 infected plants. In COS pretreated plants, *Pst* DC3000 invasion also enhanced SA and JA related proteins, including 8 proteins involved in SA pathway and 10 proteins related to JA pathway (Table S3). These significantly proteins were consistent with our previous studies that COS induces resistance to *Pst* DC3000 in *Arabidopsis* by activating both SA- and JA-mediated pathways (Jia et al., 2018).

Hierarchical cluster analysis of the proteins related to plant defense response were divided into four clusters (Fig. 5). *Pst* DC3000 invasion caused the defense related proteins differently expressed, and COS pretreatment in *Pst* DC3000-infected plants seems has no obviously effect on these defense related proteins, since the expression pattern were shown high similarity between *mock + Pst* and *COS + Pst* groups.

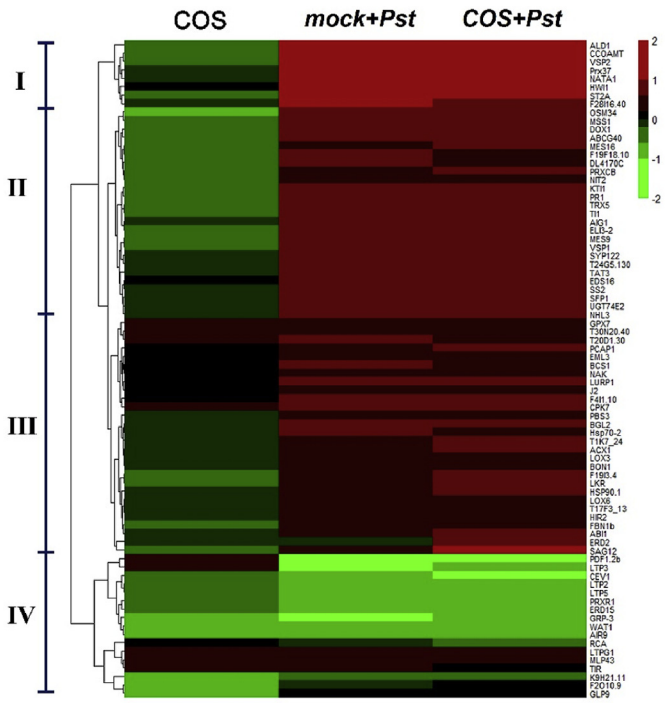


**Fig. 5.** Hierarchical cluster analysis of significantly changed proteins related to plant defense response in COS, *mock + Pst* and *COS + Pst* groups, compared with mock treated *Arabidopsis*. The "COS" group means pretreated *Arabidopsis* with 50 mg/L COS 3 d before inoculated with 10 mM $MgSO_4$. The "Mock + *Pst*" or "COS + *Pst*" group means pretreated *Arabidopsis* with water or 50 mg/L COS 3d before inoculated with *Pst* DC3000. Each group contained more than sixty *Arabidopsis* plants.

The most proteins in cluster I, II and III were shown obviously different expression pattern between COS pretreatment and *Pst* DC3000, suggesting the different regulation pattern between pre-activated and fully activated plant immune system.

### 4.4. Proteins related to cell wall biosynthesis and modification

As a physical barrier to defense for pathogens invasion, plant cell walls undergo dynamic changes during plant-pathogen interactions (Aragon et al., 2017; Ziv et al., 2018). In our proteomics analysis, 29 proteins related to cell wall biosynthesis and modification was significantly changed in COS pretreated and/or *Pst* DC3000 infected plants (Table 1). To enter the host cell, pathogens secretes cell wall-degrading enzymes to destroy the barrier, which resulting in accumulated DAMPs signal molecules. Once DAMPs recognized by plant cell, many enzymes working on cell wall components biosynthesis and modification were secreted to fortify cell walls (Franck et al., 2018; Frevert et al., 2018).

COS pretreatment differently regulated some proteins which are essential for the formation of cell walls, up-regulating RGP1, QUA1 and UGD2, and down-regulating PME2, RHD1, WAT1, TPS1, PME25 and CSLG2. Most of them showed same expression trends in *Pst* DC3000 infected plants, except CSLG2, which polymerizes the backbones of hemicelluloses of plant cell wall. CSLG2 was down-regulated in COS pretreated plants (0.63-fold), while up-regulated in *Pst* DC3000 infected plants (1.35-fold), and enhanced to an even higher amount in COS + *Pst* treated plants (2.19-fold), suggesting that COS pretreatment shown different regulation pattern of CSLG2 in different treatment background. Besides CSLG2, COS pretreatment in *Pst* DC3000 infected plants shown more obviously up-regulating trends of the proteins involved in cell wall components biosynthesis and modification process than *mock + Pst* treatment, such as XTH24, UCC2 and K19E20.1, which contribute to enhancing plant resistance to *Pst* DC3000.

**Table 1**
The cell wall biosynthesis and modification related proteins, which significantly changed in COS pretreated or *Pst* DC3000 infected plants. The "COS" group means pretreated *Arabidopsis* with 50 mg/L COS 3 d before inoculated with 10 mM $MgSO_4$. The "Mock + *Pst*" or "COS + *Pst*" group means pretreated *Arabidopsis* with water or 50 mg/L COS 3d before inoculated with *Pst* DC3000. Each group contained more than sixty *Arabidopsis* plants.

