Vegetable Research

ARTICLE

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https://doi.org/10.48130/vegres-0026-0017

Vegetable Research **2026**, 6: e024

Proteomic analysis in common bean seed coat reveals high enzymatic profile with relevance for purine nucleotide metabolism during germination

Lucia Ordoñez Pareja, Gregorio Galvez-Valdivieso, Pedro Piedras* and Mercedes Diaz-Baena

Departamento de Botánica, Ecología y Fisiología Vegetal, Grupo de Fisiología Molecular y Biotecnología de Plantas, Campus de Excelencia Internacional Agroalimentario, CEIA3, Campus de Rabanales, Universidad de Córdoba, Córdoba 14014, Spain

* Correspondence: bb2pimop@uco.es (Piedras P)

Abstract

Common bean is a major legume crop with high seed protein content and significant global importance for human consumption. Seedling establishment is a critical stage for plant survival, as the plant must develop in a potentially hostile environment where the seed coat or testa plays a crucial role. In this manuscript, we performed a proteomic study of bean seed coats and compared protein levels before and after germination. We found a significant number of proteins in the seed coat, of which 919 increased in abundance during germination, 717 decreased, and 1,567 remained unchanged. Among the various protein categories that increased in abundance, the nucleoside diphosphate kinase (NDPK) family and T2 ribonucleases, proteins involved in nucleotide metabolism, were used to analyse the transcript-translation relationship. In common bean, there are four NDPKs, three of which have been identified in the bean seed coat at both the protein and mRNA levels, and 13 ribonucleases T2, five of which have been identified as proteins and four as genes. The purine nucleotide degradation pathway in the seed coat has also been analysed, identifying proteins that could catalyze the degradation of these nucleotides to ureides. Overall, the data presented allow us to conclude that the seed coat is a functionally active tissue during germination and does not simply act as a protective barrier. Moreover, the proteomic profile reveals that this tissue exhibits a high nucleotide metabolic rate.

Keywords: Seed coat, Nucleotides, Germination, Common bean, Proteomic

Citation: Pareja LO, Galvez-Valdivieso G, Piedras P, Diaz-Baena M. 2026. Proteomic analysis in common bean seed coat reveals high enzymatic profile with relevance for purine nucleotide metabolism during germination. *Vegetable Research* 6: e024 https://doi.org/10.48130/vegres-0026-0017

Introduction

Seeds are the means by which higher plants reproduce and constitute the basic units for their dispersal. They encapsulate genetic information and nutritional reserves, ensuring the successful development of new seedlings. Seed maturation on the mother plant, dormancy, and germination are crucial processes involving meticulously coordinated molecular and cellular processes, with significant changes in gene expression[1]. Seed germination is one of the most vulnerable processes in plant development, as seeds germinate in a potentially stressful environment and are frequently exposed to both biotic and abiotic conditions. Therefore, during this stage, the seedling must be protected from these potentially hostile conditions, while simultaneously ensuring a supply of nutrients for the developing axis using the reserves accumulated in seeds during maturation.

The seed coat, or testa, is a vital maternal structure derived from the inner and outer integuments that surround the ovule. Traditionally, the seed coat has been considered a physical barrier that protects the embryo and facilitates seed dispersal. Additionally, the seed coat and associated dead tissues enclosing the embryo may function as reservoirs of nutrients, proteins, and regulatory compounds[2]. Together, these structures operate as an integrated system that ensures embryo survival during seed dormancy in the soil and promotes proper growth and establishment in the natural environment[3]. The seed coat may play roles in protection against predators, seedling establishment in the soil, moisture absorption, seed anchorage, light filtering, and seed respiration[4]. Consequently, the seed coat also regulates dormancy by acting as a permeable barrier that restricts water uptake and gas exchange, stores germination-inhibiting compounds, and provides mechanical resistance that prevents premature embryo expansion[5].

During seed maturation, several maternal tissues surrounding the embryo (such as the seed coat, pericarp, and floral bracts) undergo programmed cell death (PCD). This process involves the degradation of macromolecules such as DNA, RNA, and proteins, which are subsequently redistributed to other plant organs. Growing evidence indicates that the dead maternal tissues enclosing the embryo, in both monocots and dicots, serve as long-term stores of active hydrolytic enzymes and other compounds that can contribute to seed persistence, germination, and seedling establishment[6]. These enzymes appear to be released upon hydration[7] and include nucleases, proteases, chitinases, and enzymes involved in cell wall remodeling and reactive oxygen species (ROS) detoxification[6]. Furthermore, the seed coat has been recognized as playing an important role in directing nutrient flow to the embryo during development, including potassium, calcium, nitrate, and other cations that enter the seed during imbibition[8].

Common bean (*Phaseolus vulgaris*) is an agriculturally important legume, whose seeds are characterized by a well-defined seed coat that persists throughout maturation and germination[9]. Common bean is a ureidic legume that transports ureides from nodules to the upper parts under nitrogen fixation conditions, constituting almost all of the organic nitrogen transported by the xylem under these nitrogen fixation conditions[10]. Interestingly, ureidic metabolism also appears to be important in these plants during early seedling development[11,12] and during nutrient mobilization that occurs during leaf senescence[13]. These ureides may serve as transport

https://doi.org/10.48130/vegres-0026-0017

molecules derived from the degradation of purine nucleotides resulting from nucleic acid degradation.

In a previous study, we observed that ribonuclease activity was very high in the common bean seed coat during germination and post-germinative development, exceeding the activity detected in other seedling tissues[14]. Furthermore, we also detected high ribonuclease activity, as well as activity of enzymes involved in nucleotide metabolism during seed coat formation in the mother plant during fruit development[15]. Therefore, we hypothesized that germination induces comprehensive proteomic reprogramming in the seed coat, where the activation of ribonucleases plays a central role in the nucleotide metabolism necessary for proper seedling development. We conducted the present study to determine changes in the abundance of proteins in the seed coat during common bean germination, with a particular attention to those related to purine nucleotide degradation, since ureides could be involved as nitrogen transport molecules during germination and post-germinative development.

