

In-Depth Proteome Analysis of *Ricinus communis* Pollens

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Pollen grains are tiny structures vital for sexual reproduction and consequently seed and fruit production in angiosperms, and a source of many allergenic components responsible for deleterious implications for health worldwide. Current pollen research is mainly focused on unraveling the molecular mechanisms underlying the pollen germination and tube formation passing from the quiescent stage. In this context, an in-depth proteome analysis of the pollens from *Ricinus communis* at three different stages—that is, mature, hydrated, and in vitro germinated—is performed. This analysis results in the identification of 1950 proteins, including 1773, 1313, and 858, from mature, hydrated, and germinated pollens, respectively. Based on label-free quantification, 164 proteins are found to be significantly differentially abundant from mature to hydrated pollens, 40 proteins from hydrated to germinated, and 57 proteins from mature to germinated pollens, respectively. Most of the differentially abundant proteins are related to protein, carbohydrate, and energy metabolism and signaling. Besides other functional classes, a reasonable number of the proteins are predicted to be allergenic proteins, previously undiscovered. This is the first in-deep proteome analysis of the *R. communis* pollens and, to the best of our knowledge, one of the most complete proteome dataset identified from the pollens of any plant species, thus providing a reference proteome for researchers interested in pollen biology.

Pollen plays an important role in plant biology by harboring the male gametes in a prominent vegetative cell. It has the capacity to tolerate desiccation and distribute the plant to distant places with the help of wind and insects. Once having reached the stigma, pollen gets hydrated followed by germination and development of pollen tube that later releases the sperm cells for fertilization.^[1]

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Depending on the level of desiccation, the time of pollen hydration and tube germination is variable and may occur within minutes to an hour, both of which are critical for plant reproduction.^[2] The pollen tube grows from the tip region, with the deposition of the membrane and cell wall material on the tip, a process known as tip growth.^[3] To date, most of the proteomic studies are dedicated to either mature^[4,5] or germinated^[6,7] pollens, while comparative studies focused on mature, germinated, and other developmental stages of pollens are scarce. Zou et al.^[8] have performed a comparative proteomic investigation of the mature and germinated *Arabidopsis* pollens and reported some differentially abundant proteins which are mainly involved in biological processes like structure, signaling, transport, metabolism, and energy production. To foster a better understanding of the mechanisms underlying the molecular events during pollen germination from the quiescent stage, we provide here a dataset acquired from a quantitative proteome analysis of the pollens from *Ricinus communis* at three

different stages, that is, mature, hydrated, and germinated.

Mature, hydrated, and germinated pollens (**Figure 1**) (details in Supporting Information) were frozen in liquid nitrogen, smashed to powder, and subjected to protein extraction, performed as previously.^[5] One hundred micrograms of proteins from each sample were subjected to in-solution trypsin digestion and LC-MS/MS analysis (Supporting Information). Experiments were performed in triplicates representing three biological replicates. MS/MS ion search was performed against a database with 62 688 sequences containing the *R. communis* nuclear and chloroplast genomes downloaded from the UniProt in August 2015. Only high-confidence peptides and proteins were considered. We used a false discovery rate (FDR) less than 1% at peptide and protein level and maximum parsimony for protein grouping. Spectrum search resulted in the identification of 17 471, 16 347, and 9125 peptides of which 13 334, 12 412, and 6695 were unique to mature, hydrated, and germinated pollens, respectively, corresponding to a total of 1950 proteins, of which 743 were present in all the three stages used here (Table S1 and Figure S1, Supporting Information). The proteins considered for downstream analysis were those identified in at least one of the three technical

