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Proteomic profile of the nucellus of castor bean (*Ricinus communis* L.) seeds during development

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ABSTRACT

In this study, we performed a proteomic analysis of nucellus from two developmental stages of *Ricinus communis* seeds by a GeLC-MS/MS approach, using of a high resolution orbitrap mass spectrometer, which resulted in the identification of a total of 766 proteins that were grouped into 553 protein groups. The distribution of the identified proteins in stages III and IV into different Gene Ontology categories was similar, with a remarkable abundance of proteins associated with the protein synthesis machinery of cells, as well as several classes of proteins involved in protein degradation, particularly of peptidases associated with programmed cell death. Consistent with the role of the nucellus in mediating nutrient transfer from maternal tissues to the endosperm and embryo, a significant proportion of the identified proteins are related to amino acid metabolism, but none of the identified proteins are known to have a role as storage proteins. Moreover for the first time, ricin isoforms were identified in tissues other than seed endosperm. Results are discussed in the context of the spatial and temporal distribution of the identified proteins within the nucellar cell layers.

1. Introduction

The nucellus is the central portion of the ovule in which the embryo sac develops. This tissue has an important role in the development of endosperm and embryo [1]. The mobilization of nutrients from the nucellus to these tissues is dependent on the occurrence of programmed cell death (PCD) in the former one [2–4], even though in castor bean seeds, cell division in the nucellus takes place until endosperm and embryo are well developed [5]. The studies on PCD of this tissue in species such as *Ricinus communis* [6] and *Sechium edule* [4] have strengthened the idea that the nucellus and other maternal tissues such as the inner integument of seeds [7] may act as transient source of reserves which may be mobilized to suit the needs of the developing seed. Although better understanding of the nature of the protein components involved in triggering PCD in plants is emerging [8], very little is known about the pattern of deposition of reserves in the nucellus, the

Abbreviations: PCD, programmed cell death; LTQ, linear ion trap; FDR, false discovery rate; GO, gene ontology.

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enzymes which are used for nutrient mobilization and the chemical identities of the compounds which are conveyed to the embryo and endosperm.

Seeds of a wide diversity of species, including castor bean [9,10], have been subjected to proteome analysis. Most of these studies have used whole seeds, either at mature [11] or developing stages [10,12]. So far, the analysis of maternal seed tissues such as integuments and nucellus has deserved little attention, thus hampering the understanding of the role of these organs in shaping seed development. We are interested in studying the development of castor bean seeds, particularly those aspects related to the deposition of proteins and lipids during development and the contribution of the nucellus to these processes. In the present study, we have taken advantage of the availability of a detailed descriptive morphology of castor bean seeds during development [13] and have selected two sharply distinct developmental stages to perform a comparative proteomic analysis of nucellus using a GeLC-MS/MS approach and high resolution orbitrap mass spectrometry, in order to evaluate the dynamics of protein deposition and mobilization.

2. Material and methods

2.1. Plant materials and histological analysis

Castor bean (*R. communis* L. cv. Nordestina) developing seeds were harvested between the months of July and September 2010. Nucelli were dissected from seeds at developmental stages III and IV [13] under a binocular microscope and immediately processed. For histological studies, seeds were collected and fixed in Karnosvisky solution for 24 h at room temperature, dehydrated in an ethanol series and embedded in historesin (Leica Microsystems Nussloch GmbH, Germany) following the standard procedures [14]. Serial sections cut at 5–7 μm thickness on a rotary microtome equipped with a steel knife were stained in 0.05% toluidine blue and mounted. The slides were examined under bright field optics in a Zeiss Photomicroscope III.