| Accession No. | ATG No. | Protein name | Function description | Ratio (Mean) | | |
|---|---|---|---|---|---|---|
| | | | | COS | Mock + Pst | COS + Pst |
| Q9SRT9 | AT3G02230 | RGP1 | Required for proper cell wall formation. | 1.276 | 1.503 | 1.391 |
| Q9LIA8 | AT3G29360 | UGD2 | Required for the formation of cell wall. | 1.35 | 1.396 | 1.106 |
| Q9LSG3 | AT3G25140 | QUA1 | Involved in pectin biosynthetic process. | 1.357 | 1.083 | 1.189 |
| Q43867 | AT1G53840 | PME1 | Pectinesterase, involved in the modification of cell wall via pectin demethylesterification. | 0.713 | 0.521 | 0.524 |
| Q42534 | AT1G53830 | PME2 | | 0.479 | 0.498 | 0.395 |
| O22149 | AT2G45220 | PME17 | | 0.858 | 2.04 | 2.136 |
| Q94CB1 | AT3G10720 | PME25 | | 0.545 | 0.62 | 0.6 |
| Q9C7W7 | AT1G64440 | RHD1 | Involved in channeling UDP-D-galactose into cell wall polymers. | 0.59 | 0.57 | 0.668 |
| Q94AP3 | AT1G75500 | WAT1 | Required for secondary wall formation in fibers. | 0.547 | 0.515 | 0.51 |
| Q9SYM4 | AT1G78580 | TPS1 | Regulates cell wall deposition. | 0.605 | 0.573 | 0.564 |
| Q8VYR4 | AT4G24000 | CSLG2 | Polymerize the backbones of hemicelluloses of plant cell wall. | 0.631 | 1.346 | 2.188 |
| Q9C9W3 | AT1G67980 | CCOAMT | Involved in the reinforcement of the plant cell wall. | 0.825 | 2.242 | 2.235 |
| Q8RY29 | AT2G41850 | PGAZAT | Polygalacturonase, involved in cell wall modification. | 0.897 | 2.045 | 1.966 |
| Q941L0 | AT5G05170 | CEV1 | Involved in the primary cell wall formation. | 0.827 | 0.539 | 0.468 |
| Q9LLR7 | AT5G59320 | LTP3 | May play a role in wax or cutin deposition in the cell walls. | 1.231 | 0.483 | 0.519 |
| Q9XFS7 | AT3G51600 | LTP5 | | 0.81 | 0.567 | 0.532 |
| Q9S7I3 | AT2G38530 | LTP2 | | 0.824 | 0.581 | 0.534 |
| P24806 | AT4G30270 | XTH24 | Involved in plant-type cell wall loosening participates in cell wall construction. | 0.838 | 1.297 | 1.502 |
| Q8LEG3 | AT4G26760 | MAP65-2 | Involved in the regulation of microtubules organization and dynamics. | 0.462 | 0.548 | 0.491 |
| Q9M439 | AT1G10070 | BCAT-2 | Involved in cell wall development. | 1.062 | 2.152 | 1.918 |
| O80517 | AT2G44790 | UCC2 | Acts as an electron carrier involved in lignin formation. | 1.025 | 1.452 | 1.512 |
| Q02972 | AT4G37990 | ELI3-2 | Involved in lignin biosynthesis. | 0.869 | 1.914 | 1.665 |
| Q39034 | AT5G19890 | F28I16.40 | Peroxidase, involved in the biosynthesis and degradation of lignin. | 0.883 | 2.309 | 1.886 |
| Q9SMU8 | AT3G49120 | PRXCB | | 0.774 | 1.502 | 1.516 |
| Q9LDN9 | AT4G08770 | Prx37 | | 0.88 | 2.786 | 2.25 |
| Q9SB81 | AT4G21960 | PRXR1 | | 0.689 | 0.54 | 0.535 |
| Q43731 | AT4G37520 | F19F18.10 | | 0.832 | 1.536 | 1.45 |
| O24603 | AT2G43570 | CHI | Chitinase, which involved in cell wall macromolecule catabolic process. | 0.953 | 2.187 | 2.363 |
| Q93WF1 | AT5G48900 | K19E20.1 | Pectin lyase-like superfamily protein. | 1.122 | 1.446 | 1.517 |

There are four pectinesterases (PME1, PME2, PME17 and PME25) which have the same function in the modification of cell walls via demethylesterification of cell wall pectin, were differently expressed during *Arabidopsis-Pst* DC3000 interaction. PME17 was significantly up-regulated, while PME1, PME2 and PME25 were down-regulated in *Pst* DC3000 infected plants, implying the different roles of PMEs in plant defense response, and PME17 may plays positive role in plant defense response. Similar with pectinesterases, there are 5 peroxidases (F28I16.40, PRXCB, Prx37, PRXR1 and F19F18.10) which play roles in the biosynthesis and degradation of lignin also shown different expression during *Pst* DC3000 invasion. PRXR1 was significantly down-regulated, and the other peroxidases were up-regulated, suggesting the negative role of PRXR1 in plant defense response.

Besides reinforce the cell wall to hinder pathogens invasion, plant cell also secreting enzymes which degrading the component of pathogen cell walls to kill the pathogens (Pusztahelyi, 2018). CHI, which possess chitinase activity, was obviously increased after *Pst* DC3000 invasion, 2.19-fold in *mock + Pst* treatment, and 2.40-fold in *COS + Pst* plants, compared with mock treated plants. Chitinases which degrading chitin from the cell wall of pathogens, are involved in the early events of host-pathogens interaction, have been used as marker genes for the activated plant defense response for many years (Maldonado-Alconada et al., 2011; van Aubel et al., 2016; Yang et al., 2017). However, as bacteria, no chitin component exists in *Pst* DC3000. From the previous studies, chitinase expression is not depended on the pathogens, some key PR genes that response to pathogens attack, such as PR-3, PR-4, PR-8 and PR-11 are different types of chitinase, thus chitinase also up-regulated in *Arabidopsis–Pst* DC3000 interaction (Pusztahelyi, 2018).

### 4.5. Proteins related to plant growth and development

In *Pst* DC3000 infected plants, proteins related to plant photosynthesis were differentially modulated during *Pst* DC3000 infection. For example, proteins plays positive roles in photosynthesis, including CAB2 (AT1G29910), RPI2 (AT2G01290), RCA (AT2G39730), PPD7 (AT3G05410), GUN4 (AT3G59400), TAP38 (AT4G27800), and VAR3 (AT5G17790) were down-regulated; NYC1 (AT4G13250), MES16 (AT4G16690), and PPH (AT5G13800), which involved in chlorophyll degradation and breakdown, were significantly increased after *Pst* DC3000 infection, suggesting the suppressed photosynthesis in *Pst* DC3000 infected plants (Table S4). These results were consistent with previous studies, since some effectors of *Pst* DC3000 were aimed to destroy host plants photosynthesis, such as HopN1, which interferes with photosystem II activity in chloroplast preparations (Rodriguez-Herva et al., 2012). These phenomena also happened in other virus or bacteria infected plants (Di Carli et al., 2010; Rahoutei et al., 2000; Scharte et al., 2005; Zhong et al., 2017). And as response, *Pst* DC3000 infected plants up-regulated 21 proteins involved in plant growth to enhancing plant growth and defense capacity, such as AE7 (AT1G68310), VSP1 (AT5G24780), VSP2 (AT5G24770) and VPS20.1 (AT5G63880).

