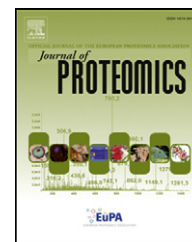


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Quantitative proteomic investigation employing stable isotope labeling by peptide dimethylation on proteins of strawberry fruit at different ripening stages☆☆☆

Li Li^a, Jun Song^{b,*}, Wilhelmina Kalt^b, Charles Forney^b, Rong Tsao^c, Devanand Pinto^d, Kenneth Chisholm^d, Leslie Campbell^b, Sherry Fillmore^b, Xihong Li^a

^aKey Laboratory of Food Nutrition and Safety (Ministry of Education), Tianjin University of Science and Technology, Tianjin 300457, PR China

^bAgriculture and Agri-Food Canada, Atlantic Food and Horticulture Research Centre, 32 Main St., Kentville, NS B4N 1J5, Canada

^cAgriculture and Agri-Food Canada, Guelph Food Research Programs, Stone Road, Guelph, ON, Canada

^dInstitute for Marine Biosciences, National Research Council of Canada, Halifax, NS B3H 4H7, Canada

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ABSTRACT

A quantitative proteomic investigation of strawberry fruit ripening employing stable isotope labeling by peptide dimethylation was conducted on 'Mira' and 'Honeoye' strawberry fruit. Postharvest physiological quality indices, including volatile production, total phenolics, total anthocyanins, antioxidant capacity, soluble solids and titratable acidity, were also characterized in white, pink and red fruit. More than 892 and 848 proteins were identified and quantified in the 'Mira' and 'Honeoye' fruit, respectively, using at least two peptides for each protein identification. Using the normalized ratio of protein abundance changes, proteins that changed two-fold or more were identified as proteins that are up- or down-regulated during fruit ripening. Among the quantified proteins, 111 proteins were common to both cultivars and represented five significant clusters based on quantitative changes. Among the up-regulated proteins were proteins involved in metabolic pathways including flavonoid/anthocyanin biosynthesis, volatile biosynthesis, antioxidant metabolism, stress responses and allergen formation. Proteins that decreased during fruit ripening were found to be responsible for methionine metabolism, antioxidant-redox, energy metabolism and protein synthesis. Our results show that strawberry ripening is a highly complex system involving multi-physiological processes made possible through changes in protein expression. This study provides new insights on the regulation of proteins during strawberry fruit ripening that lay the foundation for further targeted studies.

Biological significance

Research on the postharvest physiology and biochemistry of strawberry fruit as a model of non-climacteric fruit ripening has been conducted for many years. However, the mechanism(s) for the initiation and metabolic regulation of non-climacteric fruit ripening

☆ Identification and quantitation of proteins reveal a dynamic network of proteins in the regulation of strawberry fruit ripening and quality.

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* Corresponding author. Fax: +1 902 365 8455.

E-mail address: jun.song@agr.gc.ca (J. Song).

remains unknown. Little information on strawberry fruit ripening is available at the proteome level. This paper is the first report of a quantitative proteomic investigation of strawberry fruit ripening employing stable isotope labeling by peptide dimethylation. Postharvest physiological quality indices, including volatile production, total phenolics, total anthocyanins, antioxidant capacity, soluble solids and titratable acidity, were also characterized in ripening fruit. Significant biological changes associated with ripening were revealed and proteins that change significantly under these conditions were identified. Therefore, our study links the biological events of strawberry fruit ripening with proteomic information and provides insights into possible mechanisms of regulation. Proteins that changed during ripening were analyzed through function analysis, which provides new insights into metabolic changes occurring during ripening. Findings from this paper not only provide proteome information on fruit ripening, but also pave the way for further quantitative studies using SMR to investigate certain proteins and pathways involved in fruit ripening.

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1. Introduction

Strawberry (*Fragaria × ananassa*) fruit is one of the most popular fruit crops consumed worldwide and is recognized for its nutritional and flavor quality. Molecular biological tools have also been developed to study general plant metabolism and have been applied to investigate strawberry fruit ripening and senescence. Strawberry genes that were differentially expressed during fruit ripening were identified [1–3]. Later, DNA microarray technology was applied and identified that the alcohol acyl transferase (SAAT) gene plays a crucial role in volatile production of strawberry fruit [4]. Recently, genes related to ethylene reception [5], firmness [6], allergens [7] and anthocyanin biosynthesis [8–10] during strawberry fruit ripening and senescence have been reported. Investigations of metabolic events in the receptacle and achene tissues of strawberry fruit during fruit development and ripening revealed that both metabolic synchrony and speciation exist in strawberry fruit [11]. Despite the intensive biochemical and physiological studies using genomic and molecular approaches, very few data are available at the proteomic level to aid in our understanding of fruit ripening and senescence.

Research on fruit ripening has made great progress [12,13], but it has been hampered by the lack of appropriate genomic and other molecular biological tools. Using short-read technology without a physical map or reference genome, the genome of the woodland strawberry (*Fragaria vesca*) was sequenced and identified 33,264 protein coding genes [14]. In addition, a recent public sequence databank that contains in excess of 10,855 ESTs for strawberry (*Fragaria ananassa*), allows the identification of approximately 1004 proteins and offers new opportunities to identify unknown genes and proteins (NCBI: <http://www.ncbi.nlm.nih.gov/>).

Due to protein modifications and alterations that reflect functional states in the cell, the analysis of mRNA and gene expression may not correspond with functional proteins and truly reflect the dynamic state of the cell [15]. Proteomic techniques have emerged as a new platform to investigate biological systems. Among the proteomic techniques, 2-dimensional electrophoresis (2-DE) has been widely applied in plant proteomic research. 2-DE based proteomic technology has shown the potential to identify proteins present in strawberry fruit samples

with reasonable confidence [16–18]. Reports on strawberry, tomato, pear and grape have shown that the proteomic approach has potential to identify proteins involved in fruit ripening, the development of physiological disorders, and allergens [18–24]. These studies have demonstrated that proteomic technology is a powerful tool to reveal the complicated protein population in specific samples associated with physiological status, which can reveal the proteins involved in specific biological processes. To date, the application of proteomics has mainly used 2D gel based approaches, with only a few proteomic studies on the ripening and senescence of strawberry fruit. These studies investigated allergens in strawberry fruit [7], and the accumulated proteins in ‘Queen Elisa’ elite genotype during fruit ripening, but did not relate them to physiological changes [18]. More proteomic investigations are required to better understand the regulation of fruit ripening and to link the proteome changes with quality traits.

Quantitative proteomics is a rapidly developing field that has shown the potential to expand our understanding of biology and disease. A few quantitative proteomic strategies that employ specific labeling techniques have been developed and applied to plant proteomic studies [25–28]. Recently, a new labeling strategy that involves the methylation of peptide amino groups via reductive amination with isotopically coded formaldehydes was reported [29]. This labeling technique was previously shown to be quantitative using standard peptides [30]. In addition, a labeling scheme that allowed the simultaneous analysis of three samples, while retaining a mass difference of 4 Da with the inclusion of formaldehyde labeled with ^{13}C was developed [31]. This stable isotope dimethyl labeling technique was described to be a straightforward and cost-effective labeling strategy to detect differences in abundance ratios over two orders of magnitude for both global proteomic research and protein modifications [32].

In this study, we have conducted quantitative proteomic analysis employing stable isotope dimethyl labeling to quantitatively investigate protein profiles of strawberry fruit tissues harvested at different stages of ripeness. The objectives of this study were to determine the protein profile changes associated with different fruit ripeness and to establish links between protein profile changes and ripening physiology to provide a more in-depth understanding of strawberry fruit ripening at the proteomic level. The biological significance of protein changes is

discussed within the context of behaviors of strawberry fruit ripening.

2. Materials and methods

2.1. Plant materials

Strawberries (*Fragaria × ananassa*, cv. 'Mira' and 'Honeoye') were harvested from the Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, Kentville, Nova Scotia, Canada, at three harvest maturities (white, pink and red). The fruit maturities were determined by tagging the flowers at anthesis and after 14, 18 and 22 days fruit were harvested at the white, pink and red color stages, respectively. The experiment was conducted in the 2011 season with three harvests for replication. For each cultivar and harvest, three blocks of plants were randomly selected and harvested. On average, a minimum of 150 fruit per replicate were harvested. Harvested strawberry fruit were transferred to the laboratory, calyx and pedicel were removed, the surface was cleaned with 2% SDS solution, and the fruit was quickly frozen in liquid nitrogen and ground to a fine powder in liquid nitrogen using a stainless steel blender followed by a mortar and pestle. Ground samples were stored at -85°C until analyzed.

2.2. Biological measurements

2.2.1. Fruit quality indices

2.2.1.1. Anthocyanin analysis. Weighted frozen fruit (approx. 7.5 g) was ground in a food processor with 15 mL of a mixture of 40:40:20:0.1 (v/v) of acetone:methanol:water:formic acid. The homogenized extract was filtered through a G6 fiberglass filter and the filter cake was rinsed with the same extraction solvent. The filtrate was diluted to a final volume of 25 mL. Samples of the filtrate were freeze dried and then re-dissolved in water for HPLC analysis.

Aqueous preparations of the strawberry fruit extracts were injected on an Agilent 1100 HPLC system for analysis via PDA detection at 520 and 280 nm. The analytical column was a 2.1×50 mm Zorbax SB-C18 particle size $1.8\ \mu\text{m}$ (Agilent Technologies Inc., Mississauga, ON). A $20\ \mu\text{L}$ volume was injected onto the column and separation was carried out using a linear gradient. Initial conditions were: 95% solvent A (water adjusted to pH 1.35 with TFA) and 5% solvent B (acetonitrile adjusted to pH 1.35 with TFA). Gradient conditions were 0–12.5 min, 5–10% solvent B; 12.5–43.75 min, 10–20% solvent B; 43.75–45 min, 20–100% solvent B; 45–50 min, 100% solvent B; and 50–55 min, 100–5% solvent B. The flow rate was $0.4\ \text{mL/min}$. Cyanidin-3-glucoside (Cy-3-glc) and pelargonidin-3-glucoside (Pel-3-glc) standards were purchased from Extrasynthase (Genay, Cedex, France) and used to calibrate for Cy-3-glc and Pel-3-glc equivalents.

2.2.1.2. Total phenolics, flavonoids, and antioxidant capacity (ORAC and FRAP). Total phenolics and flavonoids were extracted from the freeze-dried powder of the strawberry fruit by 70% aqueous methanol. In brief, the freeze-dried strawberry powder (approximately 2 g) was accurately weighed and

transferred into a 50 mL tube containing 30 mL of 0.1% HCl (v/v) in 80% methanol. A slightly modified Folin–Ciocalteu (FC) reagent method was used to measure the total phenolic content and results were expressed as gallic acid (GA) equivalents. Gallic acid solutions were prepared for the generation of a standard curve [33]. Briefly, 0.2 mL of sample or solvent blank, 1.0 mL of FC reagent, and 0.8 mL of Na_2CO_3 (7.5% aqueous solution) were mixed in a 20-mL vial and allowed to stand at room temperature for 30 min. Absorption was measured at 765 nm in a Varian Cary 3C spectrophotometer (Varian Analytical Instruments, Harbor City, CA). Total anthocyanin content was determined by using a pH-differential method and results were expressed as cyanidin-3-glucoside (Cy-3-glc) equivalents [34]. Concentration was expressed as micrograms of gallic acid equivalent (GAE) mg GAE per g DW in strawberry samples. Total flavonoids were measured using a modified method employing a 96-well microplate method read at 510 nm using catechin purchased from Sigma (St. Louis, MO, USA) as a standard [35].

The assay of the aqueous strawberry extracts for the oxygen radical scavenging capacity (ORAC) was conducted according to the protocol of Ou et al. [36] with modifications [37]. The results were expressed as μmol Trolox equivalent (TE) per gram dry weight (TE $\mu\text{mol g}^{-1}$ DW).

