Comparative proteomics of round and wrinkled pea (Pisum sativum L.) during seed development period

Sintayehu Daba[1], Punyatoya Panda[2], Uma Aryal[2], Alecia M. Kiszonas[1], Sean M. Finnie[1], and Rebecca McGee[3]

[1]USDA-ARS Western Wheat Quality Lab
[2]Purdue University
[3]Affiliation not available

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Abstract

Seeds are an important part of plants, ensuring the continuation of plants' life and providing nutrient reserves for humans and animals. Seed development is controlled by the interplay of several physiological processes. We applied label-free proteomics to round and wrinkled peas using seeds sampled at five growth stages (4 days after anthesis (DAA), 7DAA, 12DAA, 15DAA, and maturity). Phenotypic results indicated that wrinkled peas had lower starch concentration compared to round peas (29.5% vs. 46.6-55.1%). A total of 4,126 high confident proteins were detected, with 22–26% shared across all sampling times within an entry. Early seed growth stages were characterized by more unique proteins compared to maturity. Two-way ANOVA revealed 1,685 proteins significantly different among samples, of which 722 proteins were characterized into 29 functional classes. The four major classes (comprising over 50 proteins) were protein biosynthesis, protein homeostasis, enzymes, and carbohydrate metabolism. Of the two types of comparisons (time-point and entry-wise), time-point comparisons yielded more differentially abundance proteins (596 proteins in total). Different protein classes exhibited different patterns of change during seed development. For example, cell division related proteins were abundant early in seed development, whereas storage proteins were abundant later in seed development (especially after 12DAA). Compared to the round pea entries, the wrinkled entry had significantly lower abundance of starch branching enzymes, a protein involved in the biosynthesis of amylopectin in starch. In conclusion, the results of this study provide valuable information to improve our understanding of seed development and form the basis for further studies.

Proteomics

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[1] USDA-ARS Western Wheat & Pulse Quality Laboratory, Pullman, Washington, USA

[2] Department of Comparative Pathobiology, Purdue University, West Lafayette, Indiana, USA

[3] Purdue Proteomics Facility, Bindley Bioscience Center, Purdue University, West Lafayette, Indiana, USA

[4] USDA-ARS Grain Legume Genetics and Physiology Research Unit, Pullman, Washington, USA

[*] Correspondence: rebecca.mcgee@usda.gov

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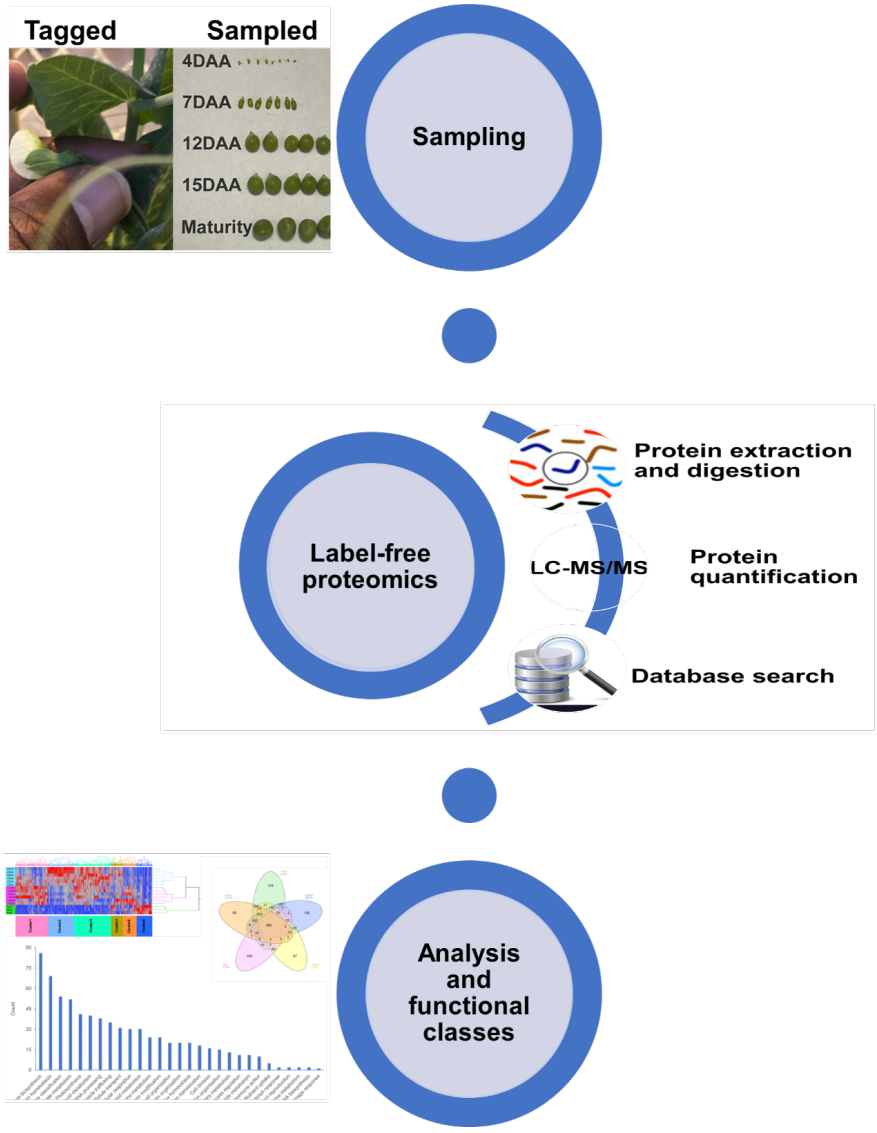
Seeds are an important part of plants, ensuring the continuation of plants' life and providing nutrient reserves for humans and animals. Seed development is controlled by the interplay of several physiological processes. We applied label-free proteomics to round and wrinkled peas using seeds sampled at five growth stages (4 days after anthesis (DAA), 7DAA, 12DAA, 15DAA, and maturity). Phenotypic results indicated that wrinkled peas had lower starch concentration compared to round peas (29.5% vs. 46.6-55.1%). A total of 4,126 high confident proteins were detected, with 22–26% shared across all sampling times within an entry. Early seed growth stages were characterized by more unique proteins compared to maturity. Two-way ANOVA revealed 1,685 proteins significantly different among samples, of which 722 proteins were characterized into 29 functional classes. The four major classes (comprising over 50 proteins) were protein biosynthesis, protein homeostasis, enzymes, and carbohydrate metabolism. Of the two types of comparisons (time-point and entry-wise), time-point comparisons yielded more differentially abundance proteins (596 proteins in total). Different protein classes exhibited different patterns of change during seed development. For example, cell division related proteins were abundant early in seed development, whereas storage proteins were abundant later in seed development (especially after 12DAA). Compared to the round pea entries, the wrinkled entry had significantly lower abundance of starch branching enzymes, a protein involved in the biosynthesis of amylopectin in starch. In conclusion, the results of this study provide valuable information to improve our understanding of seed development and form the basis for further studies.

KEYWORDS

Label-free proteomics, differentially abundant proteins, protein functional classes, round and wrinkled peas

Graphical Abstract



| INTRODUCTION

Pea (*Pisum sativum* L.), one of the common pulse crops, is an important part of the human diet, providing starch (37-49%), protein (21-33%) and other nutrients [1]. Seeds are an integral part of agriculture, and understanding their development and composition is critical to increasing agricultural productivity [2]. Seed development is an important part of the life cycle of many crops including pea and is generally divided into three stages: (1) cell division or pre-storage phase, (2) maturation or storage phase, and (3) desiccation phase [3-5]. Seed development involves different physiological processes (such as seed growth, assimilate transport process, and biosynthetic pathways), which are regulated by several genes. Cell number is determined by the rate and duration of cell division as well as the number of dividing cells, whereas non-dividing cell size is determined by cell growth and cell expansion, which are defined as increases in cell macromolecular mass and cell volume [4]. Mature seeds store protein and non-protein constituents that provide nutrition for the growing plantlets and enhance germination and provide protection against biotic and abiotic stresses [6]. In general, to alter and improve seed composition, it is necessary to understand the physiological processes that occur during seed development.

