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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

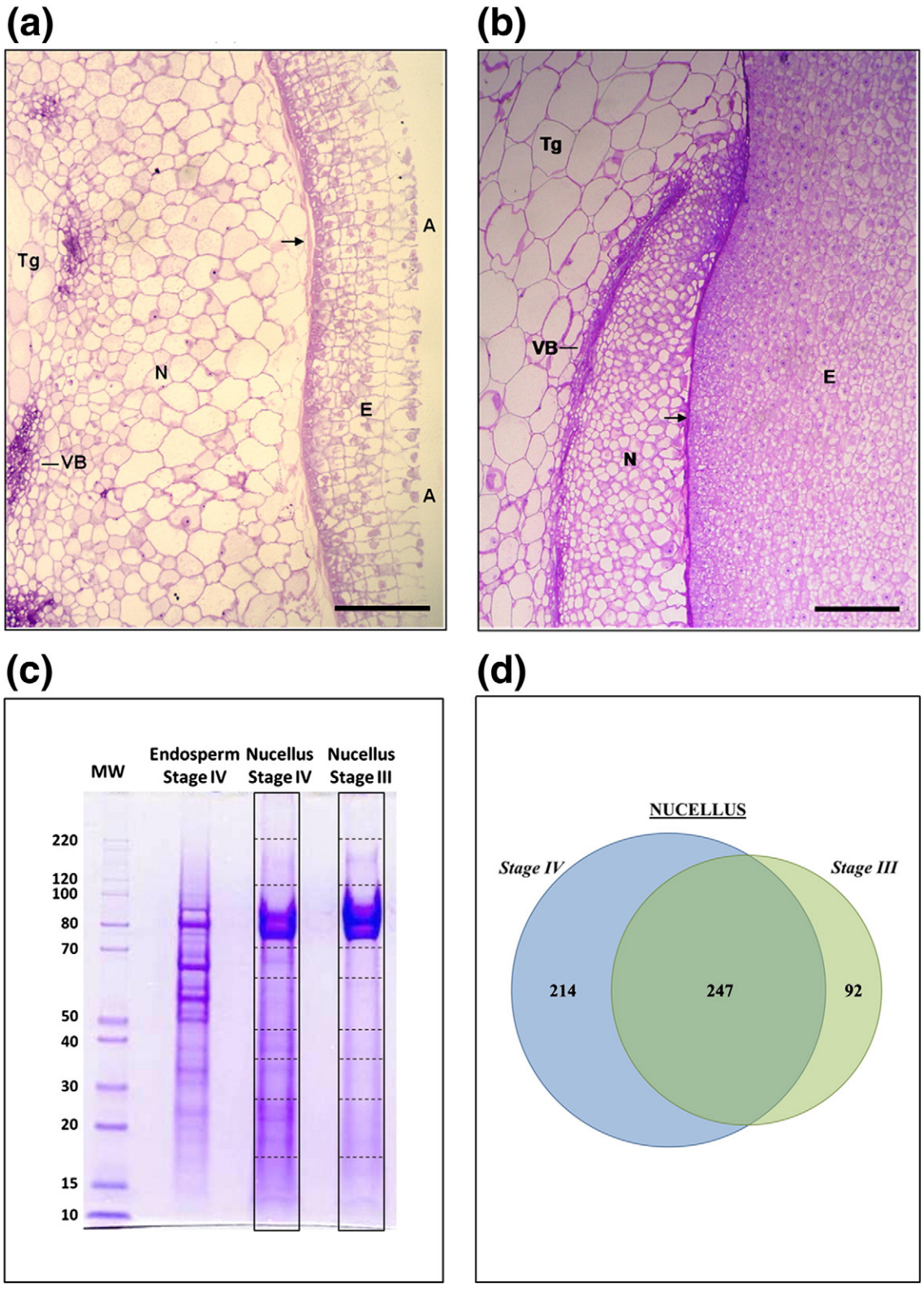

Fig. 1 – Histological analysis of developing seeds of *Ricinus communis* at developmental stages III (a) and IV (b) and 1D protein pattern of proteins extracted from nucellus of seeds at stages III and IV and from endosperm from seeds at stage IV (c); protein load was 20 μg. Venn diagram shows the number of protein groups common to both stages and specific to each one (d). Tg, tegmen; VB, vascular bundle; N, nucellus; E, endosperm; A, alveoli. The arrowheads point to the layers of cell corpses at the nucellus/endosperm boundary. Bar 200 μm.

and GOanna to determine orthologs for those proteins with no existing GO available in this reference database. The Plant GO Slim was used to summarize the sub-categories of the identified proteins.

