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Differential proteomic analysis of soluble extracellular proteins reveals the cysteine protease and cystatin involved in suspension-cultured cell proliferation in rice

Lihong Tian [1], Lei Zhang [1], Jie Zhang, Yun Song, Yi Guo *

Institute of Molecular Cell Biology, Hebei Normal University, Shijiazhuang 050016, PR China

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ABSTRACT

Extracellular matrix proteins play crucial roles in plant development, morphogenesis, cell division, and proliferation. To identify extracellular proteins that regulate cell growth, the soluble proteins of extracellular matrix were extracted from suspension-cultured rice cells for different lengths of time. The extract obtained from 3-d cultures was found to increase cells' fresh weight, while extracts from 6-d and 9-d cultures showed no effect on cells' growth. A comparative proteomic analysis was used to identify soluble extracellular proteins differentially expressed between 3 and 6 days in suspension-cultured cells. Ten unique protein spots were isolated and identified by mass spectrometry. Among them, included a cysteine protease (OsCP) and a putative cysteine protease inhibitor (cystatin, OC-I). OsCP has been down regulated in vivo using RNAi transgenic lines. The fresh and dry weight growth rates of OsCP RNAi cell lines were lower than empty vector control. Recombinant protein of OC-I could inhibit the OsCP protease activity, also it could inhibit the weight increase of suspension-culture cell as well as extracellular protease activity. These results suggest that OsCP and OC-I may be involved in the process of suspension-cultured rice cells proliferation.

1. Introduction

The plant cell wall, or extracellular matrix (ECM), is a dynamic structure that plays various important roles in development and morphogenesis. Cell wall proteins participate in cell division and proliferation, plant defense reactions, responses to stress, signal transduction, and cell–cell communication [1,2]. The proteins of the plant cell wall are usually classified into two categories: (i) structural proteins that anchor themselves to the wall and contribute less than 10% of wall dry mass, such as the extensins, arabinogalactan proteins (AGPs), proline-rich proteins (PRPs), and glycine-rich proteins (GRPs), and (ii) soluble secretory proteins, including chitinases, proteases, peroxidases, and invertases [3]. These proteins have been shown to play crucial roles in cell–cell signaling [4].

Proteomic approaches have been used for the global analysis of various subcellular organelles and physiological states of cells. Despite the difficulties in extracting and identifying proteins, cell wall proteomics has become an active field in recent years [5]. Most studies have been obtained mainly with the model plant *Arabidopsis* and many cell wall proteins were identified [6–9]. Others have also been reported in *Zea mays* [10–12], tobacco [13,14], and *Medicago sativa* [15]. Since rice is the good model plant of monocotyledon, its genome has been completely sequenced. After a comparison of all cell-wall-related genes between *Arabidopsis* and rice which has type I and type II walls respectively, a set of gene families differ greatly in the size [16].This revealed that the cell wall proteins had difference between *Arabidopsis* and rice. Until now, many proteomic studies have been carried out on various tissues and subcellular compartments of rice, and a number of differential proteomics studies have identified proteins expressed under different physiological conditions or in different tissues [17,18]. Nevertheless, studies in a specific compartment such as the ECM or cell wall using proteomic methods have been less frequently reported in rice.

Plant suspension culture has often been used as a model and convenient experimental system for functional analysis of extracellular proteins because it can rapidly regenerate and the effects of different stimuli can easily be tested. So far, a large number of proteins have been identified in the ECM of suspension-cultured cells. However, the majority of studies on extracellular proteins have focused on plant defense response to microbes and pathogens. For example, the new PR protein NtPRp27 was reported to exist in the apoplast of tobacco [13], and *Arabidopsis* secretome analysis led

Abbreviations: CBB, Coomassie Brilliant Blue, CM, conditioned medium, 2-DE, two-dimensional electrophoresis, ECM, extracellular matrix, IEF, isoelectric focusing, PSK, phytosulfokine, PVDF, polyvinylidene difluoride

* Corresponding author. The East Yuhua Road 113, Institute of Molecular Cell Biology, Hebei Normal University, Shijiazhuang, Hebei Province, PR China. Tel.: +86 311 862 67212; fax: +86 311 85894242.
E-mail address: guoyi@mail.hebtu.edu.cn (Y. Guo).

[1] These authors contributed equally to this work.

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to the discovery of GLIP1 protein, which has a function in plant resistance to *A. brassicicola* [19]. Several studies have examined the role of extracellular proteins on regulating cellular proliferation and differentiation [20,21]. In plants, a few peptides such as PSK and RALF have been identified from conditioned medium (CM) in suspension cultures and been shown to regulate cell proliferation and rapid alkalinization [22,23]. Phytosulfokine (PSK), first identified in the CM of *Asparagus officinalis* suspension cultures, also stimulates cell proliferation in rice and *Arabidopsis*. PSK appears to work as a sulfated peptide growth factor, and its receptor has been identified as a receptor-like kinase [24].

In the present study, we applied a comparative proteomic technique to rice cells in suspension culture in an effort to identify soluble extracellular proteins in ECM involved in regulating cell growth. According to the different functions of soluble extracellular proteins from different culturing periods on cell growth, specific changed proteins were identified. A cysteine protease (OsCP) and a cystatin (OC-I) were further analyzed to understand their function on cell growth and proliferation.

2. Materials and methods

2.1. Plant material

Mature rice seeds (*Oryza sativa* subsp. *japonica* var. Nipponbare) were sterilized in 70% ethanol for 1 min and 2.5% sodium hypochlorite twice for 20 min, then washed with distilled water. The sterilized seeds were placed in N6 medium in the dark to induce calli at 25 °C. After one month, the induced calli were transferred into liquid N6 medium and shaken at 125 rpm in the dark. The cells in suspension culture were subcultured weekly.

