

Article

Proteome Profiling of *Cucurbita pepo* Phyllosphere After Infection by *Podosphaera xanthii* and Application of *Reynoutria sachalinensis* Extract

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Abstract: *Podosphaera xanthii* is the main causal agent of powdery mildew (PM) disease for *Cucurbita pepo*. Disease control is attained principally by applications of chemical fungicides, along with parallel use of tolerant crop varieties and alternate application of elicitors to control development of disease resistance. To get insight into *C. pepo* molecular responses to *P. xanthii* infection and elicitor treatment we studied the proteomic profile differences at the phyllosphere of a zucchini cultivar susceptible to PM, at the onset of *P. xanthii* (PX) infection and after application of *Reynoutria sachalinensis* (RS) plant extract, respectively, using a nano-LC-HRMS/MS, Q-Exactive-Orbitrap approach. Analysis of peptide sequences regarding four treatment groups (Control; PX; RS; and RSPX (PX-infected priorly treated with RS)) resulted in 2070 CuGenDB annotations. Three comparisons (treatments vs. Control) encompassed most of the Differentially Expressed Proteins (DEPs). In these three comparisons, KEGG and Gene Ontology functional analyses highlighted unique differentially enriched pathways—some of which included highly expressed proteins—in PX-related (proteasome, pentose phosphate pathway, and carbon fixation), RS-related (biosynthesis of secondary metabolites, flavonoids, and starch and sucrose metabolism), and RSPX-related (pyruvate metabolism and polycomb repressive complex) comparisons, respectively, suggesting distinct mechanisms of early plant responses modulated by PX and RS. Furthermore, in four out of six comparisons the thiamine metabolism pathway was found to be enriched, suggesting a pivotal role in PX-induced responses.

Keywords: *Cucurbita pepo*; zucchini; *Podosphaera xanthii*; powdery mildew; *Reynoutria sachalinensis*; elicitors; proteomics; nano-LC-HRMS/MS; Q-Exactive-Orbitrap



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1. Introduction

The *Cucurbitaceae* family comprises around 1000 annual or perennial plant species, several of which are economically important cultivations worldwide. These include ‘major horticultural crops’ such as cucumber (*Cucumis sativus*), melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), pumpkins and squashes (*Cucurbita maxima*), butternut squash (*Cucurbita moschata*), and various summer squashes, zucchini, and pumpkins (all belonging to the *Cucurbita pepo* subspecies *pepo*) [1].

The genus *Cucurbita* includes three domesticated species: *C. pepo*, *C. moschata*, and *C. maxima*, with *C. pepo* being economically the most important crop of the genus. *C. pepo* is

taxonomically divided into three subspecies: (i) *C. pepo pepo* including the edible-fruited pumpkin, zucchini, cocozelle, and vegetable marrow cultivar-groups, (ii) *C. pepo ovifera* including the edible acorn, scallop, crookneck, and straightneck cultivar-groups, along with wild ornamental gourds, and (iii) *C. pepo fraterna* which comprise only wild populations. Edible cultivars are highly diverse in vine, flower, fruit shape, and color, and are valued globally as high-valued vegetables [2,3].

Powdery mildew (PM) is one of the most severe fungal diseases affecting more than 10,000 plant hosts globally. PM fungi (*Erysiphaceae*) are plant pathogens with an obligate-biotrophic lifestyle, capable usually of both sexual and asexual reproduction. Currently there are 27 genera including 724 known species reported as PM [4–8]. Cucurbit PM is considered one of the most reported and severe diseases limiting cucurbit production. The disease is caused by either *Podosphaera xanthii* or *Golovinomyces cichoracearum*, with the former species being the main causal agent of powdery mildew in cucurbits. It manifests as white mycelium on leaves, petioles, and stems, starting with chlorotic spots that progress to necrotic lesions. This leads to reduced plant growth due to inhibited photosynthesis and leaf desiccation, ultimately lowering fruit quality [5–8].

Control of PM is typically achieved through regular application of chemical fungicides [5,8,9]. However, the prolonged use of fungicides has resulted in the emergence of PM strains that are less susceptible or resistant to different chemical compounds [9–11]. Breeding programs have developed commercial varieties and breeding lines with resistance or susceptibility loci and genes (i.e., Mildew Locus O (MLO); *Pm-0*) to PM, and to ongoing evaluation of new candidates from wild relative species (i.e., *Run1*, *Run2*, *Ren2-4*; *Pm-36*; *Ren6*, *Ren7*) [5,12–16]. However, adaptation and evolution of pathogenic races even in tolerant/resistant plant genotypes hamper to a certain extent an effective control of the disease through resistant cultivars. Alternative approaches for mitigation of PM resistance development suggest a combinatory use of resistant crop varieties, limitation of chemical fungicides' applications, along with introduction of plant defense eliciting compounds [17–19].

Plant defense elicitors are natural compounds that, when applied, prime the plant's innate immune system and induce biochemical signaling cascades leading to defense responses [20–22]. The concept behind their use is to mimic signaling caused by microbial infection, and in the case of priming to promote a faster and more vigorous activation of plant defense responses once microbial infection starts [23–25]. In cucurbits, several elicitors have been used thus far, including compounds such as acibenzolar-S-methyl, chitin and chitosan, subsets of plant extracts such as laminarin and oligogalacturonides, and whole plant extracts like this of *Reynoutria sachalinensis* [26–28]. The latter has been effective against several PM fungi including *P. xanthii* in cucurbits, *Leveillula taurica* in tomatoes, and *Uncinula necator* in grapes [29,30].

Proteomics, either alone [31–34] or in combination with other omics technologies [35–38], has made a substantial contribution in plant research during the past fifteen years, particularly in host–microbe symbiosis [39,40] and plant–pathogen interactions [33,41–44]. In *Cucurbitaceae*, proteomic applications have been used to study plant–pathogen interactions with viruses [45–48], bacteria [49], and fungi [50–55]. A de novo assembly of the *C. pepo* subsp. *pepo* (zucchini cultivar) genome was released a few years ago as a high-quality draft, annotating 34,240 genes, 27,870 of which were protein coding [2]. This followed the earlier release of the first *C. pepo* subsp. *pepo* (zucchini cultivar “True French”) genome. The predicted proteome consisted of 33,966 primary protein sequences from translated transcripts and represented the leaf transcriptome of a zucchini cultivar used in breeding programs for disease resistance [3]. Proteomics studies on *C. pepo*–fungal interactions are limited in number, with studies mainly focusing on transcriptomics and selected gene expression analyses of obligate parasites such as *P. xanthii* [12,50,56,57].

The aim of this study was to investigate early proteomic changes in the phyllosphere of a *C. pepo* cultivar susceptible to powdery mildew after artificial infection with *P. xanthii* (PX) conidia and assess the impact of *R. sachalinensis* (RS) plant extract treatment.

We used a nano-LC-HRMS/MS, Q-Exactive-Orbitrap approach to generate proteomes from the phyllosphere of four different treatment groups. Differentially Expressed Proteins (DEPs) were identified in each treatment, with varying levels of up- and down-regulation. Functional pathway enrichment analyses revealed common biochemical pathways, and identified several pathways enriched in treated versus Control groups, suggesting distinct early plant response mechanisms modulated by PX and RS.

To our knowledge this is, thus far, the first report on *C. pepo* whole phyllosphere proteomic profiles under *P. xanthii* infection and *R. sachalinensis* elicitor treatment.

2. Materials and Methods

2.1. Plant Material, Treatments, and Sampling

The susceptible to powdery mildew *C. pepo* L. “Kompokolokitho” zucchini cultivar (Doulgerakis, Rethimno, Greece) was used. Zucchini seeds were sown in trays with individual 5 cm pots containing sterile peat substrate for germination and were transplanted to 2 L pots containing sterile Klamann-TS2 soil (Klamann-Deilmann, Geeste, Germany) one week later. Plants were grown under controlled greenhouse conditions (20–25 °C; Relative Humidity (RH) 70–80%; 16 h light) and fertilized twice (every 10 days) with 0.3 g/L of Nutri-Leaf 20:20:20 fertilizer (Miller Chemical & Fertilizer, Hanover, PA, USA).

The Regalia 200SC 5% *v/v* formulation of *R. sachalinensis* plant extract (provided by Syngenta, AG, Basel, Switzerland) was applied at 0.5% *v/v* (5 mL of formulation per L water) twice. The first application was performed on 21-day-old *C. pepo* plants and the second, 5 days later. Mock applications to control plants were made with water.