Mutant and omics resources provide valuable tools for deciphering the molecular mechanism underlying various physiological processes in pea development [7-10]. Proteomics analysis can be performed using either gel-free (mass spectrometry-based) or gel-based methods, however, the gel-based method is labor-intensive and often unable to detect most low abundant proteins in complex samples as compared to gel-free methods [11]. Mass spectrometry-based proteomic analysis is generally classified as labeled and label-free techniques [5] and can be generally based on two types of measurements; precursor ion intensity during chromatographic separation or counting of the matched tandem mass spectra (MS/MS counts) of the identified protein after MS/MS analysis [12, 13]. Higher chromatographic peak area/height or the number of proteolytic peptides fragments are always positively correlated with the higher protein abundance, permitting quantitative analysis of the identified peptides/proteins across samples [14]. The label-free method is a powerful proteomics technology that can be used to gain valuable insights into seed development processes.

Proteomics studies, which compare two or more states, are vital to understand different plant processes such as plant growth and development, responses to biotic and abiotic stresses, and seed germination [5, 11]. Such comparative proteomics data can elucidate the mechanisms of genetic regulation of plant growth processes and plant's responses to stresses. Pea seeds are round or wrinkled based on seed shape, and such phenotypic differences stem from the variation in starch biosynthesis genes. Several pea mutants (*lam* , *r* ,*rb* , *rug3* , *rug4* , and *rug5*) that disrupt starch biosynthesis have an impact on seed size and shape as well as concentration of total starch, amylose, and amylopectin [15]. Coordination between various proteins and pathways during seed development is essential for achieving different shapes and sizes of pea seeds. The utilization of unsupervised comprehensive proteomic analysis can contribute to elucidating the pathways involved in seed development and understanding the proteins that contribute to the phenotypes of round and wrinkled pea seeds. Such information could be crucial for future breeding efforts to improve the quality and nutritional value of the pea seeds. In this study, we included two round peas (one high and one low protein line) and one wrinkled pea and assessed the proteome differences between the high and low protein pea lines as well as the proteome profile difference between the wrinkled and round pea lines. The results suggested differential regulation of proteins involved in cell division and metabolism of seed reserves.

| Materials and Methods

| Plant materials and sampling

Three pea lines were used, two smooth types (Cameor, designated E1 and PS17100006, designated E4) and one wrinkled type (PI 357292, designated E3). PS17100006 is a low protein breeding line whereas Cameor is a high protein line. Single plant-derived seeds of PI 357292, an accession from the USDA-ARS germplasm repository (https://www.ars-grin.gov/), has high protein concentration and a wrinkled seed surface. In a greenhouse, two seeds were sown in 3.8L pots, and then thinned to a single seedling per pot after germination (about two weeks after planting). To guarantee that there would be enough pods for all sample time-points, each entry was planted in a total of 28 pots. All pots were filled with Sunshine Mix #1 (SunGro Horticulture, Agawam, MA, USA) plant media and well-watered daily during the plant growth period. About two weeks after planting, we applied about 20grams of Osmocote (14-14-14 NPK, Everris International Bv., Dublin, OH, USA).

Sufficient pea flowers were tagged with date of anthesis, and pods were sampled 4 days after anthesis (DAA), 7DAA, 12DAA, 15DAA, and at physiological maturity. Pods were considered physiologically mature when they are dried and had turned straw-colored and generally at about 37DAA, henceforth referred to as maturity. Four replicate samples were taken at each time-point. Samples were collected in 15 mL or 50 mL centrifuge tubes and flash frozen in liquid nitrogen. All samples were then transferred to -80°C freezer until seeds were extracted from the pods for proteomic analysis. Sufficient seed samples of each time-point were packaged in dry ice and sent to Purdue proteomics facility. Another set of samples were taken at 7DAA, 12DAA, 15DAA, 18DAA, 21DAA, 24DAA, 27DAA, 32DAA, and 37DAA to record the changes in fresh and dry weights and moisture content during seed development. For each entry, we harvested the remaining seeds in bulk and used them to measure protein and starch concentrations. Protein concentration (N x 6.25%) was determined using a protein analyzer FP828p (LECO Corporation, St. Joseph, MI, USA). Starch

concentration (%) was determined using the Megazyme starch test kit according to AACC Method 76-13.01 [16].

2.2 | Sample preparation, protein extraction, digestion, and LC-MS/MS analysis

Frozen pea seeds were wrapped in aluminum foil, crushed with a mortar and pestle, transferred to bead rupture pre-filled Precellys CK28-R tube (Bertin Corp., Rockville, MD, USA) with lysis buffer (5% sodium dodecyl sulfate (SDS), 50 mM triethylammonium bicarbonate (TEAB) pH 8.5) and then homogenized in a hard tissue homogenizer 6 times with 3 x 20 secs in each cycle. The lysates were transferred to new tubes and bath sonicated for 30 minutes, centrifuged at 5000 x g for 5 minutes at 4°C to get rid of the debris and then the protein concentration was measured by bicinchoninic acid (BCA) assay using BSA as a standard. For each sample, 50 µg (or equivalent volume) of protein was taken, and the TEAB buffer was added to the samples to adjust to equal volumes across all samples. The samples were reduced with 5mM tris(2-carboxyethyl) phosphine (TCEP), incubated at 55°C for 15 minutes, followed by alkylation with 20mM methyl-methanethiosulfonate (MMTS) and incubated in the dark for 10 minutes. The samples were acidified with 2.5% phosphoric acid to completely denature the proteins, followed by addition of 165uL of binding/wash buffer (100mM TEAB in 90% methanol) and mixed immediately. These were passed through the S-Trap micro spin columns (Protifi, USA) and centrifuged at 2,000 x g for 1 minute. The S-trap columns were washed three times with the binding/wash buffer with centrifugation at 2,000 x g. These columns were then transferred to clean 1.7mL Eppendorf tubes and the trapped proteins were digested overnight with Pierce Trypsin Protease, MS Grade (ThermoFisher Scientific, USA) at 1:25 (enzyme: substrate ratio) in a thermomixer at 37degC. Trypsin was resuspended in 50mM TEAB buffer. Purified peptides were eluted using 50% acetonitrile, 50 mM TEAB and 0.2% formic acid via centrifugation for 1minutes at 2000 x g and subsequently dried in a vacuum centrifuge with 45degC heat.

Dried purified peptides were resuspended in 3% ACN, 0.1% FA in water to a final concentration of 1µg/µl and 1µl of peptides was loaded into Thermo PepMap Neo trap column (5µm long, 300µm x 5mm, 1500bar) at a flow rate of 10µl/min and then, separated by reverse phase using an IonOpticks Aurora Ultimate analytical columns (25cm x 75µm with pore size 120Å and particle size 1.7µm) in the Dionex Ultimate 3000 HPLC coupled with Orbitrap Fusion Lumos Tribrid (Thermo Fisher Scientific) mass spectrometer. The peptides were separated using a 130-min mobile phase gradient with a flow rate of 400nL/min. The mobile phases A comprised 0.1% FA in water and B comprised 80% ACN, 0.1% FA in water. For the first 5mins, the gradient of B was maintained at 2%, and increased to 8% B after 5mins. Then, the percentage of B was linearly increased to 27% till the 80th min. The gradient of B was linearly increased to 45% for the next 20mins, then increased to 100% and kept constant for the next 12mins, and finally decreased to 2% and maintained at 2% B for the last 8mins. Mass spectrometry analysis was conducted using the Orbitrap Fusion Lumos Tribrid Mass Spectrometer. MS1 was acquired using an Orbitrap with a resolution of 60,000 and a scan range of 350 to 1600m/z. MS/MS was acquired using an HCD with a collision energy of 30%, resolution of 15,000, and isolation window of 1.2m/z. LC-MS/MS data were collected using four biological replicates per treatment condition for statistical analysis.