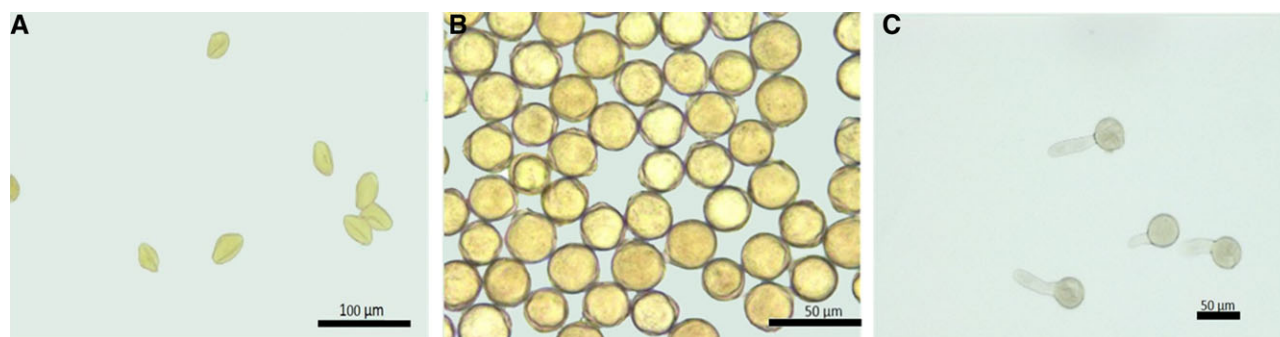


Figure 1. *Ricinus communis* pollens at mature, hydrated, and germinated stages.

replicates and minimum two biological replicates of a particular pollen stage used here.

Identified proteins were functionally classified into diverse classes using the MapMan classification system^[9] (Table S2 and Figure S2, Supporting Information). Label-free-based spectral count was used to measure the protein abundance represented by the total number of the spectra identified for a particular protein from mature to hydrated, hydrated to germinated, and mature to germinated pollens, respectively, performed as previously.^[10] Briefly, we used the TFold module of the PatternLab for proteomics. TFold module uses a *p*-value-dependent fold-change cutoff to maximize the number of identifications satisfying a theoretical FDR estimator (Benjamini–Hochberg). Fold changes were then calculated by dividing the average normalized spectral counts of the reference stage by those obtained in the other stage, respectively. One hundred and sixty four proteins were found to be significantly differentially abundant (SDA) from mature to hydrated pollens, 40 proteins from hydrated to germinated, while 57 proteins were SDA from mature to germinated pollens (Table S3, Supporting Information). Differentially abundant proteins include those involved in protein metabolism, carbohydrate and energy metabolism among others, which collaborate with the previous study of *Oryza sativa* and *Arabidopsis* pollens proteomes.^[8,11] Based on their spectral counting profile, proteins with a similar pattern of deposition (1063) among the three stages were grouped into five distinct clusters (Figure 2; Table S4, Supporting Information), as performed previously for the proteins of developing *R. communis* extrafloral nectaries.^[12]

A high proportion of the identified proteins is related to carbohydrate and energy metabolism (Tables S1 and S2, Supporting Information). We identified a complete set of the proteins involved in the glycolytic pathway, where previous studies^[5,7] detected only a few of the proteins from this pathway. Similarly, several proteins associated with the TCA cycle, electron transport chain, and pentose phosphate pathway were also identified (Table S2, Supporting Information). We identified *Arabidopsis* homologs of several enzymes involved in starch metabolism, including those involved in starch synthesis such as starch synthase, starch branching enzyme, and degradation, including alpha-glucan, water dikinase, alpha-amylase, alpha-1,4 glucan phosphorylases, and 4-alpha-glucanotransferase among others (Table S1, Supporting Information). Several enzymes involved in carbohydrate and energy metabolism such as NADH dehydrogenase, aldo-keto reductase, citrate synthase, xylose isomerase, and ATP synthase,

among others, are differentially abundant in this study (Table S3, Supporting Information), suggesting an important role of these pathways in tube formation.