C. pepo plants were artificially inoculated with an aqueous conidial suspension of *P. xanthii* following a previously standardized protocol for infection [30,57]. Briefly, freshly collected conidia were suspended in de-ionized water 0.01% Tween-20, to a concentration of 0.5×10^6 per ml of water. Plants were sprayed with the conidial suspension or with water 0.01% Tween-20 for mock controls, 2 days after the second foliar spray treatment application (developmental stage of 6 true leaves).

Four sample groups (cases) were formed: (a) control plants (C) sprayed only with water, (b) plants inoculated with *P. xanthii* conidia (PX), (c) RS sprayed plants (RS), and (d) RS sprayed plants inoculated with *P. xanthii* conidia (RSPX). Each individual plant constituted a single biological replicate. Leaf material (all 6 fully developed leaves) was collected from 4 individual plants for each of the control and treated groups, 36 h post inoculation. The leaves from each plant were piled, cut into smaller pieces (approximately 2×2 cm squares), mixed and wrapped in aluminum foil, subsequently frozen in liquid nitrogen, and stored at -80 °C before use.

2.2. Sample Preparation for Proteomics Analysis

Leaf material was pulverized with the aid of a mortar and pestle in the presence of liquid nitrogen. For each individual sample, 100 mg of ground powder was further homogenized in 250 μ L lysis buffer (7M Urea, 2M Thiourea, 4% CHAPS, 1% DTE, and 2% IPG ampholytes) in a 2 mL safelock polypropylene tube with one stainless steel bead (3 mm diameter) using the Tissue Lyser LT (Qiagen, Tegelen, The Netherlands). Two rounds of homogenization were performed at 50 Hz for 6 min each. Samples were then centrifuged at 14,000 rcf for 10 min at room temperature, and 100 μ L of the supernatant of each sample was transferred in a clean tube.

Protein concentration was determined by Bradford assay and the extract volume containing 200 μ g of total protein was calculated for each sample and transferred to a new tube. To this, protease inhibitors (Roche, Basel, Switzerland) were added at a final concentration of 3.6% *v/v* of extract volume, making the sample ready for immediate use. The rest of the protein extracts were stored at -80 °C until further use.

An aliquot of 10 μ g (approximately 3–7 μ L) of each protein extract resuspended in $4\times$ Laemmli buffer and H_2O up to a final volume of 20 μ L, was run in a FastGene Polyacrylamide Gel Electrophoresis precast 4% stacking-12% separating gradient gel (NIPPON

Genetics, Duren, Germany), with MOPS Buffer, at 100 Volts constant until the dye reached the bottom of the gel. The gels were fixed with 30% MetOH-10% acetic acid for 30', washed $3 \times 10'$ with dH₂O, stained overnight with Coomassie colloidal blue, and de-stained with $3 \times 10'$ washes of dH₂O before scanning the gels at a GS-800 imaging densitometer (BioRad, Hercules, CA, USA) in transmission mode (Supplementary Figure S1).

Protein extracts (200 µg per sample) were processed using filter-aided sample preparation (FASP) as described previously [58], with some minor modifications [59]. Briefly, buffer exchange was performed in 0.5 mL, 30 kDa MWCO, Amicon Ultra Centrifugal filter devices (Merck Millipore), mixing each sample volume with 200 µL urea buffer (8M Urea in 0.1M Tris-HCl pH 8.5) and subsequent centrifugation at 14,000 rcf for 15 min at room temperature (RT). An additional 200 µL urea buffer volume was added to the filter subsequently centrifuged at 14,000 rcf for 15 min at RT. This procedure was repeated twice. Alkylation of proteins was performed with 0.05 M iodoacetamide in urea buffer for 20 min in the dark, at RT, followed by a centrifugation at 14,000 rcf for 15 min at RT. Additional series of washes were conducted with urea buffer twice, and ammonium bicarbonate (ABC) buffer (50 mM NH₄HCO₃ pH 8.5) twice. Tryptic digestion was performed in ABC buffer using a trypsin-to-protein ratio of 1:100 *w/w*. Trypsinization was performed overnight at RT in the dark. Peptides were eluted by centrifugation at 14,000 rcf for 10 min at RT, lyophilized, and stored at −80 °C until further use.

2.3. Nano-Liquid Chromatography-High Resolution Tandem Mass Spectrometry (Nano-LC-HRMS/MS) Q-Exactive-Orbitrap Analysis

Samples were initially resuspended in 200 µL mobile phase A (0.1% Formic Acid (FA)) and 6 µL from each sample was injected into a Dionex Ultimate 3000 RSLC nano flow system coupled to a Thermo Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). A Dionex 0.1 × 20 mm, 5 µm, 100 Å C18 nano trap column with a flow rate of 5 µL/min was used. The analytical column was a PepMap C18 nano column, 75 µm × 50 cm, 2 µm 100 Å with a flow rate of 300 nL/min. Both trap and nano-flow columns were maintained at 35 °C. The nano-pump mobile phase A was H₂O, 0.1% FA, and B was ACN:0.1% FA. The gradient elution was as follows: T = 0 min, 2% B; T = 10 min, 2% B; T = 200 min, 33% B; T = 210 min, 80% B; T = 230 min, 2% B; T = 240 min, 2% B. Washing and re-equilibration of column was performed prior to each sample injection.

Ionization of the eluent was performed using a Thermo Easy Spray nanoESI source (Thermo Finnigan, Bremen, Germany) operating in positive ion mode. MS data were acquired in a data-dependent strategy selecting up to the top 10 most abundant precursor ions between *m/z* ratio 200 and 2000. The analysis was performed in the full scan mode using a resolution of 70,000. The AGC target was set at 3×10^6 and the maximum injection time was set at 100 ms. Tandem mass spectra were acquired with FT resolution of 35,000. Normalized collision energy was set to 33 and already targeted precursors were dynamically excluded for further isolation and activation for 30 s with 5 ppm mass tolerance.

2.4. MS Data Processing

Proteome Discoverer v2.2 (Thermo Finnigan, Thermo Fisher) and the Sequest search engine were used to process raw files, along with the Cucurbit Genomics Database (Cu-GenDB) (<http://cucurbitgenomics.org>; genome annotation v. 4.1), and National Center for Biotechnology Information genome assembly ASM280686v2. The parameters' settings include the protein dynamic modifications of oxidation/+15.995 Da (M), the N-terminal modifications of acetylation +42.011 Da, Met-loss −131.040 Da (M), and Met-loss+Acetyl −89.030 Da (M). Carbamidomethyl/+57.021 Da (C) was set as a static modification. Two missed cleavage sites were permitted and a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da were allowed. Unique and razor peptides were used for quantification based on the intensity values. The minimum peptide length was set at 6. The filters configuration was set as: (i) peptide: high confidence (FDR q value based = 0.01)

and medium confidence (FDR q -value based = 0.05), (ii) peptide rank: maximum rank = 1, (iii) peptide grouping: enabled, and (iv) protein grouping: enabled. The results were filtered for high confidence peptide identifications.

2.5. Data Analysis and Statistics

Proteins were identified by Proteome Discoverer (vs 2.2, Thermo Fisher Scientific Inc.) and corresponding abundances were extracted for each sample. Proteins with valid values for each sample in at least 2 out of 4 replicates were processed, while the rest were filtered out. Data were normalized and log2 transformed using variance stabilizing normalization with the vsn package in R [60,61]. Missing values were imputed under a Missing Not At Random (MNAR) assumption by random draws from a left-shifted distribution using the impute function of the DEP package [62]. Differential expression of proteins across experimental conditions was revealed by tests based on protein-wise linear models and empirical Bayes statistics incorporated in the DEP package. Differentially Expressed Proteins (DEPs) with p -value less than 0.05, that exhibited 2-fold absolute change at least in pairwise treatment comparisons, were selected and further processed.

2.6. KEGG and Gene Ontology Enrichment Analyses

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology terms (GO-terms) were used for functional enrichment analysis. A custom database was constructed, combining CuGenDB (<http://cucurbitgenomics.org/> Cucurbita pepo (MU-CU-16) v4.1) and NCBI genome assembly ASM280686v2. Specifically, accessions of both databases were cross-linked by applying a local blastn protocol. Subsequently, gprofiler2, the R version of g:Profiler tool [63], and limma [64] packages were implemented.

2.7. Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [65] partner repository with the dataset identifier PXD057221 and 10.6019/PXD057221. The list of 2070 annotated proteins identified by MS is available in Supplementary Table S1.