In COS pretreated plants, 40 proteins related to plant growth and development were significantly changed, 21 of them were up-regulated, and 19 proteins were down-regulated (Table S4). The key proteins involved in photosystem, including PSAE-1 (AT4G28750), PSAN (AT5G64040), YCF3 (ArthCp023) and LHCA1 (AT3G54890) from photosystem I, PSB28 (AT4G28660), PSBO1 (AT5G66570), and PSBH (ATCG00710) from photosystem II were significantly increased after COS pretreatment compared with mock treated plants. The proteins regulating photosynthesis such as CP12 (AT3G62410), the proteins related to chlorophyll biosynthesis and binding such as CHLG (AT3G51820) and LHCB3 (AT5G54270) were also up-regulated in COS pretreated plants. All these up-regulated photosynthesis-related proteins indicating COS has positive effect on protecting the

photosynthetic machinery and improving photosynthetic efficiency during pathogens infections. Thus, except for the widely known effect on inducing plant innate immunity, COS also shown obvious effect on enhancing plant growth by increase the content of chlorophyll, enhance the photosynthesis in multiple plants, including *camellia* (Li and Zhu, 2013), *Buddhist pine* (Wang et al., 2017), *wheat* (Zhang et al., 2016, 2018c) and *rice* (Yang et al., 2017).

Despite the positive roles of COS on plant photosynthesis, the differently modulated photosynthesis related proteins during *Pst* DC3000 invasion still remains the same trends in COS pretreated groups (*COS* + *Pst*), suggesting COS pretreatment has no significantly positive effect on these plant photosynthesis-related proteins during *Pst* DC3000 invasion. However, 27 proteins related to plant growth and development were highly up-regulated, together with the pre-activated photosynthesis before *Pst* DC3000 in COS pretreated plants, all have positive effects on plant defense against photosynthesis destroy of *Pst* DC3000 (Table S4). To better understand the dynamic regulation of these proteins, 83 significantly changed proteins that involved in plant growth were selected and analyzed by artificial division carefully, and a hierarchical clustering analysis were carried out.

The significantly changed proteins related to plant growth were divided into six clusters based on the regulation pattern in different treatment groups (Fig. 6). Most proteins in cluster III, IV and VI shared concordant expression patterns, protein abundance in cluster III was up-regulated and that in cluster IV and VI was significantly down-regulated, suggesting that COS pretreatment and *Pst* DC3000 infection resulted in similar protein abundance variation for many proteins related to plant growth. Thus, COS pretreatment seems to pre-enhancing photosynthesis and pre-promoting plant growth before pathogen infection, which providing stronger photosynthesis capacity of plants to defense when pathogen attack.

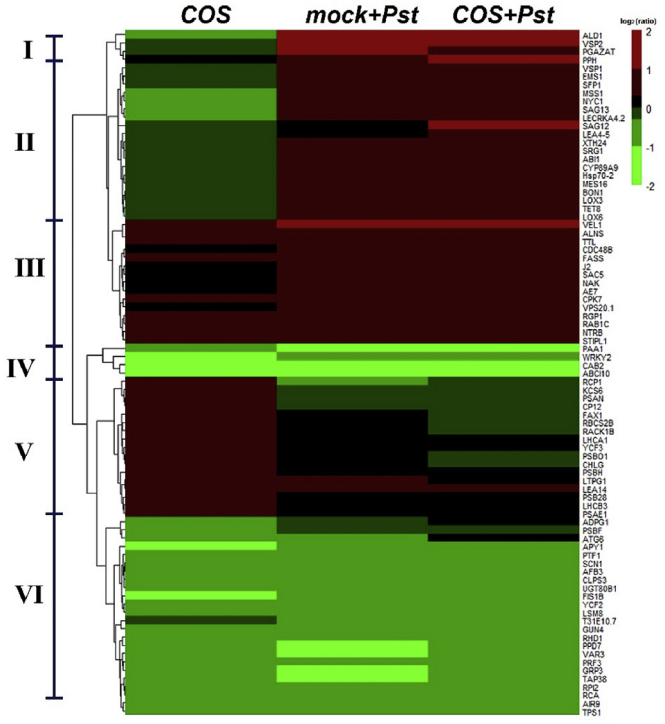


**Fig. 6.** Hierarchical cluster analysis of significantly changed proteins related to plant growth and development in COS, *mock* + *Pst* and *COS* + *Pst* groups, compared with *mock* treated *Arabidopsis*. The "COS" group means pretreated *Arabidopsis* with 50 mg/L COS 3 d before inoculated with 10 mM $MgSO_4$. The "Mock + *Pst*" or "COS + *Pst*" group means pretreated *Arabidopsis* with water or 50 mg/L COS 3d before inoculated with *Pst* DC3000. Each group contained more than sixty *Arabidopsis* plants.

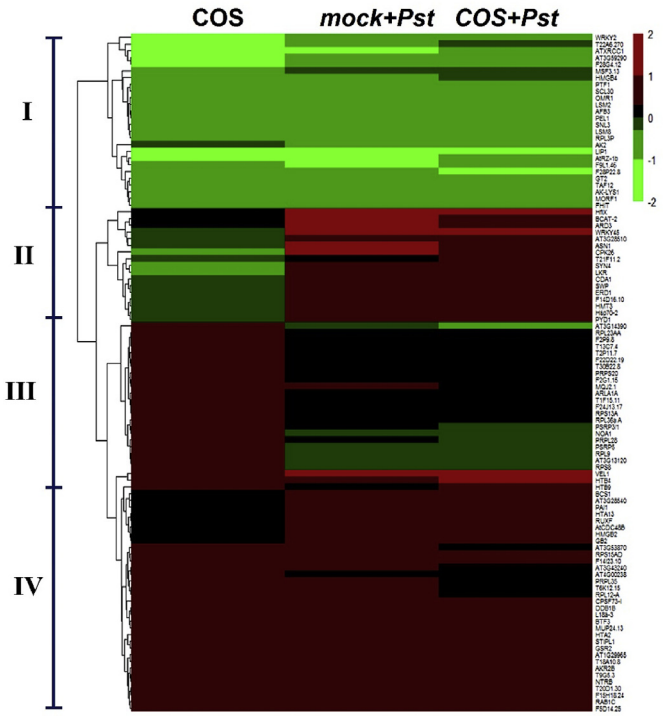


**Fig. 7.** Hierarchical cluster analysis of significantly changed proteins related to transcription and translation in COS, *mock* + *Pst* and *COS* + *Pst* groups, compared with *mock* treated *Arabidopsis*. The "COS" group means pretreated *Arabidopsis* with 50 mg/L COS 3 d before inoculated with 10 mM $MgSO_4$. The "Mock + *Pst*" or "COS + *Pst*" group means pretreated *Arabidopsis* with water or 50 mg/L COS 3d before inoculated with *Pst*DC3000. Each group contained more than sixty *Arabidopsis* plants.