2.3 | Database searching for protein identification

The raw MS/MS data were searched using MaxQuant version 2.0.3.0 against the cultivar Cameor *Pisum sativum* reference genome database that has 57,835 protein sequences [9]. The following search criteria were modified: Trypsin/P enzyme selectivity permitting up to two missed cleavages; oxidation of methionine (M) as a variable modification, alkylation at cysteine residues was set as fixed modification using methyl-methanethiosulfonate and precursor mass tolerance was set to 10ppm. Peptide spectral match (PSM) and protein identification's false discovery rate (FDR) was set at 1%. For peptide quantification, non-redundant, non-unique peptides assigned to the protein group with most other peptides-the unique plus razor peptides-were employed. Intensity measurements from label-free quantification, or LFQ, were utilized to calculate relative protein abundance.

2.4 | Data cleaning up, filtering, and missing imputation

At each sampling time-point, the total identified proteins were further evaluated across replicates. For specific time-point, non-zero values should be recorded in at least two replicates to consider a protein as detected with high confidence. Once proteins were classified as detected and not-detected according to the above criteria, the first round of filtering was performed by removing all the proteins that were not detected in at least one sample. The second round of filtering was done with missing threshold of 30% using Perseusv2.0.7.0 [17], and missing values were imputed from normal distribution.

2.5 | Statistical analysis and data visualization

2.5.1 | Explanatory analysis

A set of proteins after the first round of filtering was used to construct Venn diagrams using InteractiVenn [18], an online tool, for each entry across the sampling time-points. The Venn diagrams are important to investigate the shared and unique proteins across sampling time-points.

2.5.2 | Multi-factor analysis of variance

Proteins with significantly different abundance among the sampling time-points and entries were identified through two-way analysis of variance performed using Perseusv2.0.7.0 [17]. Proteins which were significantly different among the treatments (entry-time) at $p < 0.01$ were used for further downstream analyses.

2.5.3 | Principal component and cluster analyses

Principal component and cluster analyses were performed on a set of proteins that were significantly different among time-points and entries based on analysis of variance. Both principal component and cluster analyses were critical in determining the reproducibility of the quantification procedure and the reliability of the data generated for subsequent analyses. Cluster analysis was used to identify groups of proteins with similar profiles during the seed growth period. JMP(R) Genomics 9.0 (SAS Institute Inc., Cary, NC) was used to perform the principal component and cluster analyses. It was also used in grouping sampling time-points.

2.5.4 | Differentially abundant proteins

Two criteria were applied to identify differentially abundant proteins (DAPs) in pair-wise comparisons. A *p [?] 0.001* and*fold-change [?] 2.5 or [?] -2.5* was used to declare DAPs. The analyses were performed in two ways, i.e., (1) pair-wise comparisons among time-points for each entry, hereafter referred as time-point comparisons and (2) pair-wise comparisons among the three entries at each time-point, hereafter referred to as entry-wise comparisons. In total, 30 time-point and 9 entry-wise comparisons were performed.

2.5.5 | Protein functional characterization

Protein functional characterization was performed using Mercator4v5.0 protein classification and annotation framework [19]. We also looked at the pattern of protein abundance change over the seed growth period for storage proteins reported by Kreplak and co-workers [9] and starch biosynthesis genes published by Yu and co-workers [15].

| Results

3.1 | Seed growth, protein, and starch concentrations

The changes in fresh weight (FW), dry weight (DW), and moisture content during the seed growth period are shown in Figures 1A-1D. Fresh weight (FW) increased steadily until 32DAA for E1 and until 27DAA for E4, and then started to drop afterward. Stickland and Wilson [20] reported that maximum FW in pea was achieved at about one month after flowering. In comparison to the round pea lines (E1 and E4), the wrinkled pea (E3) exhibited a different FW growth pattern, with a significant increase between 27DAA and 32DAA (Figure 1C), primarily because of increased seed moisture. In all the three lines, a considerable dry weight (DW) increase was observed between 15DAA and 27DAA. There was a significant difference between FW and DW at most growth stages, except maturity. At approximately 37DAA with moisture content of 12-13%, which corresponds to maturity, the difference between FW and DW was almost non-existent. The

seed moisture content was more than 85% of the fresh weight at 4DAA, and it progressively decreased over the seed growth period, with rapid seed drying starting at about 32DAA for all entries (Figure 1D).

Mean protein concentration for Cameor (E1), a smooth pea, was 26.7±0.29% (Figure 1E). The other smooth pea, PS17100006 (E4), was a low protein line having a mean protein concentration of 20.9±0.18%. Wrinkled peas are expected to have high protein and low starch concentrations [21], which was consistent with the current study in which we found high mean protein (25.4±0.56%) and low starch (29.5±3.2%) for the wrinkled pea (E3: PI 357292) (Figures 1E and 1F). As expected, the low protein entry (PS17100006) had the highest starch concentration (mean = 55.1±3.9%) of the three, followed by Cameor (46.6±1.9%).

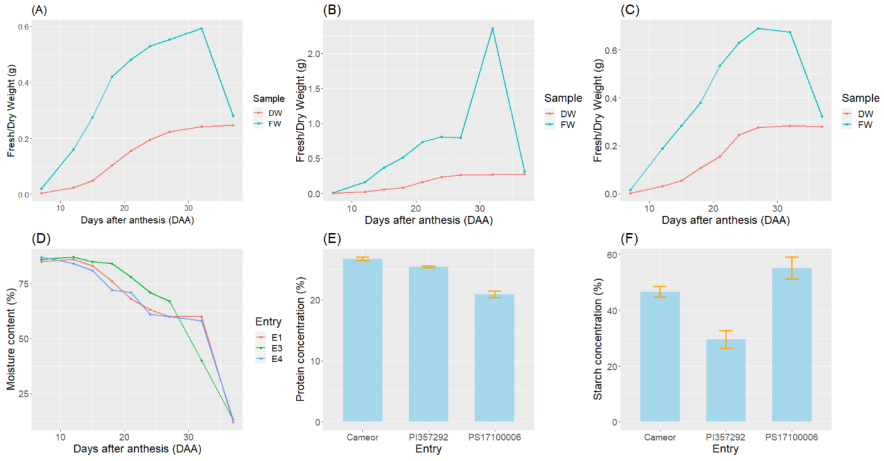


Figure 1 Fresh and dry weight changes during seed growth period for (**A**) E1: Cameor, (**B**) E3: PI 357292, and (**C**) E4: PS17100006, (**D**) moisture content changes during seed growth period, (**E**) protein concentration of mature seed, and (**F**) starch concentration of mature seed.

3.2 | Overview of the proteomics results in three pea lines

A search of the Cameor-based pea reference genome [9] resulted in a total of 5,871 protein groups across all the samples (Table S1a). These protein groups in total comprised of 11,382 proteins (average of 2 proteins per group with a range of 1-28) and a total of 52,223 peptides (average of 9 peptides per group with a range of 1-137). After removing protein groups identified in fewer than two replicates per sample, 4,126 protein groups were retained (Table S1b). The early seed growth stages, such as 4DAA for E3 (n = 2770), 7DAA for E1 (n = 2852), and 12DAA for E4 (n = 2637), had the highest protein detection, and the least at maturity (1198 for E3, 1396 for E1, and 1467 for E4) (Figure 2). Neto and co-workers [22] also reported a fewer number of proteins in late seed development of acai palm seed, due to the presence of high abundance proteins and the difficulty of extracting proteins from the thick and lignified tissues. Proteins that were shared among all the time-points ranged in number from 895 for E3 to 1089 for E4 (about 22-26% of the detected protein groups). Unique protein groups at specific time-points ranged from 66-316, 77-256, and 56-340 for E1, E3, and E4, respectively. The most unique protein groups were detected at 4DAA for E3 (n=256) and E4 (n=340), and 7DAA for E1 (n=316). For the maturity time-point, 143-165 protein groups were found to be unique for the three entries, and 89 of these protein groups were common to all three entries (Figure 2).