3. Results

3.1. Identification of Cucurbita pepo Differentially Expressed Proteins

The raw peptide sequences from all four sample groups, obtained via LC/MS-MS were mapped against protein fasta files, downloaded from NCBI and CuGenDB databases using the Proteome Discoverer 2.2 software package, resulting in 2070 annotations. After peptide identification, entries from both databases were matched against each other to determine common and unique entries. Nearly 9.5% (175) of CuGenDB entries lacked a corresponding annotation in NCBI. While the comparison of peptide descriptions did not show substantial differences between the two databases, 196 NCBI entries were labeled as “uncharacterized proteins” (Supplementary Table S1). Since CuGenDB had slightly more detailed annotations it was used in all downstream comparisons.

Three experimental conditions were tested against controls (C) with empirical Bayes statistics through the DEP package in R; (a) plants inoculated with *P. xanthii* conidia (PX vs. C), (b) RS sprayed plants (RS vs. C), and (c) RS sprayed plants inoculated with *P. xanthii* conidia (RSPX vs. C). Additionally, three pairwise comparisons were performed between the treatments: RS vs. PX, RSPX vs. PX, and RSPX vs. RS. To gain insight into the test behavior across comparisons and decide the level of the significance thresholds, p -value histograms were created (Supplementary Figure S2). An anti-conservative pattern was revealed for the p -value distributions for the three treated samples, whereas the p -value distributions for the remaining three comparisons between treated samples appeared uniform. Based on the uniform part of the anti-conservative distributions, we adopted a p -value threshold of <0.05 combined with an absolute fold change of more than two, which is a commonly cited threshold in proteomics analyses [46,48–50,52,53].

A summary of the differentially expressed proteins (DEPs) for a p -value < 0.05 and $\log_2$ fold change > 1 (absolute value for 2-fold change and above) is presented in Table 1. An elevated number of DEPs was observed in all three comparisons to the Control, with all other comparisons having four to five times less DEPs. The highest number of proteins exhibiting at least a 2-fold difference was found in the comparison of infected to control plants (PX vs. C), with 199 proteins in total, of which nearly two thirds were upregulated in the infected plants. Similar numbers for DEPs with differences above 2-fold were observed in the comparisons of RS-treated plants with and without infection versus the Control (comparisons RS vs. C and RSPX vs. C, respectively) with up- and down-regulated proteins ranging at similar orders of magnitudes in these comparisons. All relevant information regarding p -value significance and differential expression levels for all 2070 annotated proteins in all samples is provided in Supplementary Table S1.

Table 1. *C. pepo* Differentially Expressed Proteins (DEPs) in bilateral comparisons of the four sample groups. Proteins numbers with a statistical significance threshold (p -value) < 0.05 are shown, further filtered with an absolute value of 2-fold change and above expression difference, and further categorized as up- and down-regulated in case.

Comparison Case 1 vs. Case 2	DEPs ¹ p -Value < 0.05	DEPs $> 2\text{-fold} $	Up-Regulated ² in Case 1	Down-Regulated ² in Case 1
PX vs. C ³	316	199	140	59
RS vs. C	438	163	126	37
RSPX vs. C	394	148	129	19
RS vs. PX	75	31	10	21
RSPX vs. PX	76	20	15	5
RSPX vs. RS	82	25	18	7

¹ DEPs: Differentially Expressed Proteins; ² DEPs $> |2\text{-fold}|$ up- or down-regulated in Case 1, out of 2070 features;

³ C: Control plants; PX: plants inoculated with *P. xanthii* conidia; RS: *Reynoutria sachalinensis* extract sprayed plants; RSPX: RS sprayed plants posteriorly inoculated with *P. xanthii* conidia.

In Figure 1, a heatmap of the top 100 proteins with the highest fold in expression displays, in a visual way, various grouped DEPs in relation to the different treatments. The horizontal dendrogram shows a clear separation of the Control sample from all treated (PX, RS, and RSPX) samples, and a closer hierarchical relation of the RS and RSPX samples compared to PX. Moreover, a uniform intra-grouping (four sample replicates) for all different cases is observed. The vertical dendrogram presents clusters of DEPs with a similar pattern of expression along the four different cases. In clades 6 and 4, indicative groups of up- or down-regulated proteins irrespective of the treatments are shown to be clearly differentiated in all three treatments from the Control. In clades 5 and 1, examples of the effects of RS on the expression of indicative DEPs compared to all other sample groups, and its related effect also in the corresponding RSPX samples, are shown. Clades 2 and 3 indicatively show up- or down-regulated DEPs in PX samples compared to other treatments and the Control.

A Volcano-type graphical presentation of significant (p -value < 0.05) and non-significant (p -value > 0.05) DEPs, combined with $\log_2$ fold change (>1 and >2 criteria) in gene expression for all comparisons, is shown in Figure 2. This provides a visual overview of the proteins' expression magnitudes for the up- and down-regulated DEPs in all comparisons presented collectively in Table 1 and in detail in Supplementary Table S1. Moreover, DEPs having a difference in expression of 4-fold (absolute value) and above are shown with their corresponding IDs. A description of the DEPs state in each comparison follows, combining information from all three sources (Figure 2, Table 1, and Supplementary Table S1), and with a reference to indicative DEPs with high (>4 -fold) expression.

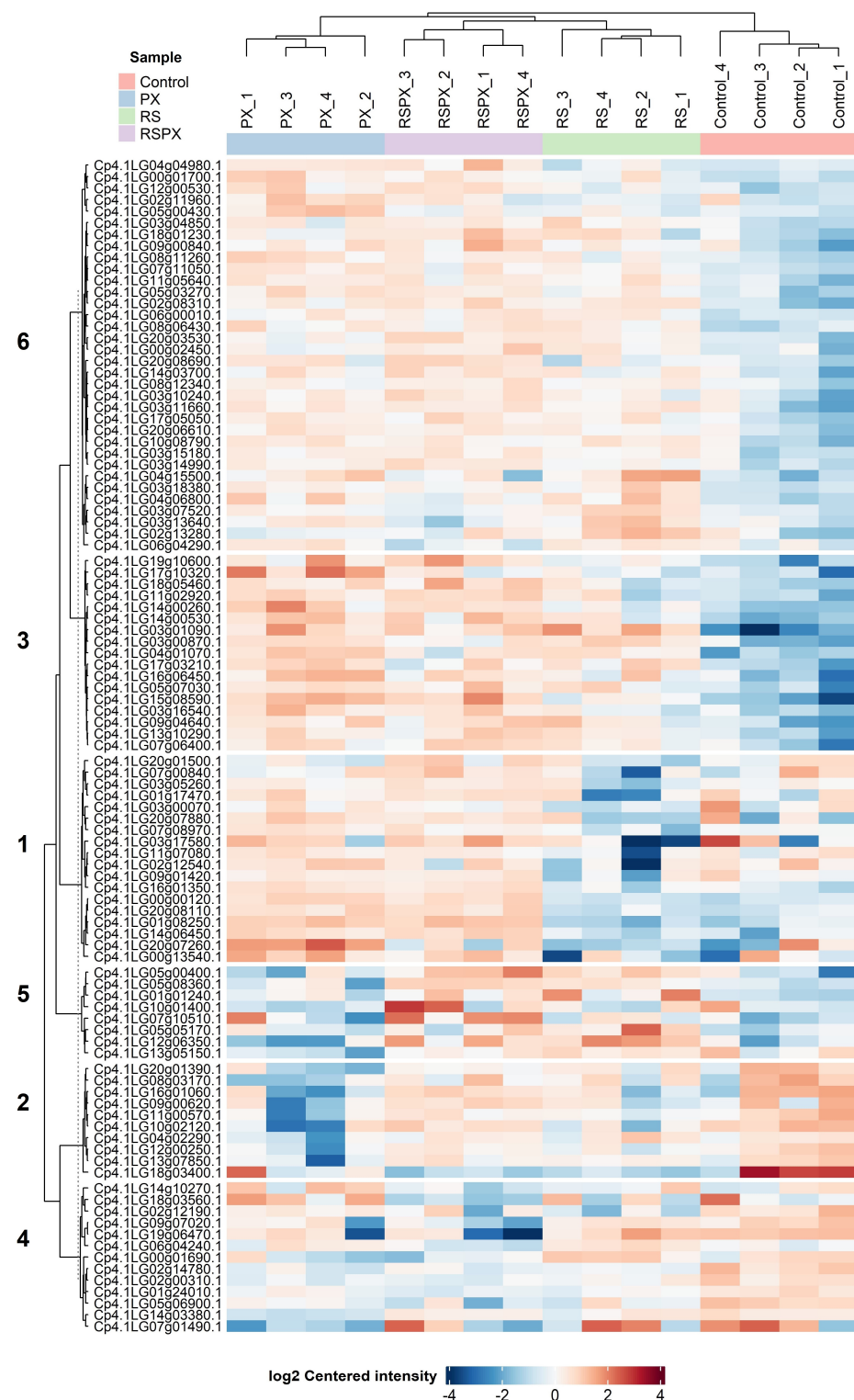


Figure 1. Heatmap of the top 100 Differentially Expressed Proteins (DEPs) in all *C. pepo* phyllosphere samples. The horizontal coordinate (dendrogram) on the top represents the clustering of each sample replicate for each treatment. Treatment names are as described in Table 1. The vertical coordinate (dendrogram) on the left represents the clustering of DEPs along with protein CuGenDB IDs. The heatmap specifies the relative up- or down-regulation of DEPs according to the log2 Centered Intensity index at the bottom of the heatmap. For each individual heatmap box, the dark red and dark blue colors represent high and low expression, respectively.

