Journal of Plant Physiology 171 (2014) 1671–1678

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Physiology

A proteomic approach to *Physcomitrella patens* rhizoid exudates

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ARTICLE INFO

Article history:
Received 17 March 2014
Received in revised form 9 August 2014
Accepted 11 August 2014
Available online 19 August 2014

Keywords:
Defence
Exudate
NanoLC-MALDI-TOF/TOF
Physcomitrella patens
Proteome
Rhizoids

SUMMARY

The interaction between plants and the surrounding environment has been widely studied, specially the defence reactions and the plant–plant interactions. One of the most remarkable metabolic features of plant roots is the ability to secrete a vast array of compounds into the rhizosphere, not only of low molecular weight but also polysaccharides and proteins. Here, we took advantage of proteomics to study the rhizoid exudates of *Physcomitrella patens* at early and late development stages (7 and 28 days of culture in liquid medium). Samples were extracted, separated and detected with nanoLC-MALDI-TOF/TOF MS/MS, identifying 47 proteins at the development stage of 7 days, and 66 proteins at 28 days. Moreover, 21 proteins were common to the two analyzed periods. All the identified proteins were classified into 8 functional categories: response to stress, response to stimulus, oxido-reduction, cell wall modification, photosynthesis and carbohydrate metabolism, transport, DNA metabolic process and regulation/signalling. Our results show important differences in the protein expression profile along the development of *P. patens*, mainly at the level of regulation- and senescence-related proteins. Defence-related proteins, such as chitinases, thaumatins and peroxidases have a major role in the interaction of *P. patens* with the environment.

Introduction

The way the different organisms interact with each other has been the object of numerous scientific investigations, which in the particular case of the interaction among plants is constricted to competition for space, nutrients, water and light (Badri et al., 2012). The bulk of plant interactions take place in the rhizosphere, which comprises the millimetres of soil surrounding the roots. This is a densely populated area in which plant roots must compete with invading root systems of neighbouring plants for space, water, mineral nutrients and with other soil-borne organisms, including bacteria and fungi (Bais et al., 2004). One of the most remarkable metabolic features of plant roots is the ability to secrete a vast array of compounds into the rhizosphere, broadly defined as root exudates (Marschner, 1995), through which a significant quantity of photosynthetically fixed carbon is being transferred to the rhizosphere, representing a significant carbon cost to the plant. Most of the root exudates comprise compounds such as amino acids, sugars, organic and phenolic acids and other secondary metabolites, all of them of low molecular weight. Nonetheless, high molecular weight compounds such as polysaccharides and proteins have also been found (Walker et al., 2003). Root secretions of secondary metabolites and volatile organic compounds have been widely studied, as they have shown to play offensive, defensive and symbiotic roles (Leitner et al., 2008). However, more attention has been paid recently to the analysis of extracellular root proteomes.

Proteomics has become an important tool in the study of plant biology, by opening up new perspectives to analyze the complex functions of model plants and crop species at different levels (Rossignol et al., 2006; Cui et al., 2012). Proteomic approaches have succeeded in identifying proteins of organs, tissues, cell suspensions (Jamet et al., 2006; Albenne et al., 2013) and also subcellular fractions, mainly mitochondria and chloroplasts (Polyakov et al., 2010). Proteomic analyses have also been of great importance for studies of plant development and the effect of hormones and signalling molecules (Sarnighausen et al., 2004; Wasternack et al., 2006). Other proteomics studies have focused on the plant–microbe interactions, due to the fact that proteins found in the root secretome are thought to play a role in the interactions among plants and soil organisms (Liao et al., 2012a,b; Vincent et al., 2012). These studies examined root secretion of proteins under inducible conditions, but there is no information available about the

Abbreviations: CBB, coomassie brilliant blue; LRR, leucine-rich repeat; PR, pathogenesis-related; TCA, trichloroacetic acid.

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http://dx.doi.org/10.1016/j.jplph.2014.08.004
0176-1617/

protein profiles in the root secretome under normal, non-stressed developmental conditions. Such information is critical to determine the baseline of protein secretion from roots and whether this process is developmentally controlled. De la Peña et al. (2010) have found that protein root exudation in plants is constitutive, although it represents a significant cost to the plant.

Proteomic analyses have detected novel proteins in the secretome of different model species, mainly *Arabidopsis thaliana* and rice (Jamet et al., 2006; Jung et al., 2008; Badri et al., 2012). The moss *Physcomitrella patens*, whose complete genomic sequence has been recently released (Rensing et al., 2008), is emerging as a complementary model system to *Arabidopsis* and rice to study various aspects of plant evolution, metabolism, development and physiology (Cove et al., 2006), mainly due to the fact that highly standardized culture techniques, as well as the necessary tools for computational biology, functional genomics and proteomics have been established. In fact, current studies regarding *P. patens* physiology, including proteomics, are performed with *in vitro* cultures, both in solid and liquid medium (Benito and Rodríguez-Navarro, 2003; Sarnighausen et al., 2004; Rensing et al., 2008; Cui et al., 2012). In this work, we have used *P. patens* plants grown in liquid culture to recover the secreted proteins from rhizoids. The present report was designed to better understand the variation of protein secretion throughout development. Proteins secreted by *P. patens* rhizoids cultured at 7 and 28 days were identified and functionally classified. These results provide a first comprehensive insight into the physiological and molecular functions involved in rhizoids interaction with rhizosphere.

