

Quantitative proteomics analysis of high and low polyphenol expressing recombinant inbred lines (RILs) of peanut (*Arachis hypogaea* L.)

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ABSTRACT

To facilitate selective breeding of polyphenol-rich peanuts, we looked for mass spectrometry-based proteomic evidence, investigating a subset of recombinant inbred lines (RILs) developed by the Australian peanut breeding program. To do this, we used label-free shotgun proteomics for protein and peptide quantitation, statistically analyzed normalized spectral abundance factors using R-package, as well as assayed important antioxidants. Results revealed statistically significant protein expression changes in 82 proteins classified between high or low polyphenols expressing RILs. Metabolic changes in polyphenol-rich RIL p27-362 point towards increased enzymatic breakdown of sugars and phenylalanine biosynthesis. The study revealed phenylpropanoid pathway overexpression resulting in increased polyphenols biosynthesis. Overexpression of antioxidant enzymes such as catalase, by 73.4 fold was also observed. A strong metabolic correlation exists with the observed phenotypic traits. Peanut RIL p27-362 presents a superior nutritional composition with antioxidant-rich peanut phenotype and could yield commercial profits. Data are available via ProteomeXchange with identifier PXD015493.

1. Introduction

Polyphenolic antioxidant compounds including flavonoids prevent oxidation of other molecules, which is a process that if not stopped, would cause the production of reactive oxygen species (ROS) in cells that damage cellular material (Kurutas, 2016). Polyphenolic compounds have been known for their antioxidant and anti-inflammatory properties in reducing platelet aggregation (Bansode, Randolph, Plundrich, Lila, & Williams, 2019), as well as anti-diabetic properties due to their reducing effect on glycemic index (Christman, Dean, Allen, Godinez, & Toomer, 2019). In particular, resveratrol is known for its vaso-relaxing role through protection from ROS damage and cancer prevention (Chaplin, Carpena, & Mercader, 2018). With growing evidence from animal models and epidemiologic studies, a positive relationship is now well established between dietary intake of phenolic antioxidants and health benefits and disease. In peanuts, resveratrol concentrations were reported to be low in leaves of natural tetraploid and diploid plants, compared to natural polyploids (Carvalho et al.,

2017). This is likely due to secondary natural selection. Resveratrol genetic sequences showed extensive variation between species, which demonstrates their intra-specific variation.

Peanut genome sequences of the two diploid progenitors *Arachis ipaensis* and *A. duranensis* were made publicly available in 2016 (Bertioli et al., 2016). Unlike rice, wheat or soybeans, which have extensive proteome maps across different tissues, such data are lacking for peanut. Despite the genomic studies of *Arachis hypogaea* being hindered by the lack of heterogeneity (Jiang et al., 2007; Moretzsohn, Hopkins, Mitchell, Kresovich, Valls, & Ferreira, 2004), proteomic methods are being applied to help tackle research challenges. Proteomics studies in peanuts mainly involve characterization of crop diseases like late leaf spot disease (Kumar & Kirti, 2015), analysis of new cultivars and growth stages (Wang, Ma et al., 2016), gene transcript aided proteomics on drought stress (Carmo et al., 2019; Kottapalli et al., 2013) and allergome characterization (Stevenson, Chu, Ozias-Akins, & Thelen, 2009). Peanut polyphenols – peanut protein interaction studies have gained momentum and show beneficial anti-allergenic antioxidant

Abbreviations: AAPH, 2,2'-Azobis (2-amidinopropane) dihydrochloride; CoA, Coenzyme A; CCV, Continuing Calibration Verification; HPLC, High Pressure Liquid Chromatography; MIAPE, Minimum Information About a Proteomics Experiment; NSAF, Normalised Spectral Abundance Factors; ORAC, Oxygen radical absorbance capacity; PCA, Peanut Company of Australia; ROS, Reactive Oxygen Species; RIL, Recombinant Inbred Line; TFA, Trifluoroacetic acid; Trolox, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid

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effects of peanut skin polyphenols in imparting reduced allergenicity of peanut. In 40% peanut skin polyphenols enriched peanut aggregates (w/w), IgE binding, histamine and β -hexosaminidase levels were reduced significantly by at least 20%, 49% and 58% respectively compared to plain peanut flour, and also inhibited calcium ion mediated cell degranulation (Bansode, Plundrich, Randolph, Lila, & Williams, 2018). Furthermore, polyphenols-peanut protein aggregates using higher concentrations of cranberry and blueberry extracts significantly reduced plasma IgE allergic response in mice previously sensitized to peanut (Bansode, Randolph, Plundrich, Lila, & Williams, 2019). Increased levels of polyphenols further decreased CD63 antigen protein expression in spleen lysate of these sensitized mice and may indicate reduced basophil activation. In a similar note, cranberry fruit polyphenols were reported to significantly reduce cell degranulation and IgE responses to peanut allergens, and suggested quercetin to present hypoallergenicity properties (Plundrich, Bansode, Foegeding, Williams, & Lila, 2017). Furthermore, resveratrol has been noted for its role in protecting important enzymes from oxidative damage, and upon bioaccumulation, it could also improve activities of antioxidant enzymes like catalase and peroxidases indirectly (He, Xue, Zhang, Guo, Ma, & Yang, 2018).

Studies on flavonoid biosynthetic pathway using omics technologies have been performed in soybean, barley seedlings and strawberry mutants (Hanhineva, Kokko, Siljanen, Rogachev, Aharoni, & Karenlampi, 2009; Hjerno et al., 2006; Kaspar, Matros, & Mock, 2010), and such studies in peanuts are beginning to gain momentum (Sales & Resurreccion, 2014). In peanuts, antioxidant biosynthesis deployed to regulate stress from selenite toxicity was observed when key enzymes phenylalanine ammonia-lyase and cinnamate 4-hydroxylase of the phenylpropanoid biosynthesis were reported to show increased activity and up-regulated gene expression in response to selenium-stress (Wang, Wu et al., 2016). The first steps into the biosynthesis of prenylated stilbenoids were recently elucidated in peanuts, where prenylation of resveratrol and stilbenoids including arachidins 1, 2 and 3 were investigated in five prenyltransferase gene candidates, of which two were found to be stilbenoid related (Yang et al., 2018). With the newly available peanut genome information and the protein database of model plants, a data mine of peanut proteins and pathways are pioneered in this study.

Protein abundance changes among genotypes would expect to affect protein abundance in many key metabolic pathways behind the phenotypic trait. Therefore, to facilitate selective breeding of polyphenol-rich peanuts, this study aimed to identify key proteomic signatures underlying the antioxidant-rich phenotypic trait by quantitatively analyzing the proteomic profiles of five peanut genotypes phenotypically differing in their phenolic antioxidant distribution and total antioxidant capacities.

2. Materials and methods

All laboratory experiments were conducted in biological and analytical triplicates. The methods described in this manuscript particularly gel electrophoresis, protein extraction, in-gel digestion, mass spectrometry, and database search and analysis are compliant with Minimum Information About a Proteomics Experiment (MIAPE) reporting guidelines for proteomics. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD015493.

2.1. Reagents

All reagents including polyphenol standards were purchased from Sigma Aldrich, St. Louis, MO, USA unless indicated differently. Fluorescein and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were sourced from Cayman chemicals (Ann Arbor, Michigan,

MI, USA). 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was sourced from AMSBIO LLC, Cambridge, MA, USA). Alcalase 2.4L FG, a subtilisin protease preparation, and Celluclast 1.5L, a cello-oligosaccharide cellulase preparation were sourced from Novozymes Corp, Denmark, partnered by Sigma Aldrich, Australia. Sequence grade modified trypsin lyophilized 20 μ g vials were purchased from Promega Corp, USA. All solvents used were HPLC analytical grade.

2.2. Peanut samples

A cross was made between two selected peanut genotypes, one expressing mid- to high-polyphenol contents and antioxidant capacity (D147-p3-115, Runner type) sourced from the Australian peanut breeding program (Peanut Company of Australia (PCA), Kingaroy, Queensland, Australia) and the other expressing low- to mid-levels of the same traits (Farnsfield, Runner type) sourced from ACI Seeds, Georgia, USA, as parents to develop recombinant inbred line (RIL) population with genetic diversity. This cross resulted in the 'p27' peanut RILs with a normal distribution of antioxidant capacity expression (Phan-Thien, Wright, Tillman, & Lee, 2014). A population of 20 selected p27 RILs (including the two parents D147-p3-115 and Farnsfield) were evaluated in multi-location trials from 2013 to 2015, and RILs showed normally distributed antioxidant capacity expression and polyphenol content (data not shown).

In the randomized block design trial 2014 used for samples in this study, crops were planted in 2 m by 5 m in length plots in duplicates, containing rows of over 100 plants each, grown at the Queensland Department of Agriculture and Fisheries, Taabinga Research Station, Kingaroy, Australia in southern inland Queensland. Field geographical coordinates are 26°35'37.5 S 151°49'45.7 E. Growing conditions were typical with soil temperature reaching 18 °C for planting, fields not grown peanuts for at least 3 years and not having a history of soil-borne diseases. Kernels were harvested upon crop maturity. Kernel samples were sourced from plots separated from each other to account for the randomization of sampling and biological variation for our experiments. At harvest, pods were removed from plants of each plot and dried down to safe pod moisture (~10%) for storage. Pods were then shelled out using a mechanical sheller and kernels were stored in at –80 °C.