Materials and methods

Plant material

Phaseolus vulgaris seeds were sterilized in ethanol by immersion for 30 s, followed by 5 min in 0.2% sodium hypochlorite. To remove any residual bleach from the seeds, six successive washes with distilled water were performed. For germination, seeds were placed in a Petri dish (150 mm in diameter) with filter paper moistened with distilled water under sterile conditions. The seeds were located at a density of ten seeds per dish, and distilled water was added daily to maintain moisture. Samples were collected 2 h (day 0) and 96 h (day 4) after starting imbibition. For each condition, four biological samples were considered, each comprising a pool of six seeds. Plant material was ground using a mortar with the continuous addition of liquid nitrogen to prevent sample degradation until a fine powder was obtained and stored at −80 °C.

Sample preparation for proteomic analysis

Sample preparation was performed at the Proteomics Facility at Central Service for Research Support (SCAI) at the University of Cordoba. Pulverized material was homogenized in Laemmli buffer at a 1:4 (w/v) ratio, and extracts were cleaned up in 10% SDS-PAGE. Electrophoresis was performed until proteins were separated 1 cm in the resolving gel. After Coomassie blue staining, gel portions were diced and kept in water until digestion. Afterwards, the gels were firstly destained in 200 mM ammonium bicarbonate (AB)/50% acetonitrile for 15 and 5 min in 100% acetonitrile. Protein was reduced by the addition of 20 mM dithiothreitol in 25 mM AB and incubated for 20 min at 55 °C. The mixtures were cooled to room temperature, followed by alkylation of free thiols by the addition of 40 mM iodoacetamide in 25 mM AB in the dark for 20 min. Gels were washed twice in 25 mM AB. Proteolytic digestion was performed by the addition of Trypsin/LysC (Promega, Madison, WI), 12.5 ng/μl of enzyme in 25 mM AB, and incubated at 37 °C overnight. Protein digestion was stopped by the addition of trifluoroacetic acid at 1% final concentration, and samples were desalted with C18 ZipTip columns.

Analysis by high-resolution mass spectrometry

LC-TIMS-MS/MS was carried out using a nanoElute nanoflow ultrahigh-pressure LC system (Bruker Daltonics, Bremen, Germany) coupled to a timsTOF Pro 2 mass spectrometer, equipped with a CaptiveSpray nanoelectrospray ion source (Bruker Daltonics). For most analyses, 200 ng of peptide digest was loaded onto an Aurora C18 capillary column (25 cm length, 75 μm ID, 1.6 μm particle size, Ion Opticks). Peptides were separated at 30 °C using a 20 min gradient at a flow rate of 300 nL/min (mobile phase A (MPA): 0.1% formic acid; mobile phase B (MPB): 0.1% formic acid in acetonitrile). A step gradient from 0% to 30% MPB was applied over 24 min, followed by a 30% to 90% MPB step for 1 min, and finished with a 90% MPB wash for an additional 5 min for a further time. The total run time was 30 min per analysis. The timsTOF Pro 2 was run in DIA-PASEF mode with isolation windows of 25 Da in a mass range of 450–950 Da without mass overlapping. Ion mobility resolution was set to 0.85–1.30 V s/cm^2 over a ramp time of 100 ms. The collision energy was increased stepwise as a function of the ion mobility ramp, from 27 to 45 eV. This analysis was performed at the Proteomics Facility at SCAI of the University of Cordoba.

Data analyses, protein identification, and functional analysis

Mass spectra data were analyzed in Spectronaut (Biognosys). The reference library was acquired from UniProt (30.501 sequences, www.uniprot.org). The direct DIA algorithm was used for raw data analysis, and library generation was created with the Pulsar search algorithm using the database itself. To account for post-translational modifications and chemical labelling, the following adjustments were used: carbamidomethylation of cysteine residues was set as fixed modification, methionine oxidation and acetylation (N-terminal protein) were set as variable modification.

The analysis of the database generated from the proteomic study was performed using Python scripts. Protein identification was carried out by SCAI of the University of Cordoba using the Uniprot database. The Phytozome Database (V.12) was used to correlate Uniprot IDs and phytozome IDs.

The correlation between GO codes (from Phytozome) and their descriptions was obtained from the Gene Ontology database (https://geneontology.org/docs/download-ontology). The correlation between KOG codes and their descriptions was obtained from NCBI (https://ftp.ncbi.nlm.nih.gov/pub/COG/KOG/kog). GO and KOG codes were assigned to proteins based on Phytozome information. For all the analyses, proteins with a q-value > 0.05 were excluded.

Preparation of crude extracts

Pulverized plant material stored at −80 °C (approximately 0.1 g) was mixed with extraction buffer at a 4:1 v/w ratio (4 volumes of extraction buffer per gram of plant tissue). The extraction buffer consisted of 50 mM TES (pH 7) and 0.15% sodium deoxycholate. Once homogenized, samples were centrifuged at 24,000 × g and 4 °C for 10 min. The resulting supernatant was collected, divided into aliquots, and stored at 4 °C as crude extracts.

Total soluble protein determination

Soluble protein concentration was estimated using the commercial Bio-Rad protein assay (Bio-Rad, Madrid, Spain) based on the Bradford dye-binding method[16] and using bovine serum albumin as the standard.

Allantoinase activity determination

Allantoinase activity was determined in crude extracts following the production of allantoate, as indicated by Raso et al.[17]. The reaction mixture consisted of 50 mM Tris–HCl (pH 7.8), 1 mM $MnSO_4$,

12 mM allantoin, and an appropriate amount of crude extract. The reaction was carried out at 35 °C, and aliquots were taken at 0 and 30 min of reaction to determine allantoate concentration. One unit of enzymatic activity is defined as the amount of enzyme catalyzing the production of 1 μmol of allantoic acid per minute.

Ureide content determination

The concentration of ureides (allantoin and allantoate) was determined by the colorimetric analysis of glyoxylate derivatives as described by Piedras et al.[18]. In this method, ureides are determined after their chemical transformation to glyoxylate. The values of total ureides in crude extracts indicated in the paper are the sum of both allantoin and allantoate.

