RESEARCH PAPER

Proteomic analysis reveals dynamic regulation of fruit development and sugar and acid accumulation in apple

Mingjun Li¹,²,*; Dongxia Li¹; Fengjuan Feng¹,²; Sheng Zhang³; Fengwang Ma¹ and Lailiang Cheng²,*

¹ State Key of Crop Stress Biology in Arid Areas/College of Horticulture, Northwest A&F University, Yangling, Shaanxi, China
² Section of Horticulture, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853, USA
³ Institute of Biotechnology, Cornell University, Ithaca, NY 14853, USA

* Correspondence: limingjun@nwsuaf.edu.cn, LC89@cornell.edu

Received 20 January 2016; Accepted 29 June 2016

Editor: Ariel Vicente, CONICET, National University of La Plata

Abstract

Understanding the fruit developmental process is critical for fruit quality improvement. Here, we report a comprehensive proteomic analysis of apple fruit development over five growth stages, from young fruit to maturity, coupled with metabolomic profiling. A tandem mass tag (TMT)-based comparative proteomics approach led to the identification and quantification of 7098 and 6247 proteins, respectively. This large-scale proteomic dataset presents a global view of the critical pathways involved in fruit development and metabolism. When linked with metabolomics data, these results provide new insights into the modulation of fruit development, the metabolism and storage of sugars and organic acids (mainly malate), and events within the energy-related pathways for respiration and glycolysis. We suggest that the key steps identified here (e.g. those involving the FK2, TST, EDR6, SPS, mtME and mtMDH switches), can be further targeted to confirm their roles in accumulation and balance of fructose, sucrose and malate. Moreover, our findings imply that the primary reason for decreases in amino acid concentrations during fruit development is related to a reduction in substrate flux via glycolysis, which is mainly regulated by fructose-bisphosphate aldolase and bisphosphoglycerate mutase.

Key words: Amino acid, apple, fruit development, functional annotation, organic acid, sugar, tandem mass tag.

Introduction

Apple (Malus domestica L. Borkh.) is one of the most widely planted tree fruits in the temperate zone. With the health benefits of apple increasingly understood by consumers, demand is on the rise for cultivars with desired quality characteristics, e.g. size, texture, flavor and nutritional value (Khan et al., 2014). For any apple cultivar, fruit quality characteristics are underlain by cell number, size and arrangement, and metabolite composition at maturity, all of which are determined by the fruit developmental process. Therefore, understanding fruit development is critical for quality improvement.

Apple is a representative of a pome fruit in the Rosaceae family, an accessory fruit derived from enlargement of the flower receptacle (Eccher et al., 2014). Apple fruit development consists of an exponential cell division phase in the first 4–6 weeks after bloom and a cell expansion phase for the remainder of fruit development (Goffinet et al., 2005). Genotypic variations in final fruit size within Malus is caused by differences in total cell number and cell size (Malladi and Hirst, 2010), whereas final fruit size for a given genotype under different crop load levels and nitrogen supply is largely...
determined by total cell number (Goffinet et al., 2005; Xia et al., 2009). However, our current understanding on how cell division and expansion are regulated in apple is very limited.

Both metabolism and accumulation of carbohydrates, organic acids and amino acids in apple are developmental stage-dependent (Zhang et al., 2010; Li et al., 2012). At the early stage of fruit development, most of the imported carbohydrates are rapidly metabolized via glycolysis and the tricarboxylic acid (TCA) cycle. At this stage, low levels of sugars but high levels of organic acids and amino acids accumulate in fruit. As the fruit continues to grow due to cell expansion, carbohydrate metabolism slows down; both fructose (Fru) and sucrose (Suc) accumulate and excess carbon is converted to starch for storage in plastids. At the late stage of fruit development, starch breaks down and Suc continues to accumulate, which, along with concomitant further decline in organic acid levels, elevates fruit sweetness to a maximum at maturity. Compared with many other species, apple and other fruits in the Rosaceae are unique in that both sorbitol (Sor) and Suc are synthesized in leaves and then utilized in sink organs. Sor is mainly converted to Fru in fruit (Yamaguchi et al., 1994), which feeds into the Suc-Suc cycle (Li et al., 2012) or ‘futile Suc recycles’ (Nguyen-Quoc and Foyer, 2001). Because Sor accounts for ~60–80% of all photosynthates produced in apple leaves (Bieleski, 1969; Cheng et al., 2005) and almost all of the Sor and half of the Suc are converted to Fru, it is estimated that at least 80% of the total carbon flux goes through Fru in apple fruit (Li et al., 2012). However, the mechanism by which the carbon flux is directed toward sugar accumulation or glycolysis/TCA cycle during fruit development is still not completely understood.

Recent release of the apple genome sequence (Velasco et al., 2010) has made it possible to apply genome-wide transcriptomic and proteomic analysis techniques to apple. So far, efforts in global mRNA profiling in apple have been focused on understanding fruit development (Janssen et al., 2008; Malladi and Johnson, 2011), cell wall construction (Legay et al., 2015), fruitlet abscission (Ferrero et al., 2015), fruit color (Elsharkawy et al., 2015) and fruit ripening (Segonne et al., 2014). However, transcriptomic analyses alone may not adequately predict exact protein abundance and activities. By contrast, proteomics can be used to obtain quantitative and structural modification information about functional proteins and the dynamic state of the cell. The few proteomic studies on apple fruit development and maturation (Zheng et al., 2013; Shi et al., 2014) have improved our understanding of the ripening process at the proteome level, but the results have been limited by the intrinsic inaccuracy of single staining-based quantitative two-dimensional gel electrophoresis. Utilization of more robust quantitative proteomics approaches, such as mass spectrometry (MS)-based stable isotopic labeling, should lead to better coverage and accuracy with regard to changes in protein levels (Zhou et al., 2012; Megger et al., 2014). Additionally, very few attempts have been made to link metabolites and protein abundance to the mechanistic relationships that regulate apple fruit development and quality.