A subset of three peanut RILs (p27-272, p27-363, p27-036) with high to low total antioxidant capacities as well as their two-parent cultivars (D147-p3-115 and Farnsfield) harvested in Taabinga in 2014 (PCA Kingaroy, Queensland, Australia) were selected from the normally distributed population for subsequent proteomics and polyphenol antioxidant content analysis in this study. For the two-parent cultivars D147-p3-115 and Farnsfield, kernels were sourced from harvests of rep1plot 15 and rep1plot 21 respectively. For the three peanut RILs p27-p272, p27-p362 and p27-p036, kernels were sourced from p27rep1plot 13, p27rep1plot 20 and p27rep1plot 9 respectively.

2.3. Polyphenolic antioxidant compounds extraction and oxygen radical absorbance capacity (ORAC) assay

All kernels used for the ORAC assay were separated from their testa and germ, frozen in liquid nitrogen and ground to a powder in an analytical mill (IKA Works, Malaysia). Five grams (5–7 kernels per replicate) of ground peanut were defatted in two cycles using *n*-hexane at 1:8 w/v ratios, vortexed for 2 min, sonicated at 50 kHz at 40 °C for 5 min, and centrifuged at 9,500 $\times$ g for 10 min (Prior et al., 2003). The defatted residue was gently flushed with nitrogen to a dry flour in a fume hood. Polyphenols were extracted by sonicating the defatted flour of each sample in a 10 mL solution of acetone/ water/ acetic acid at 70:29.5:0.5 (v/v/v) at 50 kHz and 37 °C for 5 min, followed by centrifuging at 3500 rpm for 15 min. The supernatant was collected and 25 μ L of this hydrophilic antioxidant extract used for the subsequent

ORAC antioxidant assay performed similarly to the method reported by Phan-Thien, Wright, & Lee (2013). Using 75 mM phosphate buffer pH 7.4, a 70 nM Fluorescein solution and 140 mM AAPH solution were freshly prepared, and the two were incubated at 37 °C for 60 min and 30 min respectively. Trolox working standards were prepared from 1.5 mM standard vials at 10, 25, 50, 75 and 100 μ M concentrations. An external Continuing Calibration Verification (CCV) standard of Trolox was used at 25 μ M and 75 μ M concentrations as quantitation validation. The hydrophilic extracts, the internal Trolox standards, and the external CCV standards were dispensed to their respective microwells in a black microtiter plate first, followed by 150 μ L Fluorescein and 25 μ L AAPH. The ORAC microtiter plate assay was performed using quadruplicates and each assay was run for 2.25 h with 121 cycles after injection at cycle 1, and absorbance measurements were commenced at cycle 5 to allow for complete dispensing. Excitation and emission wavelengths were 485 nm and 535 nm respectively and the ORAC measurements for samples were extrapolated from the Trolox standard curve as Trolox Equivalents (TE) represented as μ g TE g⁻¹.

2.4. Extraction of polyphenols with and without enzyme-treatment

For polyphenols profiling by High-Pressure Liquid Chromatography (HPLC) analysis, extractions with and without dual enzyme treatment were prepared in neutral pH to obtain polyphenols unbound to peanut matrix (denoted as free polyphenols) and total polyphenols consisting of matrix-bound and -unbound polyphenols. The matrix-bound polyphenols were calculated from their difference. Extracts of free polyphenols were prepared from 5 g of defatted peanut flour by shaking in 50 mL of 80% methanol overnight on a roller mixer, with two repeats. The volume of combined supernatant was reduced by evaporating with nitrogen gas at 40–50 °C and filtering through a 0.45 μ m polytetrafluoroethylene (PTFE) filter. Enzyme-treated extracts were prepared by incubating 5 g defatted peanut flour with Alcalase 2.4L (protease enzyme activity 2.4 Anson units/ g, 1 mL), Celluclast 1.5 L (cellulase enzyme activity $\geq$ 700 beta-glucanase units/ g, 1 mL) and MilliQ water (8 mL), overnight at 40 °C. Optimum temperature recommended for Alcalase was 37 °C at pH 8, while Celluclast was at 52 °C at pH 6, hence the experiment was conducted at 40 °C neutral pH with a higher concentration of Celluclast than Alcalase for an increased length of time (15 h). Methanol (40 mL) was added and the mixture was agitated overnight on a ten-pin roller mixer. The tube was then centrifuged (9,500 $\times$ g, 10 min) and the supernatant collected. This step was repeated twice with 90% aqueous methanol (25 mL). The volume of combined methanolic extract was reduced as described before. The extracts were centrifuged (9,500 $\times$ g, 10 min), filtered through a 0.45 μ m PTFE filter and then diluted with acidified water to pH 2 to result in a 5% methanol solution. The extracts were acidified to pH 2 in a method previously demonstrated on 19 key polyphenolic compounds resulting in improved recovery and optimal extraction of phenolics and flavonoids composition. Extracts contained minimal degradation at pH 2 (Phan-Thien et al., 2013).

2.5. Solid-phase extraction of polyphenol antioxidant compounds

Solid-phase extraction was performed with Strata-X (33 μ m, 85 Å, 100 mg) polymeric reversed-phase sorbent tubes (Phenomenex, NSW, Australia) using method previously published (Phan-Thien et al., 2013). Briefly, the SPE column was pre-conditioned with acidified methanol (5 mL, pH 2) followed by water (5 mL). Each extract was loaded at 1 mL min⁻¹. After washing the column with water, elution was performed by loading HPLC grade methanol (2 mL) under gravity. The eluate was evaporated to 1 mL and filtered through a 0.45 μ m PTFE syringe filter for HPLC analysis.

2.6. HPLC analysis of dominant flavonoid and polyphenolic compounds

HPLC of peanut polyphenol extracts was monitored at 320 nm for the optimized profiling of key antioxidant compounds among RILs. Polyphenol antioxidant standards used for this analysis were caffeic acid, *p*-coumaric acid, ferulic acid, *o*-coumaric acid, salicylic acid, resveratrol, daidzein, *t*-cinnamic acid, and quercetin, sourced from Sigma Aldrich (St. Louis, Missouri, USA). The separation was conducted on a Gemini reversed-phase C18 column (5 μ m, 110 Å, 250 $\times$ 4.6 mm) with SecurityGuard C18 guard cartridge (4 $\times$ 3.0 mm) (Phenomenex, NSW, Australia) using a Shimadzu Prominence HPLC instrument (Shimadzu, Kyoto, Japan). The mobile phase solution A was 0.1% trifluoroacetic acid (TFA) in MilliQ water and solution B was 0.1% TFA in acetonitrile. About 30 μ L per sample extract was subjected to HPLC analysis with the following elution gradient: 0 min 15% B, 30 min 30% B, 31 min 15% B, 35 min 15% B, the flow rate at 1.5 mL min⁻¹. An HPLC standard mixture was injected for every 20 injections as quality control for correction of drift in the retention time, and peak intensity (Phan-Thien et al., 2013).

2.7. Peanut protein extraction

A mixed lot of 15 peanut kernels for each RIL sample in biological triplicates (total 15 samples) were powdered in liquid nitrogen in a mortar and pestle and were defatted in two cycles as described previously. For each sample, proteins were extracted from 0.5 g of defatted kernel flour with 5 mL of 50 mM Tris HCl in 200 mM NaCl buffer (pH 8.2) by shaking on a rotary mixer overnight at 4 °C, and the supernatant was collected upon centrifugation at 10,000 $\times$ g for 10 min. The protein concentration was estimated using the Bradford assay (Bio-Rad, Hercules, CA, USA). Samples were subjected to subsequent shotgun proteomics analysis using an established protocol (Neilson, et al., 2014) with modifications.

2.8. Electrophoresis and in-gel tryptic digestion for shotgun proteomics analysis

Fifteen aliquots of protein samples each containing 200 μ g solubilized proteins representing biological triplicates for the two parents and three RILs, were fractionated by SDS-PAGE using Bio-Rad 4–15% TGX mini protean precast gels. Gels were electrophoresed in the Bio-Rad mini protean tetra electrophoresis system (Hercules, CA, USA) at 70 V for 5 min, followed by 150 V for 60 min, then fixed for 1 h in 7% acetic acid, 20% methanol (v/v) before staining with colloidal Coomassie brilliant blue G-250.

Each protein lane was sliced into 16 approximately equal fractions and transferred into a 96-well microtiter plate for in-gel tryptic digestion of the proteins. The gel pieces were first pH adjusted with 100 mM NH₄HCO₃ and then washed with 50 mM NH₄HCO₃, 50% acetonitrile, (w/v) pH 8.3 until gel pieces appeared stain free. They were dehydrated with 100% acetonitrile and reduced with 50 μ L 10 mM DTT, 50 mM NH₄HCO₃ solution (w/v) at 55 °C for 30 min before alkylating in the dark with 50 μ L 55 mM iodoacetamide, 50 mM NH₄HCO₃ (w/v) at 24 °C for 30 min. The gel pieces were briefly washed with 100 mM NH₄HCO₃, then with 50 mM NH₄HCO₃, 50% acetonitrile, (w/v) pH 8.3, dehydrated with 100% acetonitrile (v/v) and air-dried. Samples were subjected to tryptic digestion using 30 μ L trypsin (freshly prepared at 12.5 ng/ μ L in 50 mM NH₄HCO₃) for 30 min at 4 °C and then incubated overnight at 37 °C. Peptides were extracted in a solution of 2% formic acid, 50% acetonitrile (v/v) in three cycles and the digests were combined for each fraction. Peptide extracts were dried via vacuum centrifuge, resuspended in 0.1% formic acid (v/v), desalted with C18 ZipTips (Merck Millipore, Darmstadt, Germany) and finally reconstituted in 10 μ L 2% formic acid (v/v).