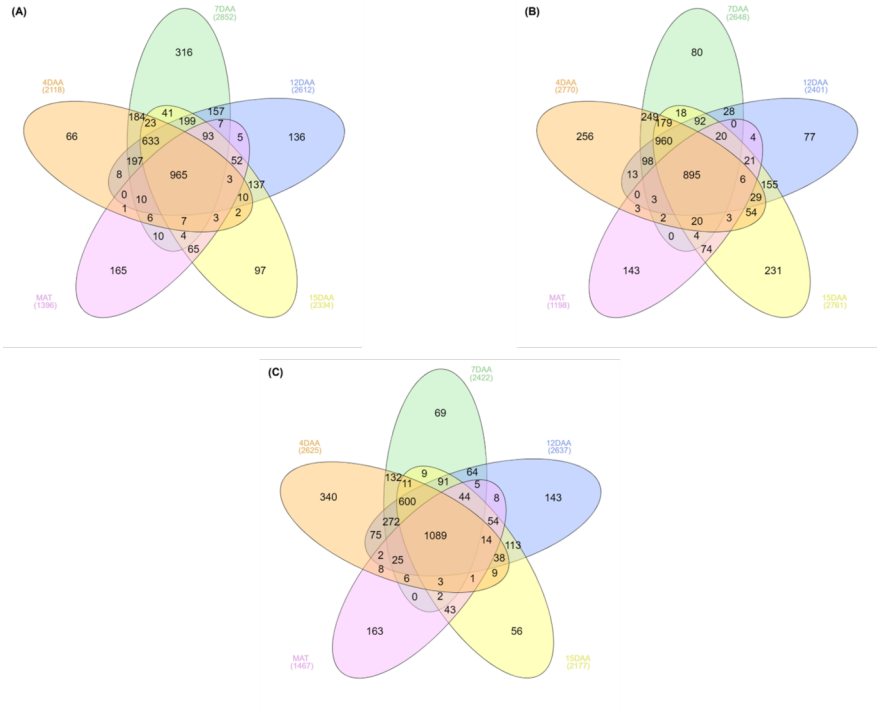


Figure 2 Venn diagrams to showing the number of common and unique proteins detected at each growth period for (**A**) E1: Cameor, (**B**) E3: PI 357292, and (**C**) E4: PS17100006.

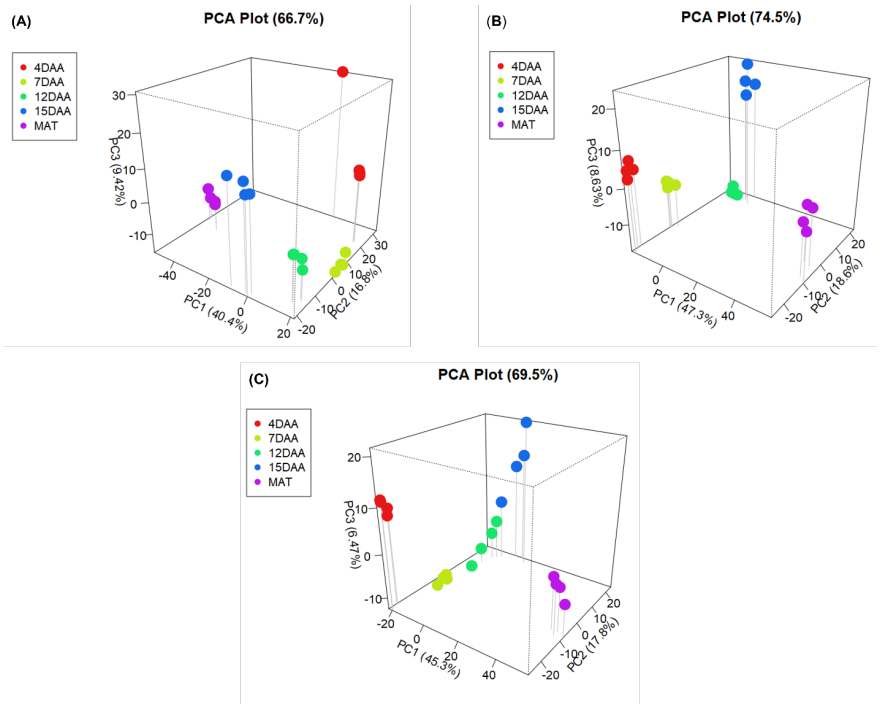


Figure 3 3D-plots showing replicate-to-replicate variation at each seed growth period for (**A**) E1: Cameor,

(**B**) E3: PI 357292, and (**C**) E4: PS17100006.

Two-way analysis of variance revealed that 1,685 of the 4,126 protein groups were statistically significant at $p < 0.01$ across the 15 treatments (entry-time combinations) (Table S1c), which were used for further downstream analyses. The 3D-plots constructed from principal component analysis of 1,685 protein groups (Figure 3) were applied to assess the reliability and repeatability of label-free proteome quantification. The first three principal components (PCs) collectively accounted for about 67-75% of the variations. Overall, the samples were discriminated according to time-points for each entry, reflecting the reliability of the proteomics quantification method. Replicate 1 at 4DAA for E1 was the only obvious outlier, so it was excluded from further analyses.

Using the MapMan4 [19], we classified 722 proteins into 29 functional classes (Figure 4A). Approximately 70% of the 722 protein groups were classified as protein biosynthesis (86), protein homeostasis (69), enzymes (54), carbohydrate metabolism (52), photosynthesis (41), amino acid metabolism (40), RNA processing (38), vesicle trafficking (n = 35), solute transport (n = 31), cellular respiration (n = 30), and lipid metabolism (n = 30). On average, protein homeostasis as well as lipid and carbohydrate metabolism related proteins tended to be more abundant late in the seed development while cell division and RNA processing related proteins tended to be more abundant early in the seed development (Figure 4B). Details of abundance changes over seed development period for individual protein in each functional class are given in Table S1d.

Hierarchical cluster analysis revealed three clusters for treatments (entry-time combinations) (Figure 5). Overall, 4DAA and 7DAA were grouped in the first cluster; 12DAA and 15DAA in the second cluster; and maturity separately in the third cluster. The cluster analysis of 1,685 protein groups generally resulted in six clusters. Cluster 1 contained 396 protein groups, that generally had the highest abundance at 12DAA and the lowest abundance at maturity (Table S1d). Using MapMan4 [19], 170 of the 396 protein groups were classified into 29 protein classes with the three dominant classes being carbohydrate metabolism (n = 19), enzyme (n = 15), and solute transport (n = 15). The proteins in cluster 2 (n = 322) had maximum abundance at 4DAA and generally decreased during seed development period. A total of 136 of the 322 protein groups were classified into 22 classes, primarily in protein biogenesis (n=27), RNA processing (n=27), and chromatin organization (n=17). Cluster 3 contained proteins commonly depleted during the seed development period and consisted of 460 protein groups, of which 209 were grouped into 23 protein classes. The classes with the highest number of proteins were protein homeostasis (n=29), protein biosynthesis (n=17), cellular respiration (n=15), and vesicular transport (n=15). Sixty-five of the 144 protein groups in cluster 4 were grouped into 20 protein classes, with nearly half of the classified proteins involved in protein biosynthesis (n=27). The proteins in cluster 4 increased slightly until 15DAA and subsequently declined at maturity. Cluster 5 (n=171) and cluster 6 (n = 192) contained proteins more abundant at 15DAA and maturity, respectively. Of the 70 classified proteins into 22 classes for cluster 5, 15 were involved in photosynthesis. For cluster 6, 70 proteins were classified into 18 classes, with carbohydrate metabolism (n=13) and protein homeostasis (n=10) contributing the most. In general, maturity had the lowest abundance for proteins in clusters 1 to 4. The list and functional characterization of the 1,685 proteins along with their cluster assignments are given in Table S1d.

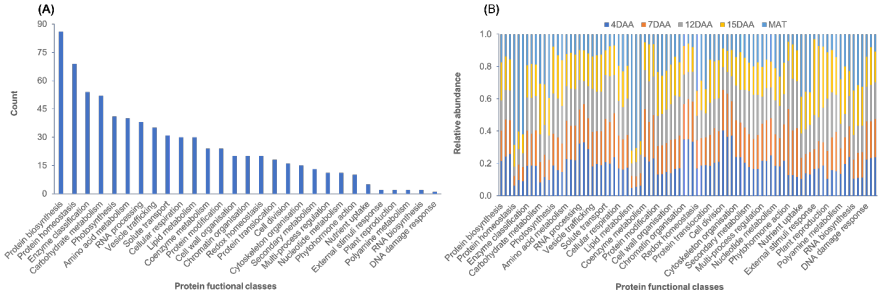


Figure 4 Functional classifications for 722 of the 1685 protein groups (identified as significant based on two-

way analysis of variance) according to MapMan4 protein classification and annotation tool: (**A**) counts of proteins in each class and (**B**) relative abundance over the seed development period, with three consecutive bars (for E1, E3, and E4, respectively) represent a functional class.