Synthesis and loosening of the cell wall are essential processes in germination and development of the pollen tube. Multiple isoforms of the proteins from such processes like pectate lyases (PLs), pectin acetylesterases, pectin methylesterases (PME), pectin methylesterase inhibitors (PMEI), glycosyl hydrolase, 1,4- β -glucan synthase, β -galactosidase, endo-1,3- β -glucanase, and α -expansin, etc., were identified here (Table S1, Supporting Information). Two isoforms of PLs were identified in all the three stages while only one in mature pollens. Although later identified in other tissues, PLs were first reported from pollen^[13] where they are thought to be involved in the initial loosening of the cell wall to aid in pollen tube emergence.^[14] PME and PMEI are important for pollen tube growth, as the former is involved in methylesterification of homogalacturonans,^[15] the main component of the pectins, and the latter regulates the post-translational modification of the former. PMEI is downregulated in this analysis (Table S3, Supporting Information) showing the promotion of tube growth during germination. Along with the PME and PLs, *exo*- and *endo*-PGs and β -galactosidase have been proposed^[16] to have the activity to reduce the size of pectic polymers through the removal of the neutral side chain residues. Similarly, glycosyl hydrolases and 1,4- β -glucan synthases are also required for the pollen tube growth and development.^[17] Isoforms of UDP-glucose dehydrogenase (UGDH) were also identified. This enzyme plays a principal role in cell wall synthesis, and about 60% of the cell wall polysaccharide components are either directly or indirectly derived from UDP-glucuronate.^[18] These results indicate the important role of pectin in germination and fertilization.

Plants produce reactive oxygen species (ROS) during normal metabolism in response to diverse physiological stimuli, including pollen germination, pollen tube formation, and fertilization, among others.^[19] Although their excessive production can cause oxidative damage, mechanisms for the appropriate balance between production and quenching of ROS are present in the plant. Several enzymes involved in detoxification of ROS including superoxide dismutases, ascorbate peroxidases, dehydroascorbate reductases, glutathione transferases, glutathione peroxidases, glutathione reductases, catalases, and glutaredoxin, among others, were identified in this study (Table S1, Supporting Information). Homologs of the different kinds of heat shock proteins (HSP) were also identified. Two HSP isoforms appeared in