3. Results and discussion

Early work [13] has shown that based on morphological features, the development of castor bean seeds can be divided into 10 stages. For this work, we have chosen stages III and IV because seeds at these developmental stages have contrasting anatomical and physiological characteristics: stage III is marked by cell proliferation, while stage IV is marked by the deposition of storage proteins. Histological analysis highlights that seeds at stage III are undergoing a sharp transition from a free nuclear endosperm to a cellular endosperm, with the formation of alveoli resulting from the deposition of cell walls around each nucleus (Fig. 1a). At this stage vascular bundles separate nucellus from tegmen and the nucellus cells are small and proliferate actively. As they distance themselves from the vascular bundle and approach the endosperm region they became vacuolated and eventually collapse, so that the corpses of cells form a dark thin layer surrounding the endosperm. At stage IV the endosperm is fairly compact and surrounded by the nucellus which was considerably reduced if compared with that at stage III (Fig. 1b). In the proximal region to the growing embryo the nucellus cells are proliferating actively, but those more distant, except the ones close to the vascular bundles, become vacuolated and no longer proliferate. As seen in stage IV, the cells closer to the endosperm eventually collapse giving rise to a dark layer of cell corpses surrounding the endosperm, thicker than the one observed in stage III.

The protein patterns of nucellus from seeds at stages III and IV are undistinguishable; however, the protein patterns of nucellus and endosperm taken from seeds at stage IV are very different (Fig. 1c). A salient feature of the protein profiles of nucellus taken from seeds at stages III and IV is that the most abundant proteins are in the molecular weight range of 70 to 100 kDa. For in-gel trypsin digestion, each lane corresponding to nucellus from seeds at stages III and IV was segmented in nine fractions, III.1–III.9 and IV.1–IV.9 respectively (Fig. 1c).

