Proteomic Analysis of Rice Plasma Membrane-Associated Proteins in Response to Chitooligosaccharide Elicitors

Fang Chen, Qun Li and Zuhua He*

(National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China)

Abstract

Chitooligomers or chitooligosaccharides (COS) are elicitors that bind to the plasma membrane (PM) and elicit various defense responses. However, the PM-bound proteins involved in elicitor-mediated plant defense responses still remain widely unknown. In order to get more information about PM proteins involved in rice defense responses, we conducted PM proteomic analysis of the rice suspension cells elicited by COS. A total of 14 up- or down-regulated protein spots were observed on 2-D gels of PM fractions at 12 h and 24 h after COS incubation. Of them, eight protein spots were successfully identified by MS (mass spectrography) and predicted to be associated to the PM and function in plant defense, including a putative PKN/PRK1 protein kinase, a putative pyruvate kinase isozyme G, a putative zinc finger protein, a putative MAR-binding protein MFP1, and a putative calcium-dependent protein kinase. Interestingly, a COS-induced pM5-like protein was identified for the first time in plants, which is a transmembrane nodal modulator in transforming growth factor-β (TGFβ) signaling in vertebrates. We also identified two members of a rice polyprotein family, which were up-regulated by COS. Our study would provide a starting point for functionality of PM proteins in the rice basal defense.

Key words: defense response; plasma membrane; proteomics; rice; chitooligosaccharide.


Plants have evolved a sophisticated and effective system to defend themselves against invading pathogens. In a typical disease resistance reaction, the co-presence of a pathogen Avr (avirulence) gene and corresponding plant disease resistance (R) gene triggers an incompatible interaction characterized by rapid programmed cell death called the hypersensitive response (HR) at sites of infection, and other defense-related responses such as cell wall reinforcement, activation of pathogenesis-related (PR) genes, rapid production of reactive oxygen intermediates (ROIs) and antimicrobial compounds (Staskawicz et al. 1995; Lamb and Dixon 1997; Dangl and Jones 2001). Following the HR, systemic acquired resistance (SAR) develops in distal plant tissues to limit the spread of invading virulent pathogens (Ryals et al. 1996). Both HR and SAR can be induced by microbial and chemical elicitors (Morris et al. 1998; Nakashita et al. 2002).

Chitooligomers or N-acetylchitooligosaccharides (COS), derived from cell walls of fungi or degradation of insoluble chitin, could induce various defense responses including generation of ROIs (Tsukada et al. 2002), production of defense-related enzymes, deposition of lignin and necrosis (Vander et al. 1998), and induction of defense-related genes (Minami et al. 1996; Nishizawa et al. 1999; Ramonell et al. 2002; Rabea et al. 2003). Interestingly, the Arabidopsis mutants of loss-of-function in chitin responsive genes showed increased susceptibility to the powdery mildew pathogen Erysiphe cichoracearum
(Ramonell et al. 2005). Our previous study showed that COS-induced cell death, rapid production of \( \text{H}_2\text{O}_2 \), and defense gene expression in rice suspension cells (Ning et al. 2004). It was shown that the COS-mediated oxidative burst depends on an NADPH oxidase. Consequently, rice disease resistance was enhanced by COS against the blast fungus (Ning et al. 2004). Those results strongly suggested that COS activates the rice defense responses via a mechanism similar to the hypersensitive response involved in the plant-microbe interactions. Similarly, bacteria-derived lipopolysaccharides could also induce defense responses associated with programmed cell death in rice cells (Desaki et al. 2006). Expression patterns of rice defense-related genes in response to an acetylchitooligosaccharide elicitor were studied with a gene chip containing randomly selected 8987 cDNAs (Akimoto-Tomiyama et al. 2003). These studies also provide the feasibility to dissect elicitor-mediated activation of non-specific defense in the model cereal crop. Interestingly, it was also reported that COS could bind to the plasma membrane (PM), suggesting that the COS signal might be perceived on the cell surface (Baureithel et al. 1994). Most recently, it was demonstrated that the rice protein CEBiP, a glycoprotein, serves as the PM receptor to recognize chitin fragments for defense signaling (Kaku et al. 2006). However, the PM-bound proteins involved in elicitor-mediated defense responses still remain widely unknown.