### 4.6. Proteins related to stress response, transcription and protein homeostasis

Except for the plant resistance enhancement and plant growth promotion effect, COS also worked effectively in improving plant stress resilience, such as cold, drought, salinity stresses (Cheplick et al., 2018; Safikhan et al., 2018; Zhang et al., 2018b; Zhou et al., 2018). From our proteomics analysis, 13 proteins that involved in response to salt stress, cold conditions, drought, and herbicides, were changed significantly after COS pretreatment (Table S5). Among these proteins, three up-regulated proteins that is T18K17.10 (AT1G73230), KTI1 (AT1G73260), GSTU27 (AT3G43800), and five down-regulated proteins that is CRK2 (AT1G70520), GSTU2 (AT2G29480), PIP2B (AT2G37170), T6A23.9 (AT2G38710) and RAB18 (AT5G66400), were not participate in plant defense response to *Pst* DC3000 infection.

And from previous studies, many stress-related proteins also play important roles in conferring protective defenses against pathogen infections (Zhong et al., 2017). Therefore, expression of stress response proteins also significantly changed during *Pst* DC3000 infection, 30 and 34 stress-related proteins were differently regulated in *mock* + *Pst* and *COS* + *Pst* treated plants, respectively (Table S5). Although 25 proteins shared similar regulation pattern between *mock* + *Pst* and *COS* + *Pst* treatment, there are also exist some proteins has distinct regulation. Some proteins were only up-regulated in *COS* + *Pst* treated plants, such as FD1 which response to karrikin, HMGB2 which confers sensitivity to salt and drought stresses, Hop3 which response to high light intensity and heat, and etc.

Numerous proteins are required to carry out the pre-activated plant defense response caused by COS pretreatment and the activated plant immune system caused by *Pst* DC3000 infection, thus the transcription and translation process were reprogramming. 101 proteins related to

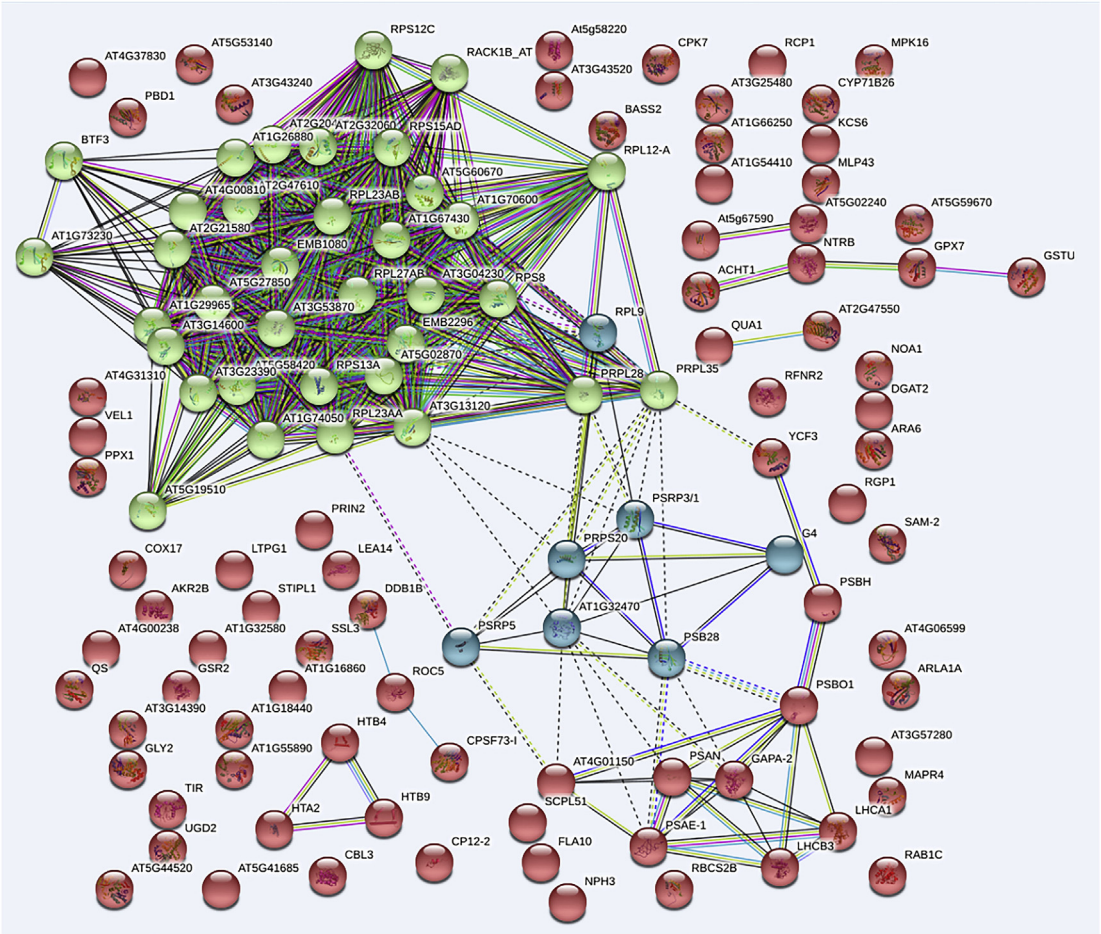


**Fig. 8.** The potential protein-protein interaction network of the up-regulated proteins response to COS pretreatment by using the search tool for the retrieval of Interacting Genes/Proteins (STRING) construction. The network, made with a medium confidence cutoff (0.7) using the k-means clustering method, includes three clusters presented as different colors. Line color indicates the type of interaction evidence. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

gene transcription and translation process differently expressed in COS pretreated and/or *Pst* DC3000 infected plants from proteomics analysis (Table S6). These significantly changed proteins were divided into four clusters according to the hierarchical clustering analysis (Fig. 7). The regulation pattern of most proteins from cluster I and IV are shown high similarity in different treatment groups, suggesting the pre-reprogramming effect of COS on gene expression process are similar with *Pst* DC3000 infection. Some proteins from cluster II and III showed evident difference between COS pretreated and *Pst*DC3000 infected plants, which may be due to the difference between pre-activated and activated plant immune response.