In the PX vs. Control comparison (Figure 2A; Table 1; Supplementary Table S1), of the 199 DEPs, 172 had a 2-to-4-fold change (increase or decrease) in their expression respectively, and the remaining 27 had a difference above 4-fold, with the highest at 12-fold. Highly up-regulated proteins included a starch branching enzyme (Cp4.1LG03g01090.1), an acetylornithine deacetylase (Cp4.1LG05g00150.1), v-type proton ATPase subunits A (Cp4.1LG04g01070.1) and G (Cp4.1LG08g11040.1), a putative carbonic anhydrase (Cp4.1LG20g07260.1), a glutaredoxin (Cp4.1LG11g06470.1), and a malate dehydrogenase (Cp4.1LG10g01820.1). Highly down-regulated proteins included a lipid transfer protein (Cp4.1LG01g16110.1), an acetyl-coenzyme A synthetase (Cp4.1LG03g06490.1), and a proline-rich protein-like isoform X1 (Cp4.1LG02g02430.1).

In the RS vs. Control comparison (Figure 2B; Table 1; Supplementary Table S1) 126 DEPs were up-regulated after RS application, with 114 proteins having a 2-to-4-fold differential expression, and the remaining 12 a 4-fold and above increase in expression. Notably, two out of the 12 proteins, the previously-reported starch branching enzyme (Cp4.1LG03g01090.1) and a plastid disulfide isomerase (Cp4.1LG08g02890.1) had a 15-fold and nearly 8-fold increase, respectively. Five out of the 27 down-regulated proteins had an above 4-fold decrease in their expression, including a 60S ribosomal protein L7 (Cp4.1LG03g14990.1) and a glycerate dehydrogenase (Cp4.1LG15g05930.1), both with an 8-fold decrease.

In the RSPX vs. Control comparison (Figure 2C; Table 1; Supplementary Table S1) the pattern regarding the trend of up and down-regulated proteins was like that of PX vs. Control, with 17 and three proteins having above 4-fold changes in expression (increase and decrease, respectively). Interestingly, the highest expression decrease (9-fold) observed was for the glyoxalase-like domain-containing protein (Cp4.1LG18g03400.1) also reported in the RS vs. Control comparison, followed by a GDSL-lipase protein (Cp4.1LG19g06470.1) with a 6-fold decrease. Interestingly, nine out of the 17 highly expressed DEPs are common in the PX vs. Control comparison as well. Among the remaining eight DEPs (unique in this comparison), a 14-3-3 protein (Cp4.1LG11g03840.1), a pyruvate kinase family protein (Cp4.1LG19g03720.1), an ascorbate peroxidase (Cp4.1LG20g08860.1), and a protein of unknown function (Cp4.1LG05g00400.1) were reported.

Regarding the comparisons between treated sample groups (PX, RS, and RSPX) the total numbers of DEPs with a 2-fold difference in expression were much less than those in the treated vs. the Control comparisons, as shown in Table 1. For the RS vs. PX comparison (Figure 2D, Table 1; Supplementary Table S1) most DEPs with a difference of 4-fold and above were grouped as down-regulated, including two carbonic anhydrases (Cp4.1LG20g07260.1; Cp4.1LG02g12540.1) and a thiamine thiazole synthase (Cp4.1LG01g08250.1). A tolB protein-related protein (Cp4.1LG12g06350.1) was the only highly up-regulated DEP with a 7-fold increase in expression in this comparison and was found also to be the highest up-regulated protein in the RSPX vs. PX comparison, with a 5-fold up-regulation (Figure 2E, Table 1; Supplementary Table S1). A WD-40 repeat protein MSI4 (Cp4.1LG18g03560.1) and the previously-reported GDSL-lipase (Cp4.1LG19g06470.1) protein were significantly down-regulated (nearly 5- and 6-fold, respectively) in RSPX vs. PX and RSPX vs. RS, respectively (Figure 2E,F, Table 1; Supplementary Table S1).

The overlap and differences shared between the three main comparisons for DEPs with p -values < 0.05 at three different levels of expression magnitude (>1 -, >2 -, and >4 -fold, respectively) are shown in Figure 3. A small number of highly expressed proteins (13, seven, and eight DEPs) are unique in each treatment (PX, RS, and RSPX respectively) and even less (below six DEPs) are shared between groups (Figure 3C). The numbers of shared and unique DEPs between all groups' comparisons are shown in Supplementary Figure S3.