Recently, great progress has been achieved in plant proteomic studies, including proteomics of development, biotic and abiotic stress responses and subcellular organelles (Peck et al. 2001; Park 2004; Agrawal et al. 2005; Hajheidari et al. 2005; Yan et al. 2005; Yan et al. 2006). Organelles-based proteomics could provide more valuable information on protein compartmentalization and functionality, for example, PM, vacuolar membrane, mitochondria and chloroplasts (Tanaka et al. 2004). PM is particularly important for studies of defense responses, given the fact that most receptors and early responsive proteins for pathogens or elicitors are located in the PM. For example, proteomic analysis of the Arabidopsis PM identified components involved in transport, signal transduction, membrane trafficking and stress responses (Alexandersson et al. 2004).

To gain an insight into rice PM proteins involved in the early response in the COS-elicited defense, we conducted a PM proteomic analysis (2D-MS) of the rice suspension cells treated with COS elicitor in the present study. Through a modified method of PM protein purification, we could reproducibly display PM proteins on 2-D gels. We report here that 14 proteins of a total 250–300 PM proteins were differentially regulated in the COS-elicited cells, of which eight were determined by MS. We found for the first time that a putative pM5 protein could be involved in the rice early response to COS elicitors.

**Results**

**Differential expression of plasma membrane proteins in response to COS**

Our previous study showed that COS could effectively activate defense responses and programmed cell death in rice suspension cells (Ning et al. 2004). Through the improved procedure for PM protein preparation by combining the two-phase Figure 1. Representative 2-DE images of rice plasma membrane (PM) proteins at 12 h and 24 h after chitooligosaccharide (COS) inoculation.

Three biological repeats were carried out for data analysis. Regulated spots were marked with arrows. Only those with twofold and above change were treated as regulated proteins. Protein molecular mass (MW) markers and \( pI \) region are indicated.
partition method and the ProteoPrep membrane extraction kit, and monitoring PM purity with visible GFP fluorescence in the transgenic rice cells (Chen et al. 2007), we successfully made PM protein preparation elicited by COS, with purity over 70% and about 80% reproducibility among biological replicates. Substantially, this purity and reproducibility in PM protein preparation could meet the criterion of 2-D analysis in proteomics of a biological process (Chen et al. 2007).

We observed a total of about 250–300 visible protein spots on each 2-D gel with silver staining (Figure 1), similar to the report by Tanaka et al. (2004) and our previous study (Chen et al. 2007). Based on biological (independent separation of PM proteins from different branches of cell cultures) and technical (parallel 2D gel runs) replicates, we screened proteins that were differentially regulated in response to the COS elicitor. Screening by the image software indicated potential differentially regulated spots at 12 h and 24 h after COS eliciting (Figure 1). We determined the regulated proteins based on the criterion: regulated spots could be reproduced in all biological and technical experiments, and their levels (percentage volumes) changed at least twofold. Finally, we observed a total of 14 protein spots with reliable expression regulation in the COS-elicited defense response (Figures 1, 2). These proteins were regulated differentially in the COS-elicited cells (Table 1). There were three (spots 1, 4, 5) and two (spots 2 and 3) spots up- or down-regulated at 12 h after COS treatment, respectively (Table 1). Seven (spots 8, 9, 10, 11, 12, 13, 14) and two (spots 6 and 7) spots were up- or down-regulated at 24 h after COS treatment, respectively (Table 1).

**Identification of regulated PM proteins**

![Figure 2](image-url)  
**Figure 2.** Expression patterns of differentially regulated spots.  
(A) Profiles of total 14 regulated proteins in response to elicitor chitooligosaccharides (COS). C, control; T, COS treatment; 12 and 24, 12 h and 24 h for treatment.  
(B) Relative abundance and standard deviation of the 14 proteins in three biological comparisons with the controls was quantified by the Melanie 4 software. Only those with twofold and above changes are shown.  
*Protein 6 was completely suppressed at 24 h in COS inoculation.*
We conducted MS protein identification of the 14 protein spots, whose levels changed at least twofold (Figure 2A, B). Of 14 regulated spots, eight were successfully identified by MS with putative functions (Figure 3, Table 2); others either could not be determined by MS or matched non PM-associated proteins. Interestingly, we observed that a putative PKN/PRK1 (protein kinase N/protein kinase C-related kinase 1) protein kinase (spot 1), a putative zinc finger protein (spot 9), a putative MAR (matrix attachment region)-binding protein MFP1 (MAR binding filament-like protein 1) (spot 11) and a putative calcium-dependent protein kinase (spot 12) were up-regulated in response to the COS elicitor at 12 h and 24 h, respectively, while a putative pyruvate kinase isozyme G (spot 7) was down-regulated at 24 h after COS treatment. It has been shown that the MAR-binding protein MFP1 and calcium-dependent protein kinase might function in rice defense responses (Chuong et al. 2005; Li et al. 2006), and zinc finger proteins are also commonly known to function in diverse stress responses.