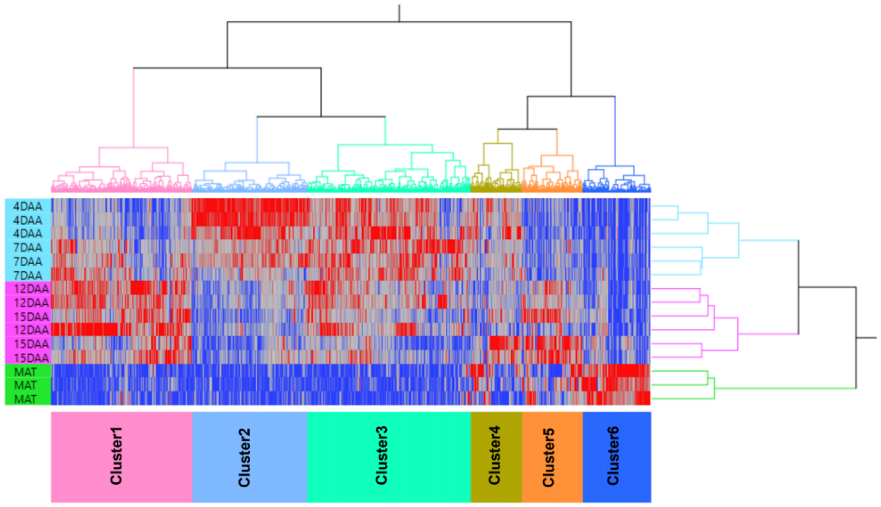


Figure 5 Hierarchical clustering with heat map of 1,685 proteins significant based on ANOVA for the five seed development period across the three entries.

3.3 | Differentially abundant proteins (DAPs) in pair-wise comparisons

3.3.1 | Comparison among time-points

Ten time-point comparisons were performed for each entry, revealing that approximately 80–99.7% (1341-1680 of 1685) of the proteins were unchanged in the pair-wise comparisons among time-points (Table 1). However, focusing only on DAPs, relatively more proteins became differentially abundant as the time intervals between time-points compared widened. Particularly, comparison of all early time-points (4DAA to 15DAA) with maturity (about 37DAA) resulted in more DAPs. For E1, the lowest number of DAPs (total of 28) were found in 4DAA vs 7DAA comparison while the highest number of DAPs (total of 344) were found in 7DAA vs maturity comparison. Besides summary of the ten comparisons, the volcano plots among 7DAA, 15DAA, and maturity for E1 are given Figures 6A-6C. In 7DAA vs 15DAA comparison for E1, 57 proteins were significantly abundant at 7DAA, and 66 proteins were significantly abundant at 15DAA (Table 1 and Figure 6A). When 7DAA and maturity were compared, 251 proteins were more abundant at 7DAA and 93 proteins more abundant at maturity (Table 1 and Figure 6B). Comparing 15DAA with maturity, 145 proteins were more abundant at 15DAA, and 48 proteins were more abundant at maturity (Table 1 and Figure 6C).

A total of 26 and 67 proteins were found to be differentially abundant at 7DAA and 15DAA, respectively, when 7DAA compared to 15DAA in E3 (Table 1 and Figure 6D). Maturity vs 7DAA comparison resulted in 246 proteins differentially abundant at 7DAA while 87 proteins differentially abundant at maturity (Table 1 and Figure 6E). A total of 288 DAPs (i.e., 231 more abundant at 15DAA and 57 more abundant at maturity) were identified in a 15DAA vs maturity comparison (Table 1 and Figure 6F).

For E4 (PS17100006), 7DAA vs 15DAA comparison resulted in 25 proteins more abundant at 7DAA while 49 proteins were more abundant at 15DAA (Table 1 and Figure 6G). In 7DAA vs maturity comparison, 184 proteins were more abundant at 7DAA, and another 78 proteins were more abundant at 15DAA (Table 1 and Figure 6H). When 15DAA compared to maturity, 133 proteins were found to be more abundant at 15DAA and 62 proteins were found to be more abundant at maturity (Table 1 and Figure 6I). The detail of functional characterization of the proteins differentially abundant among 7DAA, 15DAA, and maturity for the three entries are given in Table S1e.

Table 1 Number of differentially abundant (DAPs) between pair of time-points in the three entries.

Comparisons (Time 1 vs Time 2)	Abundant at Time 1	Abundant at Time 2	Total DAPS	Unchanged
	E1: Cameor	E1: Cameor	E1: Cameor	
4DAA Vs 7DAA	1	27	28	1657
4DAA Vs 12DAA	15	69	84	1601
4DAA Vs 15DAA	28	82	110	1575
4DAA Vs Maturity	130	99	229	1456
7DAA Vs 12DAA	22	42	64	1621
7DAA Vs 15DAA	57	66	123	1562
7DAA Vs Maturity	251	93	344	1341
12DAA Vs 15DAA	7	36	43	1642
12DAA Vs Maturity	239	89	328	1357
15DAA Vs Maturity	145	48	193	1492
	E3: PI 357292	E3: PI 357292	E3: PI 357292	
4DAA Vs 7DAA	1	4	5	1680
4DAA Vs 12DAA	46	52	98	1587
4DAA Vs 15DAA	40	85	125	1560
4DAA Vs Maturity	245	97	342	1343
7DAA Vs 12DAA	17	27	44	1641
7DAA Vs 15DAA	26	67	93	1592
7DAA Vs Maturity	246	87	333	1352
12DAA Vs 15DAA	4	38	42	1643
12DAA Vs Maturity	257	80	337	1348
15DAA Vs Maturity	231	57	288	1397
	E4: PS1710006	E4: PS1710006	E4: PS1710006	
4DAA Vs 7DAA	4	13	17	1668
4DAA Vs 12DAA	23	44	67	1618
4DAA Vs 15DAA	50	66	116	1569
4DAA Vs Maturity	204	90	294	1391
7DAA Vs 12DAA	6	13	19	1666
7DAA Vs 15DAA	25	49	74	1611
7DAA Vs Maturity	184	78	262	1423
12DAA Vs 15DAA	2	13	15	1670
12DAA Vs Maturity	181	74	255	1430

Comparisons (Time 1 vs Time 2)	Abundant at Time 1	Abundant at Time 2	Total DAPS	Unchanged
15DAA Vs Maturity	133	62	195	1490

3.3.2 | Comparison among entries

Three entries (E1: Cameor, E3: PI 357292, and E4: PS1710006) were considered in this study. As previously stated, E1 represented a high protein smooth pea, E3 a wrinkled pea, and E4 a low protein smooth pea. Fewer numbers of proteins were differentially abundant at 4DAA compared to the other time-points for all entry-wise comparisons (Table 2). In E1 vs E3 comparisons at all other time-points, a total of 8-20 proteins were found to be differentially abundant. The most DAPs in entry-wise comparisons were recorded for E3 vs E4 at 12DAA (n = 43), with 39 of which were more abundant for E3, and 4 proteins were more abundant for E4. Relatively more proteins were differentially abundant at 15DAA in E1 vs E4 comparison (n = 19), where 15 proteins were more abundant for E1 and 4 proteins more abundant for E4. In general, more proteins were differentially abundant in time-point comparisons than in entry-wise comparisons (Tables 1 and 2). Table S1e provides the list of proteins differentially abundant among the three entries at 7DAA, 15DAA, and maturity along with their functional characterization.