As the direct performer of vital movement, protein homeostasis which including protein biosynthesis, modification, transport and degradation are important to achieve unified cellular responses to developmental and environmental cues (Hinkson and Elias, 2011). Therefore, except for the activated gene transcription and translation processes, 12 proteins related to protein transport, 6 proteins related to protein folding, 33 proteins related to protein modification and 17 proteins involved in protein degradation were differently expressed in the plants response to COS or/and *Pst* DC3000 infection (Table S7). These significantly changed proteins in COS pretreated or *Pst* DC3000 infected plants suggesting both COS pretreatment and *Pst* DC3000 infection can reprogram protein homeostasis processes, and which may contribute to enhancing defense response and increasing survival during *Pst* DC3000 infection.

### 4.7. Key proteins involved in COS induction

To further reveal the induction mechanism of COS, the protein-protein interaction network of the up-regulated proteins in COS pretreated plants were analyzed by using STRING database with a cutoff confidence score of $\geq 0.7$. The protein-protein interaction network which contained 122 nodes and 580 edges has significantly more interactions than expected (Fig. 8). By using k-means clustering method, the network includes three clusters presented as different colors. 36 proteins in green cluster are all high-degree hub nodes with node degree range from 13 to 36. Among these proteins, one protein is transcription factor (BTF3, AT1G17880), one protein is elongation factor which involved in translational elongation (T20D1.30, AT5G19510), and 33 proteins are ribosomal proteins which involved in protein translation, suggesting the reprogramed gene transcription and translation process in COS pretreated plants.

RACK1B (AT1G48630) is a high-degree hub node with node degree of 28 in green cluster. RACK1 (including RACK1A, RACK1B and RACK1C) is a receptor for activated kinase that plays a role in multiple signal transduction pathways, but it possesses unknown enzymatic activity (Adams et al., 2011; Chen et al., 2006). Evidence is accumulating that RACK1 acts as a versatile scaffold protein and regulates multiple biological processes, including development, phytohormone responses, protein translation, micro RNA biogenesis and multiple environmental stress responses, also functions in disease resistance and the innate immune pathway (Chen et al., 2006; Guo et al., 2009; Guo and Sun,

2017; Hyodo et al., 2019; Speth et al., 2013). The important roles in plant immunity and the high interaction with the key proteins related to protein translation makes RACK1B has the potential to be the key kinase receptor for COS signals. RACK1 is involved in the protease IV and ArgC (proteases from pathogens) induced signaling pathway but not the flg22 (peptides from bacterial flagellin) pathway according to previous study (Cheng et al., 2015). Thus, the involvement of RACK1B in COS induced signaling pathway suggesting the difference induction mechanism between COS and flg22.

## 5. Conclusions

By using *Arabidopsis-Pst* DC3000 interaction, the underlying mechanism of COS induced resistance were partly revealed by proteomic data presented in this study. COS pretreatment pre-activating plant defense response before pathogens invasion by gene transcription and translation reprograming, and many proteins related to signaling transduction, ROS homeostasis, defense response, cell wall biosynthesis and modification, plant growth and development, and stress response were differently regulated. Many proteins are completely opposite regulated between COS pretreatment and *Pst* DC3000 infection, which reveals the different regulation pattern of many biological processes between the “primed state” immunity caused by COS and the “activated state” immunity caused by *Pst* DC3000 invasion.

In conclusion, as a broad-spectrum immunity elicitor, COS treatment enable plant to fine-tuning its defenses for a more rapid and robust response to subsequentially abiotic and biotic stresses, which makes COS worked effectively in inducing resistance in multiple plants against various pathogens, and also showed obvious effect in promoting plant growth and stimulating the resistance to abiotic stresses. Moreover, some key proteins worked in COS induced signaling pathway were also find out by this proteomic analysis, which will facilitate the sequent research on COS induction mechanism.

## Author contributions

X.C.J: carried out the proteomics analysis, interpreted the data and wrote the manuscript. H.Q.Q: carried out the proteomics analysis. S.K.B: corrected the language. T.M.L: planted Arabidopsis and sampled. J.X.H: planted Arabidopsis and sampled. S.Q.X: planted Arabidopsis and sampled. M.L.Y: revised and approved the final manuscript. H.Y: designed the experiments, revised and approved the final manuscript.

## CRediT authorship contribution statement

**Xiaochen Jia:** Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Hongqiang Qin:** Data curation, Investigation, Methodology, Validation. **Santosh Kumar Bose:** Formal analysis. **Tongmei Liu:** Investigation. **Jinxia He:** Investigation. **Shangqiang Xie:** Investigation. **Mingliang Ye:** Resources, Software, Supervision. **Heng Yin:** Funding acquisition, Project administration, Resources, Supervision.

## Declaration of competing interest

The authors declare that the submitted work was not carried out in the presence of any personal, professional or financial relationships that could potentially be constructed as a conflict of interest. This paper is not being submitted to any other journal and all authors approve this submission.

## Acknowledgments

We are grateful to Prof. Jianmin Zhou (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for kindly providing *Pst* DC3000. This work was supported by National Key R&D Program of China (2017YFD0200900) Subject 2 (2017YFD0200902), Chinese National Natural Science Foundation of China (31672077), Liao Ning Revitalization Talents Program (XLYC1807041), and Dalian Key R&D Program Projects (2018YF23SN069).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.01.037.

## References

Abdallah, C., Sergeant, K., Guillier, C., Dumas-Gaudot, E., Leclercq, C.C., Renaut, J., 2012. Optimization of iTRAQ labelling coupled to OFFGEL fractionation as a proteomic workflow to the analysis of microsomal proteins of *Medicago truncatula* roots. Proteome Sci. 10, 37.

Adams, D.R., Ron, D., Kiely, P.A., 2011. RACK1, A multifaceted scaffolding protein: structure and function. Cell Commun. Signal. 9.

Ali, M., Cheng, Z.H., Ahmad, H., Hayat, S., 2018. Reactive oxygen species (ROS) as defenses against a broad range of plant fungal infections and case study on ROS employed by crops against *Verticillium dahliae wilts*. J. Plant Interact. 13, 353–363.

Aragon, W., Reina-Pinto, J.J., Serrano, M., 2017. The intimate talk between plants and microorganisms at the leaf surface. J. Exp. Bot. 68, 5339–5350.

Aranega-Bou, P., Leyva, M.D., Finiti, I., Garcia-Agustin, P., Gonzalez-Bosch, C., 2014. Priming of plant resistance by natural compounds. Hexanoic acid as a model. Front. Plant Sci. 5.