2.9. NanoLC-MS/MS

An LTQ-XL linear ion trap mass spectrometer (Thermo Scientific, USA) was used to analyze the peptide extracts. Peptides were loaded using a Surveyor autosampler onto a reversed phase nanocolumn (75- μ m id $\times$ 9 cm length) packed in-house using C18 resin (3- μ m, 200 Å, Michrom Bioresources Inc, USA) with a fused silica capillary integrated with electrospray tip, and separated by nanoLC-MS/MS using a nano-LC Surveyor 4.0 (Thermo Scientific, USA). The column tip was positioned ~0.5 cm from the heated capillary ($T = 200^\circ\text{C}$) of the LTQ-XL, and column eluate was subjected to 1.8 kV at the low volume tee junction. Peptides were subjected to an initial wash step with buffer A (5% acetonitrile, 0.1% formic acid (v/v)) for 10 min, then eluted with a 0–50% linear gradient of buffer B (95% acetonitrile, 0.1% formic acid (v/v)) to A at 250 nL min⁻¹ over 60 min. Automated peak recognition, the dynamic exclusion for 45 s and MS/MS of the top 6 most intense ions (> 2000 counts) at 35% normalized CID were successively isolated and fragmented in data-dependent acquisition mode. A survey scan of m/z 350–2000 amu was acquired.

2.10. Peptide to spectrum matching and protein database analysis

Raw files of nanoLC-MS/MS data were converted to mzXML format were used for peptide to spectrum matching against the genome sequence of the diploid ancestors of the cultivated peanut, namely *Arachis duranensis* and *Arachis ipaensis* (Bertioli et al., 2016). The database encompassed 41,562 and 48,410 protein sequences respectively. In the peanut genome, 31.6% protein sequences of this release were uncharacterized proteins and homology returned unknown. Both forward and reverse database searches were performed using the X! Tandem algorithm run with the Global Proteome Machine software (version. 3.2.2) (Craig & Beavis, 2004). Parent ion mass tolerance was 2 Da and fragment mass tolerance was 0.4 Da. Carbamidomethylation of cysteine and oxidation of methionine was accounted for as complete and variable modifications respectively. Peptide identification data were filtered at $\log(e) - 1.6$, and only proteins identified in all three replicates with a minimum spectral count of three were retained in the final list of reproducibly identified proteins found in each sample. Using this threshold, protein FDR was less than 1% and peptide FDR was less than 0.2% in each of the final lists of reproducibly identified proteins across all samples.

2.11. Quantitation based on normalized spectral abundance factors (NSAFs) and statistical data portioning

Protein quantitation in terms of NSAFs (Zybailov et al., 2006) were calculated as the ratio of spectral counts (SpC) of a protein divided by its length (L) to the sum of all SpC/L for all proteins identified. Scrappy program with the R-package was used for this simultaneous quantitation and subsequent analyses of NSAFs (Neilson, Keighley, Pascovici, Cooke, & Haynes, 2013). Log NSAF values were subjected to further statistical *t*-test and one-way ANOVA analyses to identify proteins that may have significant abundance differences among samples, as well as to apportion proteins across samples to analyze their distribution patterns. Proteins with *p*-values of less than 0.05 were selected as statistically significant and those found to be significantly different in abundance > 2 fold were further analyzed to identify changes in expression patterns. Changes in abundance of a protein in RIL p27-362 were determined based on the statistical significance of NSAF values relative to the corresponding protein in RIL p27-272.

2.12. Protein ontology classification

Protein ontology information was extracted from the UniProt database (UniProt, 2019) and KEGG Mapper reference pathways (Kanehisa & Sato, 2020) to map the biological processes information.

Accession numbers were extracted using RefSeq IDs from NCBI. This information was further supplemented with metabolic pathway information from geneontology.org and Interpro based on reference KEGG identifiers. Proteins were then functionally classified into 23 categories based on their ontology.

3. Results and discussion

3.1. Total antioxidant capacity and polyphenols profiling of five peanut samples

Quantification of total antioxidant capacities in Trolox Equivalence (TE) using the ORAC assay confirmed the observed phenotypic traits of the five genotypes ($P = 0.0032$ between genotypes), where RIL p27-362 showed significantly greater total antioxidant capacity (217% higher) when compared to RIL p27-272. A similar trend was also observed among the parent cultivars where the total antioxidant capacity for mid-high parent D147-p3-115 was greater than mid-low parent Farnsfield. These observations made for the two peanuts parent lines are in agreement with the patterns of the high- and low- antioxidant capacities measured in Phan-Thien et al. (2013) and during the three harvest seasons between 2013 and 2015 (Table 1), and confirms that the antioxidant capacities of these peanut lines are likely to be under strong genetic control.

Polyphenolic compounds from kernels of the five selected peanut lines analyzed by reverse-phase HPLC were indicative of the quantities and distribution of antioxidant compounds in the kernel matrix (Table 1). Our optimized concentrations of Alcalase and Celluclast were approximately 0.13 U/mL⁻¹ and 17.5 GU/mL⁻¹ respectively (Phan-Thien et al., 2013), which were comparable with recent reports of the optimum enzyme concentration (168 FGB/100 g) for extraction of polyphenols from green yerba mate (Heemann, Heemann, Kalegari, Spier, & Santin, 2019). The increased incubation time and extraction conditions pH 2 and 40 °C were found optimum in this study because the doubling of cellulase concentration improved polyphenol yields (Ghandahari Yazdi, Barzegar, Sahari, & Ahmadi Gavlighi, 2019) as well as the plateau of extraction yield over time (Heemann, Heemann, Kalegari, Spier, & Santin, 2019), and showed minimal effects on degradation of polyphenolics.

The HPLC chromatograms from this study are presented as Supplementary information Fig. S1. Differences in total polyphenols levels between the high and low antioxidant capacity genotypes were reflected in the HPLC chromatograms and correlated well with ORAC values (Table 1). Antioxidant activity is directly proportional to total phenolic content and a recent study on germinated peanuts also found that phenolic compounds were the main contributor of antioxidants (Yang et al., 2019). HPLC of extracts from non-enzymatic and enzymatic methods revealed quantitative differences among individual polyphenolic content across samples and confirmed the presence of free and matrix-bound polyphenols, in agreement with our previous study (Phan-Thien et al., 2013).

Polyphenols quantified in D147-p3-115 ($P = 0.3258$), p27-362 ($P = 0.0629$) and p27-036 ($P = 0.9570$) were not affected by extraction methods unlike Farnsfield ($P < 0.0001$) and p27-272 ($P = 0.0006$). There seems a genotype-based influence of matrix-bound polyphenols, and particularly notable is an increase in matrix-free expression in the antioxidant-rich genotypes. HPLC analysis of non-enzymatic extracts illustrated abundant matrix-free composition of polyphenols in RIL p27-362 for each of the nine antioxidant compounds when compared to RIL p27-272. This observation validates the phenotypic trait observation in part for RIL p27-362. On the other hand, enzymatic extraction of polyphenols in RIL p27-362 significantly improved concentrations of all antioxidant compounds compared to non-enzymatic quantitation, except *o*-coumaric acid, indicating the matrix-bound nature of prevalence for most of these compounds.

p-Coumaric acid ($P = 0.0414$), ferulic acid/sinapic acid

Table 1

Quantitation of polyphenols by HPLC analysis of non-enzymatic and enzymatic extractions, and antioxidant capacity by ORAC assay across five peanut lines.

Phenolic compounds	D147-p3-115 (mid-high)	Farnsfield (low-mid)	RIL p27-272(low)	RIL p27-362(high)	RIL p27-036 (variably high)
<i>Non-enzymatic extraction (μg/g)</i>					
Caffeic acid	11.9 ± 4.4	19.5 ± 0.2	9.7 ± 2.4	15.6 ± 6.6	20.4 ± 8.4
p-Coumaric acid	6.3 ± 0.7	3.8 ± 0.2	1.5 ± 0.3	4 ± 1.1	8.1 ± 0.4
Ferulic acid/sinapic acid	3.8 ± 0.1	1.8 ± 0.1	0.7 ± 0.2	2 ± 0.7	6 ± 0.1
o-Coumaric acid	2.3 ± 1.0	7.3 ± 0.7	2.4 ± 0.8	6.3 ± 3.3	7.6 ± 0.4
Salicylic acid	12 ± 1.0	–	–	–	–
Resveratrol	2 ± 0.6	0.7 ± 0.2	0.6 ± 0.2	1.2 ± 0.1	1.2 ± 0.1
Daidzein	–	–	–	–	–
t-Cinnamic acid	42.5 ± 7.0	31.4 ± 1.3	19.4 ± 13.8	26.3 ± 12.8	60.5 ± 27.2
Quercetin coeluting unknown	71.7 ± 6	36.2 ± 4.7	16.6 ± 4.4	33.1 ± 11.3	59.6 ± 2.6
Sub-total	173.2	100.8	51	88.6	163.4
<i>Enzymatic extraction (μg/g)</i>					
Caffeic acid	26.9 ± 9.7	18.4 ± 2.8	13.1 ± 2.5	22 ± 10.2	19.5 ± 8.1
p-Coumaric acid	24.0 ± 14.7	21.7 ± 15.4	11.5 ± 7.3	6.7 ± 4.8	23.4 ± 14.4
Ferulic acid/sinapic acid	26.8 ± 6.8	22.5 ± 1	21.2 ± 0.9	18.6 ± 2.8	34.2 ± 4.8
o-Coumaric acid	2.2 ± 0.7	18.2 ± 0.4	2.3 ± 0.3	2.1 ± 0.5	3.8 ± 1
Salicylic acid	9.9 ± 3.1	24.6 ± 1.2	7 ± 1.2	11.3 ± 2.8	10.4 ± 1.6
Resveratrol	6.3 ± 1.7	5.2 ± 0.2	5.5 ± 0.1	5.0 ± 0.9	4.8 ± 0.5
Daidzein	5.9 ± 1.6	–	5.3 ± 1.5	6.8 ± 1.5	7.5 ± 1.6
t-Cinnamic acid	28.7 ± 9.2	86.5 ± 0.1	52.3 ± 9.5	99.9 ± 67.6	29.3 ± 9
Quercetin co-eluting unknown	72 ± 17	75.1 ± 4.8	26.7 ± 12	58.9 ± 11.2	28.6 ± 2.8
Sub-total	202.5	272	145	231.3	161.5
ORAC value of 2014 harvest (Trolox Equivalent μMg⁻¹)	1529 ± 0.7	1489 ± 0.8	930 ± 0.6	2026 ± 5.4	3354 ± 0.9
ORAC Range 2013–2015 (Trolox Equivalent μMg⁻¹)	1369–2035	967–2239	776–1073	1470–2228	1521–3354