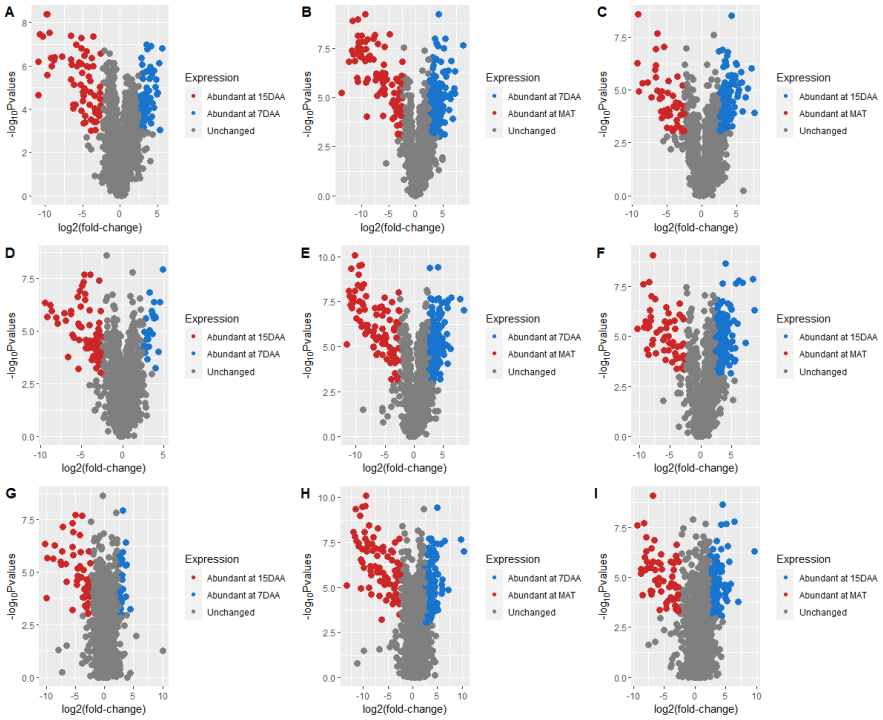


Figure 6 Volcano plots for the three entries: (A) 7DAA vs 15DAA for E1, (B) 7DAA vs Maturity for E1, (C) 15DAA vs Maturity for E1, (D) 7DAA vs 15DAA for E3, (E) 7DAA vs Maturity for E3, (F) 15DAA vs maturity for E3, (G) 7DAA vs 15DAA for E4, (H) 7DAA vs Maturity for E4, and (I) 15DAA vs Maturity for E4.

Table 2 Number of differentially abundant proteins (DAPs) in entry-wise comparisons at all five time-points.

Entry-wise comparisons (Entry 1 vs Entry 2)	Abundant in the first entry	Abundant in the second entry	Total DAPs	Unchanged
	4DAA	4DAA	4DAA	
E1 vs E3	0	3	3	1682
E1 vs E4	1	4	5	1680
E3 vs E4	3	1	4	1681
	7DAA	7DAA	7DAA	
E1 vs E3	4	16	20	1665
E1 vs E4	4	4	8	1678
E3 vs E4	3	1	4	1681
	12DAA	12DAA	12DAA	
E1 vs E3	4	9	13	1672
E1 vs E4	5	3	8	1678
E3 vs E4	39	4	43	1642
	15DAA	15DAA	15DAA	
E1 vs E3	2	6	8	1678
E1 vs E4	15	4	19	1666
E3 vs E4	9	3	12	1673
	Maturity	Maturity	Maturity	
E1 vs E3	1	10	11	1674
E1 vs E4	3	7	10	1675
E3 vs E4	3	6	9	1677

3.4 | Functional characterization of proteins

3.4.1 | Cell division

Cell division in the early stages of seed development determines the number of cells that dictates seed size [3], and hence genes regulating cell division are of great importance in seed development. The abundance of cell division-related proteins did not differ significantly among the three entries, suggesting that these proteins are equally required for all three entries. However, five DAPs associated with cell division were identified in at least one time-point comparison (Table S2a), including one involved in cytogenesis (physical division of 1 cell into 2) - microtubule-associated protein and four in DNA replication (the actual physical division of a cell into two). Microtubule-associated protein (controlled by Psat2g009880.2) was relatively more prevalent than the other four. All proteins associated with cell division were more abundant during the early stages of seed development (especially at 4DAA) and showed a decreasing trend during seed development.

3.4.2 | Photosynthesis

Based on Mercator4v5.0 functional characterization tool [19], a total of 20 proteins were related to photosynthesis (Table S2b), which were sub-divided into photophosphorylation (n=10), Calvin cycle (n=5), photorespiration (n=4), and CAM/C4 photosynthesis (n=1). Most photosynthesis related proteins decreased significantly at maturity as compared to the early seed growth stages (up to 15 DAA). In most cases, the abundance of these proteins peaked either at 12DAA or 15DAA.

3.4.3 | Carbohydrate metabolism

According to the MapMan4v5.0 [19], there were 21 DAPs in at least one pair-wise comparison that were involved in carbohydrate metabolism (Table S2c). These proteins were further classified as starch metabolism (n=5), sucrose metabolism (n=5), fermentation (n=5), nucleotide sugar biosynthesis (n=4), gluconeogenesis (n=1), and oligosaccharide metabolism (n=1). The five proteins involved in starch metabolism varied among entries and during the seed development period. All three entries showed a similar pattern of

change in starch-debranching isoamylase (regulated by Psat5g050480.1) and glucose transporter (regulated by Psat1g080960.2), with a general increase in abundance during seed development and reaching the highest at maturity. The remaining three proteins involved in starch metabolism varied somewhat among the three entries. Starch synthase 2 (SS2) regulated by Psat1g073520.1 increased between 7DAA and 12DAA in E1, and between 7DAA and 15DAA in E3 and E4, before declining afterwards. ADP-glucose pyrophosphorylase was more abundant for E1 and E3 as compared to E4, particularly at 15DAA. In most seed development periods, the round peas (E1 and E4) had a higher abundance of starch branching enzyme as compared to wrinkled peas (E3) (Table S2c and Figure 7).

During the seed development period, various patterns of change were observed in the proteins related to sucrose metabolism (Table S2c). Of the three differentially abundant sucrose synthases, two of them (regulated by Psat1g139760.1 and Psat4g019440.3) increased mainly until 15DAA, whereas the third one (controlled by Psat3g021440.1) increased steadily during seed development period with the highest at maturity. Sucrose synthase catalyzes the reversible cleavage of sucrose into fructose and uridine diphosphate glucose (UDG-G) or adenosine diphosphate glucose (ADP-G), which are inputs for production of energy, primary-metabolite, and complex carbohydrates such as starch [15, 23]. Hexokinase (controlled by Psat4g197200.1) showed a decreasing trend, whereas cytosolic phosphoglucose isomerase (controlled by Psat1g077120.2) showed an increasing trend over the seed development period.

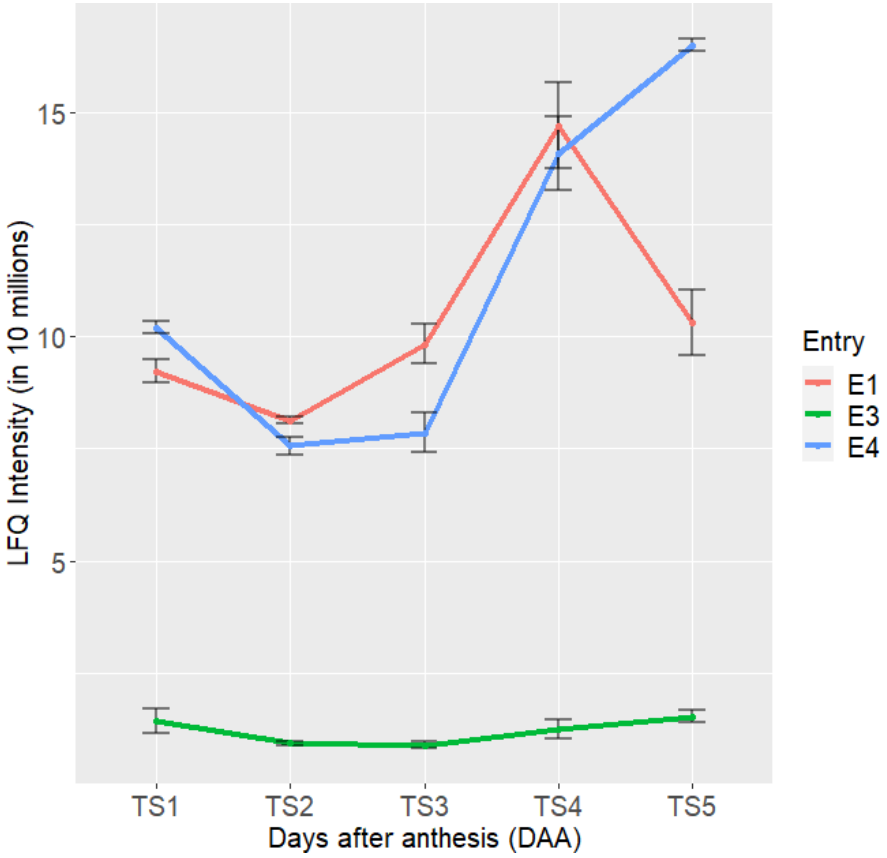


Figure 7 Abundance of starch debranching enzyme (regulated by Psat3g034640.1) during seed growth period in the three entries.