The ferric-reducing antioxidant potential (FRAP) assay was determined according to the method of Benzie and Strain [38], which was modified for the 96-well microplate reader [39]. The FRAP assay measures the ability of the antioxidants in the strawberry phenolic extracts to reduce the ferric-tripyridyl-triazine (Fe^{3+} -TPTZ) complex to the blue colored ferrous form (Fe^{2+}), which absorbs light at 593 nm. Briefly, standard or sample extracts ($10\ \mu\text{L}$) were mixed with $300\ \mu\text{L}$ of ferric-TPTZ reagent prepared by mixing 300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in the ratio of 10:1:1 (v/v/v) and added to the wells. The plate was incubated at 37°C for the duration of the reaction. The absorbance readings were taken at 593 nm at 30 min using a UV-vis micro-plate kinetic reader (EL 340, Bio-Tek Instruments, Inc., Winooski, VT, USA). Six concentrations of 25, 50, 100, 200, 400 and $800\ \mu\text{mol L}^{-1}$ of L-ascorbic acid were used to prepare the standard curve. The antioxidant activities were expressed as μmol ascorbic acid equivalent (AAE) per gram dry weight ($\mu\text{mol AAE/g DW}$). Stronger absorption therefore indicates higher reducing power of fruit.

2.2.1.3. Volatile compounds. Volatile compounds of strawberry fruit were measured from 10 g of ground tissue powder. The weighed samples were homogenized with 10 mL saturated NaCl solution and sealed in a 20 mL vial. Sampling was accomplished by placing the SPME fiber (DVB/Car/PDMS, Supelco, Bellefonte, PA) through the Mininert valve and into the headspace of the 20 mL vials. An internal standard of 2-octanone at concentration of $524\ \text{ng mL}^{-1}$ was added to each sample. Temperature for sample extraction was 60°C and sampling duration was 60 min. Volatiles were separated on a 30 m $0.32\ \text{mm}$ ($\text{df} = 1.0\ \mu\text{m}$) i.d. capillary column (VF-WAXms, Agilent Technologies Canada Inc., Mississauga, ON) having a film thickness of $0.25\ \mu\text{m}$. Ultra-purified helium (99.999%, Praxair Canada Inc.) was used as carrier gas at a flow rate $1.5\ \text{mL min}^{-1}$. Linear velocity was $44\ \text{cm s}^{-1}$. The initial temperature of the column was 35°C and increased at $15^{\circ}\text{C min}^{-1}$ to a final temperature of 240°C , which was

maintained for 4.5 min. Identification of volatile components was confirmed by comparison of collected mass spectra with those of authenticated chemical standards (Sigma-Aldrich Canada Ltd, Oakville, Ontario L6H 6J8) and to reference spectra in a mass spectral library (National Institute for Standard Technology, Search Version 1.8, Gaithersburg, MD).

2.2.1.4. Soluble solids and titratable acidity. Soluble solids of fresh squeezed juice from ten fruit were determined with a hand-held temperature-compensated refractometer (Atago Co., Tokyo). Titratable acidity was measured with a 2 mL juice sample collected from strawberry and 0.1 mol L⁻¹ NaOH was used to titrate the juice using a semi-automatic titrator (Multi-Dosimat E-415 titrator; Metrohm AG, Switzerland) to a phenolphthalein endpoint of pH 8.1. Titratable acidity was expressed as citric acid equivalents per g DW.

2.2.2. Sample preparation for proteomics

Protein was extracted and purified from ground berry samples using a modified phenol extraction followed by ammonium acetate-methanol precipitation [16]. Protein concentration in all extracts was determined using the RC/DC™ protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and interfering compounds were compensated for using the manufacturer's protocol. Bovine serum albumin [40] was employed as a standard.

2.3. Stable isotope dimethyl labeling

The resulting peptides of total tryptic digestion from different biological samples were labeled with isotopomeric dimethyl labels as described by Boersema et al. [41]. Two hundred µg of protein from each harvest maturity was re-suspended in 200 µL OFFGEL buffer composed of 5% glycerol, and ampholytes pH 3.0–10.0 IPG buffer (GE Healthcare, Bio-Sciences AB, Sweden). Samples were reduced with 5 mM DTT (Sigma D9163) at 60 °C for 30 min and alkylated with 15 mM iodoacetic acid (Sigma I4386) for 30 min at room temperature. Samples were then digested overnight at 37 °C, with sequencing-grade modified trypsin (Catalog# V5111, Promega, Madison, WI) at 1:50 (w/w) protein to trypsin ratio. The resulting peptides were labeled by reductive methylation of primary amino groups (N-term and K). Samples of fruit at the white, pink and red ripeness stages were distinguished as 'light' (L), 'intermediate' (M) and 'heavy' (H) labeled by incubating with CH₂O (light formaldehyde), CD₂O (intermediate formaldehyde) and ¹³CD₂O (heavy formaldehyde), respectively, in the presence of sodium cyanoborohydride (NaBH₃CN, light and intermediate) and sodium cyanoborodeuteride (NaBD₃CN, heavy). After labeling, three differentially labeled samples were pooled in a ratio of 1:1:1 (w/w) and desalted prior to the off-gel peptides fractionation. The schematic demonstration of the sample preparation procedure is illustrated in Fig. 1.

2.4. OFFGEL fractionation

The pI-based separation of total strawberry labeled peptides was conducted on a 3100 Off-gel Fractionator (Agilent Technologies, Palo Alto, CA, USA) with 24-wells according to

the manufacturer's protocol. Fifteen minutes prior to sample loading, 24-cm-long IPG gel strips (GE Healthcare, Bio-Sciences AB, Sweden) with a linear pH range of 3 to 10 were rehydrated in the assembled device with 40 µL of focusing buffer per well. All peptide samples, with or without labeling, were evaporated to dryness and re-dissolved in 3.6 mL of OFFGEL electrophoresis (OGE) buffer and then 150 µL of peptide solution was dispensed in each well. After 1 h of hydration with a voltage at 300 V, the electrofocusing of the peptides was performed until the 50 kVh level was reached. The current limit, power and temperature were set at 50.0 µA per strip, 300 mW and at 20 °C, respectively. After the peptide OGE, the withdrawn fractions (volume between 50 and 200 µL) were desalted, dried and re-dissolved in 2% (v/v) ACN and 1% (v/v) formic acid just prior to LC/MS analysis.

2.5. Mass spectrometry — QTOF

Mass spectra were acquired on a Waters Q-TOF Premier (Milford, MA) equipped with a nano-electrospray source and nano-lockspray interface. A lock mass solution of [Glu¹]-Fibrinopeptide B (200 fmol/µL) was infused at a frequency of 60:1 (sample-to-reference ratio). A potential of 2800 V was applied to a 30 µm id tapered electrospray tip (FS360-75-30-N-C12, New Objective, Woburn, MA). Cone voltage was set to 30 kV and source temperature at 100 °C.

Chromatographic separations were conducted using a Waters nano-Acquity UPLC system (Milford, MA) equipped with two 15 cm × 100 µm Onyx Monolithic C₁₈ columns in tandem (Phenomenex, Torrance, California). The separation was carried out using a linear gradient from 5% B to 30% B over 53 min then increasing to 90% B over 5 min (A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile) at 1 µL/min.

All data acquisition was performed using MassLynx version 4.1 (Waters, Milford, MA). Data was acquired using data directed analysis [42] where the m/z values of tryptic peptides were measured using a MS scan, followed by MS/MS scans of the four most intense peaks. Peak detection, database searching and quantification were performed using Mascot Distiller version 2.3.2 (Matrix Science Ltd., England). The relative intensities of extracted ion chromatograms (XICs) for isotopically differentiated peptide precursors were compared using Distiller Quantitation Toolbox (Matrix Science, London, UK).

2.6. Data analysis

MS/MS peak lists from the 24 OGE fractions were combined and a single database search was performed. Peptide sequences were then assigned using MASCOT (Matrix Science, London, UK) to search the NCBI with the following search parameters: The raw MS/MS data were searched against NCBI *Viridiplantae* entries, 903,371 sequences, updated Dec. 31th 2011 (NIH, Bethesda, MD, USA) and SwissProt database, 515,203 sequences, updated Dec 11th 2011 (Sprot version 57.15) using the MASCOT algorithm version 2.3.02 (Matrix Science, London, UK). The MS and MS/MS mass tolerance were both as 0.3 Da respectively and one missed cleavage was allowed. Carboxamidomethyl cysteine and oxidized methionine were set as fixed and variable modifications, respectively. Proteins with significant peptide matches were selected for error tolerant searching. MASCOT

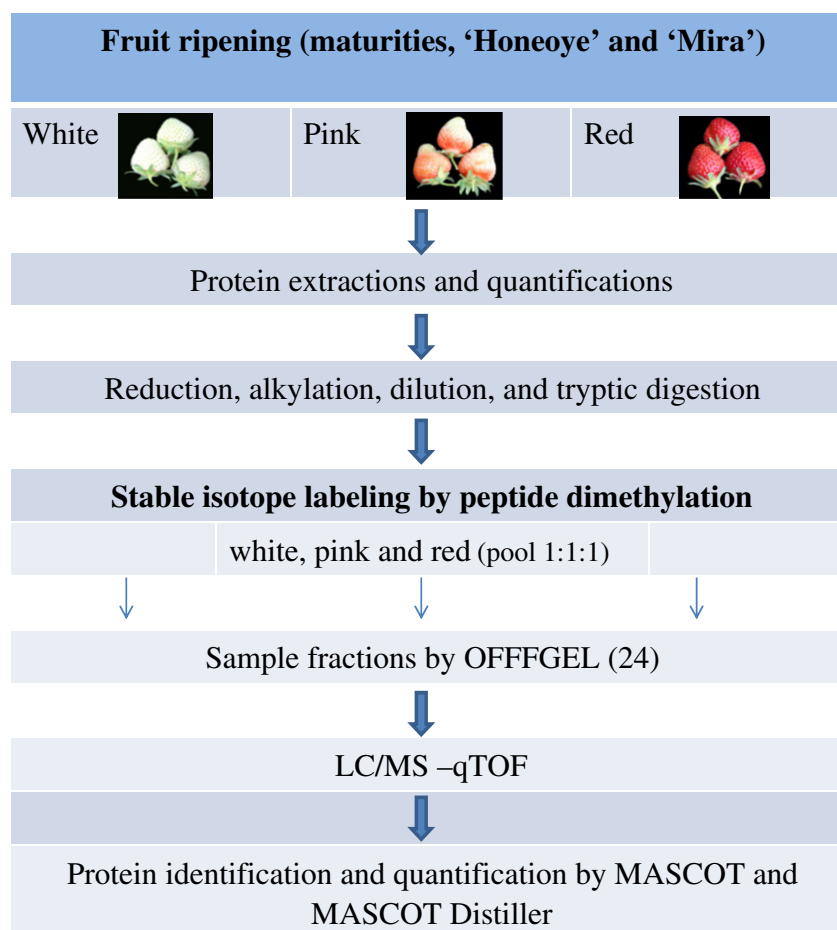


Fig. 1 – General workflow of the strawberry fruit ripening study employing the quantitative proteomic technique of stable isotope labeling by peptide dimethylation labeling experiments and OGE peptides fractionation.

searches were based on a significance threshold of $p < 0.05$ and the MudPIT scoring system was used, whereby protein hits are removed that have scores based exclusively on a large number of low-scoring peptide matches. Automated quantification was performed with a MASCOT Distiller version 2.3 (Matrix Science, London, UK). Only peptides that matched the identified protein better than any other protein in the database (#1 rank; red font) with MASCOT scores of 25 or greater were selected for quantification. False positive peptide identification rates were also calculated using the decoy option provided by MASCOT and estimated as below 1.5%. Quantification was based on peak area in the TOF-MS survey scan, using the sum of the areas of the monoisotopic peak and the first three ^{13}C -containing peaks. The remaining peptides were used to calculate the mean protein ratio and SD. The raw data was manually inspected and a protein ratio was calculated. Protein quantification ratio (medium/light, M/L and heavy/light, H/L) was calculated by Mascot Distiller that uses the average peak area of the first 3 isotopic peaks across the elution profile for quantification.

2.7. Statistical analysis

This study was conducted as a randomized block design with two cultivars, 3 harvests over time and each with 3 blocks of plants as replicates. The data was analyzed using GenStat

12th edition and ANOVA procedure with p value at 0.05. Principal component analysis (PCA) was also employed to outline the relationships among cultivars, fruit maturities and significant physiological differences. The proteomic study was conducted on two cultivars, two harvests over time as biological replicates and two replicate plants. Data were processed using EPCLUST software (<http://www.bioinf.ebc.ee/EP/EP/EPCLUST/>) which allowed clustering of proteins according to their expression profile. A complete linkage hierarchical clustering (Euclidean distance) was demonstrated with Pearson's distance and Ward's algorithm used for data aggregation.