RNA isolation and cDNA synthesis

Total RNA was extracted using the NZYol Reagent (NZYTECH) following the manufacturer's instructions, with an additional LiCl precipitation step to enhance RNA quality. Two micrograms of total RNA were treated with RNase-free DNase I (NEB), and the absence of contaminating genomic DNA was confirmed by PCR. First-strand cDNA synthesis was performed using DNase-treated RNA, RevertAid Reverse Transcriptase (ThermoFisher), and random hexamer primers.

qRT-PCR analysis

Quantitative RT-PCR (qRT-PCR) was performed on a MyiQ system (Bio-Rad) using the iTaq Universal SYBR Green Supermix (Bio-Rad) and the primers listed in Supplementary Table S1. The amplification protocol consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. After completion of the final cycle, a melting-curve analysis was conducted from 60 to 100 °C in 0.5 °C increments to assess reaction specificity. Gene expression levels were normalized using the geometric mean of two housekeeping genes (ubiquitin and actin) and quantified using the $2^{-\Delta C_T}$ method[19]. Primer specificity was verified by sequencing the corresponding PCR products.

Statistical analysis

Data are presented as means from four independent biological replicates, each with three technical replicates. Statistical significance for the *in vitro* assays was determined using Student's *t*-test. Statistical significance levels were indicated as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

Proteomic profiling of the seed coat of *Phaseolus vulgaris* during germination and analysis by gene onthology (GO) codes

We performed a comprehensive proteomic analysis of the seed coat of the common bean (*Phaseolus vulgaris)* throughout germination. A total of 3,203 proteins were identified, representing approximately 10% of total protein entries annotated for this species in the UniProt database (www.uniprot.org, February 2025) (Supplementary Table S2). Gene Ontology (GO) codes were annotated for all significantly identified proteins in seed coats and categorized into three main domains: cellular components (CC), molecular functions, (MF) and biological processes (BP). The distribution across categories was similar with 30.4% assigned to CC, 38.2% to MF and 31.4% to BP (Fig. 1a). The five most frequent GO terms within each category were identified (Fig. 1b). For CC, the most abundant categories were membrane, cytoplasm, cytosol, nucleus and plasma membrane (Fig. 1b). Within MF the predominant categories were ATP binding, metal ion binding, structural constituent of ribosome, RNA binding and GTP binding (Fig. 1b). In this category, the proportion of proteins that bind nucleoside triphosphate is noteworthy: 2,38% of identified proteins bind ATP and 0.75% bind GTP. The five most abundant categories for BP are translation, proteolysis, intracellular protein transport, carbohydrate metabolic process, and vesicle-mediated transport (Fig. 1b). It is surprising that the most abundant category in BP is translation, which supports an important role for new synthesis of proteins in this tissue. Protein abundance was compared between days 0 and 4 post-imbibition and classified as upregulated ($log_2FC > 0$) or downregulated ($log_2FC < 0$) based on the fold change, using a significance threshold of q-value < 0.05 to assess dynamic changes throughout the process (Supplementary Table S3). Among the approximately 3,200 proteins identified, 919 were upregulated, 717 were downregulated, and 1,567 showed no statistically significant changes (Fig. 2).

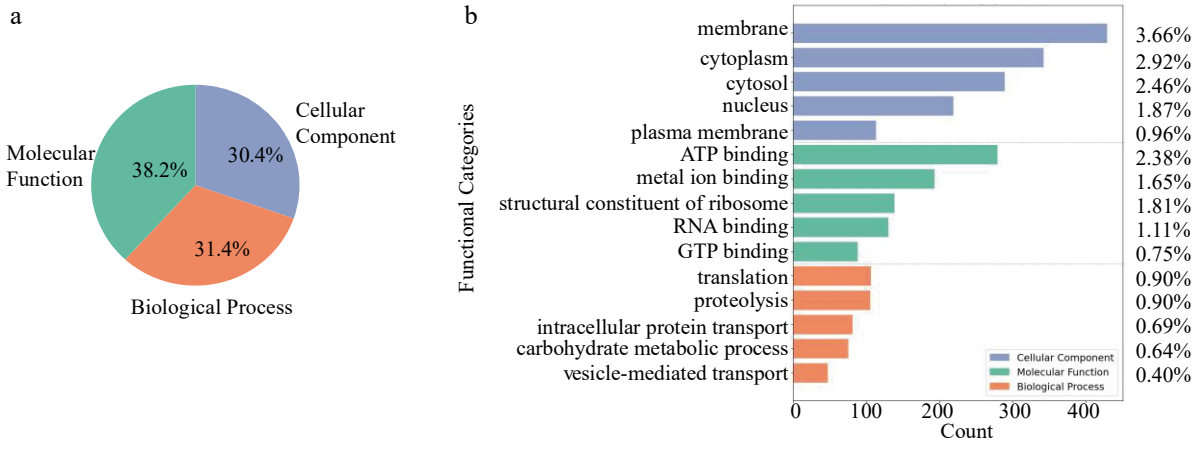

Fig. 1 Gene Ontology (GO) classification of proteins identified in the seed coat tissue of common bean. (a) Distribution of GO terms by ontology, showing the proportion of proteins assigned to the categories: Cellular Component, Molecular Function, and Biological Process. (b) Top five GO terms within each ontology ranked by frequency of annotation for each category.

https://doi.org/10.48130/vegres-0026-0017

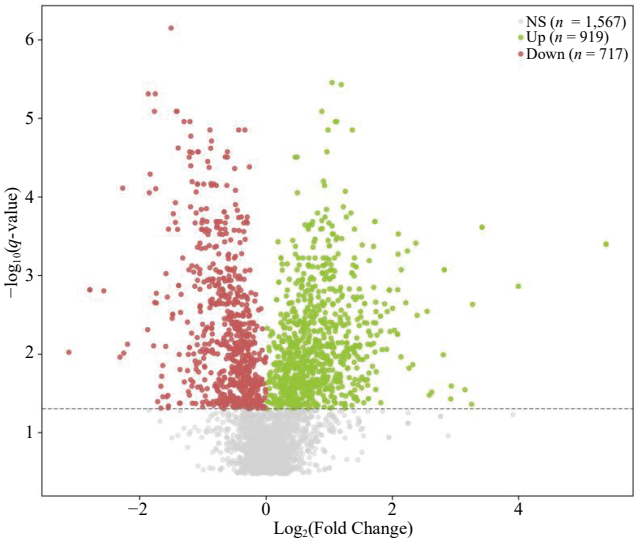

Fig. 2 Volcano plot showing the number of up- and down-regulated proteins (q-value < 0.05) in the seed coat tissue of common bean between day 0 and 4 after seed imbibition. Each point represents one protein, plotted according to its $\log_2$FC change (x-axis) and $-\log_{10}$ (q-value) (y-axis). Proteins with q-value ≥ 0.05 were classified as not significant (NS, gray). Among significant proteins (q-value < 0.05), those with positive $\log_2$FC were considered upregulated (green), while those with negative $\log_2$FC were considered downregulated (red). The numbers shown in the legend correspond to the count of proteins in each category. Proteomic profiles were compared using samples from four independent biological pools for each condition.