The oligosaccharide metabolism protein ∝-galactosidase (controlled by Psat3g118320.1) increased steadily during seed development period with the maximum at maturity (Table S2c). This is consistent with what

has been reported for the expression of acid α-galactosidase in peas [24]. Gluconeogenesis protein, phosphoenolpyruvate carboxykinase (regulated by Psat2g135640.1), showed a large increase between 4DAA and 7DAA, followed by a decrease afterward. The five nucleotide sugar biosynthesis proteins were shown to be abundant at the early stages of seed development, in most cases at 7DAA or 12DAA. The fermentation related proteins were either decreased (aldehyde dehydrogenases) or increased (pyruvate decarboxylase and alcohol dehydrogenases) throughout the seed development period.

3.4.4 | Amino acid and protein metabolism

In the current study, a total of 20 proteins related to amino acid metabolism were identified (Table S2e), as functionally characterized by MapMan4v5.0 [19]. These proteins were further classified into aspartate group amino acid biosynthesis (n=8), amino acid degradation (n=5), shikimate group amino acid biosynthesis (n=4), serine group amino acid biosynthesis (n=1), glutamate group amino acid biosynthesis (n=1), and pyruvate group amino acid biosynthesis (n=1). Even though amino acids are primarily useful for protein biosynthesis, they also serve as a building block for several other biosynthesis pathways and play roles in signaling and stress responses [25, 26]. Three of the amino acid degradation proteins (proline dehydrogenase, threonine aldolase, and succinate semialdehyde dehydrogenase) identified in the current study were among the 66 enzymes regulating amino acid catabolic pathways in Arabidopsis [25].

Three of the proteins (regulated by Psat1g063160.1, Psat4g053760.1, and Psat4g158760.1) that are involved in amino acid degradation decreased over the seed development period. One protein involving in amino acid degradation (regulated by Psat1g015240.3) increased highly after 15DAA and another one (regulated by Psat3g006560.1) reached peak abundance at 12DAA. Proteins involving in aspartate group amino acid biosynthesis were all less abundant at maturity, but more abundant early in the seed development phases such as at 4DAA (proteins regulated by Psat2g010880.1, Psat4g016840.1, and Psat5g003520.1), 7DAA (a protein regulated by Psat2g172600.1), 12DAA (proteins regulated by Psat2g000800.1 and Psat3g073640.1), or 15DAA (proteins regulated by Psat3g114440.1 and Psat5g051480.1). Two proteins involved in shikimate group amino acid biosynthesis (regulated by Psat2g028840.1 and Psat2g153160.1) decreased during the seed growth period, while the other two (regulated by Psat1g216320.1 and Psat2g070680.1) increased until 15DAA before dropping sharply at maturity. Serine, glutamate, and pyruvate group amino acids biosynthesis proteins were generally less abundant at maturity.

We found 11 differentially abundant proteins in at least one pair-wise comparison that were known to involve in protein biosynthesis (Table S2f). These proteins were further classified into five sub-groups: ribosome biogenesis (n=6), translation initiation (n=2), translation elongation (n=1), organelle machinery (n=1), and aminoacyl-tRNA synthetase activity (n=1). Like amino acid metabolism proteins, those involved in protein biosynthesis decreased in abundance at maturity.

| Protein homeostasis and storage proteins

According to MapMan4v5.0 [19], 17 of DAPs in the current study were classified as protein homeostasis (Table S2g). These proteins were further sub-classified as proteolysis (n = 10), protein quality control (n = 3), storage proteins (n = 2), and ubiquitin-proteasome system (n = 2). While most of these proteins were found to be less abundant at maturity, storage proteins and three proteolysis proteins (C13-class asparaginyl endopeptidase as well as Cystatin and Kunitz protease inhibitors) increased during seed development period and reaching the highest abundance at maturity. Plant protease inhibitors are found mostly in storage organs such as seeds and act as anti-metabolic proteins that disrupt the digestion processes of insects and phytopathogenic microorganisms, and hence provide defense against these organisms [27].

4 | Discussions and conclusions

We applied label-free proteomics to study proteome profiles of three pea lines (2 round and 1 wrinkled types) during the seed development period. Label-free proteomics methods have gained significant attention in recent years due to their simplicity and the ability to quantify protein abundance based on ion intensity or spectral counts [12, 13, 28]. Ensuring the reliability and reproducibility of proteomics data is critical to

achieving research goals. Variations in sample preparation and injection, as well as variations in retention time and m/z, can lead to loss of precision in ion intensity-based proteomics methods [13], requiring careful execution of LC-MS/MS. In our study, intensity-based measurement of precursor ions was applied to quantify protein abundances and the results showed exceptionally good reproducibility of the data. Daba and co-workers [29] also demonstrated the reproducibility and reliability of label-free proteomics quantified as chromatographic ion intensity in wheat.

In general, pea seeds reach maturity at approximately 37DAA. However, four of the five sampling timepoints were concentrated in the early stages of seed development (within 15DAA) and only maturity was included from the later stages of seed development. This sampling design helps identify trends in protein profiles at early stages of seed development but cannot clearly reveal trends throughout seed development period. Therefore, it is of utmost importance in the current study to carefully discuss the results of proteomic profiling during the seed development stage. Cluster analysis assigned 4DAA and 7DAA to the same group, and 12DAA and 15DAA together in another group. This may indicate that sampling of one of the timepoints within each cluster may be sufficient, and additional sampling time points may be added from later stages of seed development. Based on the observations made in the current study, two general strategies for sampling design can be proposed: (1) target a specific seed development stage (e.g., early seed development stage) and limit sampling within that stage; or (2) select a specific interval (e.g., every 7 days) to examine the entire seed development process by sampling throughout the seed development period.

The two round peas exhibited somewhat similar seed growth patterns, with a steady increase in fresh weight until 27DAA or 32DAA and a sharp decrease after 32DAA. Dry weight increased rapidly between 15DAA and 27DAA. However, wrinkled peas had a different seed growth pattern. Fresh weight gain was somewhat similar for both round and wrinkled types until 27DAA. After 27DAA, fresh weight of wrinkled pea increased rapidly during the next 7 days of seed development period, mainly due to increased moisture content. We found that the wrinkled pea line had a much lower abundance of starch branching enzyme I (SBEI). This is consistent with the fact that wrinkled peas have defective SBEI and SBEII [21, 30]. In agreement with previous reports [31-33], we found that the wrinkled pea entry contained less starch than the round pea entries. Wrinkled peas are not only low in starch, but they are also low in legumin protein levels; however, they have a high amylose/amylopectin ratio, and high sucrose and lipid levels [30]. Increased sucrose accumulation increases the osmotic pressure resulting in greater water uptake and, ultimately, increased cell size and fresh weight. However, as seeds mature, they lose a significant amount of water, resulting in a wrinkled phenotype.