3. Results

3.1. Physiological characterization of fruit at different ripening stages

3.1.1. Anthocyanins, total phenolics, total flavonoid compounds and antioxidant capacity

Significant differences in anthocyanin concentration as a result of fruit ripening was shown in both cultivars, when expressed as total anthocyanins content [43] of total 520 nm area count equivalent to either Cy-3-glc or Pel-3-glc (Fig. 2A and B).

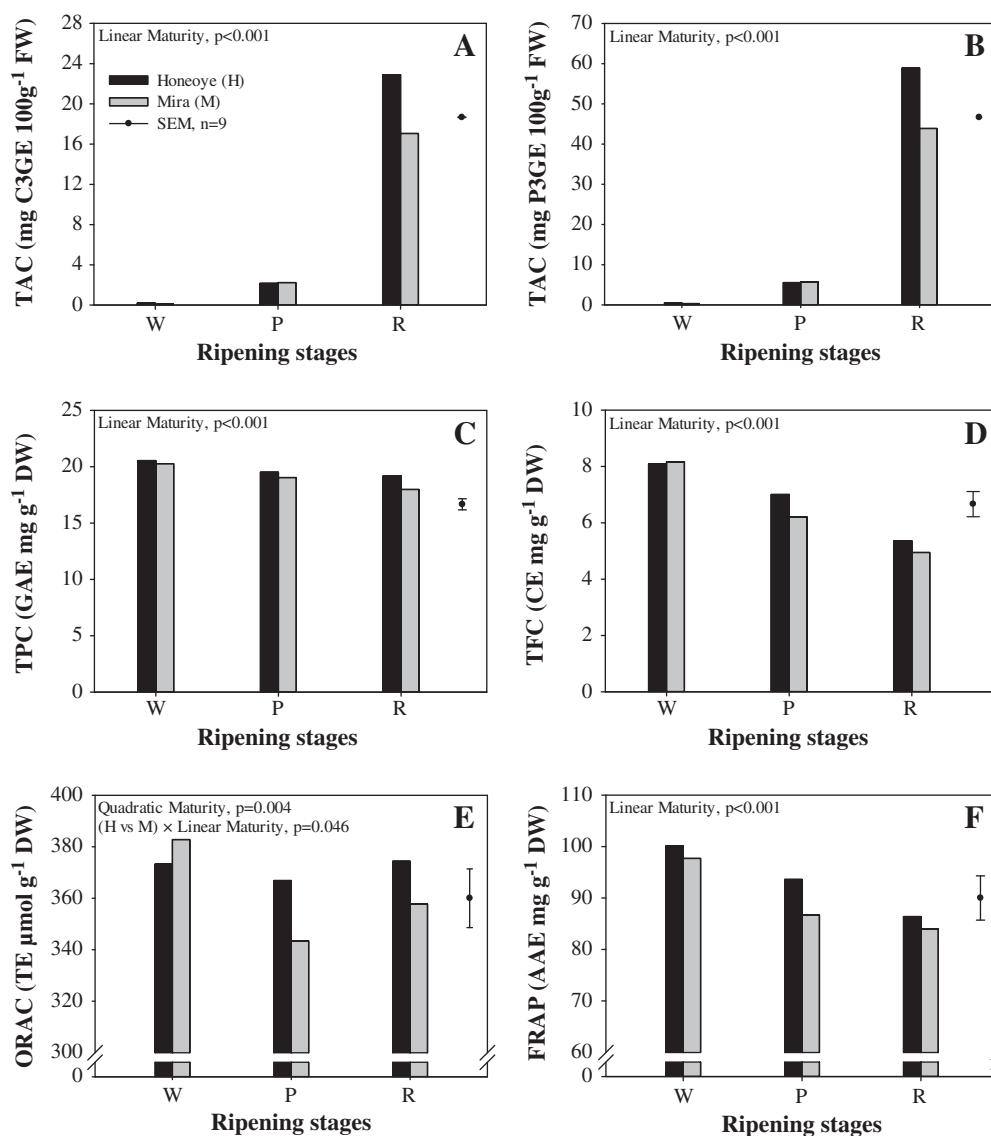


Fig. 2 – Postharvest physiological quality indices of strawberry fruit at different ripening stages. **A:** Total anthocyanins expressed as C3GE 100 g⁻¹ FW; **B:** Total anthocyanins expressed as P3GE 100 g⁻¹ FW; **C:** Total phenolics; **D:** Total flavonoids; **E:** ORAC; **F:** FRAP.

Anthocyanin content was significantly higher in red fruit of both 'Honeoye' and 'Mira' cultivars being 22.9 and 17.07 mg Cy-3-glc 100 g⁻¹ FW, or 58.93 and 43.9 mg Pel-3-glc 100 g⁻¹ FW, respectively. In red ripe fruit, 'Honeoye' showed higher anthocyanin content than 'Mira'. Meanwhile, significant decrease in total flavonoid compounds during ripening was observed in this study, while no significant decrease in total phenolic compounds was found (Fig. 2C and D). A quadratic difference in the antioxidant capacity measured as ORAC was found in 'Mira' at three ripeness stages (Fig. 2E). While, a significant decrease of 15% of the antioxidant capacity measured as FRAP was found in both cultivars with advanced ripeness (Fig. 2F).

3.1.2. Volatile compounds, soluble solids (SS) and titratable acidity (TA)

In order to link protein profiles from the proteomic study with physiological activities and metabolites in strawberry fruit at

different ripening stages, chemical measurements including fruit volatile production, soluble solids (SS) and titratable acidity (TA) were determined to reveal significant physiological changes (Fig. 3A–F). From white to pink and red stages, total volatile production increased significantly. At the red stage, more than 30 major volatile compounds were identified. Total volatile concentration increased as much as 400% (Fig. 3A). Esters were the major class of volatile compounds, comprising 87% of total volatiles and increased by 800% as fruit changed from white to red (Fig. 3B). Furanol (DMMF) also increased significantly from trace amount to 17–18 normalized area counts in red fruit of both cultivars (Fig. 3C) and gamma-decalactone increased to 48 normalized area counts in red 'Honeoye' fruit (Fig. 3D). Total soluble solids content (TSS) increased from 5.8 to 7.6 and 7.1 to 8.6% in 'Honeoye' and 'Mira', respectively, as fruit ripened from white to red. Titratable acidity (TA) decreased significantly as fruit ripened

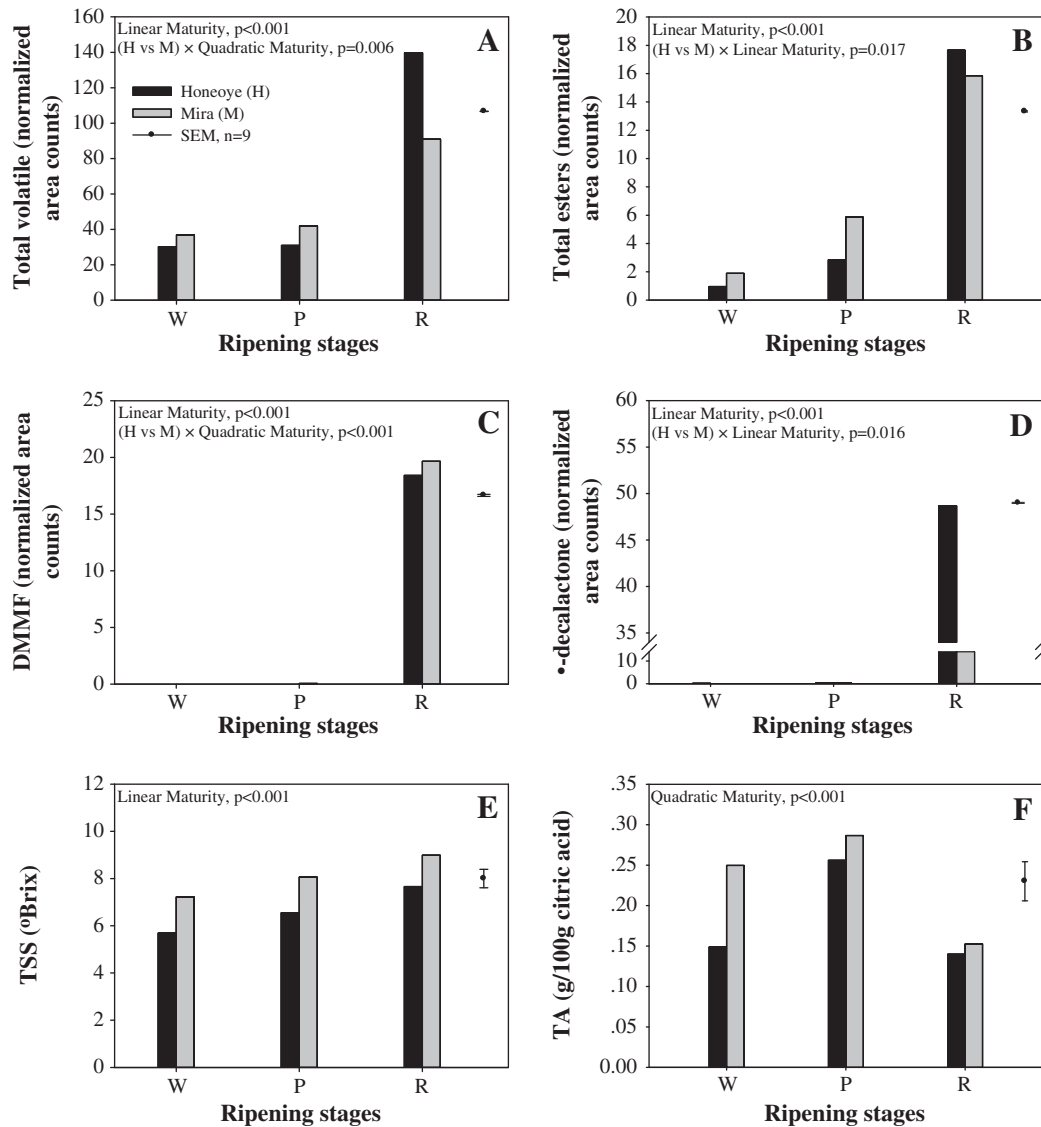


Fig. 3 – Postharvest physiological quality indices of strawberry fruit at different ripening stages. A: Total volatiles, B: Total esters; C: DMMF; D: Decalactone; E: TSS and F: TA Organic acids.

from pink to red (Fig. 3E). The highest amount of TA was found at the pink stage; however, it decreased by 50% in both ‘Honeoye’ and ‘Mira’, respectively, as fruit turned red (Fig. 3F).

3.1.3. Principal component analysis of quality indices in ‘Honeoye’ and ‘Mira’ fruit at different ripening stages

To determine and compare the overall relationship among the strawberry fruit quality indices for ‘Honeoye’ and ‘Mira’ at different ripening stages, a principal component analysis was conducted to reveal the relationships among cultivars, fruit ripening and physiological measures. The PCA plots indicate clearly that fruit ripening contributes significantly to the variation in volatile production, accounting for 84% of the total variability (Fig. 4A). The PCA plot score 2 indicates the difference in the cultivars at the different ripening stages, which only accounts for 15% of the total variability, indicating fruit ripening played a more important role than cultivars in strawberry fruit volatile production. The PCA of anthocyanins,

phenolic compounds, and other quality indices showed that 66% of the variability could be accounted for by fruit ripening. A close relationship among anthocyanin compounds and SS was found. The relationship between ORAC and TA with fruit ripening stages maturity was the only negative relationship, accounting for the 23% of variability.