We analyzed the variation in protein abundance across GO categories to better understand the functional dynamics of the seed coat proteome during germination. For each functional term, the average fold change in protein abundance was calculated by comparing day 4 to 0. Positive values represent GO categories with an overall increase in protein abundance at day 4, while negative values indicate a net decrease. To focus the analysis on functional activity, GO terms related to the cellular component (CC) domain were excluded, and only categories from the biological process (BP) and molecular function (MF) domains were analyzed. Among the top 40 Gene Ontology (GO) functional categories, most exhibited a net increase in protein abundance between day 0 and 4 of imbibition (Fig. 3). Again, categories related to ATP and GTP binding and hydrolysis showed strong positive changes, suggesting increased energy metabolism activity. Categories related to transport (protein export from nucleus, nuclear export signal receptor activity, intracellular protein transport, vesicle mediated transport), metal binding (metal ion binding, copper binding, iron ion binding, zinc binding) and to stress responses (response to heat, defence response, response to light stimulus) were also very representative among the GO categories with changes during germination (Fig. 3).

Analysis of differentially abundant proteins by eukaryotic orthologous groups (KOG)

To complement the GO-based analysis, we performed a functional classification of differentially abundant proteins (DAP) using Eukaryotic Orthologous Groups (KOG). The analysis was only conducted with functional groups that showed a net increase in protein abundance at 4 DPI compared to 0 DPI. Proteins showing increases can provide valuable information about the true functional and biochemical processes occurring in the seed coat during germination. The top 40 categories contributing with most proteins changing in abundance were listed in Fig. 4. The five top groups with the most proteins that showed changes in abundance were glucose-6-phosphate/phosphate and phosphoenolpyruvate antiporter, histone 4, aspartyl protease, 40s ribosomal protein s10 and predicted membrane protein (Fig. 4). The overall increase in proteins abundance for each KOG category is indicated by the length of each bar in Fig. 4. The five top groups reflecting the greatest changes in seed coat during germination were predicted E3 ubiquitin ligase, glucose-6-phosphate/phosphate and phosphoenolpyruvate antiporter, aspartyl protease, Mitochondrial/chloroplast ribosomal protein L22 and histone H4. Notably, ribonuclease T2 family and nucleoside diphosphate kinase categories also appeared among the most frequent KOG groups and showed a net increase in protein abundance on day 4. These enzyme families are involved in nucleotide metabolism and may contribute to nucleotide mobilization during germination, and these groups have been further studied.

Analysis of the transcription–translation relationship for validation of protein analysis by qRT PCR analysis of ribonucleases T2, nucleases S1/P1, and nucleoside diphosphate kinases in the seed coat during germination

The identification of ribonuclease T2 and nucleoside diphosphate kinases as KOG categories that increase in abundance after germination prompted us to analyse the presence of members of these families among the identified proteins. In addition, S1/P1 nucleases were also included in the analysis as other proteins involved in nucleotide metabolism.

Proteomic analysis identified five ribonucleases T2 among the 13 proteins belonging to this family[20] (Table 1). PvRNS3, PvRNS4 and PvRNS5 did not show significant changes in abundance, while PvRNS7 and PvRNS8 increase their abundance after germination (118 and 34%, respectively) (Table 1). Nucleoside diphosphate kinases, which are involved in the balance of cellular NTP pools, were also detected. Three NDPKs showed differential abundance in the seed coat between 0 and 4 DPI (Table 1). In the common bean genome database (phytozome, searched at 01/12/2025), we identified a total of four candidate genes encoding NDPKs in common bean. NDPKI showed a slight decrease in abundance after germination (−17, 55%), NDPKII a slight increase (10, 34%), whereas NDPKIII increased its abundance by approximately 65% (Table 1). The nuclease S1/P1 family is composed of five members in common bean[21], and we identified three proteins belonging to this family, PVN3, PVN4, and PVN5 (Table 1), none of which showed statistically significant changes.

The presence of mRNA transcripts encoding all proteins identified in seed coats and listed in Table 1 was analysed by qRT-PCR at 0 and 4 DPI (Fig. 5). We determined the presence of mRNA for all the identified ribonucleases T2 except for *PvRNS5* (Fig. 5a). Expression for all the genes remained constant during germination, with higher expression values for *PvRNS4* than for the other genes. Expression was determined for the three genes encoding identified nucleoside diphosphate kinases (Fig. 5b). Statistically significant changes were observed only for NDPKIII (Fig. 5b). Similarly, transcripts corresponding to the three identified nucleases S1/P1 genes were detected, although without statistically significant changes (Fig. 5c). Therefore, the data correlate transcript and proteomic analysis, suggesting that the identification of proteins is not a consequence of proteins that remained in the seed coat from development in