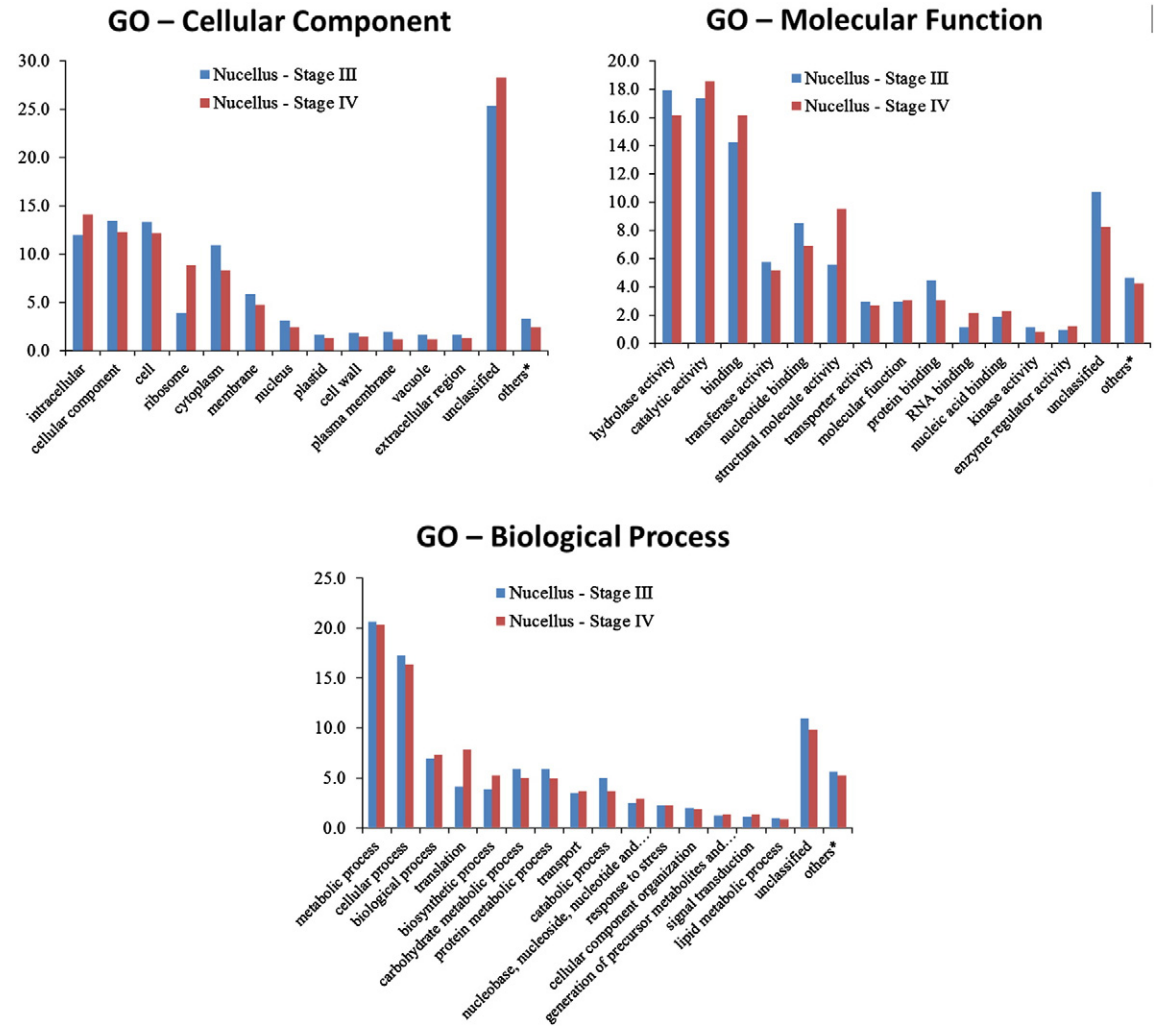

Fig. 2 – Gene Ontology categories for all protein groups present in nucellus from seeds of *Ricinus communis* at stages III and IV. The Plant GO Slim was used to summarize the sub-categories of the identified protein groups. Y axes represent percentage of protein groups.

By using a Gel LC-MSMS strategy, Proteome Discoverer with Mascot as a search tool and filters of 1% FDR and rank 1, we identified a total of 3036 peptides from nine gel fractions for each nucellus sample (peptide ion score higher than 30) (Supplementary Table 1). Proteins sharing peptides were grouped and ranked according to their protein score and sequence coverage and this reduced the number of proteins from 766 to 553 groups (Supplementary Table 1). After Protein Center analysis of the identified protein groups, 247 were common to both stages while 92 and 214 were unique to stages III and IV respectively (Supplementary Table 2, Fig. 1d). Despite the higher number of protein identification in nucellus at stage IV, annotation for each of the three GO categories (Fig. 2, Supplementary Table 3) failed to highlight any major proportional differences between classes within each category of the two stages, suggesting a fairly stable protein pattern between them.

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 (Supplementary Table 4). The high number of proteins involved in the metabolism of cysteine and methionine highlights the prominent role of sulphur metabolism in seed development, as well as the role of these amino acids in generating precursors for the biosynthesis of S-adenosylmethionine, an important methyl-group donor in plant cells [19]. None of the identified proteins are known to have a role as storage proteins, thus suggesting that the transfer of nitrogen and carbon sources to the endosperm and embryo does not depend on the mobilization of a specific group of proteins.

In line with the histological analysis which revealed a complex pattern of cell division and differentiation and cell death in nucellus tissues from both stages (Fig. 1), a fairly large number of proteins associated with the protein synthesis machinery of cells (ribosomal proteins, translation initiation factors, aminoacyl-tRNA synthetases, etc.) were identified, as well as several classes of proteins involved in protein degradation. Prominent among the latter were proteasome components and ubiquitination proteins, which indicate a proper functioning of the proteasome, a *sine qua non* condition for the occurrence of PCD in plant cells [20]. Besides proteasome catalytic subunits, peptidases belonging to the other four major mechanistic classes of peptidases were identified (Table 1) and among these, peptidases which are known to have a role in PCD in castor bean and in other species [5], notably vacuolar processing enzyme, vignain and xylem serine proteinase I (Table 1). The vacuolar processing enzyme is a cysteine peptidase which share several enzymatic properties with caspases and metacaspases [21] and which mediates cell death in maternal seed tissues of barley [1] and in other plant tissues [21]. Vignain is a KDEL-tailed cysteine peptidase which is directly involved in PCD in the endosperm of germinating castor bean seeds [6] and which has several homologs in senescing tissues of other plants. The xylem serine proteinase I is a member of a class of peptidases that display caspase-like activity and

Table 1 – Peptidases and peptidase inhibitors identified in nucellus from *Ricinus communis* seeds at developmental stages III and IV.