($P < 0.0001$), salicylic acid ($P < 0.0001$), *t*-cinnamic acid, daidzein ($P < 0.0001$), and resveratrol ($P < 0.0001$) increased with the enzyme-treatment, showing that significant amounts of these compounds were tightly associated with peanut matrix. Most abundant antioxidant compounds were *t*-cinnamic acid, quercetin co-eluting unknowns and caffeic acid in RIL p27-362 compared to others, whereas daidzein, salicylic acid, ferulic acid/sinapic acid and resveratrol formed key compounds specific to peanut matrix. *o*-Coumaric acid, however, was found predominantly in matrix-free form. Matrix-bound fractions showed greater antioxidant capacity, supporting previous findings in peanut (Rocchetti, Chiodelli, Giuberti, & Lucini, 2018). In peanuts, phenolic acids and flavonoids also function as plant self-defences when responding against important disease-causing microbial pathogens (de Camargo et al., 2017). Their effectiveness in terms of antimicrobial potency and antioxidant capacity could be further enhanced in the case of polyphenol-rich peanuts.

There was a high correlation between total phenolics and antioxidant capacity, similar to our previous studies (Phan-Thien et al., 2013). HPLC analysis between the two parents validated ORAC findings, where D147-p3-115 showed higher total polyphenols contents compared to Farnsfield in the non-enzymatic extractions, and the enzymatic method revealed that a substantial amount of polyphenols were matrix-bound ($P = 0.0566$). Among the three selected RILs, p27-272 showed the lowest content in both extractions. Upon enzyme treatment, the polyphenols content extracted from RIL p27-362 was greater by 245% ($P = 0.0629$), proving its polyphenol-rich phenotype expressing even higher matrix-bound polyphenols, also confirmed by high total antioxidant capacity measurements. Our findings confirm that peanuts have greater polyphenol contents with significant amounts as matrix-bound compounds.

3.2. Summary of protein identifications

We identified a non-redundant total of 1007 proteins among the five different peanut lines. Of these, a total of 339 proteins were identified in the low-polyphenol RIL p27-272, and 549 proteins were identified in the polyphenol-rich RIL p27-362. Protein and peptide identification summary, as well as the protein distribution pattern for the five peanut lines, are provided in Supplementary Information Fig. S2. Protein

identifications across each peanut line originated from equal amounts of protein subjected to MS/MS and the average peptide count is indicative of this. The distribution pattern of the proteins among the five peanut lines indicates the genetic variation correlating with phenotypically different polyphenolic levels expressed in these peanut lines, and show significant changes in the proteome by the unique expression of 114, 92, 101, 47, and 30 proteins exclusive to the mid-low peanut parent Farnsfield, mid-high parent D147-p3-115, stable high antioxidant RIL p27-362, stable low antioxidant RIL p27-272 and variable high antioxidants RIL p27-036, respectively. Further, the results indicate significant differential protein expression among 82 proteins in the polyphenol-rich RIL p27-362 relative to the polyphenol-low RIL p27-272. These are listed in Table 2 along with quantitation and protein ontology information. Observed protein expression changes explain the biological basis for the high- and low-antioxidant capacity phenotypic traits observed for peanut RILs p27-362 and p27-272, respectively.

3.3. Quantitative proteomics and functional distribution of differential proteins

Quantitative proteomics based on protein abundance measured by NSAFs revealed significant protein expression changes between the two RILs p27-362 and p27-272. These differences were indicative of their associated phenotypic traits whose corresponding high- and low-antioxidant capacities and polyphenol contents were also evaluated by ORAC and HPLC analysis. The 82 differentially expressed proteins among the peanut lines were distinguished from unchanged proteins based on their relative protein abundance and illustrated using LogNSAF plots (Fig. 1). Proteins with significant differential expression are shown as dark blue spots. Proteins with expression levels that did not differ statistically between high or low polyphenol/antioxidant capacity RILs are shown as light blue spots. Proteins with increased or decreased abundances in RIL p27-362 correspond to darker spots below or above diagonal respectively. These plots provide an informative visual display of the total number of differentially expressed proteins and the overall trends of differential expression. Supplementary information Table S1 provides peptide identification and quantitation information for the 82 differentially expressed proteins between the polyphenol-rich RIL p27-362 and the polyphenol-low RIL p27-272. Supplementary

Table 2

Differentially expressed proteins between high (RIL p27-362) and low (RIL p27-272) polyphenol antioxidants expressing peanut lines, based on their Log NSAF ratios.