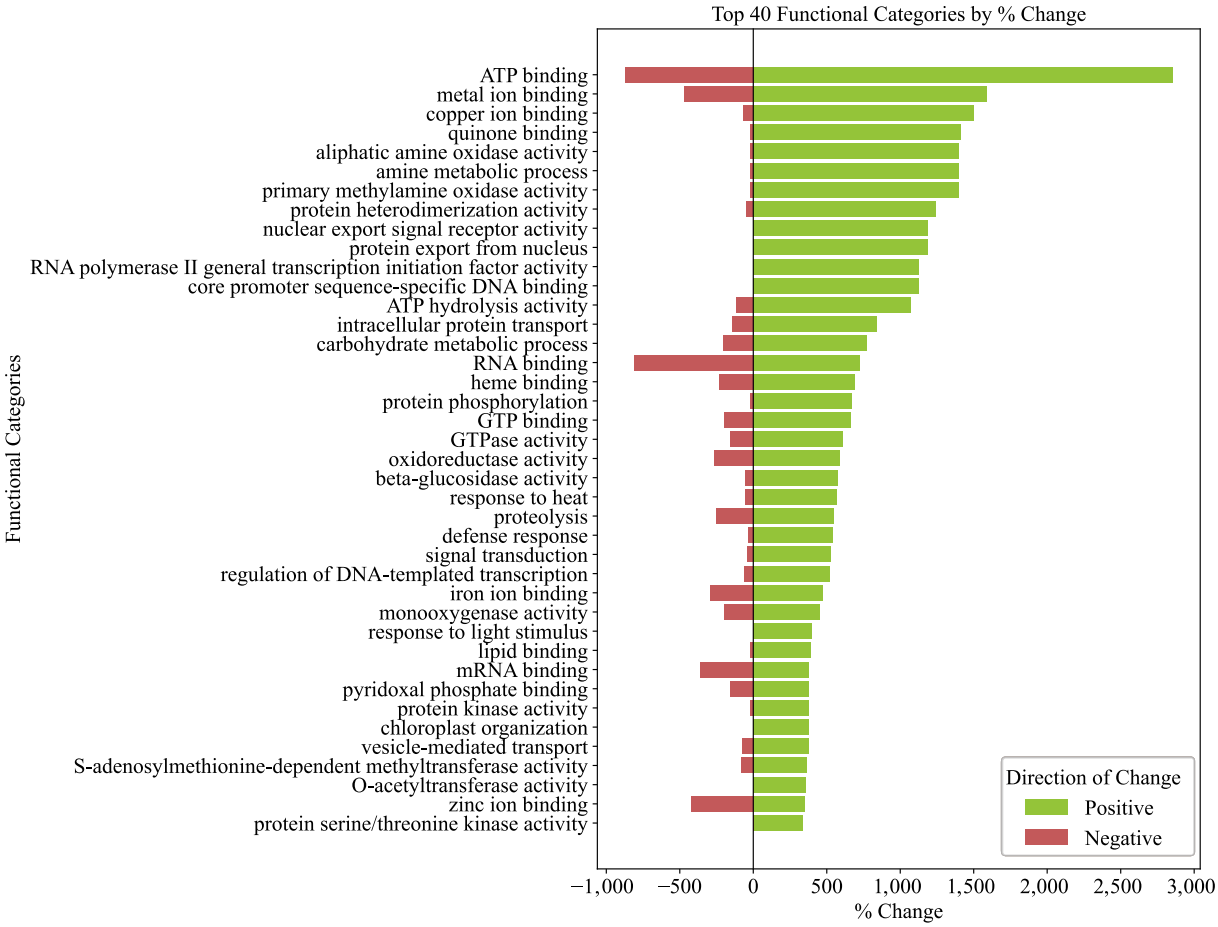

Fig. 3 Top 40 Gene Ontology (GO) functional categories showing changes in protein abundance in the seed coat of common bean between day 0 and 4 of germination. % change values represent the percentage change in protein abundance between the two times. Bars represent the summed % change for all proteins annotated to each GO term, with positive values (green) indicating overall upregulation and negative values (red) indicating overall downregulation. GO annotations were obtained from the Gene Ontology database (http://geneontology.org). Only categories from the Molecular Function (MF) and Biological Process (BP) domains were included. Proteins with significant change (q-value < 0.05) were retained, and the categories were ranked by total positive % change; the top 40 terms are shown.

mother plants, but are probably the result of their synthesis during germination.

Enzymes identified in the pathway from nucleotides to ureides

The increase in the amount of nucleic acid-degrading proteins would imply an increase in nucleotide concentration and its degradation products. The degradation pathway of purinic nucleotides involves ureides as intermediates, compounds that have been shown to participate in the germination of ureidic legumes differentially to amidic legumes[12]. Therefore, we look for the presence of proteins responsible for converting nucleotides to allantoin to check if the seed coat contains the enzymatic pathway for the degradation of purinic nucleotides. The following proteins involved in this pathway were identified in our proteomic analysis: two putative nucleotidases (PvNTD1 and PvNTD2), two nucleosidases (PvNSH1 and PvNSH2), xanthine dehydrogenase, uricase, hidroxiisourate hydrolase, and allantoinase (Table 2). For most of these proteins, the abundance either remained unchanged from 0 to 4 DPI or decreased after germination (Table 2). Statistically significant decreases were detected for both nucleosidases, hydroxyisourate hydrolase, and allantoinase 1. These enzymes could directly convert nucleoside monophosphate to allantoin or allantoate, suggesting the synthesis of ureides in the seed coat of common bean being higher at 0 than at 4 DPI.

The abundance pattern described above for proteins involved in the pathway from nucleotides to ureides prompted us to determine ureide levels, allantoinase activity, and allantoinase gene expression in the seed coat. The amount of ureides in the seed coat at 0 DPI was very high and decreased after germination (Fig. 6a). Similar patterns were obtained for allantoinase activity (Fig. 6b) and gene expression (Fig. 6c).

Discussion

The seed coat has traditionally been considered as a dead tissue that encloses the embryo and whose main role is to protect it during storage in the soil. However, recent studies have revealed that these structures can be a storage compartment for proteins that can retain their function for years[7,8]. This work describes the identification of a large number of proteins in the seed coat before and after germination. The number of proteins determined is consistent with that described in other proteomic analyses performed on metabolically active plant tissues, such as leaves, where protein identifications commonly reach around 3,000[22]. The high proportion of proteins that remain or increase in abundance after germination is