3.2. Protein identifications in strawberry fruit during ripening

Due to the unavailability of the complete strawberry genome sequence, we used the *Viridiplantae* protein database of NCBI and Swiss-Prot to identify strawberry fruit proteins with 95% confidence as the cut off. From ‘Honeoye’, 958 and 965 proteins were identified from two biological repeats respectively, while from ‘Mira’, 890 and 812 proteins were identified (Fig. S4). After combining all analyses (biological and technical repeats), a total of 3625 proteins were identified. From two

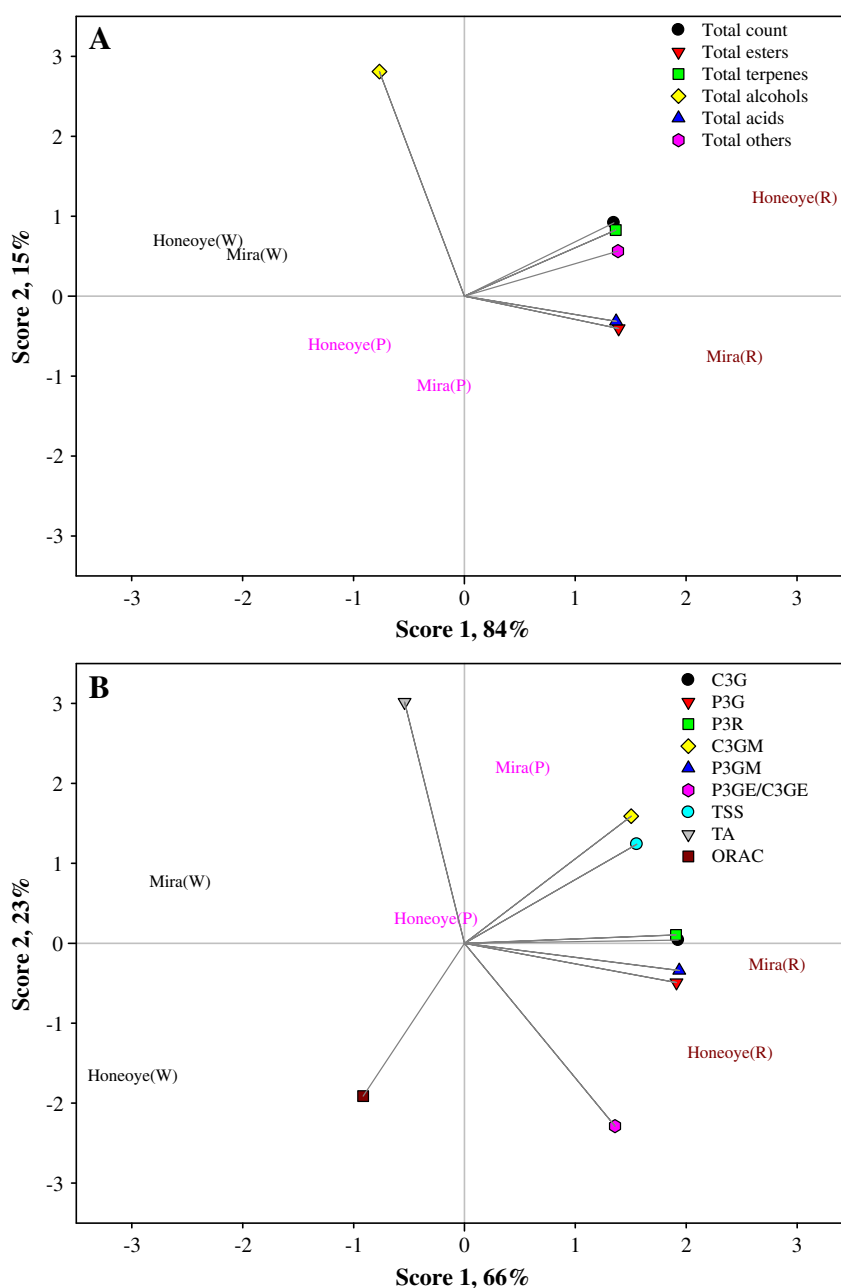


Fig. 4 – Principal component analysis among fruit quality indices in strawberry fruit at different ripening stages. A: Volatile compounds; B: Anthocyanins, phenolic compounds and antioxidant capacities.

biological replicates, we identified 209 and 331 proteins that were commonly present in all maturities and replicates in ‘Honeoye’ and ‘Mira’, respectively. Proteins identified from each cultivar are illustrated in Fig. S3 and listed in supplementary tables, respectively (Tables S2 and 3).

3.3. Protein changes associated with fruit ripening

Using white fruit, labeled light (L) as the denominator, the ratio of proteins at corresponding stages of pink labeled as medium (M) and red labeled heavy (H) were compared to assess protein abundance that was significantly changed at different fruit ripening stages. A twofold cut-off as $\log_2$ (–1

and 1) values was applied to assess changes in abundance and to be stringent enough to minimize false positive abundance changes. Analysis of the strawberry proteome datasets from the two harvests and each cultivar revealed significant regulation of proteins at the three fruit ripening stages. In ‘Honeoye’, among the 209 common proteins identified from both biological replicates, 83 and 63 proteins were found to increase and decrease significantly, respectively (Fig. S5A and Table S2). While, among the 331 common proteins in ‘Mira’, 101 and 139 proteins were found to increase and decrease, respectively (Fig. S5B and Table S3).

Analysis of the strawberry proteome dataset from the two cultivars revealed a total of 111 common proteins at the three

stages of fruit ripening (Table 1). Cluster analysis was employed to provide the groupings of changed proteins (Fig. 5). All the significantly changed proteins are listed in Table 1. The list of identified proteins is also presented in Supplemental Table S1. These results indicate that there is a complex regulatory network involving active processes in strawberry fruit ripening. The identification and quantification of proteins that change significantly revealed that many changes in protein populations occur during the change from pink to red. Identification and quantification of these proteins suggest that their change in abundance was related to fruit ripening and senescence.

3.4. Hierarchical clustering of significantly changed proteins in ripening strawberry fruit

Cluster analysis illustrated the profile of 111 proteins present in both 'Honeoye' and 'Mira' during fruit ripening (Fig. 5). Based on their relative abundance, five clusters of proteins were identified. Overall, Cluster 1 included 34 proteins that increased in pink and red fruit compared to white fruit in both cultivars (Fig. 5, Table 1). Among them, aldo/keto reductase, flavanone 3-hydroxylase, anthocyanidin synthase, quinone oxidoreductase, O-methyltransferase, pyruvate decarboxylase, aspartyl protease family protein, ascorbate peroxidase, alcohol acyltransferase (AAT), Fra a 2 allergen, nutrient reservoir, GTPase and UDP-glucosyl transferase are present. In Cluster 2, 16 proteins were identified whose relative increase was greater in 'Honeoye' than 'Mira' during ripening, including chloroplast carbonic anhydrase, synptobrevin-related protein, pectin methylesterase isoform1, aconitate hydratase and phospholipase alpha. While Cluster 3 shows 21 proteins that were lower in pink, but higher in red fruit in both 'Honeoye' and 'Mira', including catalase (CAT), heat shock protein (HSP70), 14-3-3, cytosolic aldolase and the receptor for activated protein kinase C. Cluster 4 shows 9 proteins that increased in pink but then decreased in red fruit of both cultivars. Those proteins were identified as NDK, HSP70, prohibitin, 40S ribosomal protein S16 and actin. Cluster 5 shows 31 proteins that decreased as ripeness advanced in both cultivars. Among them are aquaporin, elongation factor 1-alpha, methionine synthase, pectinesterase-3 precursor, 2-cys peroxiredoxin, isoflavone reductase related protein, 1-cys peroxiredoxin, ribosomal protein S19, major strawberry allergen Fra a 1-B, cytoplasmic Cu/Zn-superoxide dismutase, RAD23 and ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit protein. All proteins are listed in Table 1.

3.5. Protein identification and functional classifications

Based on the identifications of the most significantly changed proteins, dynamic protein networks during strawberry fruit ripening were studied using GO annotation terms and results from this study. In order to obtain a better understanding of the protein functions, we focused on the proteins that changed significantly at the three maturity stages. Among the 111 common changed proteins present in five clusters, their molecular functions, biological processes and cellular locations were classified. The categorization results obtained from the QuickGO tool demonstrated that the various molecular

functions, biological processes and cellular locations were involved in strawberry fruit ripening (Fig. 6). Clearly, the majority of up-regulated proteins in Cluster 1 have oxidoreductase, nucleotide, catalytic, transferase and magnesium ion binding activities, which are involved in cellular, biological, transport and catabolic process. Those proteins are located at the plasma membrane, chloroplast, membrane and cytoplasm. While many down-regulated proteins in Clusters 4 and 5 showed significant functions in structural constitution of ribosome, translation elongation factor activity, s-methyltransferase, GTPase activity and GTP binding, participate in the GTP catalytic, translation, translational elongation, cellular amino acid biosynthesis and methionine biosynthesis process, are located mainly in ribosome, cytoplasm intracellular, plasma membrane, chloroplast and membrane.

4. Discussion

Strawberry fruit is considered non-climacteric and therefore does not ripen in response to ethylene treatment and produces only a minimum amount of ethylene [8]. However, during ripening, physiological changes lead to significant changes in the fruit affecting aroma volatile production, anthocyanin synthesis, sugar (SS) accumulation, and firmness and acidity loss, which all affect eating quality. From a proteomic standpoint, this study identified significant changes in proteins that may be related to fruit metabolism during ripening and could lead to a better understanding of strawberry fruit ripening. Previous proteomic studies conducted on strawberry fruit have mainly employed 2-D gel based technologies to evaluate hundreds of proteins [18]. In this study, we applied the dimethyl peptide labeling technique combined with off-line OFFGEL to quantitatively evaluate changes in the strawberry proteome that relate to fruit ripening. The overlap between the two cultivars, independent experiments and technical repeats in our study was comparable to other labeling studies on other organisms with labeling techniques such as isobaric tags for relative and absolute quantitation (iTRAQ) or stable isotope dimethyl labeling [27,41,44]. Proteins identified were linked to metabolic process such as flavonoid biosynthesis, volatile production, antioxidants and redox enzymes, response to stress and protein synthesis. Our discussion will be guided by those categories.

4.1. Flavonoid biosynthesis

Flavonoids are an important group of secondary metabolites that contribute directly to the nutritional value of strawberry fruit. We found that total anthocyanin content increased significantly in ripening fruit (Fig. 3). Total phenolics showed a constant level throughout ripening, while total flavonoids decreased significantly. We also found a constant level of fruit antioxidant capacity as measured by both ORAC and FRAP assays. In strawberry fruit, the content of flavonoids showed two phases of biosynthesis during fruit development and ripening with major flavonoid compounds decreasing in red ripe fruit [45]. In most strawberry cultivars, the increase of pigmentation of the receptacle tissue is mainly due to the accumulation of glycosylated pelargonidins and a small

Table 1 – The proteins differentially expressed in five clusters of strawberry proteome during ripening.