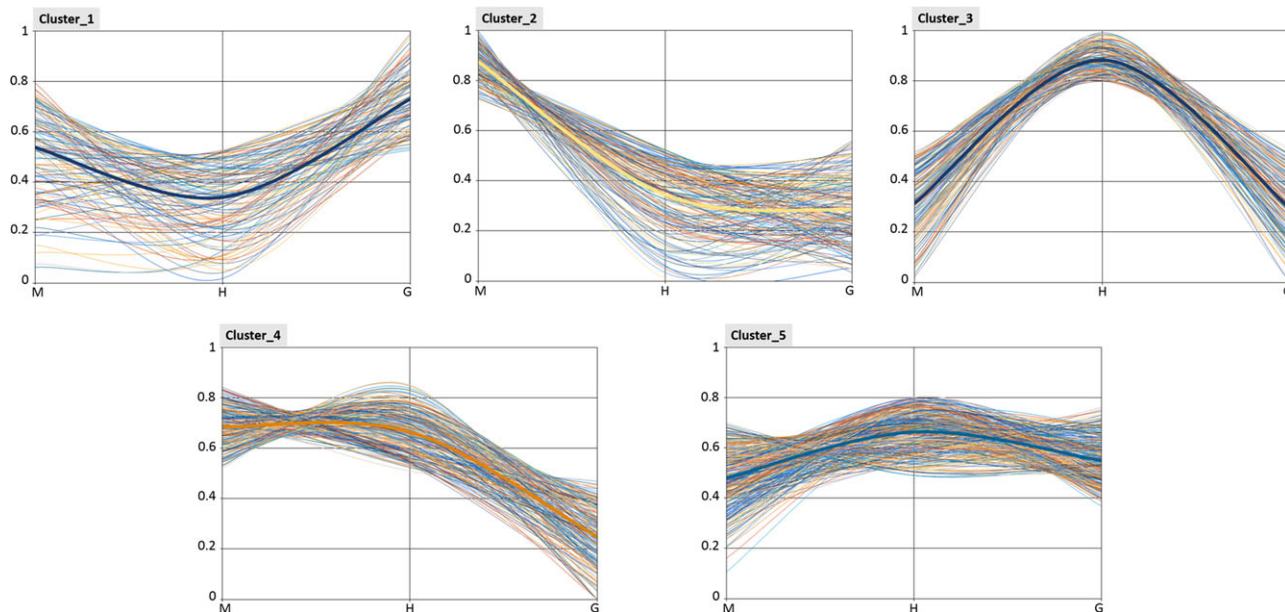


Figure 2. Cluster analysis of the proteins identified from the pollen of *R. communis* at three different stages. Proteins with an alike pattern of deposition were grouped into five different clusters. x-axes represent pollen stage, while y-axes represent signal intensity. M, mature; H, hydrated; G, germinated pollens.

mature stage only while the rest of the others were identified in more than one stage (Table S1, Supporting Information). Along with other roles, these proteins were found to be present in pollen development and embryogenesis, and thus are important for the proper functionality of the development process of pollen.^[20] *Arabidopsis* homolog of a desiccation-related protein (B9RZV0) was also identified, which was present in all the three stages and grouped in cluster II indicating its important role during pollen germination. Similarly, four late embryogenesis abundant proteins were identified of which one isoform was present in mature stage only, while others in all the three stages of pollens used here. These proteins are not only important during water deficit conditions but also to water limitation in seed and pollen development due to optimal growth conditions.^[21] Several isoforms of so-called glycine-rich proteins were also identified in this study. Expression of these proteins was found to be elevated under different stress conditions, including salinity and cold,^[22] and their presence in pollen may be another level of protection against different environmental stresses. Based on label-free quantification, several of these defense proteins were found as differentially abundant among the stages used in this study (Table S3, Supporting Information).

Pollen allergens are the major source of respiratory allergic reactions.^[23] In order to determine the allergenic proteins in the *Ricinus* pollens, the 1950 identified proteins were submitted to Structural Database of Allergenic Proteins and Allergen Online (Supporting Information). Of the identified proteins, 103 were predicted as putative allergens, of which 31 were identified for the first time and were not identified from other tissues of *Ricinus*^[12,24–27] (Table S5, Supporting Information). Ten HSPs were predicted as being allergenic, of which one was identified for the first time from this species. HSPs were shown to bind to the IgE of the allergic patients.^[28] One pectate lyase and five

pectinesterases were also identified as putative allergens, where the former was found to be the main allergen of the members from Asteraceae and Cupressaceae families^[29] and of the olive tree pollens.^[30] A number of so-called pathogenesis-related proteins (PR-proteins) including venom allergen (B9S7U5) (PR-1) and two class I chitinases (PR-3) unique to *Ricinus* pollens were also identified (Table S5, Supporting Information). Besides protective-role PR-proteins are regarded to possess allergenic properties as well.^[31] Different isoforms of serine and cysteine proteases which were identified for the first time from *Ricinus* pollen were also predicted as allergens. Serine (Cuc m 1) and cysteine (Act d) proteases with allergenic potential from the pollens of different plant species were previously described.^[32,33]

Last, we identified *Arabidopsis* homologs of the two important classes of the seed storage proteins (SSPs) named cruciferins (AT5G44120) and napins (AT2G28680, AT1G07750, and AT2G28490) (Table S1, Supporting Information). Members of these SSPs were previously also detected in the pollen proteome of *Arabidopsis*^[8] and tomato,^[34] which together with our results negate the formerly proposed notion that pollens lack SSPs.^[35]

Besides this proteomic dataset, previously we studied different biological aspects of *R. communis* through proteomic investigation of different tissues,^[12,24–26] organelles^[24] and exudates,^[27] which collectively make a part of the project to establish the proteome of this biotechnologically important oilseed. So far, we have identified 4679 proteins, in which 671 are solely present in the pollen proteomes. Major classes of the proteins among these unique pollen proteins include those involved in protein metabolism, cell wall metabolism, signaling and stress, of which several are SDA. These proteins have a prominent role in pollen germination and tube development after hydration. Many of the proteins identified here are not yet described in proteomic

studies of the pollen from other plant species. We believe that this dataset will serve as a reference proteome for the researchers interested in pollen biology.