Identifier	Protein name	Fold change	Biological processes	Mr kDa
<i>Proteins over-expressed in high antioxidant capacity peanuts RIL p27-362</i>				
gi 1021551593	Catalase isozyme 1-like	73.7 ↑	redox process	56.9
gi 10212106750	Adenosylhomocysteinase	35.4 ↑	amino acid metabolism	53.3
gi 1021508058	Aspartic protease in guard cell 2-like	19.5 ↑	proteolysis	51.3
gi 10212030985	Coatome subunit gamma-2	18.7 ↑	transport	98.7
gi 10212115401	Pyruvate decarboxylase 2	13.1 ↑	carbohydrate metabolism	64.7
gi 10212125578	Acidic 27 kDa endochitinase-like	11.3 ↑	response to stress	28.9
gi 10212217504	Glucose-6-phosphate isomerase 1, chloroplastic	11.2 ↑	carbohydrate metabolism	68.7
gi 10212115588	Ferritin, chloroplastic-like	9.3 ↑	cellular iron homeostasis	28.3
gi 10212202471	Ubiquitin carboxyl-terminal hydrolase 13-like isoform X2	8.6 ↑	protein metabolic process	131.2
gi 1021493502	26S proteasome non-ATPase regulatory subunit 12 homolog A	8.6 ↑	protein metabolic process	50.4
gi 10212173682	Biotin carboxylase 1	8.0 ↑	lipid metabolism	58.8
gi 10212031668	Uncharacterized protein LOC107476790	8.0 ↑	unknown	96.1
gi 10212224664	Phosphoribosylformylglycinamide synthase	7.3 ↑	purine metabolism	154.2
gi 1021570516	7S globulin-like	7.3 ↑	seed storage proteins	47.6
gi 10212208665	Citrate synthase, mitochondrial	6.9 ↑	carbohydrate metabolism	52.4
gi 10212035158	Transport protein SEC31 homologB	6.6 ↑	transport	121.3
gi 10212038168	26S proteasome non-ATPase regulatory subunit 2 homolog A	6.5 ↑	protein metabolic process	103.0
gi 1011997786	Malate synthase, glyoxysomal	5.9 ↑	carbohydrate metabolism	64.1
gi 10212264103	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase α	5.8 ↑	carbohydrate metabolism	67.5
gi 10212211836	Pre-mRNA-processing-splicing factor 8	5.7 ↑	transcription	275.6
gi 10212241905	Coatome subunit alpha-1-like	5.6 ↑	transport	136.7
gi 10212000205	Alpha-aminoadipic semialdehyde synthase	5.5 ↑	amino acid metabolism	115.8
gi 10212027118	Uncharacterized protein LOC107475966	5.5 ↑	unknown	49.6
gi 10212213796	26S protease regulatory subunit S10B homolog B	5.4 ↑	protein metabolic process	44.6
gi 10212128808	Putative transferase At4g12130	5.4 ↑	Fe/S biogenesis	44.9
gi 10212220952	Carbamoyl-phosphate synthase large chain, chloroplastic-like	5.4 ↑	pyrimidine metabolism	129.0
gi 10212209451	Uncharacterized protein LOC107459025	5.4 ↑	unknown	91.1
gi 1011996067	26S protease regulatory subunit 6B homolog	5.2 ↑	protein metabolic process	47.3
gi 10212129457	Isoleucine-tRNA ligase	5.2 ↑	translation	132.1
gi 10212261169	Aldose 1-epimerase-like	4.8 ↑	carbohydrate metabolism	41.2
gi 10212100357	Mitochondrial import receptor subunit TOM40-1-like	4.8 ↑	transport	34.5
gi 10212095988	Alanine-glyoxylate aminotransferase 2 homolog 1	4.8 ↑	amino acid metabolism	51.9
gi 10212108891	Stilbene synthase 3-like	4.8 ↑	secondary metabolite metabolism	42.8
gi 10212256226	Probable N-acetyl-gamma-glutamyl-phosphate reductase	4.8 ↑	amino acid metabolism	40.0
gi 1021480771	Calcium-binding protein CML49	4.8 ↑	proteolysis	31.2
gi 10212089389	Elongation factor Tu, chloroplastic	4.8 ↑	protein metabolic process	52.8
gi 10212252880	Formin-like protein 5	4.7 ↑	seed morphogenesis	80.5
gi 1011993216	Glycerophosphodiester phosphodiesterase GDPDL4	4.7 ↑	lipid metabolism	82.6
gi 10212179383	3-oxo-Delta(4,5)-steroid 5-beta-reductase-1	4.7 ↑	response to stress	44.4
gi 10212102481	T-complex protein 1 subunit delta	4.5 ↑	protein folding	57.6
gi 10212124483	Binding partner of ACD11 1-like	4.4 ↑	unknown	29.8
gi 10212239345	T-complex protein 1 subunit gamma	4.4 ↑	protein folding	60.4
gi 10212187107	Tryptophan synthase alpha chain	4.2 ↑	amino acid metabolism	33.2
gi 10212188006	Glutathione S-transferase L3-like	4.2 ↑	response to stress	27.2
gi 10212033069	Salicylate carboxymethyltransferase-like	4.2 ↑	secondary metabolite metabolism	44.8
gi 10212239766	Pyruvate kinase isozyme A, chloroplastic-like	4.2 ↑	carbohydrate metabolism	63.7
gi 10212175579	Putative 3,4-dihydroxy-2-butanone kinase isoform X1	4.1 ↑	carbohydrate metabolism	61.4
gi 10212212319	Adenylosuccinate synthetase 2,	4.1 ↑	purine metabolism	52.8
gi 1021501775	UDP-glycosyltransferase 74G1-like isoform X2	4.1 ↑	secondary metabolite metabolism	51.1
gi 1021481338	Uncharacterized protein C167.05-like	4.1 ↑	unknown	18.3
gi 10212214141	Probable 26S proteasome non-ATPase regulatory subunit 3	4.1 ↑	protein metabolic process	55.6
gi 10212244862	Transportin-1 isoform X2	4.1 ↑	transport	82.1
gi 10212262780	26S proteasome non-ATPase regulatory subunit 11 homolog	4.1 ↑	protein metabolic process	47.1
gi 10212101086	Copper-transporting ATPase RAN1	4.1 ↑	transport	107.3
gi 1021503138	2-alkenal reductase (NADP(+)-dependent)	3.8 ↑	redox process	39.0
gi 10212234151	Long chain acyl-CoA synthetase 8	3.7 ↑	lipid metabolism	79.5
gi 10212122412	Saccharopine dehydrogenase-like oxidoreductase	3.6 ↑	redox process	48.5
gi 1021563364	Histone H2A.v1-like	3.6 ↑	transcription	12.4
gi 10212201786	Dihydropyrimidinase	3.5 ↑	amino acid metabolism	58.4
gi 10212168146	ATP-citrate synthase alpha chain protein 1	3.5 ↑	lipid metabolism	46.6
gi 10212243205	Subtilisin-like protease SBT1.3	3.5 ↑	proteolysis	84.6
gi 10212191624	Signal recognition particle receptorβ	3.5 ↑	signal transduction	29.2
gi 10212212735	3-isopropylmalate dehydrogenase, chloroplastic	3.5 ↑	amino acid metabolism	43.8
gi 10212236393	26S protease regulatory subunit 6A	3.5 ↑	protein metabolic process	47.5
gi 10212202053	UBP1-associated protein 2B-like	2.9 ↑	signal transduction	56.0
gi 10212248904	DNA-directed RNA polymerase II subunit RPB2	2.9 ↑	transcription	134.3
<i>Proteins under-expressed in high antioxidant capacity peanuts RIL p27-362</i>				
gi 1021506701	Ferritin, chloroplastic-like	21.2 ↓	cellular iron homeostasis	28.2
gi 10212099909	Uncharacterized protein At4g28440	7.8 ↓	unknown	15.2
gi 10212106107	Peptide methionine sulfoxide reductase B5-like	6.0 ↓	protein repair	15.1
gi 10212020279	10 kDa chaperonin-like	5.2 ↓	protein folding	10.6
gi 1021496101	Nuclear transport factor 2-like	5.0 ↓	transport	13.6
gi 10212233930	11 kDa late embryogenesis abundant protein-like	4.5 ↓	growth and development	14.2

(continued on next page)

Table 2 (continued)

Identifier	Protein name	Fold change	Biological processes	Mr kDa
gi 1012235073	Autophagy-related protein 8i-like	4.4 ↓	transport	13.7
gi 1012228332	Absciscic acid receptor PYL9-like	3.9 ↓	signal transduction	21.3
gi 1012184864	Sugar phosphate/phosphate translocator At5g04160	3.9 ↓	transport	37.3
gi 1012202289	Argonaute 4-like	3.8 ↓	gene silencing	101.8
gi 1021495280	Protein EXORDIUM-like 2	3.8 ↓	growth and development	32.4
gi 1011992863	Peptidyl-prolyl <i>cis</i> -transisomerase CYP19-4	3.7 ↓	protein folding	21.5
gi 1012001564	Transmembrane 9 superfamily member 11	3.6 ↓	signal transduction	75.0
gi 1012176607	Proteasome subunit beta type-3-A	3.0 ↓	protein metabolic process	22.8
gi 1012127700	TS14 protein-like	2.7 ↓	response to stress	18.3
gi 1012246374	Photosynthetic NDH subunit of lumenal location 5, chloroplastic	2.1 ↓	transport	27.7

Increased or decreased protein abundances calculated in terms of fold changes are indicated using upward ↑ or downward arrow ↓ respectively.

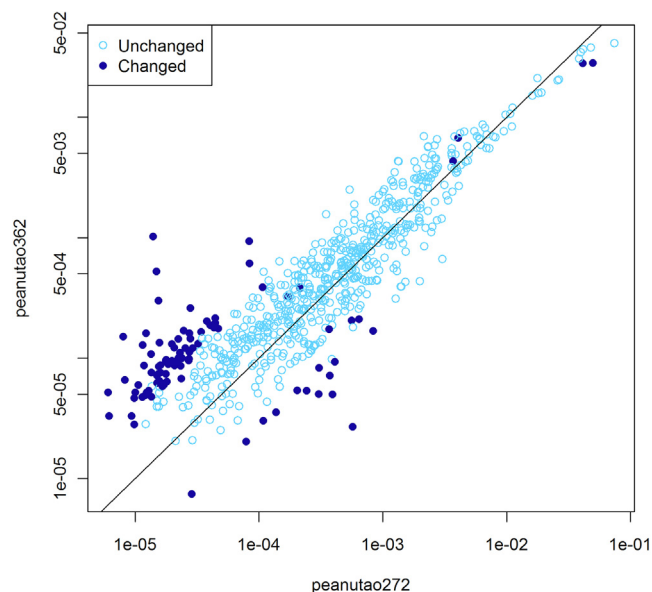


Fig. 1. Log NSAF plot of differential and unchanged proteins between peanut RIL p27-362 relative to RIL p27-272.

information Table S2 provides quantitation information in terms of NSAF values for the non-redundant list of all proteins identified as part of the peanut proteomic profile in this study.

Qualitative and quantitative distributions of the 82 differential proteins spanned 23 functional categories in terms of their protein ontology information. They were first categorized based on their function as a percentage of the total number of proteins (Fig. 2A), and then re-categorized as a percentage of protein abundance in each functional category based on sum NSAF values (Fig. 2B). This comparison showed that the total number of proteins within a functional category often followed a different trend than when quantified based on their overall protein abundance. Qualitative distribution revealed a greater number of proteins involved in protein metabolic processes (12%), transport (12%), carbohydrate metabolism (10%), amino acid metabolism (9%), followed by lipid metabolism, protein folding, signal transduction and stress response at 5% each. Seed storage and translation proteins each represented 1% (Fig. 2A). The greatest protein abundance changes were seen for: catalase isozyme-1 protein (73.7↑), an important antioxidant enzyme involved in oxidation–reduction processes; adenosylhomocysteinase (35.4↑), which is involved in amino acid metabolism; aspartic protease (19.5↑), which is involved in proteolysis; coatomer subunit gamma-2 (18.7↑), which is involved in transport; pyruvate decarboxylase 2 (13.1↑) and glucose-6-phosphate isomerase1 (11.2↑), which are involved in the carbohydrate metabolism; and ferritin (21.2↓), which is involved in cellular iron homeostasis. These are illustrated in Supplementary information in Fig. S3.

Quantitative distribution analyzed using spectral abundance factors

for the differential proteins in RIL p27-362 revealed a different pattern compared to the above (Fig. 2B). Proteins belonging to transport (15%), stress response (12%), protein metabolic processes (11%) were the most abundant. The next most abundant categories were oxidation–reduction processes (8%), carbohydrate metabolism (7%), amino acid metabolism (6%), and protein folding (6%). Among these, unknown proteins lacking ontology were 7%. Although there was only one seed storage protein differentially expressed, its an isoform of Ara h1 at low abundance. Most seed storage proteins identified in this study were not differentially expressed between samples, being naturally abundant at the peptide level with relatively 500 to 1800 peptides each, many of those are also allergen proteins and this is also typical of other tree nuts (Koppelman et al., 2016). Furthermore, there is a good agreement for peanut allergens where abundance levels were reportedly unchanged between four different cultivars. Considering the total pool of allergenic seed storage proteins naturally present in a given peanut, differential expression recorded for this low abundant isoform of 7 s globulin (19 peptides) does not necessitate newer safety considerations, and current guidelines for peanut allergy will apply for RIL p27-362. It is important to note that differentially expressed proteins involved in stilbene metabolism, oxidation–reduction processes and lipid metabolic proteins in RIL p27-362 could be indicative of the genetic changes in peanut's two most commercially vital phenotypic traits, high oleic lipids, and antioxidant polyphenol-rich composition. There is potential for peanut RIL p27-362 to be a commercial value-added health food superior in antioxidant polyphenolics, protein and lipid-rich nutritional profiles. With nutritional qualities to alleviate stress and human health issues, the polyphenol-rich RIL p27-362 could become an excellent dietary source.