The protein spot 10 was revealed as protein NP_914086 in the Database (www.ncbi.nlm.nih.gov/entrez/), which was obviously up-regulated at 24 h after COS treatment (Figures 2, 3). Through a BLAST search, we found that it could be a putative pM5 protein. The pM5 protein, now known as transmembrane nodal modulator (Nomo), is a signaling factor of the transmembrane nodal modulator (Nomo).

Table 1. Expression patterns of regulated putative rice plasma mem-
brane (PM) proteins in response to chitooligosaccharides (COS)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description of expression pattern</th>
</tr>
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<tbody>
<tr>
<td>1, 4, 5</td>
<td>Up-regulated at 12 h under COS incubation</td>
</tr>
<tr>
<td>2, 3</td>
<td>Down-regulated at 12 h under COS incubation</td>
</tr>
<tr>
<td>8, 9, 10, 11</td>
<td>Up-regulated at 24 h under COS incubation</td>
</tr>
<tr>
<td>12, 13, 14</td>
<td>Down-regulated at 24 h under COS incubation</td>
</tr>
<tr>
<td>6, 7</td>
<td>Down-regulated at 24 h under COS incubation</td>
</tr>
</tbody>
</table>

Figure 3. Identification of protein 10 by MS.

(A) Peptide mass fingerprinting (PMF) resulting from MS analysis.
(B) Amino acid sequences of peptide fragments were obtained by PMF searching in the database. Calc., calculated; observ., observation; seq., sequence.
forming growth factor-β (TGFβ) superfamily with a key role in vertebrate development (Haffner et al. 2004). Intriguingly, the protein is for the first time identified in the plant kingdom, and our results suggested that it could be involved in plant defense.

The spots 8 and 13 were revealed as retrotransposon polyprotein-like (PPL) proteins (Table 2). PPL proteins have been frequently found to be involved in plant defense to diverse environmental stresses (Wessler 1996). We searched genome-

Figure 4. Phylogenetic tree of rice polyprotein-like protein family.

Branch numbers represent a percentage of bootstrap values in 1000 sampling replicates. OsPPL1 and OsPPL2 are proteins 8 and 13 in the study, respectively.
wide homologs of these ployproteins and retrieved a total of 42 PPL proteins in the family from the *japonica* rice genome, and a phylogenetic tree was established (Figure 4). It was shown that PPL2 (protein 13 in this study), PPL12 and PPL13 are structurally distinct from others.

### Discussion

Plasma membrane proteomics analysis has been thought particularly difficult because the PM contains a large amount of lipids and saccharides, and many PM proteins contain hydrophobic peptides. Accordingly, we were able to detect only 250–300 putative PM proteins among more than 3000 proteins detected in rice proteomics (Koller et al. 2002). On the other hand, in consideration of low abundance of many PM proteins, we used silver staining to detect differentially regulated PM proteins. However, silver staining certainly affected the efficiency of MS, only about 60% of targeted proteins were successfully identified by MS in our current study. Moreover, predicted MWs (molecular weight) and pIs (isoelectric point) of some PM proteins showed inconsistencies with their positions in the gels (Figure 1, Table 2). There are two possibilities to cause these inconsistencies: first, modification of PM proteins including glycosylation and phosphorylation might be changed; second, PM proteins contain hydrophobic peptides that might be broken during protein preparation. Similar inconsistencies were also observed in many other 2D-MS studies. PM proteomics is still in its infancy in planta (Alexandersson et al. 2004; Tanaka et al. 2004; Mahmood et al. 2006; Chen et al. 2007). Many of the identified rice PM proteins have not been shown with biological functions (Alexandersson et al. 2004; Tanaka et al. 2004; Mahmood et al. 2006). However, our repeated 2-D gel analysis should reveal PM proteins are involved in the defense response induced by the COS elicitor. Because the PM is thought to first respond to environmental stimuli, we applied two time points (12 h and 24 h) to investigate PM proteomics in an early response to COS.