Mass spectrometry raw data files are available on the Chorus repository web site (chorusproject.org; project ID: 1524, project name: In-depth Proteomic Analysis of *Ricinus communis* Pollens).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

allergens, castor, defense proteins, oilseed proteomics, pollen proteomics

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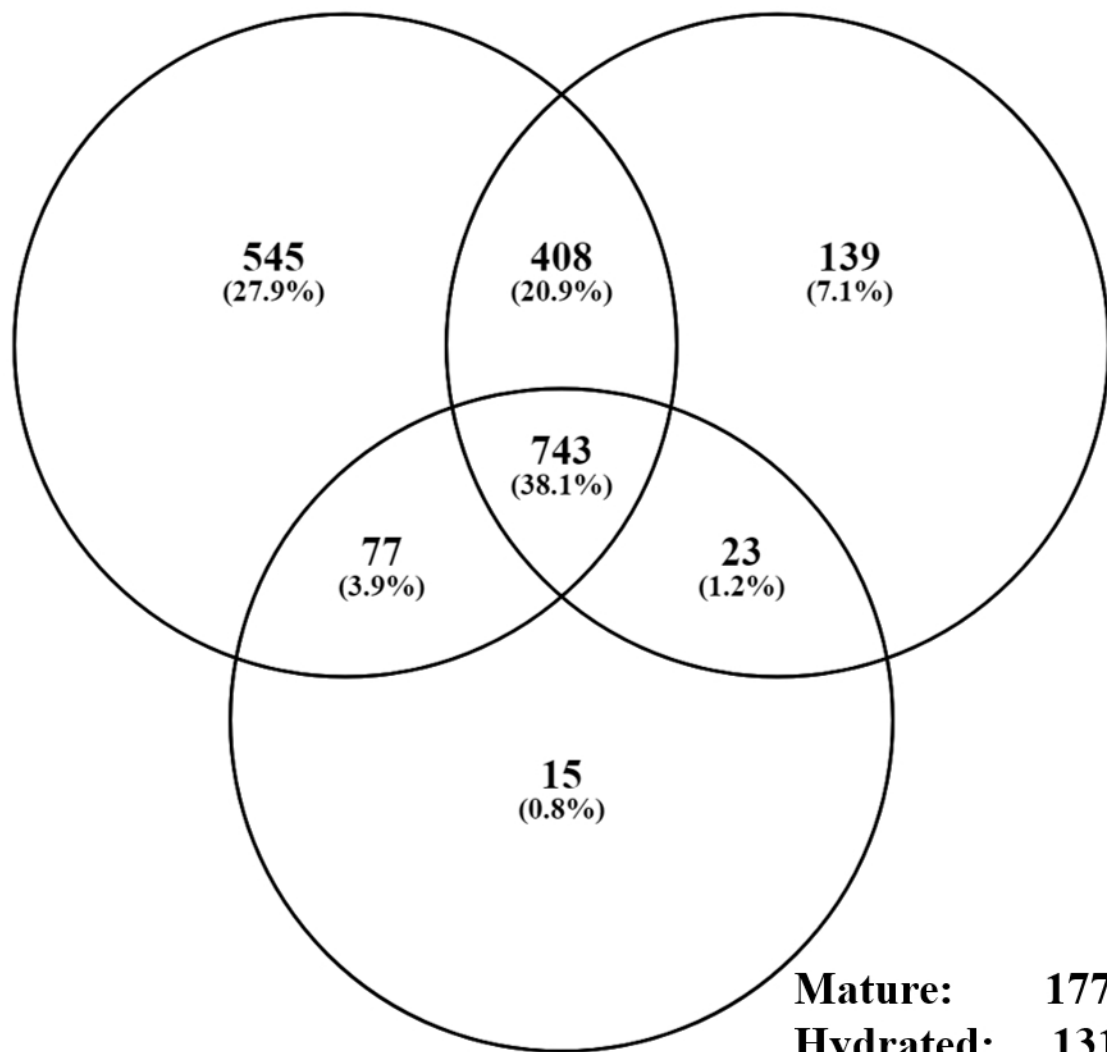
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Mature