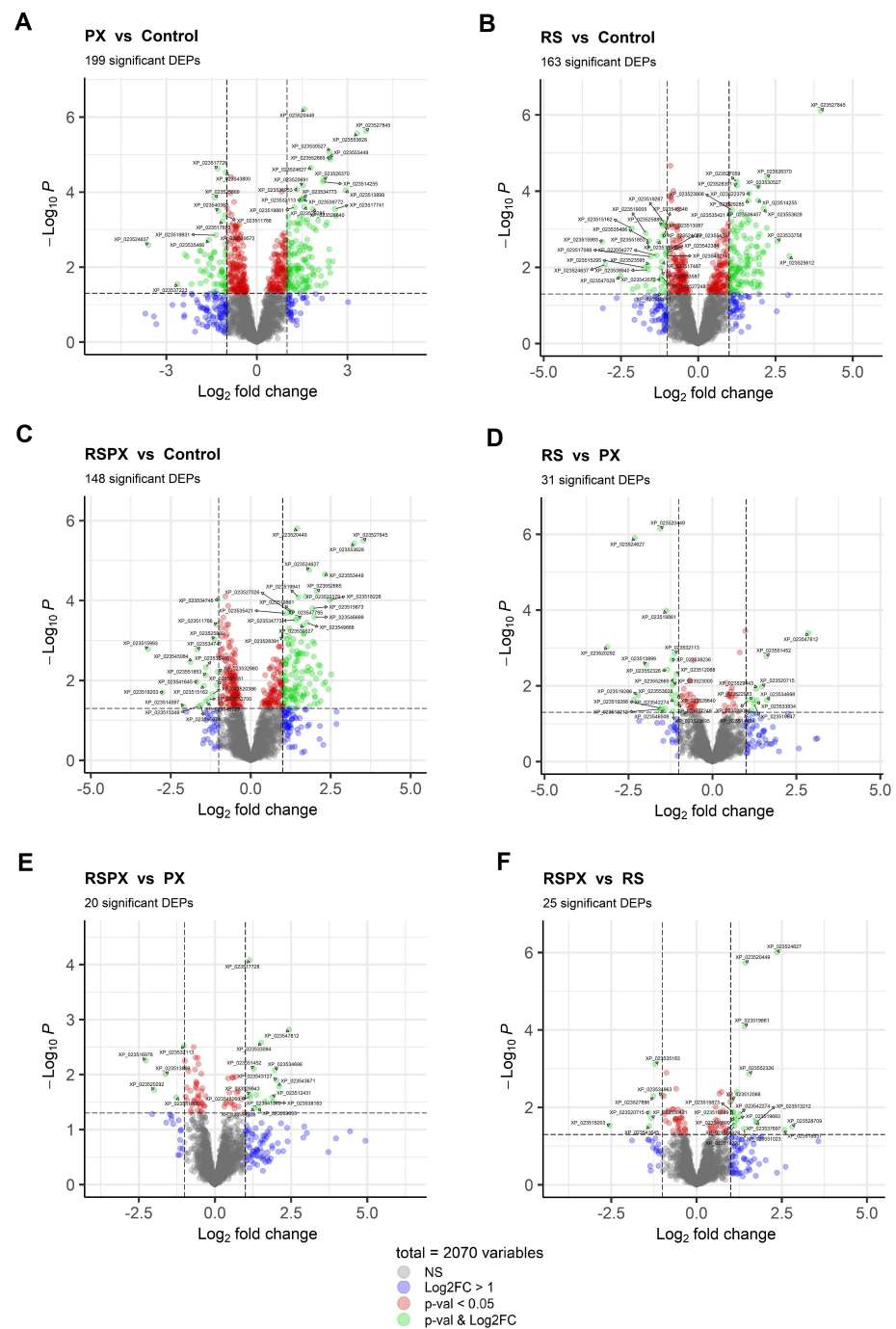


Figure 2. Identification of differentially expressed proteins (DEPs). Volcano plots show the DEPs in each of the six (A–F) comparisons for the four treatments. Treatment names are as described in Table 1. The horizontal axis represents the fold change in protein expression between the two sample groups (Log2 fold change), and the vertical axis represents the p -value-based significance of the DEPs between the two sample groups ($-\log_{10} P$). Threshold lines for DEPs screening criteria are indicated by dashed lines (placed as a visual aid), with the horizontal line representing the statistical significance threshold (p -value < 0.05) set for the current analysis and the vertical ones placed at fold-change equals two. Non-significant (NS) DEPs (p -value > 0.05) are shown in gray (<2-fold change in expression) and blue (>2-fold change in expression). Significant (p -value < 0.05) up-regulated and down-regulated DEPs are shown as red (<2-fold change) and green (>2-fold change) dots, respectively. Proteins with a 4-fold and above difference in expression are depicted with their corresponding IDs.

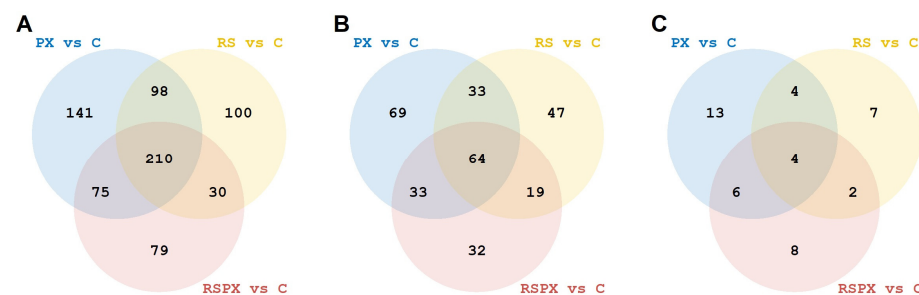


Figure 3. Venn diagrams showing the overlap and differences of DEPs with p -values < 0.05 shared between the three comparisons of treated (PX, RS, RSPX) samples versus the Control. Treatment names are as described in Table 1. Overlaps for expression magnitudes above 1-fold (A), 2-fold (B), and 4-fold (C) difference in expression are shown.

3.2. Functional Enrichment Analyses of *C. pepo* Differentially Expressed Proteins

3.2.1. KEGG Analysis

The KEGG analysis revealed pathways that were enriched either in common between different sample group comparisons, or uniquely for a certain comparison. A collective presentation of 26 enriched pathways deduced from all comparisons with p -values < 0.05 is shown in Table 2. The complete list of enriched pathways, including those with p -values ranging from 0.05 to 0.1, along with the specific gene IDs within each pathway, is provided in Supplementary Table S2.

In the PX vs. Control comparison, four (4) pathways appeared unique; “Pentose phosphate pathway; cpep00030”, “Carbon fixation in photosynthetic organisms; cpep00710”, “Proteasome; cpep03050”, and “Fatty acid degradation; cpep00071”. The first two contained DEPs with expression differences greater than 4-fold. Similarly, four (4) pathways were exclusively enriched in the RS vs. Control comparison, the “Biosynthesis of secondary metabolites; cpep01110”, “Flavonoid biosynthesis; cpep00941”, “Starch and sucrose metabolism; cpep00500”, and “Tryptophan metabolism; cpep00380”, with the first one being the most enriched, featuring 25 proteins and highly differentially expressed DEPs across three of the four groups. In the RSPX vs. Control comparison five (5) unique pathways were reported; “Pyruvate metabolism; cpep00620”, “Fatty acid biosynthesis; cpep00061”, “ABC transporters; cpep02010”, “Biosynthesis of cofactors; cpep01240”, and “Monobactam biosynthesis; cpep00261”. Five more enriched pathways the “Glutathione metabolism; cpep00480”, “Ascorbate and Aldarate metabolism; cpep00053”, “Thiamine metabolism; cpep00730”, “Glyoxylate and dicarboxylate metabolism; cpep00630”, and “Ribosome; cpep03010” were also reported, with the first three being common in the PX and RSPX vs. Control comparisons, and the remaining two being common in the PX and RS sample groups vs. the Control.

Three pathways were reported in all three comparisons of treated sample groups (PX, RS, and RSPX) with the Control, with “Metabolic pathways” being highly enriched with 47, 46, and 40 proteins annotated in the three comparisons, respectively.

A smaller number of KEGG pathways were enriched in comparisons between the treated groups (PX, RS, and RSPX) (Table 2). In the RS vs. PX comparison, three pathways were enriched, including two previously-identified (“Metabolic pathways” (cpep01100) and “Biosynthesis of cofactors” (cpep01240)). In the RSPX vs. PX comparison, three enriched pathways were identified: “Biosynthesis of amino acids” (cpep01230), “Polycomb repressive complex” (cpep03083), and the previously-reported “Carbon fixation in photosynthetic organisms” (cpep00710). Notably, “Thiamine metabolism” (cpep00730), previously reported in PX and RSPX vs. Control comparisons, was also enriched in the RS vs. PX and RSPX vs. RS comparisons, with a highly significant p -value (p .DE close to 0). Of the eight enriched pathways across comparisons, six contained DEPs with expression differences greater than 4-fold. Notably, in the RS vs. PX comparison, the “Metabolic pathways” contained almost all DEPs with highly significant differential expression.

Table 2. Functional enrichment analysis of differentially expressed proteins. Enriched KEGG pathways in all six comparisons between the four different sample groups (cases). Enriched pathways which are common or unique in the three comparisons between the treated sample groups (PX, RS and RSPX) and the Control, and in the three comparisons between the treated sample groups are presented in the upper and lower part of the table respectively. The numbers of differentially expressed proteins and corresponding *p*-values are presented under each comparison for each pathway.

KEGG Pathways				PX vs. Control ¹		RS vs. Control		RSPX vs. Control	
Group ²	Map Code	Pathway Description	N ³	DEPs ⁴	p.DE ⁵	DEPs	p.DE	DEPs	p.DE
1	cpep00030	Pentose phosphate pathway	85	4 ⁶	0.021				
	cpep00710	Carbon fixation in photosynthetic organisms	114	5	0.013				
	cpep03050	Proteasome	90	5	0.005				
	cpep00071	Fatty acid degradation	66	3	0.048				
2	cpep01110	Biosynthesis of secondary metabolites	1593			25	0.021		
	cpep00941	Flavonoid biosynthesis	31			2	0.042		
	cpep00500	Starch and sucrose metabolism	186			5	0.047		
	cpep00380	Tryptophan metabolism	67			3	0.034		
3	cpep00061	Fatty acid biosynthesis	62					3	0.020
	cpep02010	ABC transporters	70					3	0.028
	cpep01240	Biosynthesis of cofactors	311					8	0.008
	cpep00620	Pyruvate metabolism	131					4	0.035
	cpep00261	Monobactam biosynthesis	12					2	0.005
4	cpep00480	Glutathione metabolism	113	5	0.013			4	0.021
	cpep00053	Ascorbate and aldarate metabolism	81	4	0.013			4	0.007
	cpep00730	Thiamine metabolism	29	2	0.050			2	0.030
5	cpep00630	Glyoxylate and dicarboxylate metabolism	109	4	0.005	5	0.003		
	cpep01200	Carbon metabolism	367	10	0.008	8	0.040		
	cpep03010	Ribosome	475	13	0.042	10	0.028		
6	cpep01100	Metabolic pathways	3036	47	0.021	46	0.000	40	0.003
	cpep00970	Aminoacyl-tRNA biosynthesis	74	4	0.009	3	0.044	4	0.004
	cpep00902	Monoterpenoid biosynthesis	2	1	0.022	1	0.021	1	0.018
KEGG Pathways				RS vs. PX		RSPX vs. PX		RSPX vs. RS	
Group	Map Code	Pathway Description	N	DEPs	p.DE	DEPs	p.DE	DEPs	p.DE
7	cpep01240	Biosynthesis of cofactors	311	4	0.015				
	cpep00910	Nitrogen metabolism	49	2	0.004				
	cpep01100	Metabolic pathways	3036	10	0.042				
8	cpep01230	Biosynthesis of amino acids	327			3	0.013		
	cpep03083	Polycomb repressive complex	66			2	0.004		
	cpep00710	Carbon fixation in photosynthetic organisms	114			2	0.014		
9	cpep00730	Thiamine metabolism	29	2	0.000			2	0.000

¹ **PX:** *Podosphaera xanthii* inoculated; **RS:** *Reynoutria sachalinensis* treated; **RSPX:** RS treated and subsequently PX inoculated. ² **Group:** groups of pathways that appear significantly enriched in certain sample comparisons. ³ **N:** Number of proteins included in each pathway. ⁴ **DEPs:** Differentially expressed proteins enriched in each pathway. ⁵ **p.DE:** *p*-value < 0.05 for significantly enriched pathways. ⁶ Bold numbers show KEGG pathways that include DEPs with a difference in expression of > 4-fold.