3.4. Analysis of protein metabolic changes across major biological processes in peanuts

3.4.1. Redox processes

Proteomic analysis revealed significant expression changes in enzymes involved in oxidation–reduction processes. The antioxidant enzyme catalase isozyme 1-like was increased in abundance by 73.3 fold in RIL p27-362 compared to RIL p27-272. In Arabidopsis model plants, catalase genes have been well studied and are known to encode catalase 1, catalase 2 and catalase 3 proteins, where catalase 1 is predominantly involved in the enzymatic degradation of H₂O₂, and along with catalase 2 and 3 they play a vital role in maintaining cellular homeostasis of reactive oxygen species (Du, Wang, Chen, & Song, 2008). In this study, the increased level of catalase isozyme 1 protein in RIL p27-362 is likely to be one of the direct effects of the genetics behind the high total antioxidants phenotype for this RIL. Further, this may help in cellular defenses when exposed to adverse growing conditions from temperature or drought stress. Activation of stress-induced gene expression of catalase 1 is known to be mediated by the MAP kinase signaling in Arabidopsis (Xing, Jia, & Zhang, 2007). These results suggest that these kernels are likely to be equipped to withstand temperature or drought stress conditions while maintaining ROS species homeostasis, which

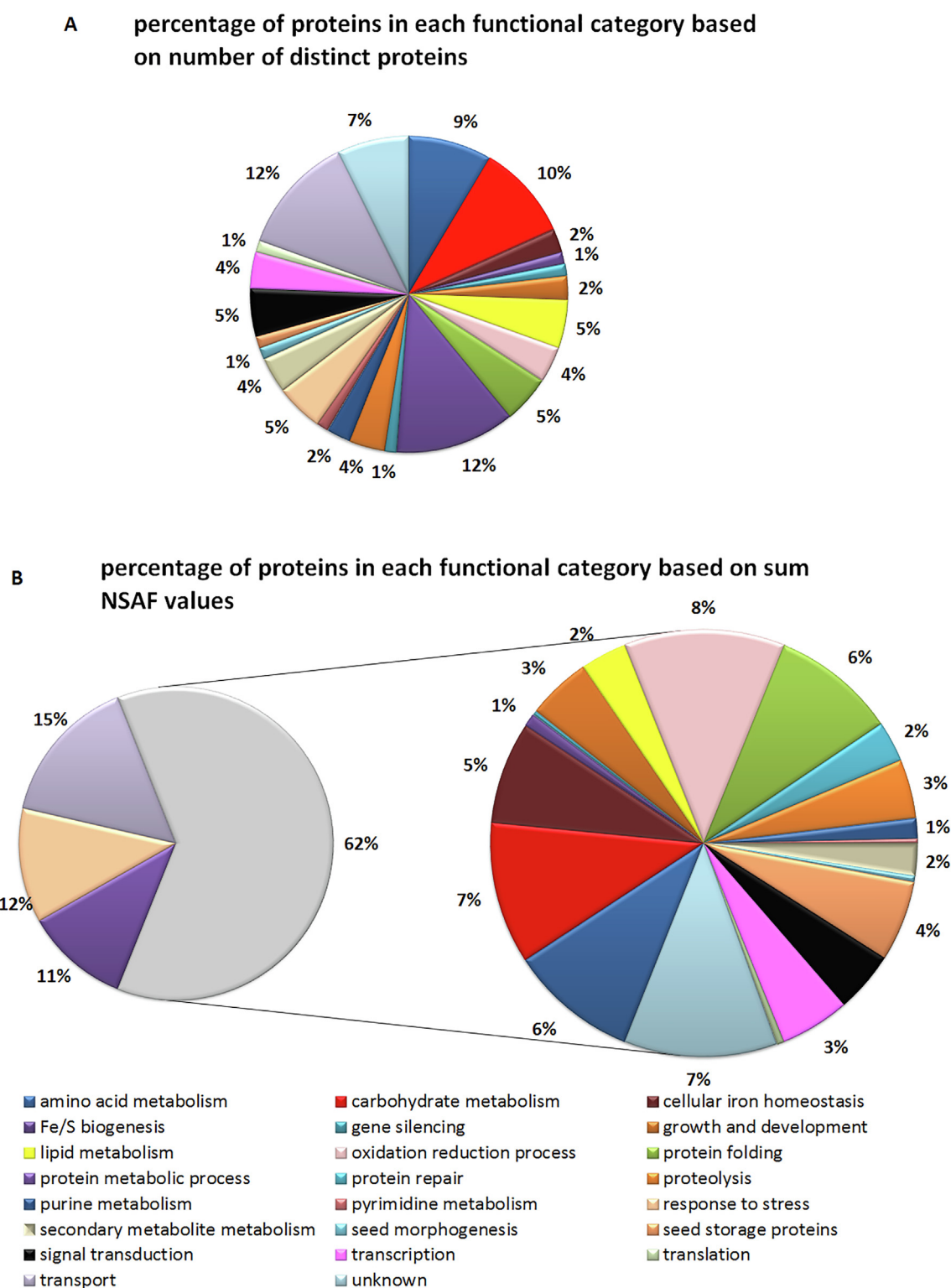


Fig. 2. Qualitative and quantitative distribution of 82 differentially expressed proteins, in terms of observed NSAF values. Proteins have been classified into 23 different functional categories. They were resorted (A) as a percentage of protein number, (B) as a percentage of protein abundance based on their sum NSAF values in each biological process category. NSAF of each protein represents the average NSAF of the biological triplicate.

could benefit the peanut industry. Other important enzymes with redox function identified in RIL p27-362 include saccharopine dehydrogenase, 2-alkenyl reductase, gamma-interferon-inducible lysosomal thiol reductase, 1-Cys peroxiredoxin, peroxiredoxin-2B-like, quinone oxidoreductase PIG3-like and Cu-Zn superoxide dismutase.

3.4.2. Glycolysis

Differentially expressed carbohydrate metabolic enzymes identified as increased in abundance in RIL p27-362 include pyruvate decarboxylase-2 (↑13.1 fold), glucose-6-phosphate isomerase 1 (↑11.2 fold), citrate synthase (↑6.9 fold), pyruvate kinase (↑4.2 fold), malate synthase glyoxysomal (↑5.9 fold), aldose 1-epimerase (↑4.8 fold),