Hydrated



Mature: 1773

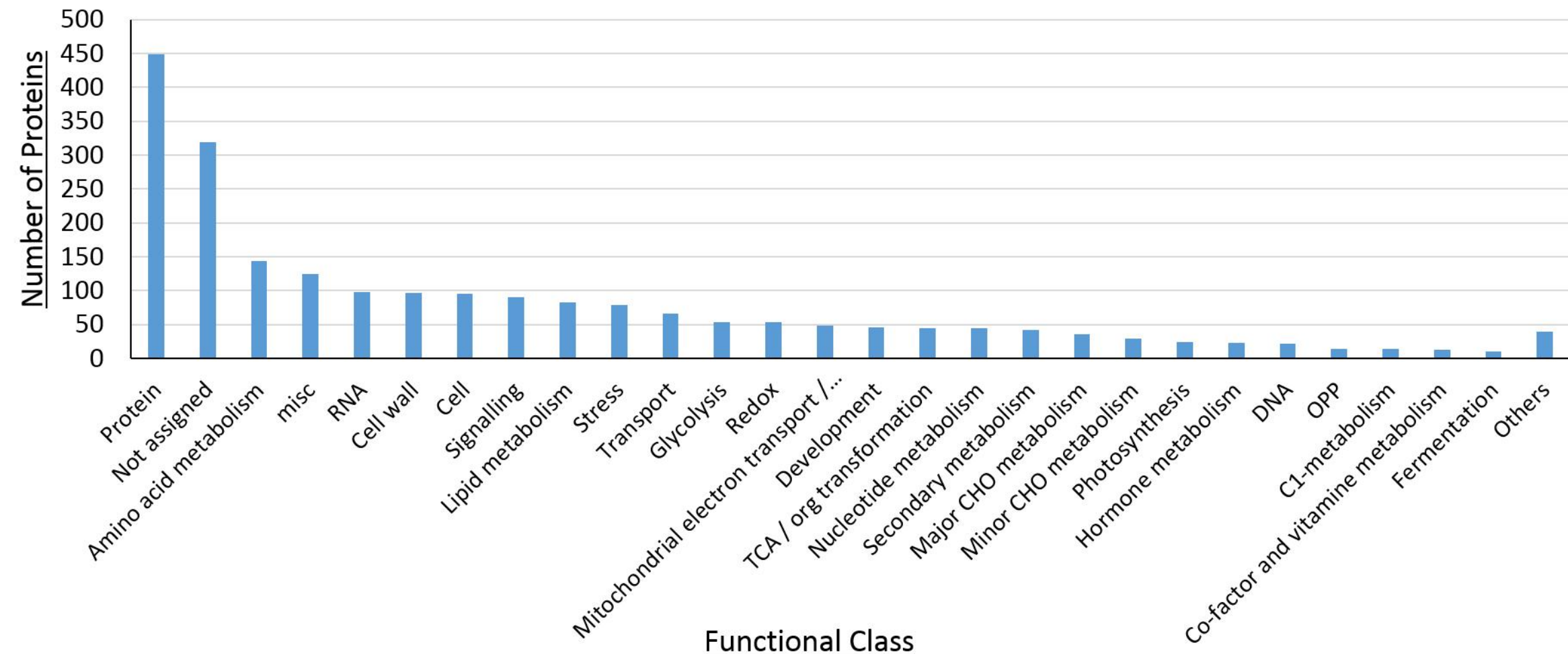
Hydrated: 1313

Germinated: 858

Total: 1950

Germinated

Proteins Functional Classification



Material and Methods

Pollens collection and in-vitro germination

Ricinus communis, BRS-Paraguaçu cultivar were harvest at agronomy experimental area of Federal University Ceará, Brazil. Male flowers were collected in paper bags (20-30 male flower) and pollen was obtained through vigorous shaking. In order to obtain hydrated and germinated pollens, 200mg of mature pollens were with germination solution: 12% sucrose, 100ppm boric acid, 300 ppm calcium nitrate, 100 ppm of magnesium sulfate and potassium nitrate at room temperature [1][2]. A solution containing 150,000 pollen/mL, estimated by quantification at Neubauer chamber, were kept at Petri dishes inside a container lined with moist filter paper. A solution containing pollen incubated till 2 hours, characterized by spherical shape and absence of protrusion through exine aperture, examined through a light microscope, were classified as hydrated pollen. Pollens incubated for 5 hours in germination solution were considered germinated when examined under a microscope with pollen tubes extended twice than the diameter of the mature pollen grain. Pollens collection, hydration and germination was performed three times as three independent biological replicates.

Protein extraction and sample preparation

Proteins were extracted according to a previously used method [3] with an acetone solution containing 10% TCA and 1% DTT in a proportion of 1:10 (w/v). After addition of extraction buffer it was incubated at -20 °C overnight and centrifuged. Pellets were washed with cold acetone, dried under vacuum, dissolved in 7M urea/2M thiourea and quantified through Qubit assay. 100 µg of proteins from each stage of the pollen were first reduced with 10 mM DTT for 30 min at 56 °C and then alkylated with 55 mM with iodoacetamide for 30 min at room temperature in dark. Urea concentration was reduced to less than 1 M and proteins were digested with trypsin (Promega) overnight at 37 °C. Proteins digestion was stopped by adding formic acid to a final concentration of 2%.

LC-MS/MS and Data Analysis

Peptides were cleaned using spin columns (Harvard Apparatus) filled with C-18 material, dissolved in 25 µl of 0.1% formic acid. 4 µl peptide solution was applied to EASY-nLC II (Proxeon) coupled online to an LTQ-Orbitrap Velos mass spectrometer