3.2.2. GO-Term Analysis

Gene Ontology term (GO-term) analysis was performed to relate potential biological functions—categorized into three groups; biological process (BP), cellular component (CC), and molecular function (MF)—to DEPs. A collective presentation of enriched GO-terms regarding all comparisons is shown in Figure 4.

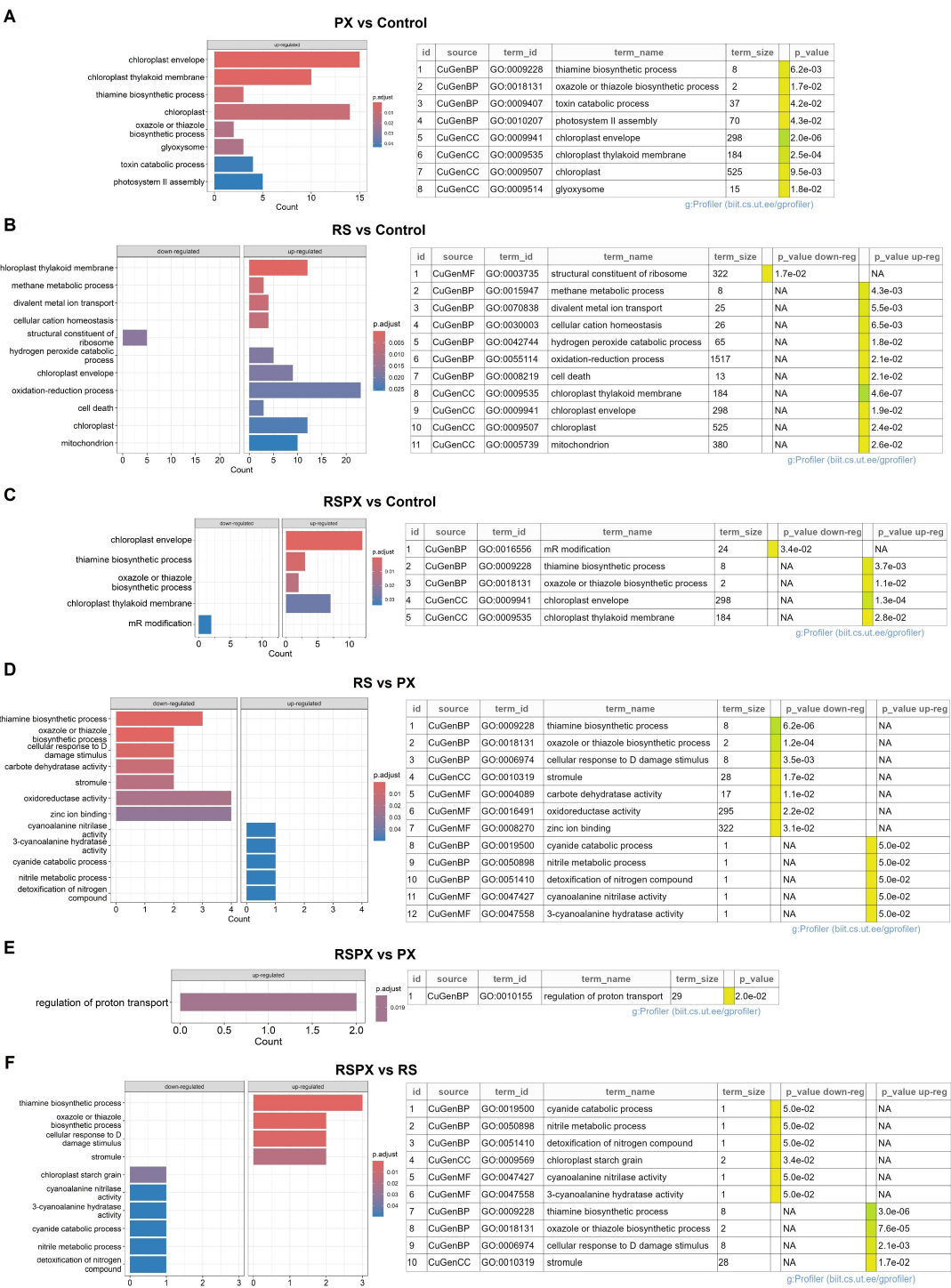


Figure 4. Gene Ontology terms (GO-terms) functional enrichment analysis of DEPs for all six comparisons (A–F) between the four treatment sample groups. Treatment names are as described in Table 1. GO-terms are presented as bar-plots on the left side of the figure, and using g:Profiler-type table outputs on the right side of the figure, respectively. The bar-plot panels on the left present quantitative data (counts) regarding up- and down-regulated DEPs, categorized from top to bottom according to significance (smaller to higher adjusted *p*-values), and irrespective classification of DEPs in main functional categories. The table panels on the right present the same GO-terms further categorized into three functional groups: biological process (BP), cellular component (CC), and molecular function (MF). GO-term IDs and sizes are also presented along with *p*-value significance scores.

Functional analysis regarding the Biological Process category revealed that 16 GO-terms were enriched, with thiamin (GO:0009228), and oxazole and thiazole (GO:0018131) biosynthetic processes enriched in positive or negative regulation in four different comparisons and positioned high up regarding significance (Figure 4A,C,D,F). Other terms included photosynthesis-related processes (GO:0010207) found in PX vs. Control (Figure 4A); ion homeostasis and transport (GO:0070838; GO:0030003), oxidation and reduction (GO:0042744; GO:0055114), and cell death (GO:0008219) all reported in the RS vs. Control comparison only (Figure 4B); cyanide and nitrile metabolic processes (GO:0019500; GO:0050898), detoxification of nitrogen compounds (GO:0051410), and cellular response to DNA damage stimulus (GO:0006974) found enriched in the RS vs. PX and RSPX vs. RS comparisons (Figure 4D,F).

In the Cellular Component category, there were seven GO-terms that were significantly enriched. These were associated with the chloroplast (GO:0009941; GO:0009535; GO:0009507; GO:0009569), with the first two spanning all three different comparisons of treated samples (PX, RS, and RSPX) versus Control (Figure 4A–C); stromule (GO:0010319), found enriched in RS vs. PX and RSPX vs. RS comparisons (Figure 4D,F), fatty acid-breakdown-related glyoxysome (GO:0009514), and mitochondrion (GO:0005739) reported in PX vs. Control and RS vs. Control comparisons, respectively (Figure 4A,B).

In the Molecular Function category, six (6) GO-terms were found to be significantly enriched. These terms were associated with cyanoalanine nitrilase and hydrolase activities (GO:0047427; GO:0047558), zinc ion binding (GO:0008270), oxidation and reduction functions (GO:0016491), and carbonyl dehydratase activity (GO:0004089), all reported in the RS vs. PX comparison (Figure 4D) with the first two also reported in RSPX vs. RS (Figure 4F). One term related to ribosomal constituents (GO:0003735) was reported in the RS vs. Control (Figure 4B).

4. Discussion

4.1. Evaluation of Proteomics Data and Selection of DEPs

For the generation of differentially expressed protein lists, we applied two criteria, a p -value < 0.05 and a 2-fold difference, as cutoff values after the examination of p -value distributions per comparison (Supplementary Figure S2), and concurrent consideration of relevant literature in proteomic analyses. All p -values regarding the three treated sample groups (PX, RS, and RSPX) against the Control (C) produced histograms that indicated the existence of cases favoring the alternative hypothesis of the test applied (i.e., significant differences), whereas the three comparisons between treated sample groups fell in sub-optimal patterns, something that is also reflected in the much lower numbers of DEPs as shown in Table 1 (approximately one fifth to one sixth when compared to total DEPs of treated sample groups against the Control). At the biological level, these observations could suggest that certain mechanisms modulated as initial plant responses after treatment with RS and/or pathogen infection (irrespective RS treatment) are not highly divergent, a hypothesis that will be further explored in the discussion of functional analyses of DEPs and the biochemical pathways modulated.