Berg, G., 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl. Microbiol. Biotechnol. 84, 11–18.

Betsuyaku, S., Katou, S., Takebayashi, Y., Sakakibara, H., Nomura, N., Fukuda, H., 2017. Salicylic acid and jasmonic acid pathways are activated in spatially different domains around the infection site during effector-triggered immunity in *Arabidopsis thaliana*. Plant Cell Physiol. 59, 8–16.

Carr, J.P., Lewsey, M.G., Palukaitis, P., 2010. Signaling in induced resistance. Nat. Eng Resist. Plant Viruses, Pt Ii 76, 57–121.

Chen, J.G., Ullah, H., Temple, B., Liang, J.S., Guo, J.J., Alonso, J.M., Ecker, J.R., Jones, A.M., 2006. RACK1 mediates multiple hormone responsiveness and developmental processes in *Arabidopsis*. J. Exp. Bot. 57, 2697–2708.

Chen, X.L., Xie, X., Wu, L.Y., Liu, C.Y., Zeng, L.R., Zhou, X.P., Luo, F., Wang, G.L., Liu, W.D., 2018. Proteomic analysis of ubiquitinated proteins in rice (*Oryza sativa*) after treatment with pathogen-associated molecular pattern (PAMP) elicitors. Front. Plant Sci. 9, 1064.

Chen, Y.F., Zhan, Y., Zhao, X.M., Guo, P., An, H.L., Du, Y.G., Han, Y.R., Liu, H., Zhang, Y.H., 2009. Functions of oligochitosan induced protein kinase in *tobacco mosaic virus* resistance and pathogenesis related proteins in *tobacco*. Plant Physiol. Biochem. 47, 724–731.

Cheng, Z.Y., Li, J.F., Niu, Y.J., Zhang, X.C., Woody, O.Z., Xiong, Y., Djonovic, S., Millet, Y., Bush, J., McConkey, B.J., Sheen, J., Ausubel, F.M., 2015. Pathogen-secreted proteases activate a novel plant immune pathway. Nature 521, 213.

Cheplick, S., Sarkar, D., Bhowmik, P.C., Shetty, K., 2018. Improved resilience and metabolic response of transplanted blackberry plugs using chitosan oligosaccharide elicitor treatment. Can. J. Plant Sci. 98, 717–731.

Cox, J., Hein, M.Y., Luber, C.A., Paron, I., Nagaraj, N., Mann, M., 2014. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol. Cell. Proteomics 13, 2513–2526.

Cui, S.J., Wang, J.Y., Gao, F.Y., Sun, G.C., Liang, J.S., 2018. Quantitative proteomic analyses reveal that RBBI3.3, a trypsin inhibitor protein, plays an important role in *Magnaporthe oryzae* infection in rice. Plant Growth Regul. 86, 365–374.

de Vega, D., Newton, A.C., Sadanandom, A., 2018. Post-translational modifications in priming the plant immune system: ripe for exploitation? FEBS Lett. 592, 1929–1936.

Di Carli, M., Villani, M.E., Bianco, L., Lombardi, R., Perrotta, G., Benvenuto, E., Donini, M., 2010. Proteomic analysis of the plant-virus interaction in *Cucumber Mosaic Virus* (CMV) resistant transgenic tomato. J. Proteome Res. 9, 5684–5697.

Evans, C., Noirel, J., Ow, S.Y., Salim, M., Pereira-Medrano, A.G., Couto, N., Pandhal, J., Smith, D., Pham, T.K., Karunakaran, E., Zou, X., Biggs, C.A., Wright, P.C., 2012. An insight into iTRAQ: where do we stand now? Anal. Bioanal. Chem. 404, 1011–1027.

Fabre, F., Vignassa, M., Urbach, S., Langin, T., Bonhomme, L., 2019. Time-resolved dissection of the molecular crosstalk driving *Fusarium head blight* in wheat provides new insights into host susceptibility determinism. Plant Cell Environ. 42, 2291–2308.

Franck, C.M., Westermann, J., Boisson-Dernier, A., 2018. Plant malectin-like receptor kinases: from cell wall integrity to immunity and beyond. Annu. Rev. Plant Biol. 69, 301–328.

Frevert, C.W., Felgenhauer, J., Wygrecka, M., Nastase, M.V., Schaefer, L., 2018. Danger-associated molecular patterns derived from the extracellular matrix provide temporal control of innate immunity. J. Histochem. Cytochem. 66, 213–227.

Guo, J.J., Wang, J.B., Xi, L., Huang, W.D., Liang, J.S., Chen, J.G., 2009. RACK1 is a negative regulator of ABA responses in *Arabidopsis*. J. Exp. Bot. 60, 3819–3833.

Guo, R.K., Sun, W.N., 2017. Sumoylation stabilizes RACK1B and enhance its interaction with RAP2.6 in the abscisic acid response. Sci. Rep. 7.

Hake, K., Romeis, T., 2019. Protein kinase-mediated signalling in priming: immune signal initiation, propagation, and establishment of long-term pathogen resistance in plants. Plant Cell Environ. 42, 904–917.

