JOURNAL OF PROTEOMICS 108 (2014) 284–294

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Phospho-proteomic analysis of developmental reprogramming in the moss *Physcomitrella patens*

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ARTICLE INFO

Article history:
Received 21 March 2014
Accepted 19 May 2014

Keywords:
Physcomitrella patens
Protoplast
Developmental reprogramming
Phospho-proteome

ABSTRACT

One of the most common post-translational modifications is protein phosphorylation, which controls many activities of plant life. However, its role in the reprogramming of developmental pathways of plant cells remains elusive. Here, using *Physcomitrella patens*, we characterize the phospho-proteome for protonemata, protoplasts made therefrom, and protoplasts regenerated for 2 d. Through a titanium dioxide (TiO_2)-based phospho-peptide enrichment method and liquid chromatography–tandem mass spectrometry (LC–MS/MS), more than 2000 phospho-proteins were identified. Among the 519 proteins with functional annotation in fresh protoplasts and protoplasts regenerated for 2 d, proteins involved in epigenetic modification, post-transcriptional gene regulation, hormone signal transduction, and meristem maintenance have been previously reported to be important for developmental reprogramming. Several novel transcription factors including SWI/SNF complex protein, SNF2 family protein and MADS-domain transcription factor appear to be important in developmental reprogramming plant cells. Phosphorylation of marker proteins such as somatic embryogenesis receptor kinase and NAC transcription factor, suggests that this post-translational modification is vital for the cell's ability to adjust its developmental program. Together, our study presents a more complete understanding of the plant cell's developmental reprogramming.

Biological significance
Protoplast regeneration is an ideal model system for investigating developmental reprogramming in plants. Here, for *Physcomitrella patens*, we characterize the phospho-proteome for protonemata, protoplasts made therefrom, and for protonemata regenerated from the protoplasts for 2 d. Among the 519 proteins with functional annotation in fresh protoplasts and protoplasts regenerated for 2 d, proteins involved in epigenetic modification, post-transcriptional gene regulation, hormone signal transduction, and meristem maintenance have been reported to be important for expression of developmental reprogramming. Together, our study presents a more complete understanding of the plant cell's developmental reprogramming.

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http://dx.doi.org/10.1016/j.jprot.2014.05.012
1874-3919/

1. Introduction

Stem cells are the precursors of differentiated cells and are, thus, indispensable for growth and development in animals and plants. Stem cells are defined both by self-renewal and by their potential to differentiate to multiple cell types [1]. In fact, many plant cells or tissues have the ability to regenerate an entire plant from a small piece of tissue, or even a single cell. However, little is known about the mechanisms or genes that play a key role in the dedifferentiation or reprogramming of a single, somatic plant cell into a totipotent cell.

For analyzing how somatic cells become totipotent, protoplasts, i.e. cells devoid of their cell wall, provide a valuable experimental tool. For example, a large population of protoplasts can be obtained from a mature plant tissue and followed as they regenerate into plants [2]. The developmental reprogramming of protoplasts has been shown to involve changes in DNA methylation, increased transcription and reorganization of specific chromosomal subdomains [2,3]. These changes lead, first, to the activation of genes whose products are involved in acquisition or maintenance of totipotency and, second, to genes involved in differentiation. During this reprogramming, among the genes that are consistently found to be up-regulated are those that contain a so-called NAC (NAM/ATAF1/CUC2) domain, implying that these proteins play a key role [2,3].

In seed plants, application of phytohormones, such as auxins and cytokinins, could stimulate protoplasts from different tissues to reenter the cell cycle, proliferate [4]. However, protoplasts from the moss do not need phytohormones for regeneration, nor do they form a callus during regeneration. Instead, the moss protoplasts have ability to regenerate directly into the filamentous protonemata and this developmental pathway is similar to moss spore germination [5]. It is interesting to find the reason for this difference. Among the moss, *Physcomitrella patens* is an ideal model to study plant development [6]. The completed sequence of the *P. patens* genome facilitates the use of genetic and molecular approaches to identify genes and the ability of the moss to undergo homologous recombination at appreciable frequency offers a powerful way to determine gene function [7]. Moreover, the moss *P. patens* regenerates readily from protoplasts, and protoplast fusion permits complementation analysis to be carried out [8].