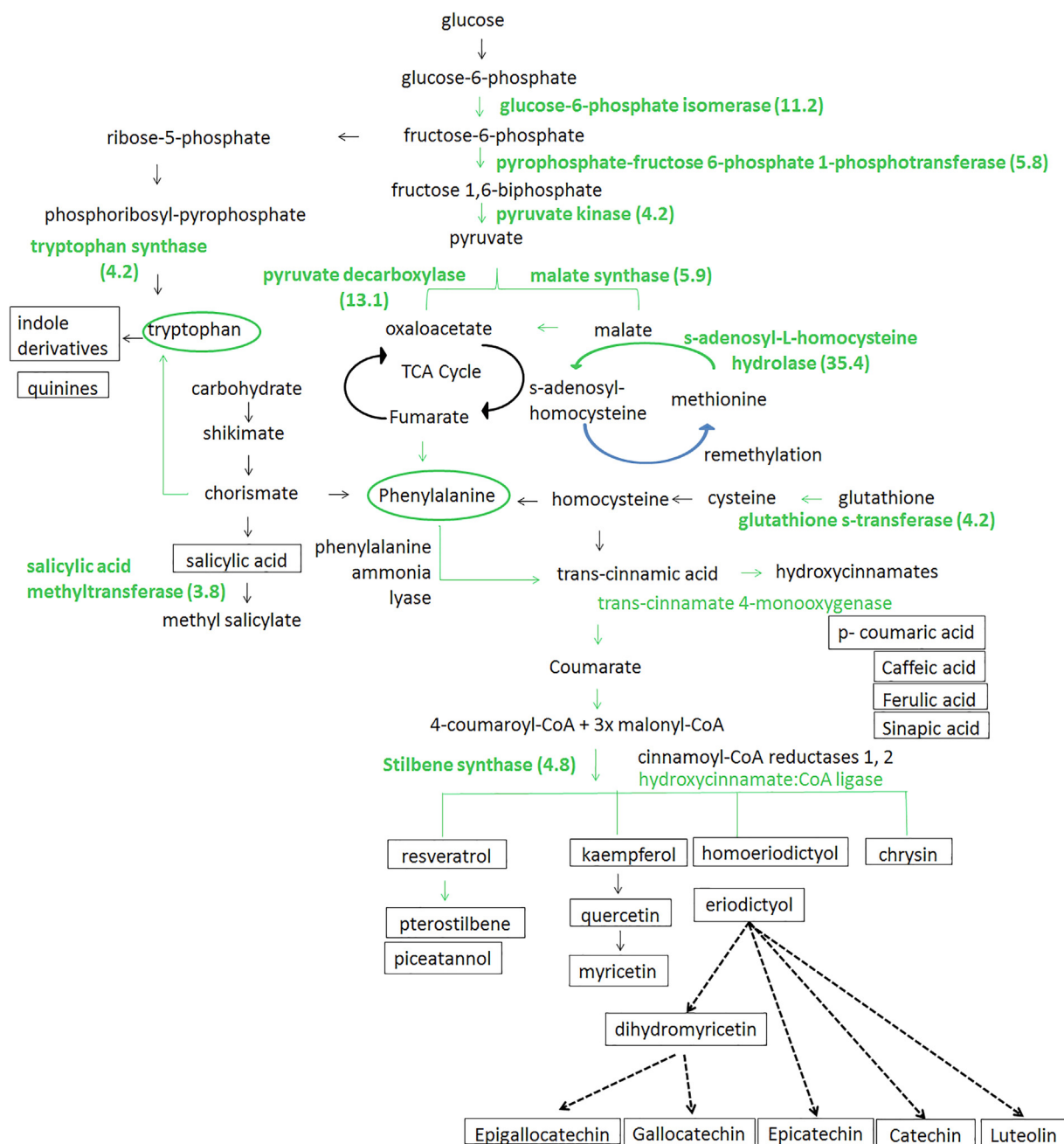


Fig. 3. Proposed cascading of the metabolic pathways towards increased phenolic antioxidants biosynthesis, as evidenced by differential protein expressions in RIL p27-362. Enzymes (fold change) and reactions increased in abundance are highlighted in green, whereas those decreased in abundance are highlighted in blue. Compounds outlined in black boxes have antioxidant properties.

pyrophosphate–fructose-6-phosphate 1-phosphotransferase (15.8 fold) (Table 2). Mapping out these enzymes based on their functional pathways (Fig. 3) shows that they seem to propel glycolysis over-expression for increased biosynthesis of polyphenolic compounds (Rojas, Senthil-Kumar, Tzin, & Mysore, 2014).

3.4.3. Phenylpropanoid pathway

Proteomics evidence revealed positive regulation of different metabolic pathways in the polyphenol-rich RIL p27-362, beginning from increased sugar metabolism resulting in an increase of sugar substrates, and co-inducing phenylalanine levels converging towards increased phenylalanine and tyrosine amino acid biosynthesis. This feeds into the phenylpropanoid biosynthetic pathway for increased synthesis of phenylpropanoid antioxidant compounds, as described in the flowchart in

Fig. 3. Several proteins identified in this study are either directly or indirectly involved in the syntheses and transport of different flavonoid compounds such as resveratrol, including acetyl-coenzyme A (CoA) carboxylase, stilbene synthase, phenylalanine lyase, and coumaroyl CoA ligase (Sales & Resurreccion, 2014). Phenylpropanoids constitute a relatively diverse family of aromatic ring molecules that are derived from enzymatic cascades beginning with phenylalanine (or tyrosine in some cases) and malonyl-coenzyme-A. Phenylalanine is initially converted to cinnamic acid by deamination, followed by hydroxylation and methylation to generate coumaric acid and other acids with a phenylpropane unit. Stilbene synthase has a conserved cysteine residue at the center of its protein sequence and this could be the active binding site for the enzymatic addition of three malonyl-CoAs to form 4-coumaroyl-CoA. Its direct involvement in driving the same condensing type

enzymatic conversion of 4-coumaroyl-CoA and three malonyl-CoAs, but producing different ring compounds, is critical to forming different stilbenes and flavonoids (Fig. 3) including resveratrol and is of great biological relevance for breeding antioxidant-rich peanuts. This supports phenolic compounds reported for peanut kernels being more p-coumaric acid derivatives occurring at greater concentrations than proanthocyanidins (de Camargo et al., 2017). Increased expression of stilbene synthase (↑4.8 fold) becomes the final branch-point for regulating the formation of different phenylpropanoid antioxidants (Fig. 3).

Interestingly, the pathway also accommodates bifunctional enzymes such as glutathione-S-transferase, possibly switching the conversion of glutathione to homocysteine to phenylalanine, accumulating more phenylalanine, thereby fueling the phenylpropanoid pathway (Fig. 3). Acting as another branch-point of the phenylpropanoid pathway, we also identified *trans*-cinnamate 4-monooxygenase, an enzyme initiating the biosynthesis of *trans*-4-coumarate from *trans*-cinnamate and belonging to the cytochrome P450 family. Key to the isoflavonoids phytoalexin biosynthesis, isoflavone reductase was also identified. This provides proteomic evidence behind the presence of daidzein, genistein, and glycitein in the peanut kernels because isoflavone reductase would have brought about the reduction of 2-hydroxyisoflavones such as 2'-hydroxydaidzein, 2'-hydroxygenistein, and 2'-hydroxyformononetin as substrates, and converted them to their isoflavones daidzein, genistein, and glycitein (Cheng et al., 2015).

3.4.4. Secondary metabolites and lipid metabolic process proteins

Among the differentially expressed proteins involved in the secondary metabolites metabolic processes, UDP-glycosyltransferase 74G1-like isoform X2, salicylate carboxymethyltransferase-like, nitrile specifier protein 5 and stilbene synthase were increased in abundance in the polyphenol-rich RIL p27-362 (Table 2). The UDP-glycosyltransferase family 1 protein is also known to have quercetin 3-O-glucosyltransferase and/or quercetin 7-O-glucosyltransferase molecular activity in Arabidopsis, where it is reportedly involved in the flavonoid biosynthetic process as well as the flavonoid glucuronidation process (Lin et al., 1999). In RIL p27-362, there is an increase in quercetin levels, and this is probably related to the increased abundance of the UDP-glycosyltransferase 74G1-like isoform X2 by 4.1 fold. This protein may code for a similar molecular utility in peanuts involving quercetin activity, warranting further research. This protein, when knocked out in transgenic Arabidopsis, resulted in plants becoming highly susceptible to pathogen infection (Liu, Yang, Pichersky, & Klessig, 2010). Increased expression of salicylate carboxymethyltransferase-like in RIL p27-362 will likely amplify the effectiveness of the crops' systemic resistance against pathogens, especially during kernel development stages. Salicylic acid is likely to tap into the classic systemic acquired resistance pathway and methyl salicylate from this conversion could impart resistance against pathogen infection as part of the peanut plant defense mechanism (Park, Kaimoyo, Kumar, Mosher, & Klessig, 2007).

Many enzymes involved in lipid accumulation and degradation were identified in our study, in agreement with some recently reported on proteomic changes across different peanut seed developmental stages (Wang, Ma et al., 2016). Increased abundance of crucial lipid metabolic proteins in RIL p27-362 is indicative of their lipid-rich genetic makeup. Notable differentially expressed proteins include the long chain acyl CoA synthetase 8 (↑3.7 fold) which catalyzes the 12–20 carbon long chain fatty acids, ATP citrate synthase α chain protein 1 (↑3.5 fold), and glycerophosphodiester phosphodiesterase GDPDL4 (↑4.7 fold). Proteomic analysis of peanuts at seed development and post-germination stages revealed initiation of fatty acid accumulation to begin after 20 days from flowering, whereas lipid degradation was significant at germination (Bertioli et al., 2016). Variants of acyl-CoA derivatives and long-chain acyl-CoA synthetases were also identified in here, which may collectively mark the types of fatty acids being synthesised, and their relative distribution in peanuts.

3.4.5. Amino acid metabolism and activated methyl cycle

The amino acid metabolic proteins that were increased in abundance in RIL p27-362 include adenosylhomocysteinase (↑35.4 fold), α aminoadipic semialdehyde synthase (↑5.5 fold), tryptophan synthase α chain (↑4.2 fold), *N*-acetyl- γ -glutamyl phosphate reductase (↑4.8 fold), and alanine-glyoxylate aminotransferase 2 homolog 1 (↑4.8 fold).

Increased expression of tryptophan synthase (14.2 fold) in RIL p27-362 coupled with amplified glycolysis and pentose phosphate pathway identified in this study suggests energy production after an increase in tryptophan availability. The excess energy produced could also be used for increased plant defense ability (Rojas, Senthil-Kumar, Tzin, & Mysore, 2014). The metabolism of tryptophan results in an abundance of indole acetic acid, which is one of the most physiologically active plant growth regulators (Ahmad, Ahmad, & Khan, 2005; Kazan & Manners, 2009). The regulation of plant growth and defense through the expected increase in auxin production due to tryptophan buildup in RIL p27-362 is further reinforced by the increased abundance of salicylate carboxymethyltransferase (↑4.2 fold).

Adenosyl-*L*-homocysteinase was over-expressed by 35.4 fold in RIL p27-362. Here, a greater flux of *L*-homocysteine and methionine signifies the co-induction of the activated *s*-adenosylmethionine cycle, and push towards plant defense (Rojas, Senthil-Kumar, Tzin, & Mysore, 2014) in the form of flavonoid biosynthesis. This is also vital for regulating plant hormones such as ethylene biosynthesis as well as cellular signalling, growth, and repair (Wang, Oh, & Komatsu, 2016). Taken together with the decreased abundance of the methionine sulfoxide reductase B5 and the increased abundance of *s*-adenosylhomocysteine reductase in polyphenol-rich RIL p27-362, increased methionine levels converting to *s*-adenosyl homocysteine and then homocysteine to phenylalanine, point towards the phenylpropanoid pathway for enhanced flavonoids biosynthesis (Fig. 3). Further saccharopine dehydrogenase (↑3.6 fold) together with α -aminoadipic semialdehyde synthase (↑5.5 fold), appear to negatively regulate lysine accumulation in kernels, according to the *L*-Lysine degradation via saccharopine pathway (Galili, Amir, & Fernie, 2016; Wang, Wu et al., 2016). This may be an underlying signaling correlation between the increased flux of lysine reduction via saccharopine dehydrogenase degradation and increased methionine conversion to homocysteine in RIL p27-362. These findings act as supportive proteomics evidence for the genotypic variation involved in stable high polyphenol-rich peanuts.