No.	Accession	Protein name and species	Score	Mass	Peptide no.	Honeoye				Mira			
						P/W	±SD	R/W	±SD	P/W	±SD	R/W	±SD
Cluster 1													
1	gi 53988164	Aldo/keto reductase (<i>Fragaria × ananassa</i>)	501	37,037.96	15	2.15	0.1955	6.93	3.2240	2.05	0.3678	5.74	3.3582
2	gi 46370000	UDP glucose:flavonoid-3-O-glucosyltransferase (<i>Fragaria × ananassa</i>)	533	51,685.66	15	3.71	1.1590	16.79	5.0722	2.35	0.6323	9.09	1.4478
3	gi 28628183	UDP-glucosyl transferase (<i>Fragaria × ananassa</i>)	384	63,491.7	16	1.84	0.7213	3.72	2.3592	1.44	0.1543	2.57	0.4344
4	gi 51493451	Flavanone 3-hydroxylase (<i>Fragaria × ananassa</i>)	1356	42,418.64	34	2.47	0.2804	8.83	2.0036	1.46	0.3108	4.22	0.3443
5	gi 51872681	Anthocyanidin synthase (<i>Fragaria × ananassa</i>)	426	44,259.13	21	2.53	1.3735	4.99	3.5939	1.16	0.2080	2.59	0.9499
6	gi 15808674	Putative quinone oxidoreductase (<i>Fragaria × ananassa</i>)	2151	37,313.11	63	1.85	0.1254	10.11	6.4222	1.52	0.1950	7.92	3.1790
7	gi 29468088	Quinone oxidoreductase (<i>Fragaria × ananassa</i>)	1293	35,288	51	1.48	0.2882	10.06	7.5589	1.40	0.0382	7.03	3.1774
8	gi 225441044	PREDICTED: hypothetical protein (<i>Vitis vinifera</i>)	261	59,222.08	9	1.04	0.0701	1.31	0.4663	1.02	0.0968	1.21	0.5012
9	gi 6760443	O-methyltransferase (<i>Fragaria × ananassa</i>)	1318	41,345.64	26	1.24	0.1814	5.45	1.0166	2.06	0.2664	4.83	0.6858
10	gi 17225598	Pyruvate decarboxylase (<i>Fragaria × ananassa</i>)	819	67,405.03	32	1.39	0.0897	4.49	0.1756	1.87	0.2791	3.49	0.2670
11	gi 10121330	Pyruvate decarboxylase (<i>Fragaria × ananassa</i>)	678	67,213.72	30	1.38	0.2335	3.92	0.9356	1.62	0.3666	3.91	1.5701
12	gi 70907497	Cystatin CPI-1 (<i>Fragaria × ananassa</i>)	254	27,169.49	5	1.00	0.1786	1.58	0.7137	1.14	0.1220	1.38	0.2169
13	gi 254771939	Alcohol acyltransferase (<i>Fragaria chiloensis</i>)	266	51,780.93	7	1.97	1.4831	14.02	17.3496	1.32	0.3347	2.70	1.9898
14	gi 224100177	Predicted protein (<i>Populus trichocarpa</i>)	127	42,714.32	4	1.47	1.4562	10.17	9.4215	1.01	0.3863	2.38	2.9631
15	gi 10121328	Alcohol acyltransferase (<i>Fragaria × ananassa</i>)	419	52,057.06	10	1.76	0.8189	14.75	18.2232	1.0197	0.1372	1.99	1.1197
16	gi 2792295	Unknown (<i>Fragaria × ananassa</i>)	197	36,708.65	11	1.60	1.1233	4.85	2.8283	1.0591	0.3173	1.35	0.5338
17	gi 238479752	GTP binding/gtpase (<i>Arabidopsis thaliana</i>)	311	93,889.68	12	1.04	0.1382	1.67	0.1616	0.96	0.1109	1.13	0.0075
18	gi 82400168	Adenosine kinase isoform 1 T-like protein (<i>Solanum tuberosum</i>)	499	38,821.07	7	1.23	0.2618	2.06	0.9297	0.94	0.5727	1.41	0.3970
19	gi 217069906	Unknown (<i>Medicago truncatula</i>)	108	17,296.03	2	1.12	0.1527	1.54	0.5171	0.91	0.0226	1.32	0.3008
20	gi 15220668	Phosphoglucumutase, cytoplasmic, putative/glucose phosphomutase, putative (<i>Arabidopsis thaliana</i>)	185	64,950.9	11	0.95	0.2314	1.39	0.5191	0.92	0.1194	1.15	0.0522
21	gi 6911551	Heat shock protein 70 (<i>Cucumis sativus</i>)	2135	73,755.81	37	0.96	0.1516	1.27	0.3028	0.94	0.1239	1.08	0.0783
22	gi 224120514	Predicted protein (<i>Populus trichocarpa</i>)	146	31,386.07	5	1.05	0.0739	1.31	0.1220	0.96	0.0878	0.98	0.1131
23	gi 224112467	Predicted protein (<i>Populus trichocarpa</i>)	364	197,619.9	13	0.97	0.0529	1.26	0.1294	0.81	0.1411	2.02	1.2453
24	gi 255574808	Clathrin heavy chain, putative (<i>Ricinus communis</i>)	426	197,803.8	21	1.08	0.0296	1.22	0.2220	0.96	0.0213	1.43	0.8358
25	gi 8885586	Unnamed protein product (<i>Arabidopsis thaliana</i>)	57	27,617.04	2	1.01	0.3462	1.22	0.7615	1.02	0.2448	1.35	0.5340
26	gi 260600660	Fra a 2 allergen (<i>Fragaria × ananassa</i>)	275	18,217.74	11	2.44	2.3956	2.39	0.8012	2.26	2.1061	1.76	1.1150
27	gi 255570801	Nutrient reservoir, putative (<i>Ricinus communis</i>)	133	53,389.02	3	1.32	0.0917	1.23	0.3593	1.28	0.1965	1.12	0.0232
28	gi 23507246	Oligomycin sensitivity conferring protein (<i>Silene latifolia</i>)	125	26,342.33	4	1.70	NA	1.76	NA	1.24	0.2420	1.53	0.1415
29	gi 67937775	Non-specific lipid transfer protein precursor (<i>Fragaria × ananassa</i>)	103	12,559.02	5	1.69	0.2532	1.58	0.1618	1.24	0.3661	1.53	0.1399
30	gi 118340975	11S globulin precursor isoform 3B (<i>Ficus pumila</i> var. <i>Awkeotsang</i>)	226	56,836.49	13	1.58	0.1413	1.59	0.1768	1.20	0.3420	1.64	0.1698
31	gi 147798422	Hypothetical protein (<i>Vitis vinifera</i>)	310	18,521.48	2	1.19	0.5024	1.28	0.8785	1.64	0.0266	1.11	0.4845
32	gi 125536152	Hypothetical protein osi_37858 (<i>Oryza sativa Indica</i> Group)	59	33,077.54	2	0.73	NA	2.49	NA	1.58	0.1884	1.36	0.7682
33	gi 15808779	Ascorbate peroxidase (<i>Hordeum vulgare</i> subsp. <i>Vulgare</i>)	93	28,439.33	4	0.86	0.7767	1.89	0.1193	1.05	0.3434	1.03	0.0586
34	gi 118481185	Unknown (<i>Populus trichocarpa</i>)	150	40,807.07	4	0.95	0.2595	1.40	0.6951	1.18	0.1508	0.92	0.2006
Cluster 2													
1	gi 62865755	Chloroplast carbonic anhydrase (<i>Nicotiana benthamiana</i>)	292	19,698.86	4	3.79	4.3724	1.78	1.6110	0.87	0.3268	1.16	0.1030
2	gi 224139550	Predicted protein (<i>Populus trichocarpa</i>)	317	27,964.88	6	2.13	0.5236	2.75	0.8336	0.87	0.0637	0.99	0.3233
3	gi 297819656	Hypothetical protein ARALYDRAFT_323570 (<i>Arabidopsis lyrata</i> subsp. <i>Lyrata</i>)	55	30,299.75	3	1.75	1.0072	2.26	1.2800	0.81	0.0508	1.10	0.2133
4	gi 225430198	PREDICTED: hypothetical protein (<i>Vitis vinifera</i>)	131	15,058.98	4	1.60	1.0730	2.28	1.6518	0.84	0.1215	0.99	0.0464
5	gi 116782330	Unknown (<i>Picea sitchensis</i>)	101	16,623.54	8	1.93	0.4501	1.83	0.5929	1.05	0.3106	0.80	0.2083

6	gi 33347409	Synptobrevin-related protein (<i>Pyrus pyrifolia</i>)	127	22,758.86	6	1.76	0.9542	2.08	0.2671	0.99	0.3850	0.81	0.2722
7	gi 224138868	Precursor of carboxylase h-protein 4, glycine decarboxylase complex (<i>Populus trichocarpa</i>)	83	18,003.91	2	1.84	NA	2.28	NA	0.96	0.6602	0.67	0.6679
8	gi 62288364	Pectin methylesterase isoform 1 (<i>Fragaria × ananassa</i>)	695	24,921.22	25	1.16	0.1845	1.35	0.2660	0.87	0.0947	0.91	0.0831
9	gi 3121731	Aconitate hydratase, cytoplasmic; Aconitase; Citrate hydro-lyase	302	68,736.63	5	1.09	0.1210	1.20	0.2832	0.89	0.0252	0.90	0.3060
10	gi 225441977	PREDICTED: hypothetical protein (<i>Vitis vinifera</i>)	717	68,816.68	23	1.11	0.0767	1.12	0.2475	0.94	0.0104	0.92	0.2424
11	gi 58891689	Phospholipase D alpha (<i>Fragaria × ananassa</i>)	532	93,417.08	24	1.04	NA	1.12	NA	0.89	0.2306	0.97	0.2324
12	gi 225450055	PREDICTED: hypothetical protein (<i>Vitis vinifera</i>)	67	40,971.12	6	1.37	0.0017	1.72	0.0044	2.94	0.0479	3.39	0.2064
13	gi 147792574	Hypothetical protein (<i>Vitis vinifera</i>)	77	78,813.01	3	1.01	0.8782	1.19	0.1401	1.40	0.1491	2.58	0.2259
14	gi 4691448	Actin (<i>Populus alba</i>)	408	10,567.16	9	0.82	0.0582	1.18	0.0474	1.73	0.1535	2.01	0.1364
15	gi 15232503	Aspartyl protease family protein (<i>Arabidopsis thaliana</i>)	73	46,679.19	4	1.03	0.2408	0.82	0.0289	2.24	0.1767	1.69	0.0657
16	gi 3914394	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase; BPG-independent PGAM; Phosphoglyceromutase; PGAM-I	96	62,579.23	4	1.07	0.0512	0.77	0.0041	1.69	0.2068	1.69	0.2123
Cluster 3													
1	gi 118486221	Unknown (<i>Populus trichocarpa</i>)	456	35,978.22	16	0.87	0.1597	0.82	0.2163	0.84	0.1611	1.32	0.1628
2	gi 54306591	Major storage protein (<i>Fragaria × ananassa</i>)	251	17,339.86	11	1.22	0.5236	0.87	0.8336	1.16	0.0637	0.85	0.3233
3	gi 6911549	Heat shock protein 70 (<i>Cucumis sativus</i>)	1305	75,908.37	21	0.92	0.1780	1.03	0.2516	0.98	1.8564	0.97	1.2835
4	gi 3202032	Leaf catalase (<i>Mesembryanthemum crystallinum</i>)	169	58,028.49	6	0.78	0.0917	1.20	0.3593	0.84	0.1965	0.94	0.0232
5	gi 57639078	ER-binding protein (<i>Malus pumila</i>)	1105	75,991.26	22	0.91	4.3724	1.10	1.6110	0.94	0.3268	0.88	0.1030
6	gi 192910870	Uridylate kinase (<i>Elaeis guineensis</i>)	128	23,611.85	4	0.81	0.0701	1.51	0.4663	0.86	0.0968	0.79	0.5012
7	gi 55375985	14-3-3 Family protein (<i>Malus × domestica</i>)	1249	30,442.78	37	0.81	0.1413	1.36	0.1768	0.82	0.3420	0.84	0.1698
8	gi 168068005	Predicted protein (<i>Physcomitrella patens</i> subsp. <i>Patens</i>)	125	51,836.81	8	0.86	0.4351	1.44	0.2964	0.73	0.0049	0.86	0.3648
9	gi 116780995	Unknown (<i>Picea sitchensis</i>)	82	32,655.52	3	0.69	0.3899	1.54	0.3292	0.59	0.2138	0.66	0.0420
10	gi 10645188	Cytosolic aldolase (<i>Fragaria × ananassa</i>)	1040	39,801.97	32	0.92	0.2921	1.03	0.1351	0.87	0.0626	0.94	0.2034
11	gi 7527721	T5E21.15 (<i>Arabidopsis thaliana</i>)	82	64,706.17	2	0.98	0.4659	1.17	0.5984	0.78	0.2180	0.90	1.2400
12	gi 25555301	D-3-phosphoglycerate dehydrogenase, putative (<i>Ricinus communis</i>)	196	64,633.47	11	0.94	0.0404	1.12	0.1525	0.61	0.4112	0.81	0.3159
13	gi 217795376	Transaldolase (<i>Dimocarpus longan</i>)	207	49,072.01	7	0.95	0.1210	1.04	0.2832	0.88	0.0252	0.87	0.3060
14	gi 211906454	Peroxisomal protein (<i>Gossypium hirsutum</i>)	530	17,841.66	5	0.85	0.0767	1.04	0.2475	0.85	0.0104	0.74	0.2424
15	gi 255577354	Receptor for activated protein kinase C, putative (<i>Ricinus communis</i>)	230	37,046.04	7	0.87	NA	0.99	NA	0.93	0.1220	0.86	0.1868
16	gi 3421123	20S proteasome beta subunit PBG1 (<i>Arabidopsis thaliana</i>)	298	28,219.47	4	0.69	0.2356	1.02	0.1400	0.71	0.1878	0.69	0.0259
17	gi 118486780	Unknown (<i>Populus trichocarpa</i>)	79	36,076	3	0.78	0.0046	1.00	0.0232	0.96	0.0036	1.10	0.0471
18	gi 297848004	Hypothetical protein ARALYDRAFT_474695 (<i>Arabidopsis lyrata</i> subsp. <i>Lyrata</i>)	247	48,539.89	13	0.84	0.1890	0.93	0.3064	0.86	0.0180	0.97	0.0713
19	gi 224109062	Predicted protein (<i>Populus trichocarpa</i>)	291	43,862.42	8	0.79	0.0650	0.92	0.3490	0.88	0.1560	0.90	0.0222
20	gi 225450227	PREDICTED: hypothetical protein (<i>Vitis vinifera</i>)	181	104,618.2	6	0.81	0.1617	1.01	0.5004	0.79	0.4366	0.95	0.3435
21	gi 255550271	Pectinesterase-3 precursor, putative (<i>Ricinus communis</i>)	106	65,717.71	8	0.33	NA	1.00	NA	0.48	0.6602	0.78	0.6679
Cluster 4													
1	gi 254558248	Heat shock protein 70 (<i>Ulva prolifera</i>)	1052	73,290.9	19	0.97	0.2056	1.05	0.1670	1.10	0.1726	0.76	0.1112
2	gi 47026989	Nucleoside diphosphate kinase (<i>Hyacinthus orientalis</i>)	633	18,206.76	20	1.25	0.4501	1.08	0.5929	0.95	0.3106	0.81	0.2083
3	gi 115479225	Os09g0422000 (<i>Oryza sativa Japonica</i> Group)	53	48,036.9	4	1.28	0.3192	1.09	0.5082	0.78	0.0814	0.67	0.1163
4	gi 255553601	Prohibitin, putative (<i>Ricinus communis</i>)	238	32,637.05	4	1.02	0.0124	0.99	0.0755	0.98	0.2017	0.85	0.2818
5	gi 113367104	Actin (<i>Aegiceras corniculatum</i>)	556	14,500.03	12	0.98	0.3842	1.04	0.1238	0.93	0.2736	0.80	0.1076
6	gi 6850918	Serine carboxypeptidase (<i>Cicer arietinum</i>)	62	41,341.33	4	1.04	2.3956	1.28	0.8012	0.87	2.1061	0.62	1.1150
7	gi 6984222	40S ribosomal protein S16 (<i>Euphorbia esula</i>)	154	17,044.3	14	1.08	0.2498	0.89	0.5195	0.86	2.2028	0.75	2.5273
8	gi 118482048	Unknown (<i>Populus trichocarpa</i>)	155	46,700.58	2	1.06	0.3894	0.8512	0.6227	0.8297	0.0727	0.6215	0.2611
9	gi 225446831	PREDICTED: hypothetical protein (<i>Vitis vinifera</i>)	149	29,207.69	5	1.62	0.0529	0.7586	0.1294	0.8331	0.1411	0.3124	1.2453