Materials and methods

Plant culture

P. patens was cultured as described (Benito and Rodríguez-Navarro, 2003), in 100 mL of liquid culture on a rotator shaker (130 rpm) in a growth chamber at 25 °C with a 16 h photoperiod. The culture medium with the rhizoid secretome was collected at 7 and 28 days after culture initiation.

Protein extraction and quantification

Proteins from rhizoid secretome were extracted from culture medium by two different precipitation methods, ammonium sulphate and trichloroacetic acid (TCA)–acetone. Precipitation with ammonium sulphate was carried out at 95% saturation followed by centrifugation. The precipitate was resuspended in 50 mM Tris-HCl pH 7.5 and dialyzed overnight in the same buffer. TCA–acetone precipitation protocol was carried out as reported by Damerval et al. (1986) with modifications. Proteins from culture medium were precipitated by adding four volumes of ice-cold acetone containing 10% TCA and 0.07% (w/v) β-mercaptoethanol. Samples were incubated at −20 °C overnight, and then centrifuged for 40 min at 1600 × *g*. The pellet was washed with ice-cold acetone containing 0.07% (w/v) β-mercaptoethanol and centrifuged again 5 min at 6000 × *g*. In both $(NH_4)_2SO_4$ and TCA–acetone precipitations the final pellets were lyophilized and resuspended for protein solubilization in a buffer containing 6 M urea, 2 M thiourea and 25 mM ammonium bicarbonate. Samples were sonicated and the insoluble material eliminated by centrifugation. Protein amount was determined according to Bradford (1976).

Electrophoretic analysis

SDS-PAGE was performed on 10% (w/v) polyacrylamide gels using a MiniProtean® 3 Cell electrophoresis kit (Bio-Rad) and an electrophoresis buffer composed of 192 mM glycine and 25 mM Tris pH 8.8 containing 0.1% SDS. SDS-PAGE was performed at 200 V for 45 min at room temperature. Proteins were stained by the ammoniacal silver method (Oakley et al., 1980).

In-gel fractioning

For each pool, 30 μg of protein were loaded and separated by SDS-PAGE in 10% acrylamide in-house gels in a Protean® mini-gel system (Bio-Rad, Hercules, (CA), USA). The gels were lightly stained with CBB for 10 min to check protein integrity. Each entire lane was size-fractioned into 3 sections that were subsequently processed independently. Each section was de-stained with methanol, divided into two identical parts to create two experimental replicates, diced into small pieces and in-gel digested following a standard procedure (Shevchenko et al., 2006). Briefly, the samples were desiccated with acetonitrile, reduced with dithiothreitol, alkylated with iodoacetamide and trypsin-digested (6 ng/μL, Promega MSgold, Madison, (WI), USA) for 16 h at 37 °C. Peptides were then extracted, dried in a speed-vac, reconstituted in 0.1% trifluoroacetic acid and de-salted using nu-tipC18 (Glygen, Columbia, (MA), USA).

nanoLC-MALDI-TOF/TOF

The peptide fractions were separated using reversed-phase chromatography in a nanoLC system (Tempo, Eksigent, Dublin, (CA), USA) by loading through a trapping column into a C18 silica-based column (New Objective, Woburn, MA, USA) with an internal diameter of 300 Å. Peptides were eluted at a flow rate of 0.35 μL/min during a 30 min linear gradient from 2 to 40% B (mobile phase A: (0.1% trifluoroacetic acid 2% acetonitrile. mobile phase B: 0.1% trifluoroacetic acid 96% acetonitrile), mixed with α-cyano matrix (4 mg/mL at a flow rate of 1.2 μL/min) and deposited onto a MALDI plate using an automatic spotter (Sun Collect, Sunchrome, Friedrichsdorf, Germany). Chromatograms corresponding to each gel section were composed of 10 spots, each one comprising a 15 s deposition.

The MS runs for each chromatogram were acquired and analyzed in a MALDI-TOF/TOF instrument (4800 ABSciex, Framingham, (MA), USA) using a fixed laser intensity of 3800 kV and 1500 shots/spectrum. Automated precursor selection was done using a Job-wide interpretation method (up to 10 precursors/fraction, signal-to-noise lower threshold of 50) with a laser voltage of 4800 and 1500 shots/spectrum at medium CID (collision-induced dissociation) collision energy range. A second Job-wide precursor selection was done excluding those precursors previously fragmented and using a lower signal-to-noise threshold of 30, to identify peptides coming from low-abundance proteins. Data from both MS/MS acquisitions were used for data processing and subsequent protein identification.

Database search and protein identification

The complete MS and MS/MS raw data for the samples were processed separately using the ProteinPilot 3.0 software platform (ABSciex). Peptide identification was performed against the last NCBI release. Search parameters included carbamidomethylation of cysteines (fixed), oxidation of methionine (variable, ≤4), 1 missed trypsin normal cleavage, precursor ion mass range 800–4000 Da ± 100 ppm tolerance on precursor and ± 0.3 Da on fragmentation of ions. The scoring model was defined by the algorithm. Likewise Mascot v.1.9 from Matrix Science (www.matrixscience.com) was also used to identify the proteins. Peak intensity of the monoisotopic peptide mass fingerprinting data obtained from MS and the amino acid sequence tag obtained