Seeds contain key components that protect and nourish the embryo and provide energy during germination before the seedling becomes photosynthetically active [34]. Seed development is controlled by a complex interplay of multiple genes. Although the early stages of seed development are characterized by high mitotic activity that determines seed size, the later stages involve accumulation of seed reserves (such as starch and protein), and seed development ends with desiccation and dormancy at maturity [3, 4, 35-38]. The transition from the stage of active cell division to cell differentiation and reserve accumulation is initiated by a shift from a hexose-rich to a sucrose-rich state [3]. During reserve accumulation, the seed also gains in photosynthetic activity. Our study identified several proteins differentially abundant during seed development period that relate to cell division and photosynthesis, as well as biosynthesis of storage products.

Because the early stages of seed development are dominated by cell division, it is expected that cell division-related proteins are more abundant early in the seed development period. That was exactly what we found in the present study for five differentially abundant proteins (DAPs) related to cell division. They were more abundant at 4DAA, and abundance declined as the seed developed. One of these proteins involves cytogenesis while the other four regulate DNA replication. Cell cycle is generally divided into four steps [4, 39]: (1) physical cell proliferation and organelle duplication (G1 phase), (2) complete nuclear DNA synthesis (S phase), (3) cell growth as well as protein and organelle formation (G2 phase), and (4) division of copied DNA and cytoplasm (M phase). A microtubule-associated protein, one of the DAPs in the current work, has been described as a self-organizing mechanism that segregates chromosomes during mitosis [40] and promotes cell proliferation and axial growth in Arabidopsis [41, 42]. Three minichromosome maintenance

(MCM) replicative DNA helicase complexes and one proliferating cell nuclear antigen (PCNA) sliding clamp loader complex were also identified as DAPs related to cell division. MCM DNA helicase complexes are important for initiation and elongation of chromosomal DNA by serving as replicative DNA helicase [43], whereas PCNA is acting as a sliding clamp and providing the essential progressivity to duplicate the entire genome by binding to DNA polymerase during the S phase [44]. PCNA is an essential component of DNA replication and repair [44].

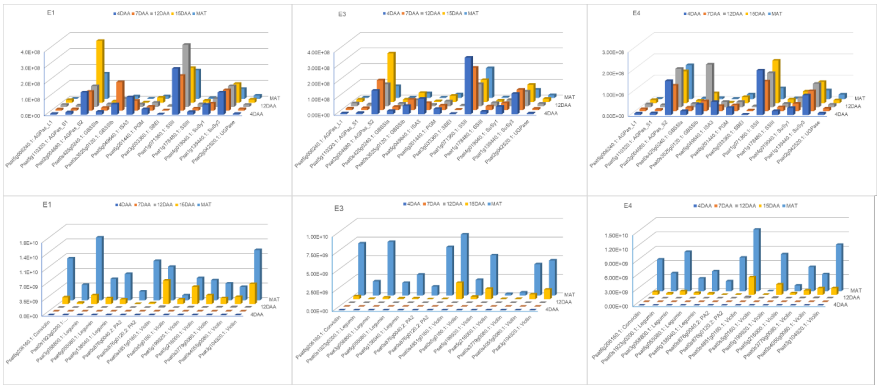
Photosynthesis produces sucrose, the main sugar that is transported in the phloem of plants [23]. Although seeds are vital for plant reproduction and to supply nutrients, they sometimes act as photosynthetic sites [45]. Seed photosynthesis is particularly important for controlling biosynthetic flux by increasing internal oxygen content and improving the energy supply of developing seeds [46-48]. Most of the proteins related to photosynthesis in the current study reached their maximum abundance at the early seed -filling phase (at 12DAA and 15DAA). Previous research [47, 48] also found that seed photosynthesis happens early in seed development, when the seeds are still green.

Using MapMan4v5.0 [19], we identified 21 differentially abundant proteins that are associated with carbohydrate metabolism, including starch, sucrose, and nucleotide sugar metabolism pathways. In fact, starch metabolism alone is directly or indirectly regulated by 22 pea genes [15]. Seventeen of these genes directly control the synthesis of two components of starch, namely, amylose (regulated by AGPase and GBSS) and amylopectin (regulated by AGPase, SS, SBE and DBE) [46]. Since MapMan4V5.0 functionally characterizes a portion of these proteins, it is important to augment it with reports [15] to better understand the processes that occur during seed development. Our search of all the detected proteins (n = 4,126) identified 19 of the starch metabolism genes reported by Yu and co-workers [15], with 13 of them among the 1,685 proteins significant based on two-way ANOVA (Figure 8). Among them, three proteins such as AGpase_S2 (regulated by Psat5g110320.1), SSII (regulated by Psat1g071360.1), and Susy3 (regulated by Psat1g139440.1) particularly more abundant than the other proteins. AGpase_S2 was found to be more abundant for E1 and E3 compared to E4 at 15DAA. Soluble starch synthase II (SSII) had different abundance in the three entries, reaching the highest at 12DAA in E1 but at 4DAA in E3 and E4. Susy3 was less abundant at maturity than early seed development stages (4DAA–15DAA).

Amino acids are synthesized in various pathways, including the shikimate pathway for biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) [49, 50]; the aspartate pathway for biosynthesis of lysine, methionine, isoleucine, and threonine [50-52]; and the pyruvate pathway for the biosynthesis of valine, leucine, and isoleucine [50]. Our study identified 15 proteins involved in the biosynthesis of aspartate, shikimate, pyruvate, and serine group amino acids. These amino acid biosynthesis proteins were generally more abundant in the early stages of seed development than in mature seeds. Free amino acids are primarily utilized for the synthesis of seed storage proteins [26, 37]. Kreplak and co-workers [9] reported a total of 40 storage protein genes (legumin, vicilin, convicilin, and albumin) in peas. Even though 22 of these storage proteins were included in the total proteins detected (n = 4,126), only 14 of them were among the 1,685 proteins that were significant based on ANOVA (Figure 8). All these proteins increased in abundance during the seed development period in all three entries, particularly starting from 12DAA, with the greatest abundance at maturity. This finding supports the hypothesis that storage protein gene expression is regarded as an indicator of seed maturity [35]. Most of storage proteins were shown to be significantly lower in E4 (low protein entry) than in E1 (high protein entry), especially at 12DAA, 15DAA, and maturity (Table S1h).

In conclusion, we identified specific proteins associated with various biological processes during seed development and seed maturity. We were able to identify key proteins involved in cell division, carbohydrate metabolism, and amino acid biosynthesis, suggesting important role of these proteins and pathways in seed formation and growth. The dataset will be a valuable resource for the research community and breeding programs aimed at improving nutritional traits, increased seed size, germination, or resistance to pathogens. The present study particularly linked the proteins to a Cameor -based reference genome [9], which can pave the way for a wide range of studies on each individual protein to further our understanding of the seed development processes and to facilitate their application in crop improvement.

Figure 8 Abundance of starch biosynthesis related proteins [15] [**upper three plots**] and storage proteins [9] [**bottom three plots**].



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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Sintayehu D. Daba contributed to conceptualizing the project, collecting the samples and phenotypic data, analyzing the data, and preparing and reviewing the manuscript. **Punyatoya Panda**contributed to reviewing the manuscript. **Uma K. Aryal**contributed to generating proteomic data and reviewing the manuscript.**Alecia M. Kiszonas** contributed to reviewing the manuscript.**Sean M. Finnie** contributed to reviewing the manuscript.**Rebecca J. McGee** contributed to conceptualizing the project, providing financial support for the project, and reviewing the manuscript.

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Captions for Supplemental Tables:

Table S1 . Different datasets of proteins: (a) raw proteomics data as acquired from maxquat, (b) LFQ intensity for the 4,126 high confidence proteins, (c) LFQ intensity for the 1,685 proteins significant based on two-way ANOVA, (d) Clustering assignment of the 1,685 proteins significant based on two-way ANOVA, and (e) list of proteins differentially abundant in time-point and entry-wise comparisons.

Table S2 . Functionally characterized proteins of different classes: (a) cell division related proteins, (b) photosynthesis related proteins, (c) proteins related to carbohydrate metabolism, (d) starch biosynthesis related proteins reported by Yu et al. (2021), (e) amino acid biosynthesis related proteins, (f) protein biosynthesis related proteins, (g) protein homeostasis related proteins, and (h) storage proteins reported by Kreplak et al. (2019).

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