(continued on next page)

Table 1 (continued)

No.	Accession	Protein name and species	Score	Mass	Peptide no.	Honeoye				Mira			
						P/W	±SD	R/W	±SD	P/W	±SD	R/W	±SD
Cluster 5													
1	gi 116787058	Unknown (<i>Picea sitchensis</i>)	83	17,395.9	4	1.08	0.2018	0.7355	1.2828	0.7953	0.1427	0.8089	0.1704
2	gi 82568687	Aquaporin (<i>Prunus mume</i>)	61	30,535.55	2	1.59	0.2120	0.4439	0.1032	0.7911	0.0787	0.5998	0.0986
3	gi 157060284	Actin (<i>Lobosphaeropsis lobophora</i>)	342	25,131.85	13	0.77	0.2017	0.7140	0.2653	0.9365	0.2220	0.8199	0.0612
4	gi 5640111	RAD23 protein (<i>Solanum lycopersicum</i> var. <i>Cerasiforme</i>)	86	41,957.17	3	0.73	NA	0.4663	NA	1.0408	0.2420	0.7835	0.1415
5	gi 95106179	Cytoplasmic Cu/Zn-superoxide dismutase (<i>Populus suaveolens</i>)	240	15,746.65	20	0.90	0.0653	0.9272	0.0214	0.8008	0.0158	0.7151	0.0396
6	gi 115361539	Methionine synthase (<i>Nicotiana suaveolens</i>)	756	87,085.52	23	0.72	0.2421	0.8681	0.3552	0.6162	0.3010	0.5052	0.0503
7	gi 116786768	Unknown (<i>Picea sitchensis</i>)	791	55,028.47	33	0.84	0.2977	0.8182	0.8650	0.6538	0.0408	0.6484	0.0138
8	gi 226530753	Elongation factor 1-alpha (<i>Zea mays</i>)	975	51,295.77	53	0.83	0.1592	0.7777	0.4110	0.6635	0.2482	0.6705	0.1763
9	gi 225455555	PREDICTED: hypothetical protein (<i>Vitis vinifera</i>)	761	49,654.75	11	0.85	0.1215	0.8041	0.4042	0.7594	0.1716	0.6662	0.1145
10	gi 297804932	Hypothetical protein ARALYDRAFT_915516 (<i>Arabidopsis lyrata</i> subsp. <i>Lyrata</i>)	647	55,217.37	35	0.84	0.1883	0.7853	0.2629	0.6941	0.0184	0.6096	0.0951
11	gi 151347486	Methionine synthase (<i>Carica papaya</i>)	1950	86,456.96	44	0.79	0.1331	0.7286	0.0527	0.6189	0.0973	0.4995	0.0419
12	gi 71000469	Methionine synthase (<i>Beta vulgaris</i>)	887	90,045.91	26	0.76	0.0511	0.7285	0.7437	0.7177	1.4289	0.5924	1.5183
13	gi 445612	Ribosomal protein S19	270	16,297.98	5	0.65	NA	0.6802	NA	0.5817	0.2081	0.5213	0.0521
14	gi 13431949	Triosephosphate isomerase, chloroplastic; TIM; Triose-phosphate isomerase	283	34,535.36	10	0.73	0.0077	0.8204	0.0083	0.7907	0.0782	0.6450	0.0275
15	gi 148524155	Elongation factor 1-alpha (<i>Chara australis</i>)	590	49,635.81	39	0.53	0.2735	0.75	0.2434	0.48	0.0557	0.43	0.1760
16	gi 159491562	Predicted protein (<i>Chlamydomonas reinhardtii</i>)	45	19,432.4	3	0.44	0.2595	0.47	0.6951	0.51	0.1508	0.45	0.2006
17	gi 116783572	Unknown (<i>Picea sitchensis</i>)	179	18,067.63	8	0.36	0.2250	0.25	0.1549	0.32	0.4837	0.45	0.1399
18	gi 1143427	Heat shock protein 70 (<i>Cucumis sativus</i>)	313	77,428.62	10	0.95	0.2531	0.79	0.2954	1.02	0.2541	0.69	0.0352
19	gi 90185678	Major strawberry allergen Fra a 1-B (<i>Fragaria</i> × <i>ananassa</i>)	963	18,581.1	20	0.87	0.0117	0.68	0.1156	0.95	0.0692	0.61	0.0284
20	gi 225447408	PREDICTED: hypothetical protein (<i>Vitis vinifera</i>)	212	28,935.49	9	0.86	0.0296	0.79	0.2220	0.91	0.0213	0.61	0.8358
21	gi 9279589	Dihydrolipoamide S-acetyltransferase (<i>Arabidopsis thaliana</i>)	120	46,130.85	2	0.98	NA	0.67	NA	0.89	0.1527	0.61	0.0312
22	gi 147789055	Hypothetical protein (<i>Vitis vinifera</i>)	90	49,592.38	3	0.84	0.2222	0.54	0.0775	0.75	0.0885	0.41	0.2216
23	gi 3885515	Similar to ribosomal protein S26 (<i>Medicago sativa</i>)	79	14,946.51	2	0.86	0.0923	0.76	0.0752	0.78	0.0174	0.59	0.0383
24	gi 255569484	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, putative (<i>Ricinus communis</i>)	1770	86,470.03	48	0.83	NA	0.71	NA	0.78	0.1884	0.56	0.7682
25	gi 78210840	Beta tubulin like protein (<i>Pyrus pyrifolia</i> var. <i>Culta</i>)	625	45,390.73	26	0.85	0.6627	0.68	0.1555	0.77	0.6627	0.59	0.6421
26	gi 3243234	Isoflavone reductase related protein (<i>Pyrus communis</i>)	92	34,475.04	2	0.51	0.0590	0.33	1.2322	0.56	0.2239	0.30	0.8627
27	gi 255087344	Predicted protein (<i>Micromonas</i> sp. RCC299)	114	32,197.38	4	0.79	NA	0.29	NA	0.79	0.3038	0.48	0.4379
28	gi 242064500	Hypothetical protein SORBIDRAFT_04g007650 (<i>Sorghum bicolor</i>)	57	48,083.22	2	0.93	0.1041	0.30	0.1545	0.66	0.1624	0.46	0.0674
29	gi 269980509	2-Cys peroxiredoxin (<i>Vigna radiata</i>)	175	29,339.63	4	0.83	0.2375	0.45	0.1930	0.79	0.0319	0.51	0.0819
30	gi 54306593	1-Cys peroxiredoxin (<i>Fragaria</i> × <i>ananassa</i>)	198	10,094.87	3	0.73	0.9542	0.39	0.2671	0.73	0.3850	0.45	0.2722
31	gi 116672873	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (<i>Camellia sinensis</i>)	80	20,485.35	4	0.72	0.3423	0.28	0.3860	0.59	0.1381	0.36	0.1282

amount of glycosylated cyanidins [11,46], which is in agreement with our analysis. At the proteomic level, we identified and quantified several key enzymes related to flavonoids and anthocyanidin biosynthesis including UDP glucose:flavonoid-3-O-glucosyltransferase (UG3T), UDP-glucosyl transferase [47], flavanone 3-hydroxylase (F-3H), and anthocyanidin synthase (ANS). These enzymes were found in both cultivars and increased during ripening (Cluster1) (Table 1 and Fig. 5). We also identified phenylalanine ammonia-lyase (PAL) in 'Honeoye' and cinnamoyl CoA reductase (CHR), chalcone synthase (CHS), and chalcone-flavonone isomerase (CHI) in 'Mira' that increased during ripening (Supplemental data, Table S2). These results indicated that the flavonoid biosynthesis pathway in ripening strawberry fruit is up-regulated from the entry point of carbons through phenylalanine. Characterization of the flavonoid biosynthetic pathways at the transcript level showed the up-regulation of PAL, ANS, FNS CHS, while the phenylpropanoid genes FaPAL, FaC4H and Fa4CL had a similar two-phase expression pattern with a low level of transcripts in white fruit [48]. Gene expression of anthocyanidin glucosyltransferase (FaGT1) was undetected in white strawberry fruit, but increased significantly in pink and red fruit. Therefore its gene expression is related to fruit ripening. Also it is negatively regulated by auxin. Down regulation of anthocyanidin glucosyltransferase (FaGT1) resulted in a significant decrease of pelargonidin 3 O-glucose malone and pelargonidin O-glucoside and revealed the fact that it is a branching point in the synthesis pathway that directs the flow of flavonoids into anthocyanin synthesis [10]. Our results provide strong evidence at the proteomic level that ANS, F3H, UFGT and UG3T are up-regulated during anthocyanin synthesis leading to the red color in ripe strawberry fruit.

In contrast, an isoflavone reductase (IFR) decreased with ripening in this study. IFR is an enzyme specific to isoflavonoid biosynthesis. It catalyzes a NADPH-dependent reduction involved in the biosynthesis of important phenylpropanoid-derived plant defense compounds, e.g. phytoalexins. Proteomic work using 2-DE on strawberry found different IFR related proteins were up-regulated and increased during fruit ripening [18]. It has been demonstrated that IFR abundance increased in response to heat stress during climacteric ripening of banana fruit [49]. Two additional IFR-like proteins were found to be up-regulated during late ripening in skin tissue of 'Barbera' grapes (*Vitis vinifera*), another non-climacteric fruit [50]. The discrepancy between our work and published results may indicate the presence of multiple isozymes of IFR in strawberry fruit that are regulated differently from other species by both genetic and environmental factors.

4.2. Proteins associated with volatile production

Strawberry fruit produce more than 300 volatile aroma compounds; the majority are esters, alcohols, aldehydes, terpenes and acids [51]. Our study clearly illustrates that total volatile production is significantly increased as fruit ripening advances from white to pink to red (Fig. 3). This increase of volatile compounds is closely related to total volatiles, esters and acids, but negatively related to alcohols, which were predominant in white fruit (Figs. 3 and 4). The increase of total volatile compounds and esters is well

correlated with the protein profiles associated with aroma production in strawberry. We identified and quantified two alcohol acyltransferases (AATs), two quinone oxidoreductases and O-methyltransferase (Table 1 and Fig. 1). The involvement of AAT in ester formation of many fruit is well known [52]. AAT is one of the most important enzymes to catalyze the formation of esters in strawberry fruit during ripening [4]. At the proteomic level, we found two AAT proteins (AAT1 and AAT2) increased more between the pink to red stage than from white to pink stage in both cultivars. This change confirmed the results at the transcript level reported by Aharoni et al. that AAT expression is induced in fruit ripening [4]. In addition, we also found significant up-regulation of two pyruvate decarboxylases (PDC) in both 'Honeoye' and 'Mira' and acyl-CoA synthetase in 'Mira'. These increases can be explained by the high demand for acyl-CoA for ester and fatty acid biosynthesis during ripening. Our work provides proteomic evidence that PDC and AAT become more abundant concurrent with rapid volatile production in ripening strawberry fruit.