Hadwiger, L.A., 2015. Anatomy of a nonhost disease resistance response of pea to *Fusarium solani*: PR gene elicitation via DNase, chitosan and chromatin alterations. Front. Plant Sci. 6.
Hadwiger, L.A., Tanaka, K., 2017. Non-host Resistance: DNA damage is associated with SA signaling for induction of PR genes and contributes to the growth suppression of a pea pathogen on pea endocarp tissue. Front. Plant Sci. 8.
Hinkson, I.V., Elias, J.E., 2011. The dynamic state of protein turnover: it's about time. Trends Cell Biol. 21, 293–303.
Hyodo, K., Suzuki, N., Okuno, T., 2019. Hijacking a host scaffold protein, RACK1, for replication of a plant RNA virus. New Phytol. 221, 935–945.
Janda, M., Ruelland, E., 2015. Magical mystery tour: salicylic acid signalling. Environ. Exp. Bot. 114, 117–128.
Jia, X., Meng, Q., Zeng, H., Wang, W., Yin, H., 2016. Chitosan oligosaccharide induces resistance to *Tobacco mosaic virus* in *Arabidopsis* via the salicylic acid-mediated signalling pathway. Sci. Rep. 6, 26144.
Jia, X., Zeng, H., Wang, W., Zhang, F., Yin, H., 2018. Chitosan oligosaccharide induces resistance to *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis thaliana* by activating both salicylic acid- and jasmonic acid-mediated pathways. Mol. Plant-Microbe Interact. 31, 1271–1279.
Koornneef, A., Pieterse, C.M.J., 2008. Cross talk in defense signaling. Plant Physiol. 146, 839–844.
Kutschera, A., Ranf, S., 2019. The multifaceted functions of lipopolysaccharide in plant-bacteria interactions. Biochimie 159, 93–98.
Lakra, N., Kaur, C., Anwar, K., Singla-Pareek, S.L., Pareek, A., 2018. Proteomics of contrasting rice genotypes: identification of potential targets for raising crops for saline environment. Plant Cell Environ. 41, 947–969.
Li, Q.F., Wang, J.D., Xiong, M., Wei, K., Zhou, P., Huang, L.C., Zhang, C.Q., Fan, X.L., Liu, Q.Q., 2018. iTRAQ-Based analysis of proteins co-regulated by brassinosteroids and gibberellins in rice embryos during seed germination. Int. J. Mol. Sci. 19.
Li, S.J., Zhu, T.H., 2013. Biochemical response and induced resistance against anthracnose (*Colletotrichum camelliae*) of camellia (*Camellia pitardii*) by chitosan oligosaccharide application. For. Pathol. 43, 67–76.
Liu, D., Jiao, S., Cheng, G., Li, X., Pei, Z., Pei, Y., Yin, H., Du, Y., 2018. Identification of chitosan oligosaccharides binding proteins from the plasma membrane of wheat leaf cell. Int. J. Biol. Macromol. 111, 1083–1090.
Liu, P., Zhang, H.M., Yu, B.Y., Xiong, L.M., Xia, Y.J., 2015. Proteomic identification of early salicylate- and flg22-responsive redox-sensitive proteins in *Arabidopsis*. Sci. Rep. 5.
Loake, G., Grant, M., 2007. Salicylic acid in plant defence-the players and protagonists. Curr. Opin. Plant Biol. 10, 466–472.
Ma, D.D., Liu, Q.X., Zhang, M.H., Feng, J.H., Li, X.M., Zhou, Y., Wang, X.J., 2019. iTRAQ-based quantitative proteomics analysis of the spleen reveals innate immunity and cell death pathways associated with heat stress in *broilers (Gallus gallus)*. J. Proteom. 196, 11–21.
Maldonado-Alconada, A.M., Echevarria-Zomeno, S., Lindermayr, C., Redondo-Lopez, I., Durner, J., Jorrin-Novo, J.V., 2011. Proteomic analysis of *Arabidopsis* protein S-nitrosylation in response to inoculation with *Pseudomonas syringae*. Acta Physiol. Plant. 33, 1493–1514.
Martinez-Medina, A., Flors, V., Heil, M., Mauch-Mani, B., Pieterse, C.M.J., Pozo, M.J., Ton, J., van Dam, N.M., Conrath, U., 2016. Recognizing plant defense priming. Trends Plant Sci. 21, 818–822.
Martinez-Medina, A., Van Wees, S.C.M., Pieterse, C.M.J., 2017. Airborne signals from *Trichoderma* fungi stimulate iron uptake responses in roots resulting in priming of jasmonic acid-dependent defences in shoots of *Arabidopsis thaliana* and *Solanum lycopersicum*. Plant Cell Environ. 40, 2691–2705.
Poncini, L., Wyrsch, I., Denervaud Tendon, V., Vorley, T., Boller, T., Geldner, N., Metraux, J.P., Lehmann, S., 2017. In roots of *Arabidopsis thaliana*, the damage-associated molecular pattern *At*Pep1 is a stronger elicitor of immune signalling than flg22 or the chitin heptamer. PloS One 12, e0185808.
Pusztahelyi, T., 2018. Chitin and chitin-related compounds in plant-fungal interactions. Mycology 9, 189–201.
Rahoutei, J., Garcia-Luque, I., Baron, M., 2000. Inhibition of photosynthesis by viral infection: effect on PSII structure and function. Physiol. Plantarum 110, 286–292.
Ramirez-Prado, J.S., Abulfaraj, A.A., Rayapuram, N., Benhamed, M., Hirt, H., 2018. Plant ommunity: from signaling to epigenetic control of defense. Trends Plant Sci. 23, 833–844.
Rodriguez-Herva, J.J., Gonzalez-Melendi, P., Cuartas-Lanza, R., Antunez-Lamas, M., Rio-Alvarez, I., Li, Z., Lopez-Torrejon, G., Diaz, I., del Pozo, J.C., Chakravarthy, S., Collmer, A., Rodriguez-Palenzuela, P., Lopez-Solanilla, E., 2012. A bacterial cysteine protease effector protein interferes with photosynthesis to suppress plant innate immune responses. Cell Microbiol. 14, 669–681.
Safikhan, S., Khoshbakht, K., Chaichi, M.R., Amini, A., Motesharezadeh, B., 2018. Role of chitosan on the growth, physiological parameters and enzymatic activity of milk thistle (*Silybum marianum (L.) Gaertn.*) in a pot experiment. J. Appl. Res. Med. Aromat. Plants 10, 49–58.
Scharte, J., Schon, H., Weis, E., 2005. Photosynthesis and carbohydrate metabolism in tobacco leaves during an incompatible interaction with *Phytophthora nicotianae*. Plant Cell Environ. 28, 1421–1435.
Seo, J.S., Diloknawarit, P., Park, B.S., Chua, N.H., 2019. ELF18-INDUCED LONG NONCODING RNA 1 evicts fibrillarin from mediator subunit to enhance PATHOGENESIS-RELATED GENE 1 (PR1) expression. New Phytol. 221, 2067–2079.