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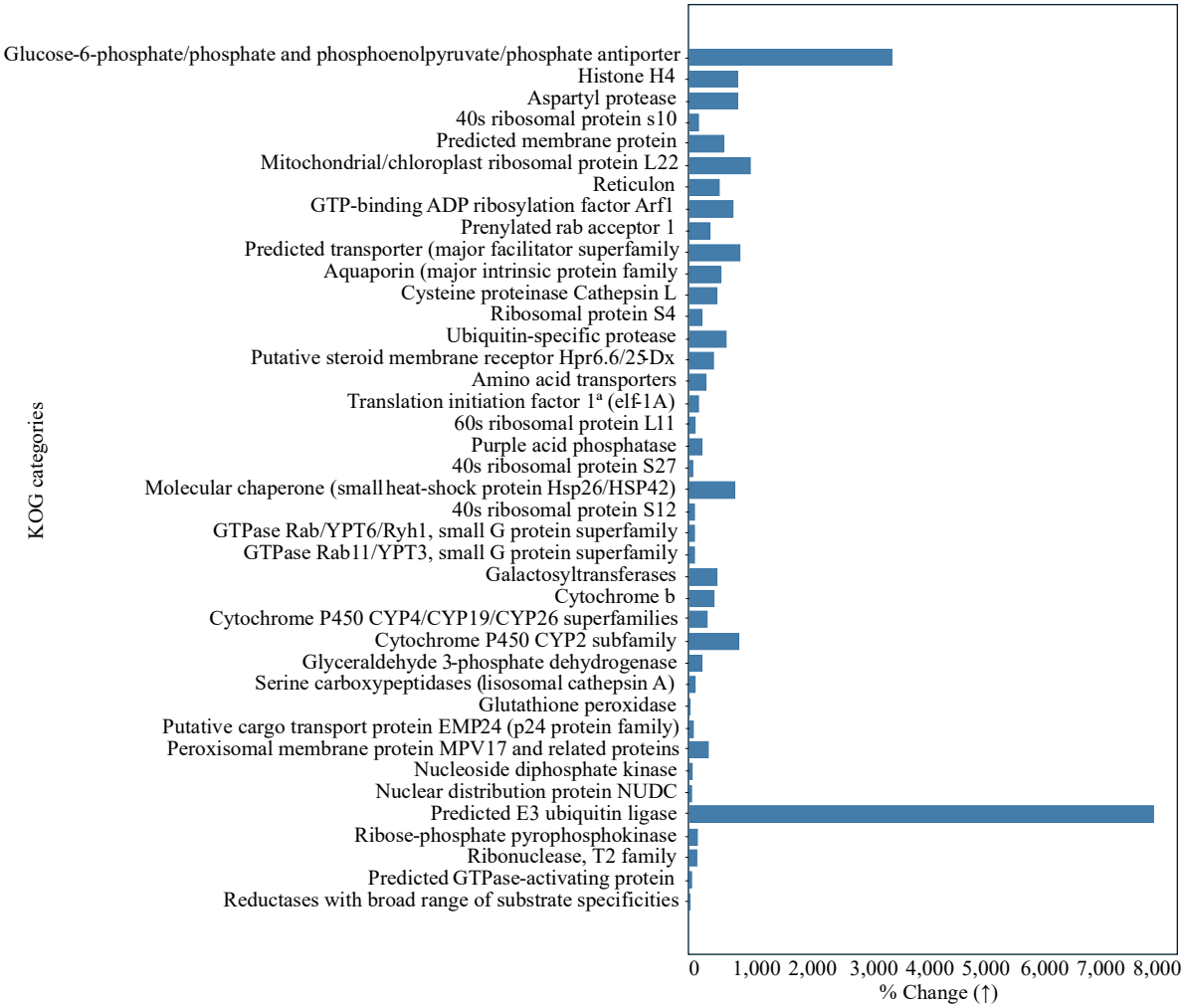

Fig. 4 Top 40 KOG categories showing increases in protein abundance in the seed coat of common bean vulgaris between day 0 and 4 after imbibition. Only proteins with positive % change were retained. When proteins were annotated with multiple KOG terms, annotations were split and evaluated pairwise with their descriptions; entries lacking a description or labeled as uncharacterized/NA were excluded. Bars represent the summed % change across all proteins assigned to each KOG description (*x*-axis). Categories on the *y*-axis are ordered by the number of contributing proteins (most proteins at the top), while bar length reflects the total % change for that category. KOG annotations were obtained from the NCBI COG/KOG resource. Only significant change proteins with a *q*-value < 0.05 were retained for analysis.

Table 1. Quantification and fold change of T2-Ribonucleases, nucleases S1/P1, and NDPK. Protein quantification at 0 df after imbibition (0 D) and 4 d after imbibition (4 D) is presented as the mean of the individual quantifications obtained from four biological replicates per condition. The fold change column indicates the percentage variation (%) between 0 D and 4 D. The *q*-value represents the false discovery rate (FDR)-adjusted *p*-value for the comparison between conditions.

ID	Protein	0 D protein quantification	4 D protein quantification	Fold change	*q*-value
Ribonucleases T2					
Phvul.002G084600	PvRNS3	12.49	11.05	−11.50	0.308
Phvul.010G110200	PvRNS4	37.07	35.79	−3.45	0.307
Phvul.006G112700	PvRNS5	17.84	7.66	−57.04	0.105
Phvul.006G112900	PvRNS7	577.12	1258.66	118.09	< 0.001
Phvul.006G112800	PvRNS8	37.67	50.31	33.54	0.024
Nucleoside diphosphate kinases					
Phvul.010G045400	PvNDPKI	93.91	77.42	−17.55	0.003
Phvul.003G208500	PvNDPKII	44.79	49.42	10.34	0.014
Phvul.002G297900	PvNDPKIII	168.90	277.90	64.54	0.001
Nucleases S1/P1					
Phvul.002G223100	PvN3	11.09	9.91	−10.06	0.141
Phvul.006G190700	PvN4	15.45	32.54	110.6	0,062
Phvul.007G028400	PvN5	21.52	24.41	13.4	0.216