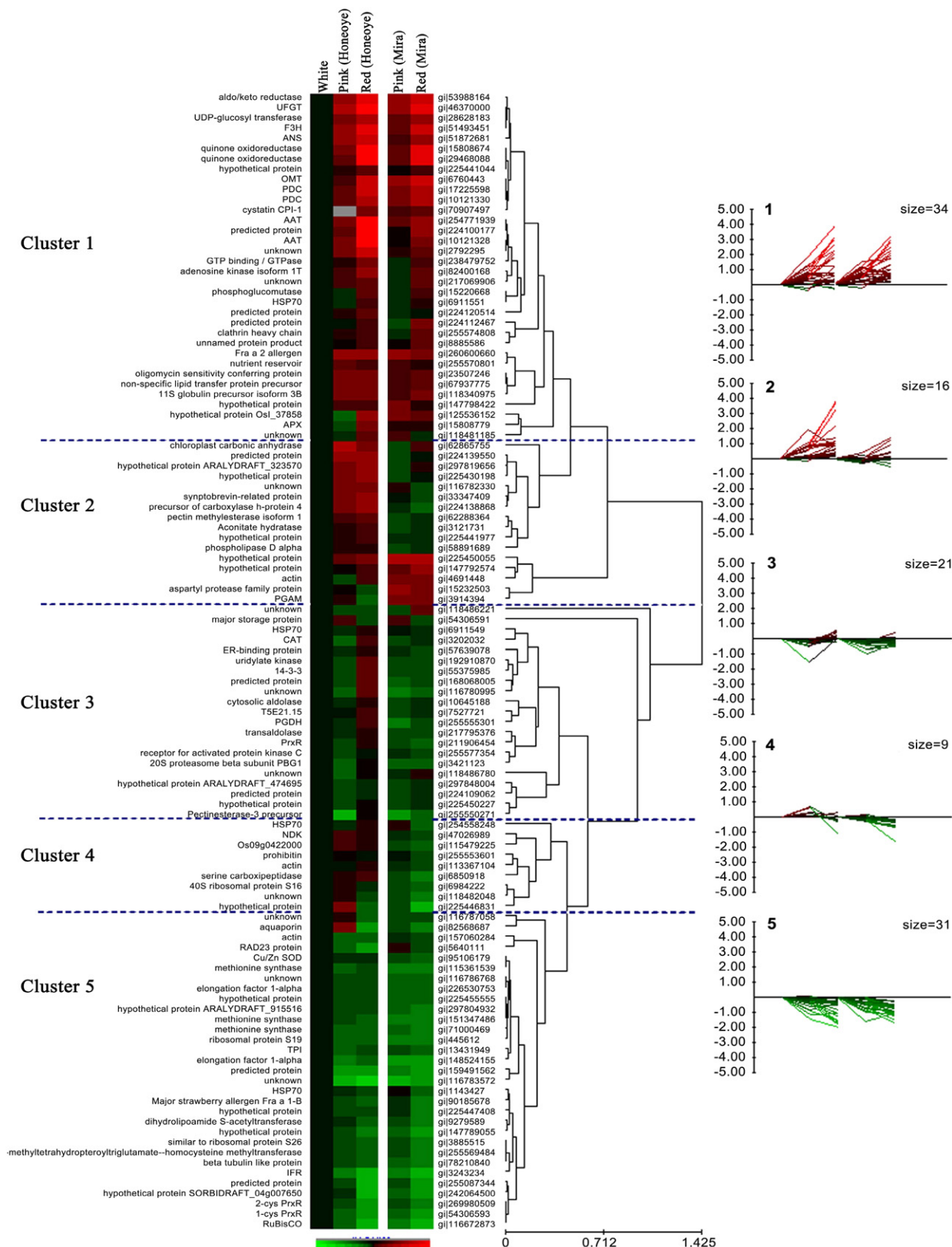
Furanones [4-hydroxy-2, 5 dimethyl (2,3H), DMHF, furaneol and 4-methoxy-2, 5 dimethyl (2,3H), DMMF, mesifurane] have been recognized as key volatile compounds in strawberry and a group of 3(2H)-furanone compounds have been identified in strawberry fruit [53,54]. Using HS-SPME, we identified DMMF (mesifurane) in both cultivars. DMMF has a signature olfactory characteristic of "sweet" and "burnt sugar" notes and its methylation is mediated by an O-methyltransferase (FaOMT), whose activity increased during fruit ripening [55]. The formation of furanones is under the control of FaOMT, a gene that is responsible for the variation of the natural mesifurane content in strawberries [56,57]. In both 'Honeoye' and 'Mira', there was a significant increase in OMT protein as fruit ripened from white to red, which coincides with the increase of DMMF concentration. In addition, two quinone oxidoreductases were identified, which both increased during ripening. The quinone oxidoreductase FaQR, has been shown to be strongly induced by ripening and to be auxin dependent [58]. This enzyme functions as an enone oxidoreductase in the biosynthesis of DMMF and is responsible for the conversion of DMHF to DMMF. In anti-ACO line kiwi fruit, it increased 5-fold in expression and peaked at 168 h after ethylene treatment [59]. These results indicate the role of quinone oxidoreductase in volatile biosynthesis during fruit ripening.

4.3. Antioxidants and redox enzymes

Antioxidant and redox related enzymes play an important role during fruit ripening and senescence [60]. Transcriptional analysis during strawberry fruit ripening indicated that the strawberry ripening transcriptional program is an oxidative, stress-induced process [61]. The balance between ROS production and the activities of these ROS-removing systems determine the type and concentration of ROS present and thus to what extent signaling and/or damage will occur. In our study, ascorbate peroxidase protein increased as fruit ripened (Table 1, Fig. 5). A homologue of cytosolic ascorbate peroxidase protein was identified as an increased protein in strawberry during ripening [18]. Ascorbate (vitamin C) has important antioxidant and metabolic functions, which may

contribute up to 25–30% of the total antioxidant capacity of the ripe strawberry fruit. In addition to APX, we identified a GDP-mannose-3'-5'-epimerase [62] protein which involves in the synthesis of ascorbate, as a protein that decreased in 'Honeye' (Support information Fig. S5, Table

S2). The final concentration of ascorbate in fruit is balanced by its biosynthesis, metabolism and recycling. Therefore, the identification of APX and GME may partially explain the reasons antioxidant capacity decreases in ripe strawberry due to higher metabolism and lower synthesis. Both down- and



up-regulation of APX in grape fruit during ripening has been reported while two CATs decreased during ripening [63]. Meanwhile, a catalase (CAT) decreased in pink but then increased in red fruit. Compared to 'Honeoye', CAT concentration was lower in 'Mira' fruit (Fig. 5).

We also identified peroxiredoxin R (PrxR), 1- and 2-cys peroxiredoxin (Prx) and Cu/Zn-superoxide dismutase (SOD), as down-regulated proteins during ripening. We further identified that thioredoxin H3 was up-regulated during fruit ripening in 'Honeoye' (Table 1, Fig. 4, and support information Table S2). These enzymes are responsible for reactive oxygen species (ROS) removal systems in plants. SOD was up-regulated during apple and papaya fruit ripening [64]. Prx plays a significant role in plant growth and development, redox-signaling, desiccation tolerance, detoxification of reactive oxygen/nitrogen species, protection from pathogen attack and tolerance to other abiotic stresses. Bianco et al. [18] reported that the decrease of 1-cys peroxiredoxin in strawberry fruit during ripening is in agreement with the present study. We found a decrease in PrxR and 2-cys peroxiredoxins during ripening. These results indicate the ROS removing systems, which derive reducing power directly or indirectly from NADPH or ferridoxin, decreased. Interestingly, a nucleoside diphosphate kinase (NDK) was also identified and grouped in Cluster 4. NDK was recently reported to be involved in ROS resistance and to interact with CAT1 [65].

4.4. Allergens and defense response proteins in strawberries

In our study, many defense proteins were identified and quantified. Among them, allergen proteins showed a strong response to fruit ripening. Both Fra a 2 allergen and non-specific lipid transfer protein (nsLTP) precursor were identified in Cluster 1 and increased with ripening, while a major strawberry allergen Fra a 1-B decreased (Cluster 2). Previous investigations on strawberry fruit allergens revealed that Fra a 2 and nsLTP are widely present in strawberry fruit, seeds and leaves. At the transcript level, the gene expression of Fra a 2 is directly linked to flavonoid biosynthesis and the Fra a 2 allergen had an essential biological function in pigment formation in strawberry fruit [66]. The nsLTP genes are developmentally regulated and the accumulation of gene transcripts and encoded protein were observed during development in several Rosacea fruits, such as apple [67] and peach [68]. Postharvest storage caused a reduction of nsLTPs content and related gene expression in apple and peach [69,70]. The decrease of the major strawberry allergen Fra a 1-B revealed the complex nature of allergens and their biological functions in strawberry fruit during ripening. Fra a1, like Bet v1, is

homologous to the group of pathogenesis-related (PR)-10 proteins that were originally characterized at the transcriptional level, showing an increased gene expression in stress situations, such as during microorganism infection [71]. Previous results on Fra a 1e also showed a decreased level of gene expression and that Fra a isoforms might bind and transport flavonoid pathway intermediates within the cytosol or through membranes [66,72]. Recent research reported that the amount of Fra a 1 protein had a relevant genotype dependent response to environmental stress conditions and had poor correlations to antioxidant, phenolic and allergenic properties of strawberry fruit in different years [73]. The identification of allergens in strawberry fruit during ripening (Fig. 2, Table 1) demonstrates the dynamic presence of allergens in this fruit and that ripening influences the formation and regulation of fruit allergens. It is interesting to consider whether fruit allergen content may be mitigated by postharvest handling techniques. With the additional information on peptides provided in this study, we can also design future quantitative proteomic analysis of strawberry allergens using the multi-reaction monitoring (MRM) approach.

Another significant enzyme, aldo/keto reductase (AKR) [74], which is responsible for the detoxification of toxic compounds generated by the interaction between ROS and polyunsaturated fatty acids of membrane lipids was also identified in Cluster 1 and increased significantly (Fig. 2). A positive role of AKR in rice was found in abiotic stress-related reactive aldehyde detoxification pathways and has been used for improvement of stress tolerance in plants [75]. More research is needed to characterize this enzyme in strawberry fruit.

4.5. Protein synthesis, degradation and folding

Fruit ripening involved many dynamic changes in proteins, including de novo synthesis and degradation. Little information is available on the regulation of protein synthesis during fruit ripening. In our study, we analyzed several proteins involved with protein synthesis, degradation and folding. Along these lines, we reported three methionine synthases, 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, RAD32 protein, ribosomal protein S19, 40S ribosomal protein S16, two elongation factor 1- α and two heat shock protein 70 as well as beta tubulin like protein and actin (Table 1, Fig. 3). This group of proteins is mainly present in Clusters 4 and 5, indicating that they were down-regulated during strawberry fruit ripening. Methionine synthase catalyzes the final reaction of the Methionine biosynthetic pathway using cysteine and O-phosphohomoserine as substrates [76]. In plants, methionine serves as a precursor for a variety of metabolic processes, including protein synthesis, as the prime methyl donor for a large number of biological

Fig. 5 – Hierarchical cluster analysis of 111 proteins in 'Honeoye' and 'Mira' strawberry fruit during ripening. The protein name is indicated on the right side of the plot and listed in Table 1. Based on the ratio between pink/white and red/white, five major clusters of protein changes were formed: Cluster 1 shows the proteins that were up-regulated in both 'Honeoye' and 'Mira' in both pink and red fruit; Cluster 2 reveals the proteins that increased in both pink and red fruit but were more abundant in 'Honeoye'. Cluster 3 demonstrates the proteins that increased in red 'Honeoye' fruit but decreased in 'Mira'. Cluster 4 presents the proteins that increased in pink fruit but decreased in red. Cluster 5 illustrates the proteins that decreased in pink and red fruit in both 'Honeoye' and 'Mira'. Increasing intensities of red or green color indicate differentially up- or down-regulated proteins compared with the control at day 0, respectively. Identified proteins are also marked and listed in Table 1.