3.5. Proteins present in all five peanut lines

There were 115 common proteins amongst all five RILs, including 23 allergenic proteins, 15 stress response proteins, 15 translation proteins, 15 proteins from protein metabolism, folding and proteolysis, 15 proteins together in carbohydrate, amino acid and lipid metabolism, and 7 proteins involved in oxidation–reduction processes among others. The 24 allergenic seed storage proteins accounted for 67% of protein abundance which is typically expected for peanuts and included proteins such as Arachin Ahy3, Arachin Ahy4, allergen Ara h1, legumin type B, conglutins, vicilins, 11 s globulin, and 7 s globulins. The globulins are well known to have nutrient reservoir activity as well as IgE binding activity. Ara h 3 is the most abundant peanut allergen, making up 63% of the 24 detected allergens common among the RILs. Other highly abundant proteins include desiccation-related protein, galactose binding lectin, P24 oleosin isoform A, auxin polar transporter aminopeptidase M1, glucose and ribitol dehydrogenase, linoleate 9S-lipoxygenase-like protein, Annexin 1, and phosphoglycerate kinase. These 115 proteins seemingly play an indispensable role in the overall cellular and metabolic maintenance.

3.6. Proteins expressed uniquely to polyphenol-rich peanut RIL p27-362

Ninety nine proteins were expressed uniquely to RIL p27-362 across 25 functional categories. Notable proteins of this group include stilbene

synthase 3-like, also known as resveratrol synthase 3, which was recently reviewed at the transcript level in peanuts (Carvalho et al., 2017) and known to catalyze *trans*-4-coumarate to synthesize 3,4',5-trihydroxystilbene biosynthesis as part of phytoalexin synthesis. The identification of this enzyme uniquely in RIL p27-362 is an important proteomic signature, and metabolic evidence behind the high antioxidant capacity phenotypic trait observed in RIL p27-362. Further, the *trans*-cinnamate 4-monooxygenase identified as part of this group is involved in the biosynthesis of *trans*-4-coumarate from *trans*-cinnamate and forms part of the phenylpropanoid pathway. The enzyme is also reported to be vital for pollen development and growth (Saito et al., 2013). We identified salicylate carboxymethyltransferase often expressed as a defense response to stress and involved in the synthesis of methyl salicylate as part of catalyzing the reaction *s*-adenosyl-*L*-methionine to *s*-adenosyl-*L*-homocysteine, as demonstrated in Arabidopsis (Chen et al., 2003). Here, *s*-adenosyl-*L*-methionine seems to also push the flux towards phenylalanine for flavonoid biosynthesis. The Cu-Zn superoxide dismutase, the 17.3 kDa class I heat shock protein and glutathione-S-transferase F9 are some others unique to RIL p27-362 that are notable for their intrinsic antioxidant functional activity in response to stress.

3.7. Proteins uniquely expressed only in polyphenol-rich RIL p27-362 and D147-p3-115

The seed biotin-containing protein SBP65 belongs to the late embryogenesis abundant group of proteins and was one of the most abundant proteins in this category. It is highly likely to act as an important biotin source required for seed development and germination. This protein could be a potential contributor towards improvement in crop vigor and yield. We also noted 3-hydroxyalkyl-CoA dehydrogenase, which catalyzes the formation of 3-oxoacyl-CoA. An increased level of 3-oxoacyl-CoA in RIL p27-362 could contribute to increase of malonyl-CoA, cascading subsequent syntheses of antioxidant polyphenols by stilbene synthase. LTI65, a low-temperature-induced water soluble 65 kDa protein identified here, was previously reported to be responsive to abiotic stressors like cold temperatures and water deprivation, and in leaf senescence in Arabidopsis. This protein is a potential biomarker indicative of possible cold tolerance imparted as an additional trait to RIL p27-362 and may be a useful subject for future temperature tolerance studies.

3.8. The overall effect of enhancing antioxidant capacity on peanut allergen composition, particularly in the polyphenol-rich RIL p27-362

The most abundant group of proteins identified in all peanut lines irrespective of antioxidant capacity are the common allergenic proteins of peanut representing 67% quantitatively at the peptide level, in this study. This is classic for all peanuts because the common allergenic proteins are the seed storage proteins and come about with the greatest peptide counts (Mamone, Di Stasio, De Caro, Picariello, Nicolai, & Ferranti, 2019). Polyphenol-rich peanuts showed 7 s globulin peanut allergen protein (NCBI accession XP_016205496) to be increased in abundance in RIL p27-362 by 7.6 fold (Table 2). However it is critical to understand that the protein abundance of this isoform of 7 s globulin is inherently low (average peptide count = 19) when compared to other isoforms of the same Ara h1 protein (NCBI accessions XP_015941445 and XP_016174800 with average peptide counts 1421 and 1715), or other allergenic proteins like Arachin Ahy-3 / Ahy-4 (NCBI accessions XP_016205866 = 1539 peptides; XP_015968312 = 1511 peptides) all of which are unchanged among RILs. Therefore this protein cannot present any newer allergenic potential in RIL p27-362 than that already presented by other allergenic proteins existing in greater abundance, not different among RILs at the peptide level. The observed protein expression levels between two other isoforms of allergenic Ahy3 were statistically insignificant (< 2 fold) and are also unlikely to influence

allergic responses in humans. High antioxidant polyphenolic trait induced no significant changes to Ara h2, h3, h6, h7 (Conglutin – 2S albumin), Ara h5 (profilin), Ara h9 (lipid transfer protein), Ara h10, h11 (oleosins), or Ara h12, h13 (defensins). Since these are previously well-known allergens in peanuts that naturally occur at higher abundances, the allergenic panel of proteins remains the same among the different RILs.

In a study using peanut proteins complexed with cranberry or blueberry polyphenols, quercetin was found to be the one bound to most peanut proteins reducing IgE binding by up to 38% and suggested to have hypoallergenic potential. Further the peanut protein – peanut skin polyphenol complexing also suppresses allergenic IgE reactions in mice and showed phosphorylation changes in proteins of the mitogen-activated protein kinase pathway (Bansode, Randolph, Plundrich, Lila, & Williams, 2019). Therefore a regulated dietary intake of polyphenol-peanut aggregate preparations from RIL-p27-362 peanuts with skin on could present therapeutic benefits for suppressed IgE sensitization, and seem to be a useful future direction for oral desensitization screening. RIL p27-362 is currently expected to contain the same, if not reduced, allergic risks to those currently allergic to peanuts, and polyphenol-rich peanuts are unlikely to induce allergy in non-allergic individuals.

3.9. Candidate biomarkers for antioxidant traits in peanuts

A panel of enzymes quantified as significantly increased in abundance in polyphenol-rich peanut RIL p27-362 were selected as highly useful metabolic signatures behind this phenotypic trait that could be used to act as successful candidate biomarkers. As evidenced by our proteomics results (Table 2), a panel of enzymes is selected as candidate biomarkers that show statistically significant metabolic changes behind the polyphenol-rich phenotypic trait. These are catalase 2 (73.7↑ fold), adenosylhomocysteinase (35.4↑ fold), stilbene synthase 3 (4.8↑ fold), salicylate carboxymethyltransferase (4.2↑ fold), pyruvate decarboxylase 2 (13.1↑ fold), glucose-6-phosphate isomerase 1 (11.2↑ fold) and pyruvate kinase isoenzyme A (4.2↑ fold). Stilbene synthase-3, which is important for switching on the stilbene and flavonoid biosynthetic pathway, has resulted in the accumulation of stilbenoids including resveratrol when over-expressed in RIL p27-362. This is in good agreement with findings reported for transgenic tobacco plants when over-expressed with stilbene synthase, and the study also demonstrated improved salt, peroxidation and stress tolerance (He, Xue, Zhang, Guo, Ma, & Yang, 2018). Therefore along with the above enzymes, the overexpressed stilbene synthase together with 4-coumaroyl CoA ligase could be useful as potent biomarkers for the screening of polyphenol-rich peanut varieties.

4. Conclusion

We found proteomic evidence in the form of differentially expressed enzymes in carbohydrate and protein metabolism as well as stilbene and flavonoid biosynthesis, signifying the biological relevance on what confers genotypic variation between stable high- and low- polyphenol contents among closely related peanut RILs. RIL p27-362 peanuts are found to be rich in antioxidant polyphenols as well as antioxidant enzymes such as catalase and superoxide dismutases; and can help protect against ROS induced health issues. Many metabolic changes were identified that clearly contribute towards increased phenylalanine biosynthesis, which is the major entry point to centrally switching on the phenylpropanoid pathway. These proteomics findings prove the basis for accumulation of antioxidant polyphenolic compounds in RIL p27-362 and were used as validation experiments necessary to assist breeders in selecting high antioxidant peanut phenotypes, and has potential for release as favorable commercial varieties.

Author contributions

NAL developed the concept, supervised the study and contributed to the writing of the ORAC and HPLC sections. GCW developed the RIL population used in this study. SM devised the methodological approach and conducted the proteomics experiments, analysis of the data and manuscript writing. YYP performed the ORAC, HPLC experiments and statistical analysis, and contributed to the writing of the ORAC and HPLC sections and analyzing proteomics data. SM, YYP, GCW, PAH and NAL contributed in reviewing and revising the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2020.127517>.

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