Under the selected criteria, a clear separation of the four treatment groups and a uniform replicates' intra-grouping of replicates within each treatment was observed in a cluster analysis (Figure 1). DEPs with an absolute fold-change in expression above two times (Figure 2, Supplementary Table S1) showed figures of similar order of magnitude regarding treated samples versus the Control, however, with a somehow higher number of proteins reported for PX ($n = 199$) compared to RS ($n = 163$) and RSPX ($n = 148$). A closer inspection of DEPs in PX vs. C and RSPX vs. C traced proteins that reached expression differences up to 12-fold in PX and RSPX, or even higher, up to 15-fold, in RS (Supplementary Table S1), suggesting that they could have principal roles in the relative *C. pepo* responses to each causative agent (either the pathogen, the elicitor, or their combination). Such indicative DEPs (rated equal to or highly above 4-fold) were highlighted and their putative roles, either as members of a biochemical pathway or singular/unique ones, are discussed in the next sections.

4.2. Enriched Biochemical Pathways After *P. xanthii* Infection

To define the pathways modulated early after *P. xanthii* infection, we focused on the PX vs. Control comparison. Of the thirteen pathways presented as clear candidates in Table 2 (groups 1, 4, 5 and 6), four (group 1) were found enriched only in this specific comparison.

The pentose phosphate pathway is a basal source for nicotinamide adenine dinucleotide phosphate (NADPH) provision to be utilized in several subsequent reductive biosynthetic processes. It is related to the carbon fixation pathway, contributing to the maintenance of carbon homeostasis [66,67]. In this comparison, both pathways include highly expressed DEPs (>4-fold). Such alterations in protein content and the related functions involved in were anticipated, since photosynthesis rate is one of the primary physiological aspects affected in plants after infection by biotrophic pathogens such as mildews and rusts [68]. The proteasome is a component of the ubiquitin proteasome system (UPS), the major regulatory mechanism for selective protein degradation in plants. UPS appears to be an imperative component of defense-hormone signaling pathways such as these of salicylic acid (SA), jasmonic (JA) acid, and ethylene [69,70]. A further proteasome role in mediation of a plant's SA-dependent systemic acquired resistance (SAR) to pathogens has been shown, via degradation of the transcription coactivator NPR1 (Nonexpressor of Pathogenesis-Related genes 1) [69,71]. Fatty acids (FAs), apart from being cell membrane constituents and an important energy source, play significant roles in pathogen defense. FAs breakdown products have been shown to induce basal- and effector-triggered immunity to act as signaling molecules following pathogen infection [72,73]. The enrichment of these four pathways (group 1) shows that common and standard mechanisms are also activated in *C. pepo* plants at 36 h post-*P. xanthii* infection, suggesting a road map of signaling and defense lines to be followed by the plant at the onset of powdery mildew infection.

4.3. Enriched Biochemical Pathways After *C. pepo* Elicitation by *R. sachalinensis* Extract

Biochemical pathways and DEPs highlighted in the RS vs. Control comparison indicate the RS eliciting effect on *C. pepo* plants, irrespective of pathogen infection. We have previously shown that application of RS on *C. pepo* plants effectively triggers plant responses [30]. Four pathways (group 2) appeared uniquely enriched in the RS vs. Control comparison, with "Biosynthesis of secondary metabolites", "Flavonoid biosynthesis", and "Starch and Sucrose metabolism" containing highly expressed DEPs (Table 2).

Plant secondary metabolites (PSMs) are a large heterogeneous class of chemical compounds utilized as part of the plants' innate immune system in their defense against abiotic and biotic stressors. PSMs function as end-products for defense, or signaling molecules often associated with expression of defense genes and accumulation of related end-product metabolites [74]. Flavonoids are PSM-synthesized by plants to serve diverse physiological roles such as phytoalexins, antioxidants, antimicrobials, allelopathy, and signaling molecules [75]. A variety of different enzymes including catalases, isomerases, reductases, hydroxylases, and dioxygenases can act in and selectively divert the flavonoid biosynthesis pathway, thus leading to a different flavonoid subclass regarding the aim to be accomplished each time [76,77]. In the RS vs. Control comparison, both pathways are significantly enriched and represented with a total of 25 DEPs (two being common) of which eight are highly expressed (>4-fold), including three dehydrogenases, two catalases, two peroxidases, two phosphatases, one phospholipase, one flavanol synthase, and three proteins yet uncharacterized. Our previous results have shown that RS application had an impact on the production of the phenolic compounds salicylic acid and p-coumaric acid and the flavonoid caffeic acid in *C. pepo* plants [30], supporting the eliciting role of RS in modulation of DEPs involved in PSM production.

Starch degradation, besides its role as carbon source for sucrose synthesis, is closely integrated with processes of a plant's secondary metabolism [78], providing sugar metabolites as skeleton molecules for defense compounds such as flavonoids, stilbenes, lignin, and callose, or as sugar signaling molecules acting directly, or in conjunction with, redox-mediated and hormone-regulated gene expression processes [79,80]. There is also evidence

supporting a direct role of these sugar metabolites as priming molecules, under the concepts of “sweet priming” and “sweet immunity”, where they stimulate plant defenses against fungal pathogens by modulating sugar efflux transporters [81]. Sucrose synthesis is catalyzed in the cytosol by sucrose-phosphate synthase and sucrose-phosphate phosphatase, two enzymes that were found enriched in the starch and sucrose metabolism pathway.

In previous research we showed that RS elicited callose formation in *C. pepo* plants along with secondary metabolites as stated previously [30]. Callose formation may be related with the modulation of starch and sucrose metabolism by RS. However, further experimentation towards an untargeted or oriented metabolomics approach to connect RS-elicited production of callose and previously-reported secondary metabolites to starch and sucrose metabolism is required. This could unravel roles of sugars as signaling molecules, and further describe groups of secondary metabolites produced.

4.4. Biochemical Pathways Enriched Due to Synergistic Action of RS-Priming Followed by *P. xanthii* Infection

Elicitors also function by priming plants, setting various biochemical mechanisms in a “stand-by” mode to be activated in a faster and/or more robust way at the onset of a subsequent pathogen infection and onwards [82,83]. In the RSPX vs. Control comparison, the putative synergistic effects of RS-priming and the pathogen’s infection-trigger could be an anticipated plant state. A total of 11 pathways were enriched in this comparison (Table 2). Five pathways (group 3) were found to be enriched in this comparison only.

Pyruvate is a product of glycolysis, a catabolic biochemical process categorized as part of the primary metabolism. The activation of primary metabolism to meet energy demands in plant defense against pathogens has been previously reported [84,85]. In the enriched pathway, four enzymes were represented, including a dehydrogenase and a pyruvate kinase, both of which were highly up-regulated (more than 4-fold). Under the hypothesis of a rapid response of RS-primed *C. pepo* plants to *P. xanthii* infection, it is likely that the increased energy demands lead to the activation of primary metabolism pathways in RSPX-treated plants.

Three additional pathways related to “Monobactam”, “Cofactors”, and “Fatty acid” biosynthesis, are represented by a total of 13 DEPs. Monobactam is a commonly-used antibiotic against bacteria, and its biosynthesis pathway has been related to antifungal activities [86,87]. The “Biosynthesis of cofactors” pathway includes two inositol monophosphatase-like enzymes and two thiamine thiazol synthases (TTSs). The former is an enzyme involved in the metabolism of inositol, a molecule shown to act as a second messenger for various plant responses [88,89]. The latter is an enzyme involved in thiamin metabolism. TTS was present in the relevant pathway which appeared significantly enriched (groups 4 and 9) in four out of six treatment comparisons and was shown to be related to the PX-induced plant responses, as discussed previously. The role of fatty acids as signaling molecules was previously noted in the context of the “Fatty acid degradation” pathway. However, further experiments are needed to validate their roles, both as degraded and newly synthesized products, in PX- and RS-mediated plant responses.

4.5. Enriched Biochemical Pathways Activated as Common Response Mechanisms or Revealing Further Differences Between Treated Groups

Several metabolic pathways (groups 4–6) were found to be common across the three treatments versus Control comparisons, suggesting that RS mimics PX in inducing *C. pepo* responses (groups 5 and 6) or that PX-induced effects are dominant regardless of RS treatment (group 4).

Regarding group 4 pathways, glutathione, along with reactive oxygen species (ROS) play important roles in the activation of plant resistance during biotic stress, mainly through the hypersensitive response [90–94]. Ascorbate has been shown to form part of a complex network for modulating basal and induced plant resistance to pathogens via defense-hormonal signaling and NPR1-dependent induction of defense genes’ expression. Furthermore, its production was shown to be activated by applications of various elicitors [95–97].