2.2. Sample preparation and protein determination

Nucelli from seeds at stages III and IV were easily separated from coenocytic and from cellular endosperm tissues by using a thin spatula and briefly washing in Tris–HCl buffer. Proteins from nucelli were extracted in duplicate for each stage according to Nogueira et al. [15]. 50 mg of isolated tissues was macerated with pyridine buffer (50 mM pyridine, 10 mM thiourea and 1% SDS, pH 5.0) and polyvinyl-polypyrrolidone, in a proportion 1:40:2 (w/v/w). The mixture is stirred for 2 h at 4 °C and centrifuged at 10,000 *g* for 40 min. The proteins were precipitated with cold 10% trichloroacetic acid in acetone and the pellet was washed with cold acetone. The last precipitate is then dried under vacuum. Protein concentration was measured using a fluorimetric method (Qubit® Quantitation Assay Kit, Invitrogen).

2.3. 1D-SDS-PAGE

20 μg of each stage and each replicate were subject to 1-D gel electrophoresis using NuPAGE® Novex 4–12% Bis–Tris precast mini gel 1.0 mm, 12 well (Invitrogen), in a XCell SureLock™ Electrophoresis system (Invitrogen) and Power Pac 300 power supplier (Bio-Rad) following the manufacturer's instructions. Gel was stained with Coomassie Brilliant Blue R-250.

For in-gel trypsin digestion, lanes corresponding to samples from nucellus at stages III and IV were divided into nine slices. Each band was de-stained, reduced with 10 mM DTT for 45 min at 56 °C, alkylated with 55 mM iodoacetamide for 30 min at room temperature in the dark and, digested overnight with trypsin (Promega). The peptides were extracted from the gels bands 3 times with 50% ACN and 5% TFA solution. Purified peptides were eluted from a custom-made Poros Oligo R3 reverse-phase microcolumn (Applied Biosystems) [16,17], dried under vacuum and stored for further analyses. For each stage we prepared two replicates in a total of 2×9 samples.

2.4. LC–MS/MS and data analysis

Samples were analyzed by an EASY-nano LC system (Proxeon Biosystems) coupled online to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). Extracted peptides from each fraction were loaded onto a 18 cm fused silica emitter (100 μm inner diameter) packed in-house with reverse phase capillary column ReproSil-Pur C18-AQ 3 μm resin (Dr. Maisch GmbH, Germany) and eluted using a gradient from 100% phase A (0.1% formic acid) to 35% phase B (0.1% formic acid, 95% acetonitrile) for 10 min, 35% to 100% phase B for 5 min and 100% phase B for 8 min (a total of 23 min at 250 nl/min). After each run, the column was washed with 90% phase B and re-equilibrated with phase A. Mass spectra were acquired in positive mode applying data-dependent automatic survey MS scan and tandem mass spectra (MS/MS) acquisition. Each MS scan in the orbitrap (mass range of *m/z* of 400–1800 and resolution 60,000) was followed by MS/MS of the five most intense ions in the LTQ. Fragmentation in the LTQ was performed by collision-induced dissociation and selected sequenced ions were dynamically excluded for 30 s. Raw data were viewed in Xcalibur v.2.1 (Thermo Scientific) and data processing was performed using Proteome Discoverer v.1.2 (Thermo Scientific). For each replicate nine Raw files were generated and these were submitted together to searching using Proteome Discoverer with in house Mascot v.2.3 algorithm against *R. communis* database downloaded from UNIPROT January 2011, which contains all *R. communis* genome sequence annotation [18]. The searches were performed with the following parameters: ms accuracy 10 ppm, MS/MS accuracy 0.5 Da, trypsin digestion with one missed cleavage allowed, fixed carbamidomethyl modification of cysteine and variable modification of oxidized methionine. Number of proteins, protein groups and number of peptides were filtered for FDR less than 1% and peptides with rank 1 using Proteome Discoverer. Protein Center software (Thermo Scientific, http://prg.proteincenter.proxeon.com/ProXweb/app) was used to interpret the results at protein level (KEGG Pathways, Gene Ontology, Gene Number, Transmembrane Domain, and Signal Peptide). Gene Ontology annotation for plant categories were obtained using AgBase tools and database (http://agbase.msstate.edu/index.html), GORetriever for retrieve proteins with GO annotation from *R. communis* deposited in UNIPROT