(Thermo Scientific). Peptides were passed through a trap column (150 μm x 2 cm) packed in-house with C-18 ReproSil 5 μm resin (Dr. Maisch) in sequence with an analytical column (75 μm x 16 cm) packed with ReproSil-Pur AQ C-18 3 μm resin (Dr. Maisch) using a gradient from 100% phase A (0.1% formic acid) to 35% phase B (0.1% formic acid, 95% ACN) for 150 min, followed by 20 min washing. Mass spectra were acquired in a positive mode using the data-dependent automatic (DDA) survey MS scan and MS/MS acquisition. Each DDA consisted of a survey scan of the m/z range 300–2000 and resolution 60 000 with a target value of 1×10^{-6} ions. Survey scan was followed by the MS/MS of the 10 most intense ions in the Orbitrap using the HCD fragmentation, and previously precursor ions were dynamically excluded for 60 s. Three biological replicates of each sample were applied three times as technical replicates as performed previously [4].

MS/MS ion search was performed using Comet [5] (PSM) inserted in PatternLab (version 4.0) [6] against *R. communis* genomic database downloaded from UniProt in October 2016. Search parameters were: full tryptic hydrolysis, three missed cleavages, oxidation of methionine as variable modification, carbamidomethylation as a fixed modification and peptide tolerance of 40 ppm. The spectrums of peptide matches obtained from the Comet were subsequently filtered through Search Engine Processor (SEPro) [7] as performed before [4]. Proteins identified in at least two biological replicates were used for downstream analysis. Functional annotation of the identified proteins was performed through the MapMan functional classification system [8]. Label-free quantification of the proteins differentially expressed from mature to hydrated and germinated pollens, respectively, was carried out using Patternlab TFold module [9] as previously performed for the developing endosperm of *Jatropha curcas* [10]. Proteins with similar deposition pattern were evaluated by clustering them into five different clusters using TrendQuest module of the PatternLab for proteomics [6]. Proteins with allergenic properties were predicted as performed for the *Populus tomentosa* [11] pollen proteins using SDAP [12], based on sequence similarity (>35%) with known allergens and having eight consecutive amino acids with respect to reported allergens, and the Allergen Online [13].

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