Protein phosphorylation is one of the most common post-translational modifications in eukaryotic cells and plays a critical role in a vast array of cellular processes. Reversible phosphorylation of serine, threonine, and tyrosine residues is a key step in the control of signal transduction pathways [9]. Recent studies show that global phosphorylation analysis can be used to investigate signaling pathways [10–13]. Here, to study the signaling pathways during developmental reprogramming in the moss, we identify phospho-proteins in fresh protoplasts and 2-day-old protoplasts of *P. patens* by using a titanium dioxide (TiO_2) phospho-peptide enrichment strategy coupled with liquid chromatography–mass spectrometry (LC–MS/MS).

2. Materials and methods

2.1. Plant material and harvesting

P. patens (Hedwig) ecotype 'Gransden 2004' was grown in BCDA medium [14] on cellophane, cultured at 25 °C under a light cycle of 16 h light/8 h darkness and a light intensity of 55 μmol s^{-1} m^{-2}. For proteomics, proteins were isolated from protonemata, freshly harvested protoplasts, and protoplasts regenerated for 2 d. For 7-day-old protonemata, plants from six plates were scraped from the cellophane and frozen in liquid nitrogen. For protoplasts, 7-day-old protonemata from 20 plates were treated with 0.5% driselase (Sigma, St Louis, MO, USA) in 8.5% mannitol for 45 min. Following filtration and washing with 8.5% mannitol, protoplasts were resuspended in liquid BCDA medium with 8.5% mannitol under the same culture conditions [14], harvested (immediately and after 2 d) by centrifugation (300 or 800 *g* for 5 min), and frozen in liquid nitrogen. Protoplast isolation was repeated three times and the three biological replicates were harvested for protein isolation.

2.2. Fluorescence activated cell sorter (FACS) and microscopy analysis

FACS measurements were performed on isolated nuclei (from protonemata, fresh protoplasts and 2-day-old protoplasts) to avoid interference of plastid and mitochondrial DNA. Nuclei were isolated in a buffer containing 10 mM 2 N-morpholino ethane sulphonic acid (MES), 0.2 M sucrose and 0.01% Triton X-100, 10 mM KCl, essentially as described [15]. The buffered solution containing tissue (cut by scalpel) was passed through 23 μm nylon mesh to collect nuclei. Nuclei were stained in 2.86 μM 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI). FACS was carried out on a two-laser FACStar Plus platform (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with an argon laser emission of 488 nm and analyzed with CELLQUEST software (Becton Dickinson, Franklin Lakes, NJ).

2.3. Protein extraction

Frozen plant material was ground (protonemata) or suspended (fresh protoplasts and 2-day-old protoplasts) in a solution containing 250 mM sucrose, 20 mM Tris–HCl, pH 7.5, 10 mM EDTA, 1 mM 1,4-dithiothreitol, protease inhibitors (Sigma, St Louis, MO), and phosphatase inhibitors (Sigma, St Louis, MO). Cell debris was removed by centrifugation at 8000 *g* for 10 min at 4 °C. The supernatant was removed and centrifuged at 120,000 *g* for 1 h at 4 °C. Then protein extraction was done in two steps. First, the final supernatant was used for extraction of soluble proteins, as described previously [16]. Second, the pellets (membrane-associated proteins) of final centrifugation were extracted overnight at −20 °C in acetone. The precipitated membrane proteins were rinsed three times with ice-cold acetone containing 13 mM DTT and subsequently lyophilized. Finally, both pellets corresponding to soluble and membrane-associated protein faction were dissolved separately in a resuspension buffer (8 M urea, 4%