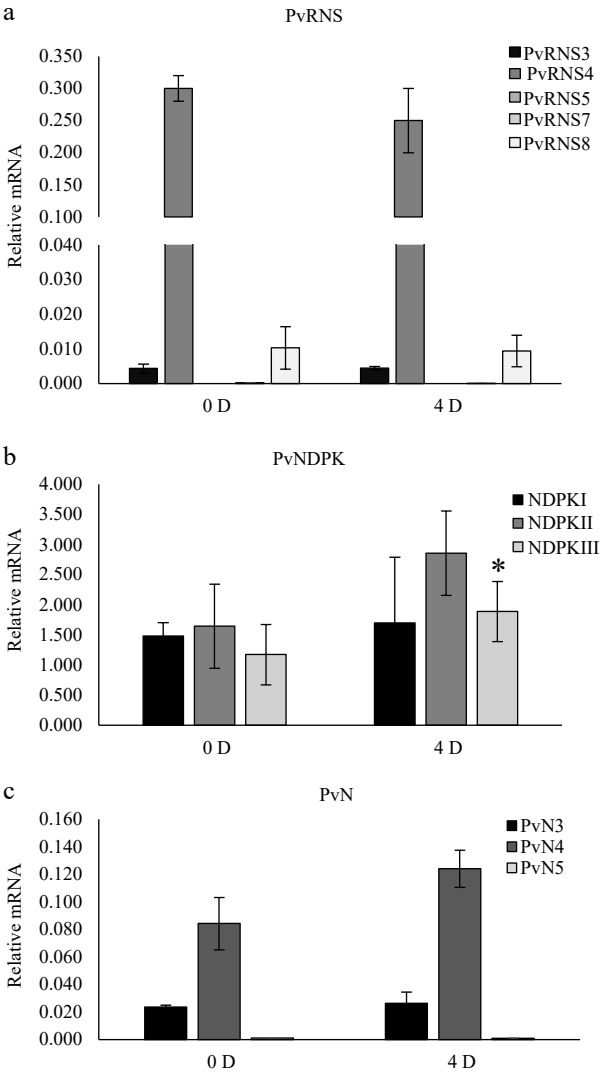

Fig. 5 Expression pattern of (a) Ribonucleases T2, (b) nucleoside diphosphate kinase, and (c) nucleases in seed coats at 0 (0 D) and 4 (4 D) d after the start of imbibition. Gene expression analysis was performed using qRT-PCR on total RNA samples extracted from the seed coat of common bean. The relative expression level was normalized using the geometric mean of two reference genes and analysed using the $2^{-\Delta C_T}$ method. Student´s *t*-test was performed comparing both conditions. Statistical significance levels were indicated as follows: * $p < 0.05$.

interesting, suggesting a crucial physiological role for the seed coat rather than its being considered a dead tissue. We focused our analysis on the proteins that increase in abundance after germination. Some reports describe how programmed cell death is induced during seed coat formation in the mother plants and how nutrients are transported to the developing embryos[23,24]. In this process, some proteins necessary for seed maturation in plants may be stored, but the increase in abundance of proteins related to physiological processes demonstrates that the seed coat is not a dead tissue but a metabolically active one during seedling development. The fact that the main GO category in biological process corresponds to translation reflects the importance of new protein synthesis during germination and clearly demonstrates the function of transcription in the seed coat during this process. This is corroborated by the fact that many differentially determined proteins showed an increase in abundance after germination.

The proteomic analysis was coordinated with an analysis of the expression of genes belonging to the ribonucleases T2 family, NADPK, and nucleases S1/P1. Most of the analysed genes maintained stable transcript levels after germination. Other KOG categories that denote the high metabolic activity for the seed coat during germination are proteins related to proteolysis, as has been described previously[7,25,26].

The analysis by Eukaryotic Orthologous Groups indicates the importance of nucleotide metabolism in this process, with NDPK and ribonucleases T2 families increasing in protein abundance during germination. Nucleotide diphosphate kinases were identified in the proteomic study as one of the most abundant families that increase in protein abundance after germination. Nucleotide diphosphate kinase transfers the phosphate group from NTPs to different NDPs, forming new NTPs and, thereby, maintaining the nucleotide metabolism balance within cells[27,28]. NDPKs are classified into four types (I to IV), and in common bean, the family is composed of one member in each class. In the proteomic analysis, the NPPK I, II, and III have been identified, and RNA was detected for each protein. NDPKs are involved in the energy balance in all organelles; therefore, with a housekeeping function. However, it has also been postulated that NDPKs participate directly in signalling and stress-related pathways[29,30]. However, research on this protein family is scarce, and given the high levels of expression found in the seed coat, they deserve further analysis in the future to determine if they have additional functions in nucleotide metabolism beyond being housekeeping proteins.

Ribonucleases T2 are also identified as a protein that increases in abundance in the seed coat after germination. The relevance of ribonuclease activity in the seed coat was demonstrated in our

Table 2. Identified proteins belonging to nucleotide catabolism. Protein abundance at 0 d after imbibition (0 D) and 4 d after imbibition (4 D) is reported as the average of measurements from four biological replicates per condition. The fold change column shows the percentage change (%) between 0 D and 4 D. The *q*-value corresponds to the FDR-adjusted *p*-value for the comparison between the two conditions.

ID	Protein	0 D protein quantification	4 D protein quantification	Fold change	*q*-value
Phvul.004G174200	PvNTD1	21.74	23.25	6.99	0.201
Phvul.011G182400	PvNTD2	30.79	27.55	−10.52	0.114
Phvul.001G188700	PvNHS1	14.87	7.22	−51.45	0.002
Phvul.003G000600	PvNHS2	22.09	10.70	−51.55	0.002
Phvul.005G148000	XDH	6.54	6.46	−1.35	0.108
Phvul.007G234300	Uricase	67.67	71.20	5.22	0.059
Phvul.010G034000	Hydroxyisourate hydrolase	31.53	19.48	−38.28	0.032
Phvul.006G186800	ALN1	167.07	73.25	−56.15	< 0.001
Phvul.006G186700	ALN2	26.17	43.11	64.73	0.063