Protein ID	Protein names	Nucellus Stage	Mechanistic class
B9RG92	Aspartic proteinase nepenthesin-1 precursor, putative	III and IV	Aspartic
B9RFR2	Aspartic proteinase precursor, putative	III	
B9RXH6	Aspartic proteinase precursor, putative	III and IV	
B9T7L5	pepsin A, putative	IV	
B9RN00	Cathepsin B, putative	III and IV	Cysteine
B9SJL8	Protease C56, putative	III and IV	
O65039	Vignain	III and IV	
B9R816	Cysteine protease, putative	IV	
B9RYC1	Cysteine protease, putative	III and IV	
B9RHA4	Cysteine protease, putative	III and IV	
B9R777	Cysteine protease, putative	III and IV	
B9SB56	Cysteine proteinase inhibitor, putative (inhibitor)	IV	
B9SYV2	Cysteine proteinase inhibitor, putative (inhibitor)	III and IV	
B9RAJ0	Aspartyl aminopeptidase, putative	IV	Metallo
B9SG76	Aspartyl aminopeptidase, putative	IV	
B9STR1	Leucine aminopeptidase, putative	III and IV	
B9SWY5	Proliferation-associated 2g4, putative	IV	
B9R7A2	Cucumisin precursor, putative	IV	Serine
B9T6I8	Cucumisin precursor, putative	III and IV	
B9R9K9	Cucumisin precursor, putative	IV	
B9RYW8	Cucumisin precursor, putative	IV	
B9S4V0	Proteinase inhibitor, putative	III and IV	
B9SMP4	serine carboxypeptidase, putative	III	
B9SJ52	Vitellogenic carboxypeptidase, putative	III and IV	
B9RBX6	Xylem serine proteinase 1 precursor, putative	III and IV	
B9SAV8	Xylem serine proteinase 1 precursor, putative	III and IV	
B9RBX4	Xylem serine proteinase 1 precursor, putative	IV	
B9T6Y9	Xylem serine proteinase 1 precursor, putative	IV	
B9R7I8	Protein Z, putative (inhibitor)	III and IV	Serine/cysteine

which is thought to be associated with cell death [8]. In addition to inhibitors of cysteine and serine peptidases (Table 1) that regulate plant peptidases through the formation of reversible complexes with target peptidases, we identified a protein called protein Z (Table 1) which is a peptidase inhibitor of the serpin class. Proteins belonging to this class are capable of forming irreversible complexes with target peptidases belonging either to the serine or to the cysteine mechanistic classes of peptidases [22]. In *Arabidopsis*, the peptidase inhibitor *At*Serpin1 which shares high degree of sequence similarity with Protein Z, was shown to target a cysteine peptidase involved in the regulation of PCD [23]. As yet we do not know whether these peptidases and peptidase inhibitors are evenly synthesized in all nucellar cell layers or whether their deposition is restricted to the proliferating or the non-proliferating cell layers. It is expected that the answer to this question will help in establishing the precise role of these proteins in PCD.

Three isoform of ricin were identified in the nucellus of seeds at stages III and IV. Isoform B9RRJ1 was found at stage III, isoform BT1S1 at stage IV and isoform B9STV4 was found in both stages (Supplementary Table 1). The number of identified peptides for these isoforms were 2 (B9T1S1), 4 (B9RRJ1) and 13 (B9STV4). As in seeds at stage III only syncitial endosperm is present and in seeds at stage IV cellular endosperm formation is only in its beginning (Fig. 1), we can rule out the possibility that the ricin isoforms identified are derived from the endosperm. So far ricin [24] or its transcripts [25] have not been detected in castor bean seed tissues other than endosperm and thus our results are unforeseen. Ricin is a ribosome-inactivating protein that acts through the hydrolysis of the N-glycosidic bond between the base and the ribose at position A4324 in the 28S rRNA of rat and which during seed development is deposited within protein bodies of the endosperm along with storage proteins [26]. The sequestration of ricin within the protein bodies protects castor bean cells from its toxicity and as the targeting of ricin to the protein bodies involves the proteolytic processing of a ricin precursor, it would be of interest to determine the subcellular targeting of ricin in nucellus tissues, as well as the proteolytic system involved in ricin maturation. In this connection a cysteine peptidase called vacuolar processing enzyme which is involved in the processing of pro-ricin into mature ricin in the developing endosperm was also identified in this study (Table 1). As it is the case with the peptidases and peptidase inhibitors discussed above, we do not know whether the synthesis of ricin takes place in all of nucellar cell layers or whether it is restricted to the proliferating or to the non-proliferating cell layers which are undergoing PCD. The uptake of ricin by mammalian cells is known to induce apoptosis [27] and the deposition of ricin in castor bean tissues which will eventually be subjected to PCD, suggests that any event that would result in the release of mature ricin into the cytoplasm could well be the trigger to PCD, especially in light of the fact that plant ribosomes are known to be sensitive to ricin action [28]. As previously noted, in the degenerating endosperm of castor bean, after the hydrolysis of storage proteins and transport of the resulting amino acids to the seedling, the release of a KDEL-tailed cysteine endopeptidase from the ricinosomes is the final stage of PCD [6], in spite of the fact that the chain of events leading to PCD is still largely unknown.

In conclusion, besides providing a glimpse into the complex metabolism of the nucellus in the developing seed of castor bean, our analysis has pointed out various issues which are relevant for the understanding of the role of the nucellus in shaping seed development, especially the questions related to the spatial and temporal distribution within nucellar cell layers of proteins putatively related to PCD and therefore constitute an important basis for further studies on the mechanisms of PCD in seed tissues.

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2012.01.002.

Conflict of Interest

The authors have declared no conflict of interest.

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