It is well known that COS binds to the plasma membrane of plant cells (Baureithel et al. 1994; Day et al. 2001; Okada et al. 2002; Day et al. 2004). The observation of rapid HR-like stimulation in rice cells suggests the existence of COS receptor(s) at the cell surface (Baureithel et al. 1994; Okada et al. 2002). A rice 75-kDa COS-binding membrane protein was identified to be a functional receptor for the elicitor (Ito et al. 1997). Moreover, a novel tobacco membrane-bound chitinase-related receptor-like protein has been identified, which contains both chitinase and serine/threonine kinase domains (Kim et al. 2000), and the rice glycoprotein CEBiP was identified to serve as the plasma membrane receptor to recognize chitin fragments for defense signaling (Kaku et al. 2006). It is quite possible that the plant cells are equipped with several different COS perception mechanisms. Functional identification of these COS receptors and COS-regulated PM proteins and further characterization of COS signaling would shed light on the mechanisms of plant basal defense responses that are usually activated by elicitors such as COS. Our current study for the first time shows preliminary proteomic information of the rice plasma membrane in response to the COS elicitor. Interestingly, we identified some important putative membrane-associated proteins, including a PKN/PRK1 protein kinase, a MAR-binding protein MFP1, and a putative pM5 protein. The further functionality of these proteins in rice defense will provide more clues as to how the PM-associated proteins are involved in elicitor-mediated defense responses.

### Materials and Methods

#### COS inoculation and purification of PM protein samples

Rice *Xa21*-transgenic suspension cells were incubated with 1 μg/mL COS. Isolation of plasma membrane fraction and purity and assessment of PM samples were deter-
mined as described previously (Chen et al. 2007).

**Electrophoresis and detection of changed proteins**

Rehydration was carried out for 10 h at 17 °C with buffer consisting of 7 mmol/L urea, 2 mol/L thiourea, 0.5% Triton X-100, 0.5% IPG buffer (pH 4–7), 20 mmol/L dithiothreitol (DTT) and Bromophenol blue before protein samples (100 μg) were loaded onto immobilized pH gradient strips (linear pH 4–7, 17-cm, Bio-Rad (the city and country of manufacturer). The first dimension electrophoresis (isoelectric focusing, IEF) was conducted as follows: 200 V for 2 h, 250 V for 30 min, 1000 V for 1 h and 10 000 V for 10 h. After IEF, strips equilibration was carried out twice in 50 mmol/L Tris–Cl (pH 8.8), 6 mol/L urea, 30% glycerol, 2% sodium dodecyl sulphate (SDS) and Bromophenol blue, the first time with 2% DTT for 10 min and the second time with 2.5% iodoacetamide for 3 min. The second dimension electrophoresis was carried out on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 5 h at a constant current 60 mA. Differentially expressed protein spots were analyzed with Melanie 4 software after silver stain (Blum et al. 1987).

**Protein identification by MS**

Silver-stained gel pieces containing proteins interested were destained in 50 mmol/L sodium thiosulfate and 15 mmol/L potassium ferricyanide at room temperature for 20 min, followed by washing twice with ddH2O, dehydrated in 100% ACN for 10 min and complete drying at 37 °C overnight for in-gel tryptic digestion. Incubation with 50% CAN and 0.1% TFA was carried out twice (30 min each) before trypsin-digested peptides were collected and dried for subsequent MS identification. The mass range of peptide mass fingerprinting (PMF) was determined as described previously (Chen et al. 2007).

**Phylogenetic tree establishment of the OsPPL family**

Open reading frames of rice PPL genes gained from a genome-wide search in the public GenBank database (http://www.ncbi.nlm.nih.gov/) were multi-aligned by Megalign and ClustalX 1.81. Phylogenetic analysis was carried out by Puzzle 5.2 and TreeView 1.6.1 software.

**References**


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