Silva, M.S., Arraes, F.B.M., Campos, M.D., Grossi-de-Sa, M., Fernandez, D., Candido, E.D., Cardoso, M.H., Franco, O.L., Grossi-de-Sa, M.F., 2018. Potential biotechnological assets related to plant immunity modulation applicable in engineering disease-resistant crops. Plant Sci. 270, 72–84.
Spence, C.A., Lakshmanan, V., Donofrio, N., Bais, H.P., 2015. Crucial roles of abscisic acid biogenesis in virulence of rice blast fungus *Magnaporthe oryzae*. Front. Plant Sci. 6.
Speth, C., Willing, E.M., Rausch, S., Schneeberger, K., Laubinger, S., 2013. RACK1 scaffold proteins influence miRNA abundance in *Arabidopsis*. Plant J. 76, 433–445.
Strange, R.N., Scott, P.R., 2005. Plant disease: a threat to global food security. Annu. Rev. Phytopathol. 43, 83–116.
Sun, Y.C., Guo, H.J., Yuan, E.L., Ge, F., 2018. Elevated CO2 increases R gene-dependent resistance of Medicago truncatula against the pea aphid by up-regulating a heat shock gene. New Phytol. 217, 1696–1711.
van Aubel, G., Cambier, P., Dieu, M., Van Cutsem, P., 2016. Plant immunity induced by COS-OGA elicitor is a cumulative process that involves salicylic acid. Plant Sci. 247, 60–70.
Vellosillo, T., Vicente, J., Kulasekaran, S., Hamberg, M., Castresana, C., 2010. Emerging complexity in reactive oxygen species production and signaling during the response of plants to pathogens. Plant Physiol. 154, 444–448.
Wang, M.Y., Chen, Y.C., Zhang, R., Wang, W.X., Zhao, X.M., Du, Y.G., Yin, H., 2015. Effects of chitosan oligosaccharides on the yield components and production quality of different wheat cultivars (*Triticum aestivum L.*) in Northwest China. Field Crop. Res. 172, 11–20.
Wang, Z., Zhao, Y., Wei, H.X., 2017. Chitosan oligosaccharide addition affects current-year shoot of post-transplant Buddhist pine (*Podocarpus macrophyllus*) seedlings under contrasting photoperiods. iFor. Biogeosci. For. 10, 715–721.
Wu, X., Ding, C., Baerson, S.R., Lian, F., Lin, X., Zhang, L., Wu, C., Hwang, S.Y., Zeng, R., Song, Y., 2019. The roles of jasmonate signalling in nitrogen uptake and allocation in rice (*Oryza sativa L.*). Plant Cell Environ. 42, 659–672.
Wu, Y.Q., Tang, Y.H., Jiang, Y., Zhao, D.Q., Shang, J.L., Tao, J., 2018. Combination of transcriptome sequencing and iTRAQ proteome reveals the molecular mechanisms determining petal shape in herbaceous peony (*Paeonia lactiflora Pall.*). Biosci. Rep. 38.
Xin, X.F., He, S.Y., 2013. *Pseudomonas syringae* pv. *tomato* DC3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. Annu. Rev. Phytopathol. 51, 473–498.
Xu, Y.Q., Charles, M.T., Luo, Z.S., Mimee, B., Tong, Z.C., Veronneau, P.Y., Roussel, D., Rolland, D., 2019. Ultraviolet-C priming of strawberry leaves against subsequent *Mycosphaerella fragariae* infection involves the action of reactive oxygen species, plant hormones, and terpenes. Plant Cell Environ. 42, 815–831.
Yang, A.M., Yu, L., Chen, Z., Zhang, S.X., Shi, J., Zhao, X.Z., Yang, Y.Y., Hu, D.Y., Song, B.A., 2017. Label-free quantitative proteomic analysis of chitosan oligosaccharide-treated rice infected with *southern rice black-streaked dwarf virus*. Viruses-Basel 9.
Yin, H., Du, Y., Dong, Z., 2016. Chitin oligosaccharide and chitosan oligosaccharide: two similar but different plant elicitors. Front. Plant Sci. 7, 522.
Yin, H., Li, S., Zhao, X., Du, Y., Ma, X., 2006. cDNA microarray analysis of gene expression in *Brassica napus* treated with oligochitosan elicitor. Plant Physiol. Biochem. 44, 910–916.
Yin, H., Li, Y., Zhang, H.Y., Wang, W.X., Lu, H., Grevsen, K., Zhao, X.M., Du, Y.G., 2013. Chitosan oligosaccharides-triggered innate immunity contributes to *oilseed rape* resistance against *sclerotinia sclerotiorum*. Int. J. Plant Sci. 174, 722–732.
Yin, H., Zhao, X.M., Bai, X.F., Du, Y.G., 2010a. Molecular cloning and characterization of a *brassica napus L.* MAP kinase involved in oligochitosan-induced defense signaling. Plant Mol. Biol. Rep. 28, 292–301.
Yin, H., Zhao, X.M., Du, Y.G., 2010b. Oligochitosan: a plant diseases vaccine-A review. Carbohydr. Polym. 82, 1–8.
Zhang, T., Meng, L., Kong, W.W., Yin, Z.P., Wang, Y., Schneider, J.D., Chen, S.X., 2018a. Quantitative proteomics reveals a role of JAZ7 in plant defense response to *Pseudomonas syringae* DC3000. J. Proteom. 175, 114–126.
Zhang, X.Q., Li, K.C., Liu, S., Xing, R.G., Yu, H.H., Chen, X.L., Li, P.C., 2016. Size effects of chitooligomers on the growth and photosynthetic characteristics of wheat seedlings. Carbohydr. Polym. 138, 27–33.
Zhang, X.Q., Li, K.C., Liu, S., Zou, P., Xing, R.E., Yu, H.H., Chen, X.L., Qin, Y.K., Li, P.C., 2018b. Relationship between the degree of polymerization of chitooligomers and their activity affecting the growth of wheat seedlings under salt stress. J. Agric. Food Chem. 66 7551-7551.
Zhang, X.Q., Li, K.C., Xing, R.G., Liu, S., Chen, X.L., Yang, H.Y., Li, P.C., 2018. miRNA and mRNA expression profiles reveal insight into chitosan-mediated regulation of plant growth. J. Agric. Food Chem. 66, 3810–3822.
Zhong, X.T., Wang, Z.Q., Xiao, R.Y., Wang, Y.Q., Xie, Y., Zhou, X.P., 2017. iTRAQ, analysis of the tobacco leaf proteome reveals that RNA-directed DNA methylation (RdDM) has important roles in defense against *geminivirus-betasatellite* infection. J. Proteom. 152, 88–101.
Zhou, J.C., Chen, Q., Zhang, Y., Fan, L.Q., Qin, Z., Chen, Q.M., Qiu, Y.J., Jiang, L.H., Zhao, L.M., 2018. Chitooligosaccharides enhance cold tolerance by repairing photodamaged PS II in rice. J. Agric. Sci. 156, 888–899.
Ziv, C., Zhao, Z.Z., Gao, Y.G., Xia, Y., 2018. Multifunctional roles of plant cuticle during plant-pathogen interactions. Front. Plant Sci. 9.