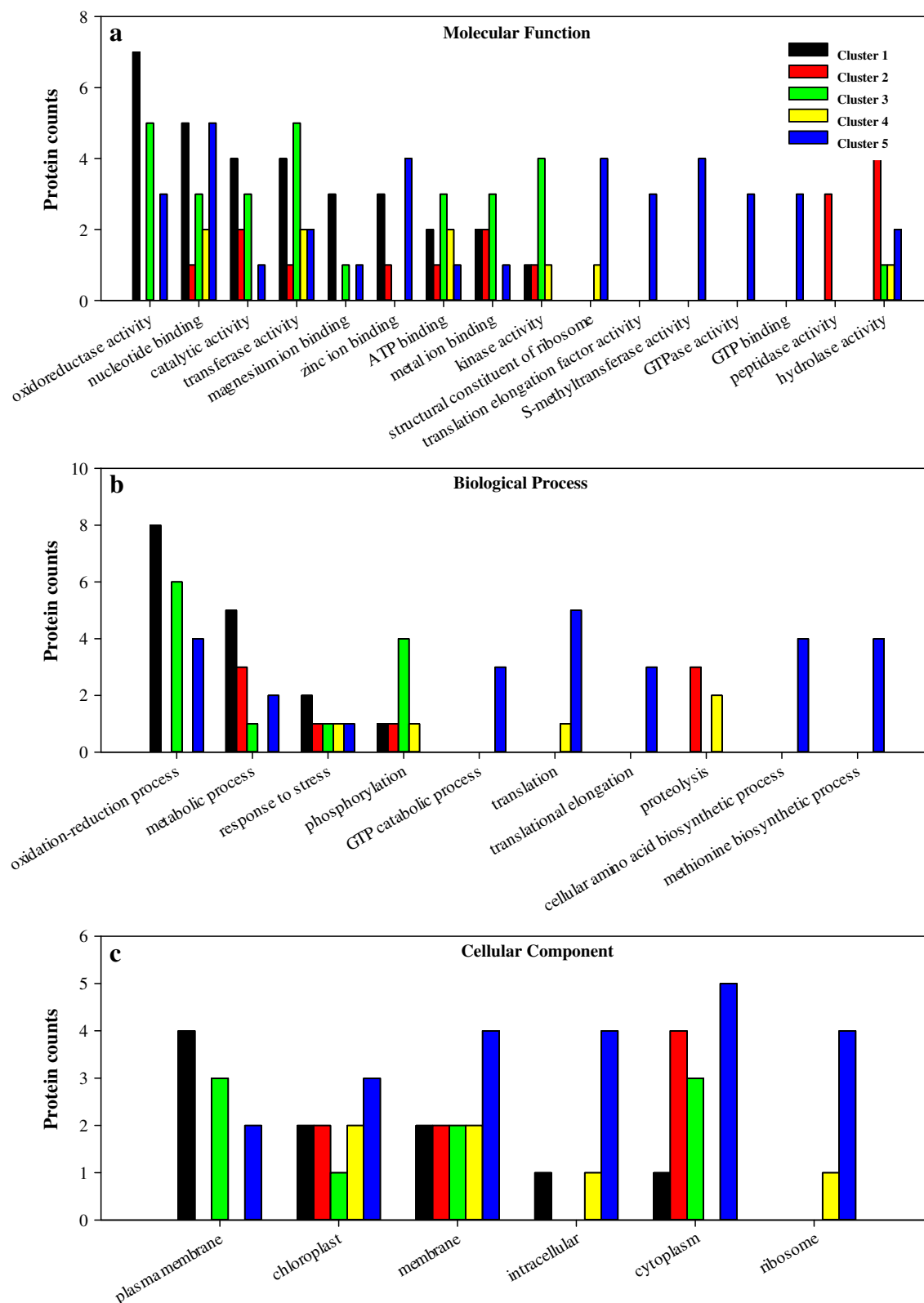


Fig. 6 – Assignment of the significantly changed proteins into putative functional group categories. Proteins were categorized into 15 biological processes according to the Gene Ontology annotation (<http://www.ebi.ac.uk/QuickGO/>). The protein counts indicate the number of protein hits that match each function category. A: Molecular functions; B: Biological process; C: Cellular components.

methyations, polyamine synthesis [77] and ethylene synthesis [78]. The decrease of methionine synthase coincided with a decrease of 5-ethyltetrahydropteroyltriglutamate-homocysteine methyltransferase, another enzyme catalyzing the formation of methionine. These results indicate that methionine synthesis was indeed significantly reduced in red strawberry fruit as compared with white and pink fruit. These proteomic results support the metabolic analysis reported previously [11] that methionine content in strawberry receptacle tissue was similar between white and red fruit; however, it was significantly lower in pink fruit. While in achene tissue, the methionine content with several other amino acids were significantly lower in red fruit than in white [11]. This composition profile illustrated the dynamic metabolism of methionine related not only to different tissues but also to development stages. Proteomic studies with citrus fruit showed a decrease in methionine synthase (MS) during early fruit development and then an increase during late fruit development of citrus [79]. In grapes, an increase of methionine synthase was found 28 days after 50% of the fruit started to change color [50]. Investigation at the transcript level in receptacle tissue of wild strawberry (*Fragaria vesica*) revealed an increase of methionine synthesis arose from the increased gene expression of cystathionine- γ -synthase [1,80], which might be co-regulated with MS to meet the demand for methionine. In climacteric fruit, where ethylene is autocatalytically synthesized and acts as the major regulator of the ripening processes, there is proteomic evidence of an increase of MS in apple [81] and papaya [82]. In contrast, strawberry fruit produce lower amounts of ethylene as compared with climacteric fruit [83]. The ethylene receptor genes expressed in ripe fruit were mainly type II receptors which have a weak link with CTR1 proteins due to the degenerated histidine-kinase domain [84]. The expression of these receptors suggests that even low amounts of ethylene can be enough to trigger the ripening process of strawberry fruit [5]. Therefore, our results suggest ethylene synthesis through methionine to S-adenosyl-L-methionine is not a major mode of methionine metabolism in strawberry fruit. Certainly, other regulatory mechanisms such as the methylation of macromolecules (proteins, DNA) or hormone interaction could also participate in the important cellular changes which cause the fruit to ripen. It is too early to draw any conclusion, but proteomic studies provide significant evidence to stimulate further investigation on the role of methionine metabolism in both climacteric and non-climacteric fruit ripening.

A RAD23 protein was identified and found in Cluster 5. The RAD23 proteins are responsible for both the binding and repairing of DNA molecules, and shuttling the proteolytic targets containing an ubiquitin like domain and interact with 26S proteasome. The RAD32 protein has been reported in 'Annurca' apple flesh [85]. In *Arabidopsis thaliana*, RAD23 proteins were discovered as having multiple functions in the cell cycle, morphology, and fertility of plants through their delivery of UPS substrates to the 26S proteasome. 40S and 60S proteosomes are important for the translation process from mRNA to protein synthesis. Furthermore, two elongation factors 1- α have also been found to decrease in our study. Elongation factor is an essential component of eukaryotic protein synthesis, which catalyzes the translocation of

peptidyl tRNA from the aminoacyl site to the peptidyl site on the ribosome in a GTP-dependent reaction. At the transcript level, an elongation factor 2 (EF-2) was induced during ripening [2]. Despite this controversy, the consistent down-regulation of these two proteasomes, together with methionine synthase and others may reflect the slower demand of protein biosynthesis in strawberry fruit during ripening.

4.6. Additional significantly changed proteins

One of the important eating quality indices of strawberry fruit ripening is the increases of SS content and decrease of acidity. In general, the accumulation of sweetness (sugar) in fruit results from the accumulation of soluble sugars such as glucose, fructose and sucrose, which is determined by an elaborate system involving both synthesis and metabolism [86]. In our study, several proteins involving sugar metabolism were identified. A phosphoglucosyltransferase (PGM), which converts glucose 1-phosphate to glucose 6-phosphate, was identified and quantified as a protein in Cluster 1, that increased significantly in both cultivars (Fig. 5). Meanwhile, a sucrose synthase 2 (SuSy) was identified as a protein in 'Mira' that decreased during fruit ripening, while sucrose phosphate phosphatase (SPP) and sucrose phosphate synthase (SPS) were identified to increase during ripening (Support information Table S3). One of the adenosine kinase isoforms (ADK; EC 2.7.1.20) in Cluster 1 was also found to increase in both cultivars (Fig. 5). This provides the proteomic evidence of the complex nature of the sugar metabolic network in strawberry during ripening. The sugar/acids ratio is considered as the major organoleptic quality trait of the mature strawberry fruit. The major organic acid in strawberry is citrate. During fruit ripening, the TA decreased by almost 40% in ripe fruit compared to pink fruit (Fig. 3F). The decrease of TA may coincide with the increase of the protein PDC and a decrease in one component of the pyruvate dehydrogenase complex, dihydrolipoamid S-acetyltransferase, which occurred in both cultivars (Fig. 5). In 'Mira', we also identified acetyl-CoA carboxylase as a protein that increased (Support information Table S3). However, several other TCA related proteins such as citrate synthase, aconitate hydratase, isocitrate dehydrogenase 2, malate dehydrogenase and succinate dehydrogenase did not significantly change during fruit ripening. This is in agreement with the protein identification reported in citrus fruit development [87].

Adenosine kinase catalyzes the phosphorylation of adenosine (Ado) to AMP and is an essential component for maintaining the adenylate metabolic network [88]. In plants, adenylate nucleotides are formed either by de novo synthesis or recycled from nucleosides and nucleobases via salvage pathways; the resulting nucleotide monophosphates are used in the synthesis of ATP, nucleotide cofactors, as well as cytokinins [89]. Therefore, these findings reveal the crucial role of adenylate metabolism in strawberry fruit ripening.

One of the cystatins CPI-1 was also identified and quantified as a Cluster 1 protein (Fig. 5). Cystatins are a group of proteins in plants that specifically inhibit cysteine-proteases. They serve two major functions: the regulation of the endogenous protein turn-over and defense against biotic and abiotic stress. In strawberry, a gene *Cyf1* encodes a phytocystatin that was

found to be a defense related gene expressed primarily in the achenes and expanded leaves [90]. Our results provide additional evidence that the cystatin CPI-1 is related to fruit ripening.

5. Concluding remarks

As a non-climacteric fruit, strawberry fruit shows significant changes in the production of volatiles, anthocyanins, phenolic compounds, TSS and titratable acidity during ripening. These physiological changes not only reveal the fruit's ripening metabolism, but also provide a vital link with dynamic protein profiles and changes obtained by using a quantitative proteomic approach. This is the first quantitative proteomic study employing stable isotope labeling by peptide dimethylation to study protein populations in strawberry receptacle tissues at different maturities and provides further information on the ripening process of this fruit. Quantitative proteomics combined with LC/MS/MS identification revealed some important details of the protein population and provided a deeper insight into understanding the ripening and senescence processes of this fruit. The proteins identified in this study not only demonstrate dynamic changes in protein populations in association with biosynthesis of volatile and flavonoids, but also strongly suggest that the antioxidant redox system, allergens and defense mechanisms, and primary and secondary metabolism are dominant metabolic activities during strawberry fruit ripening. The identification of significantly changed proteins indicates a dynamic network of proteins in the regulation of strawberry fruit ripening. Topics of further study include a quantitative analysis of targeted proteins employing multiple reaction monitoring (MRM) or gene expression analysis to determine the key regulatory pathways and determine whether the protein changes are reflected in gene expression and changes in enzyme activities.

Abbreviations

LC-MS/MS	liquid chromatography–tandem mass spectrometry
Mr.	molecular weight
pI	iso-electric point
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
ASB-14	amidodisulfobetaine-14
IPG	immobilized pH gradient
2-DE	2-dimensional electrophoresis
OGE	OFFGEL gel-electrophoresis
TAC	total anthocyanin content
TPC	total phenolic compounds
TFC	total flavonoids compounds
ORAC	oxygen radical absorbance capacity
FFAP	ferric reducing antioxidant power assay

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.09.004>.

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