2.2. Isolation of soluble extracellular proteins

This method has been described by Ndimba et al. [7]. For harvesting soluble extracellular proteins, the matrix of suspension-cultured cells was filtered through a 0.22-μm filter in a vacuum apparatus that retains intact cells. The filtered protein was precipitated in four volumes of chilled acetone at −20 °C overnight. After centrifugation at 10 000 × *g* for 15 min at 4 °C, the pellets were washed with 80% acetone. The pellets were centrifuged again, lyophilized, and stored at −80 °C before use.

2.3. Malate dehydrogenase assay

The extracted protein was solubilized and tested in the malate dehydrogenase assay to reveal the level of intracellular contamination. According to the modified method described by Husted et al. [25], aliquots of sample were added to a reaction mixture containing 0.094 mM β-NADH disodium salt and 0.17 mM oxalacetic acid in 0.1 M Tris buffer, pH 7.5. Malate dehydrogenase activity was measured by the decrease in absorbance at 340 nm for 3 min time scan using a spectrophotometer (Hitachi U-2001 Japan).

2.4. Protein immunoblot analysis

Immunoblotting against tubulin was also used to determine contamination with cytoplasmic components. After SDS-PAGE, the total cellular proteins and the soluble extracellular proteins in each extract were blotted onto a PVDF membrane. The membrane was blocked for 2 h in TTBS [20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, and 0.025% (v/v) Tween 20] containing 5% (w/v) nonfat dry milk. After blocking, the membrane was incubated at 4 °C overnight with anti-α-tubulin monoclonal antibody (Sigma, 1:2000 dilution) in TTBS. A secondary horse anti-mouse IgG antibody conjugated with alkaline phosphatase was used for immunodetection.

2.5. Treatment of suspension-cultured cells with different soluble extracellular proteins

Soluble extracellular proteins isolated from suspension culture on different days were resuspended. The soluble proteins were quantified using the 2-D Quant kit (Amersham Biosciences) and a total of 15 mg were added per liter of suspension culture in N6 medium. The effect of the additives on cell growth and proliferation was assessed by weighing the fresh weight of treated cells every three days until the twelfth day, using the medium without extracted protein as a control. The growth rates were calculated from three independent experiments.

2.6. Two-dimensional gel electrophoresis (2-DE)

After quantification, 500 μg of proteins in 250 μL of 2-D sample buffer were loaded onto dry, 13-cm IEF strips (Amersham Biosciences, linear gradient of pH 4-7) using the overnight in-gel reswelling method. The reswelled gel strips were subjected to IEF at 20 000 Vh at 20 °C using IPGphor (Amersham Biosciences). Running conditions were as follows: 500 V for 1 h, then 1000 V for 1 h, and finally 8000 V for 2 h. The focused strips were equilibrated twice in 10 mL equilibration solution for 15 min. The first equilibration buffer contained 50 mM Tris–HCl buffer pH 8.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, and 1% (w/v) DTT. In the second equilibration, DTT was replaced with 2.5% (w/v) iodoacetamide. After equilibration, the strips were loaded on top of 12.5% polyacrylamide gels for SDS-PAGE by Hoefer SE600 Ruby (Amersham Biosciences), which was performed at constant current. The electrophoresed gels were stained with Coomassie Brilliant Blue (CBB) R250 overnight and destained in 50% methanol and 10% acetic acid. The scanned gels were analyzed using Image Master 2D Platinum V6.0 software (Amersham Biosciences).

2.7. Protein identification by mass spectrometry

Each protein spot that differed between the electrophoretic patterns of the 3-day and 6-day extracts was excised from the CBB-stained gel and digested with trypsin. After digestion at 37 °C overnight, the peptides were extracted three times with 0.1% TFA in 60% acetonitrile. Extracts were pooled and lyophilized for mass spectrometric analysis.

The spots showing significant changes were analyzed first by MALDI-TOF/TOF MS, and LCQ or LTQ was used to analyze the other spots that were not positively identified by MALDI-TOF/TOF MS. For MALDI-TOF/TOF analysis, all mass spectra were acquired on an AutoFlex MALDI-TOF/TOF mass spectrometer with LIFT technology (Bruker Daltonics, Bremen, Germany). Tryptic digests were prepared on an AnchorChip sample plate (Bruker Daltonics, Bremen, Germany) according to the manufacturer's protocol. Both MS and MS/MS data were acquired with a N_2 laser at 25-Hz sampling rate. PMF data and MS–MS data were combined using FlexAnalysis, and the combined data set was submitted to MASCOT for protein identification. Using *O. sativa* as the taxonomy, the National Center for Biotechnology non-redundant (NCBInr) database was searched. Other search parameters were as follows: tryptic digestion, one missed cleavage, fixed modifications of carbamidomethyl (C), and variable modifications of oxidation (Met). The settings of peptide tolerance (50 ppm), fragment mass tolerance (±0.5 Da), and peptide charge (1+) were used. Only significant hits, as defined by MASCOT probability analysis ($P<0.05$), were accepted.

For LCQ/LTQ analysis, separation and identification of digested proteins was conducted using an LCQ DecaXP plus (Thermo Finnigan, San Jose, CA)-Finnigan LTQ mass spectrometer (ThermoQuest, San Jose, CA, USA), coupled to a Surveyor HPLC system (ThermoQuest). First, a Microcore reverse-phase column (C18, 0.15 mm × 120 mm; Thermo-Hypersil, San Jose, CA, USA) was used to separate the protein digests.