https://doi.org/10.48130/vegres-0026-0017

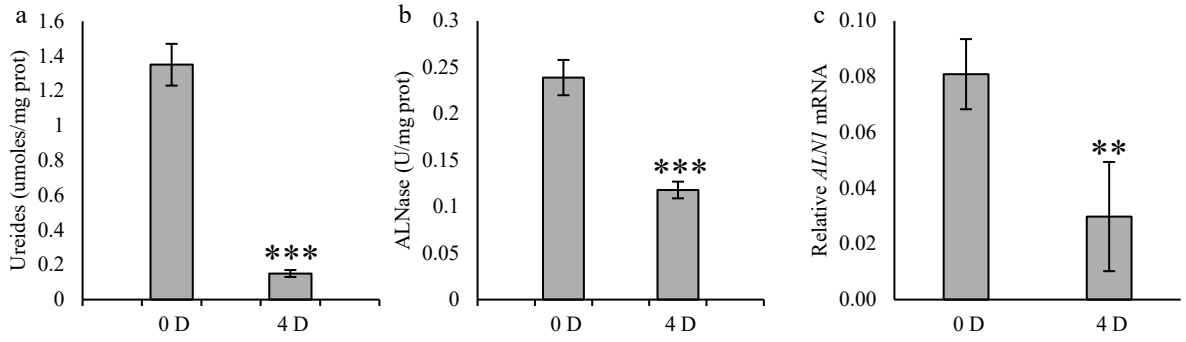

Fig. 6 Ureide content, allantoinase activity, and ALN1 gene expression in the seed coat at 0 (0 D) and 4 (4 D) d post-imbibition. Ureides were quantified and normalized to total soluble protein. (a) Allantoinase activity was determined by an enzymatic assay and expressed as specific activity. (b) ALN1 transcript levels were determined by qRT-PCR. (c) Relative expression levels were normalized to the geometric mean of two reference genes and then calculated using the $2^{-\Delta CT}$ method. Bars represent mean values ± standard error (SE) of four biological replicates. Student's *t*-test was performed comparing both conditions. Statistical significance levels were indicated as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

previous study, which showed high ribonuclease activity in the seed coat and identified several activities in a gel assay[14]. A total of five proteins were determined in the seed coat: two S-like ribonucleases T2 (PvRNS3 and PvRNS4), and three S ribonucleases T2 (PvRNS5, PvRNS7, and PvRNS8). The two identified S-like ribonucleases T2 have been previously analysed in common bean. PvRNS3 is induced in radicles under salt stress[20], and PvRNS4 is the only Class II ribonuclease T2 in common bean[20]. Class II ribonucleases are ubiquitous enzymes[31–33] involved in RNA turnover in vacuoles[34,35]. The presence of PvRNS5, PvRNS7, and PvRNS8 in seed coats is particularly interesting. All three proteins belong to Class III ribonucleases T2, which are not found in Arabidopsis[36]. Class III ribonucleases have been understudied and are mainly associated with self-incompatibility[36], although other functions have also been assigned to them, such as defense[37] and phosphorus starvation[38]. PvRNS7 increases in abundance, both protein quantity and rRNA level, after germination. This protein is an ideal candidate for further functional analysis because, due to its possible extracellular location[20], it could be involved in protecting the developing seedling or in nutrient utilization from the seed coat or soil.

Nucleotides derived from ribonuclease and nuclease activity can be either recycled or degraded. In this manuscript, we analyse the purine degradation pathway, which converts purine nucleotides to ureides, molecules rich in nitrogen of particular relevance in ureidic legumes such as common bean. Most of the enzymes involved in this pathway were detected in the seed coat immediately after imbibition, and most of the proteins remained unchanged or decreased after germination. This result suggests that at least part of the nucleotides is completely degraded in the seed coat or converted to ureides, which are then likely transported to other parts of the seedlings. Ureides are nitrogen-rich compounds that are the predominant nitrogen transport molecules in the xylem of nitrogen-fixing tropical legumes[10]. However, ureides have been implicated in nitrogen mobilization in various situations[11,13]. Purine metabolism is not only a way to recycle nitrogen from nucleic acids, but it also produces ureides that may act as antioxidants[39–41]. Interestingly, the differentiation between amidic and ureidic metabolism has recently been shown to occur in seedlings, and a correlation between ureides and antioxidant activities has been described[12]. Our results show very high ureide levels in the seed coat at 0 DPI, accompanied by high allantoinase activity and gene expression. These results indicate an important role during the early stages of imbibition. However, both activity and gene expression decrease after germination, as do ureides. This decrease coincides with an induction of ureide metabolism in both cotyledons and embryonic axes of common bean[11]. This decrease in allantoinase activity differs markedly from the other lytic activities observed in the seed coat during germination, where ribonuclease, and nuclease do not decrease during the analyzed process. Therefore, this result suggests that nucleic acid degradation in the seed coat is not complete but rather that recycling occurs. Nucleoside transport could play an important role in this process[42]. The decrease in the two nucleosidases determined in the seed coat after germination (Table 2) supports this idea. It has been postulated that the hydrolysis of nucleosides to nucleobases by nucleoside hydrolases results in a shift toward catabolism of nucleotides, since nucleosides are more effectively salvaged compared to nucleobases[42].

Conclusions

We demonstrate that the seed coat exhibits a high metabolic rate during germination with a significant change in the concentration of numerous proteins, many of which play important metabolic roles. These changes cannot be attributed solely to the degradation of proteins or nucleic acids remaining in the seed coat during its formation in the mother plants. Among the activities analysed, we observed high ribonuclease activity that remains elevated during this process, as well as an active purine degradation pathway in the seed coat. Notably, the latter is downregulated after germination, in contrast to other lytic activities, highlighting the dynamic and regulated metabolic role of the seed coat in supporting seedling establishment.

Author contributions

The authors confirm their contributions to the paper as follows: study conception and design, draft manuscript preparation: Diaz-Baena M, Piedras P; data collection: Pareja LO, Diaz-Baena M; analysis and interpretation of results: Pareja LO, Diaz-Baena M, Galvez-Valdivieso G, Piedras P. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[43] partner repository with the dataset identifier PXD075619.

Acknowledgments

This research was funded by Ministerio de Ciencia e Innovación (Government of Spain) (Grant No. PID2020-117966RB-I00), Consejería de Economía, Conocimiento, Empresas y Universidad (Junta de Andalucia) (Grant No. P20_00440), and Fundación Torres Gutierrez (Spain). L.O Pareja acknowledges the support of a predoctoral contract from Ministerio de Ciencia, Innovación y Universidades (Government of Spain). We acknowledge Servicios Centrales de Apoyo a la Investigación (SCAI-UCO) for the proteomic data.

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary information accompanies this paper online at: https://doi.org/10.48130/vegres-0026-0017.

Dates

Received 28 January 2026; Revised 19 March 2026; Accepted 27 April 2026; Published online 13 July 2026

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