Thiamine (vitamin B1) has been reported to activate plant resistance against *Sclerotinia sclerotiorum*, *Plasmopara viticola*, and *Rhizoctonia solani* by modulating several key regulators of NADPH oxidase, HR-like cell death responses and the phenylpropanoid pathway [98–103]. Furthermore, in arabidopsis plants, thiamine-induced priming was shown to be dependent on hydrogen peroxide and the NPR1 gene [104].

Our previous findings on NPR1-related gene expression in *C. pepo* plants following *P. xanthii* infection, and their regulation through defense-related hormones [30], align with the proposed roles of ascorbate and thiamine in NPR1-dependent up-regulation of defense genes in PX-infected plants.

Regarding the comparisons between different treatments, the thiamine metabolism pathway is also found enriched in the RS vs. PX and RSPX vs. RS cases, deductively suggesting a pivotal role in pathogen-induced *C. pepo* responses.

Of the four pathways reported in the RSPX vs. PX comparison, the “Polycomb repressive complex” stands out, as it is composed of proteins that epigenetically repress gene expression. This pathway is represented by two DEPs, a WD-40 repeat-containing protein MSI4, which is a histone-binding subunit targeting chromatin assembly factors with a role in epigenetic control [105], and a ubiquitin-conjugating enzyme E2 with a role in ubiquitin-proteasome system in plant-immune responses [106]. Previous research demonstrated that altered epigenetic control of *C. pepo* cultivar with intermediate resistance to *P. xanthii* led to SA-mediated physiological and SAR responses [57]. As for the RSPX vs. Control comparison (previously commented on) the RSPX vs. PX comparison could provide information regarding the primed state of the plant due to RS treatment. Epigenetically controlled processes align with the concept of priming, highlighting the need for further investigation into the role of these modifying enzymes and related biochemical pathways in RS-primed plants.

4.6. Relation of KEGG and GO-Term Functional Analyses

We used Gene Ontology term analysis as a parallel approach to KEGG, aiming to categorize DEPs through a different grouping framework and compare both outcomes. Many pathways categorized under biological process, such as thiamin metabolism, photosynthesis, nitrogen-containing metabolites processing, and cell death were also reflected in the KEGG analysis (Figure 4; Table 2). Similarly, spatial connections of cellular processes occurring in the chloroplast, stromule, mitochondrion, and glyoxysome process, are related to several biochemical reactions described by KEGG pathways, since these take place in the relevant cell organelles. Both approaches for functional categorization seemed to be in line, suggesting specific pathways to focus on for future in-depth study regarding *C. pepo* early spatiotemporal responses to *P. xanthii* infection and RS elicitation.

4.7. Highly Expressed Proteins in *P. xanthii*-Infected and RS-Treated Plants and Other Defense-Related Proteins of Interest

Of the 297 proteins with an expression of above 2-fold (Figure 3B), 44 had an expression level ranging from 4- to 15-fold. Of these, 28 proteins were unique in treated plants (PX, RS, and RSPX) compared to Control (13, 7, and 8 DEPs, respectively), with the rest being shared among different groups (Figure 3C). Several of these 44 highly expressed proteins (Supplementary Table S1) including a starch branching enzyme, a thiamin thiazol synthase, ATPase subunit proteins, carboxylases, carbonic anhydrases, and kinases, were reported as members in different enriched KEGG pathways, and their putative functional roles through those biochemical pathways of various treatment cases have been discussed previously. Among the unique DEPs reported in the three treatments, which furthermore do not fall in a previously-reported KEGG pathway, two ABC-1 domain transporter proteins also annotated with a kinase activity, a glutaredoxin, and a lipid transfer protein in PX-related group, a tolB-related protein, an alcohol dehydrogenase, and a phosphoenolpyruvate carboxylase protein in the RS-induced group, and four proteins in the RSPX-related group,

an ascorbate peroxidase, a 14-3-3 protein, a GDSL-lipase, and a protein of unknown function, are of further interest (Supplementary Table S1).

In PX- and RS-treated samples, several defense-related proteins were among the significant DEPs, albeit with relatively low expression levels. These included a pathogenesis-related thaumatin-like protein (Cp4.1LG20g08560.1), a pathogenesis-related protein PR-4 (Cp4.1LG11g09910.1), and two leucine-rich repeat (LRR) family proteins (Cp4.1LG15g04840.1; Cp4.1LG01g02450.1). These proteins have been reported to play crucial roles in plant responses to pathogens [107,108]. Although their roles are essential, the relatively low-fold changes in expression observed in different comparisons may be due to spatiotemporal influences, which will be discussed further.

Among the 2070 annotated proteins, 70 were categorized under the term “kinase”, with 28 of these being significant DEPs in at least one treatment group comparison (Supplementary Table S1). These include, among others, an ATP-dependent 6-phosphofructokinase (Cp4.1LG01g09130.1), a pantothenate kinase (Cp4.1LG09g10200.1), two previously reported ABC-1 domain-containing proteins (Cp4.1LG05g14000.1; Cp4.1LG11g05640.1), a mitogen-activated protein kinase (Cp4.1LG13g01390.1), and an FGGY carbohydrate kinase (Cp4.1LG07g08350.1) with fold expression levels ranging from 2- up to 6-fold in certain comparisons. Kinases belonging to these family types have been reported to play important roles in plant defense and hormone signaling [108–114]. It is worth noting that among the 70 reported kinases there is a receptor-like kinase 1 (Cp4.1LG20g01680.1), a receptor lectin kinase (Cp4.1LG15g03810.1), and a leucine-rich repeat protein kinase family protein (Cp4.1LG03g03340.1). Notably, three kinases—receptor-like kinase 1 (Cp4.1LG20g01680.1), receptor lectin kinase (Cp4.1LG15g03810.1), and leucine-rich repeat protein kinase (Cp4.1LG03g03340.1)—though showing higher *p*-values and low to moderate expression levels in all comparisons, may still be of interest because they belong to kinase families that mediate plant defense responses [108,115,116].

Further experimentation at the individual protein or biochemical pathway level is required for the verification of the functional roles of these DEPs in pathogen infection, elicitation, and priming, including a spatiotemporal framework. Moreover, although certain DEPs seem to be modulated by RS, our understanding of the molecular mechanisms via which those proteins participate in *C. pepo* resistance and priming is still limited. A unique RS mode of action seems unlikely, given that multiple biochemical pathways are activated.

The spatiotemporal pattern of protein expression in *C. pepo* plants should also be considered. In this research, protein expression was studied at the very early stage of *P. xanthii* infection and at whole phyllosphere level, under the concept of shaping up the plant's molecular responses at the onset of infection. Extending the time course beyond the first 36 h post-infection up to several days would allow us to track changes in DEPs composition and expression levels over time, in relation to the progression of powdery mildew disease, the pathogen's life cycle [5], and differences in the sequel of plant defense responses. Furthermore, the responses in leaves of different age [117] or due to activation of systemic acquired resistance (relation between the site of infection and responses far away from it) are also parameters of interest. As noted earlier, several defense-related proteins showed moderate to low expression levels. As noted earlier, there are various defense-related proteins with a moderate to low level of expression. A relative high concentration accumulation of such proteins locally (i.e., at a certain leaf or leaf section) would be diluted when plant responses are studied at whole phyllosphere level.

5. Conclusions

A systematic profiling of 2070 annotated peptides was conducted from the phyllosphere of *C. pepo* plants after *P. xanthii* (PX) infection and *R. sachalinensis* (RS) application. KEGG and Gene Ontology pathway enrichment analyses revealed several PX-modulated biochemical pathways, the most important ones including proteasome, pentose phosphate, carbon fixation, and thiamin metabolism, and RS-induced pathways such as biosynthesis of

secondary metabolites, and starch and sucrose metabolism. Additionally, highly expressed proteins outside of specific enriched pathways, as well as specific protein groups such as kinases, were revealed, suggesting potential key roles in *C. pepo* responses. Further research, including untargeted and targeted metabolomics, along with epigenetic regulation studies, is proposed to unravel these roles.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app142110061/s1>, Figure S1. Polyacrylamide gel electrophoresis of protein extracts from all different treatments' samples. Figure S2. Histograms of *p*-value frequencies of the six comparisons for the different sample treatments. Figure S3. Histogram of shared and unique DEPs (intersections) among the six different comparisons. Table S1. List of the 2070 annotated proteins and analysis output. Table S2. Lists of KEGG pathways and Genes by pathway.

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