In this work, we conducted an integrated metabolomic and tandem mass tag (TMT)-based proteomics analysis. Our objective was to gain a global overview of the dynamics of apple fruit development and identify key regulatory networks and proteins that contribute to fruit development and the metabolism and accumulation of sugars and acids for fruit quality improvement.

Materials and methods

Plant materials

Six-year-old trees of ‘Greensleeves’ apple (M. domestica Borkh.) on M.26 rootstocks were used for experiments conducted at Cornell University, Ithaca, NY, USA. There were five biological replicates with three trees per replicate in this study. All trees were grown under natural conditions outdoors in 55-l plastic containers filled with a 1:2 mixture of sand:MetroMix 360 (v:v) (Scotts, Marysville, OH, USA). They were trained as a spindle system at a spacing of 1.5 × 3.5 m. During the growing season, they were supplied twice weekly with 15 mM N, using Plantex® NPK (20–10–20) with micronutrients (Plantex Corp., Ontario, Canada) in a supply regime similar to that described previously (Cheng and Raba, 2009). At 16, 41, 70, 94, and 128 days after bloom (DAB) (Fig. 1; Supplementary Fig. S1 at JXB online), fruit samples were randomly taken from the south side of the tree canopy between 14:00 and 15:00 h, under full sun exposure. On each sampling date, five biological replicates were harvested, with at least six fruits collected from three trees per replicate. The fruits were immediately weighed, then cut into small pieces after removing the core before freezing them in liquid nitrogen on-site. All samples were stored at −80 °C.

Fresh weight and soluble solids content

Fresh weight and soluble solids content (SSC) were recorded from eight fruits on each sampling date. SSC was measured with a digital refractometer (Model PR-1; Atago, Tokyo, Japan).

![Fig. 1.](https://academic.oup.com/jxb/article-abstract/67/17/5145/2197688) Diagram of fruit development showing timing of major growth and carbohydrate accumulation in ‘Greensleaves’ apple, based on our current data (see Supplementary Fig. S1) and model described by Eccher et al. (2014). In the present study, fruit samples were taken on days marked with red.
Soluble sugars, organic acids and starch

As described previously (Li et al., 2012), soluble sugars/alkohols and organic acids were extracted and then derivatized sequentially with methoxyamine hydrochloride and N-methyl-N-trimethylsilyl-trifluoroacetamide, followed by analysis on an Agilent 7890A GC/5975C MS system (Agilent Technology, Palo Alto, CA, USA). The tissue residue that remained for GC-MS analysis was used for starch determination (Li et al., 2012).

Analysis of free amino acids

Flesh samples (0.5 g) were ground in 1.5 ml of 20 mM HCl, with 20 μl of norleucine (250 μg ml⁻¹) added as an internal standard. The procedure followed that described by Zhang et al. (2010). Individual amino acids were separated and quantified on an Agilent 1100 Liquid Chromatograph equipped with an Agilent 1200 fluorescence detector (Agilent Technology). Excitation at 250 nm and emission at 395 nm were used for fluorescence detection. Concentrations of individual amino acids were quantified based on peak areas and calibration curves derived from the authentic standards.

Protein extraction

Proteins were extracted and purified from ground samples using a standard phenol extraction method, then followed by ammonium acetate-methanol precipitation (Zheng et al., 2013). Concentrations in all extracts were determined with an RC/DCTM protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The proteins extracted from replicates 1–3 were pooled with the same quantity for proteomics biological replicate 1, whereas replicates 4–5 were for biological replicate 2. Further quantification was conducted on a pre-cast NOVEX 12% Tris/Glycine mini-gel (Invitrogen, Carlsbad, CA, USA) along with a series of Escherichia coli lysates (2, 5, 10 or 20 μg per lane) (Supplementary Fig. S2). The SDS gel was visualized with colloidal Coomassie blue staining (Invitrogen), then imaged by a Typhoon 9400 scanner and Image Quant Software version 5.2 (GE Healthcare).

Identification and quantification of proteins

Proteins were identified and quantified at the Cornell MS Facility, using the technique of TMT-based comparative proteomic analysis as previously described (Supplementary Fig. S3; Wang et al., 2014). Briefly, the tryptic peptides (100 μg each) were labeled with TMT 6-plex with 126-tag (16 DAB), 127-tag (41 DAB), 128-tag (70 DAB), 129-tag (94 DAB) 130-tag (128 DAB) and 131-tag (as a technical replicate for 16 DAB). After checking label incorporation by MALDI-TOF/TOF 4700 (Applied Biosystems, Foster City, CA, USA), the six labeled samples per replicate set were pooled, evaporated to dryness, and subjected to cation exchange chromatography using a PolyLC strong cation-exchange cartridge (PolyLC Inc. Columbia, MD, USA) and desalted by Sep-Pak SPE cartridges (Waters, Milford, MA, USA) for subsequent high-pH reverse phase (hprP) fractionation.

High pH reverse phase fractionation and nanoLC-MS/MS analysis

High pH reverse phase (hprP) chromatography was carried out using a DionexUltimate 3000 HPLC system with a built-in micro fraction collection option in its autosampler and UV detection (Sunnyvale, CA, USA) as reported previously (Yang et al., 2011). The TMT 6-plex tagged tryptic peptides were reconstituted in buffer A (20 mM ammonium formate pH 9.5 in water), and loaded onto an Xterra MS C18 column (3.5 μm, 2.1 × 150mm) from Waters, (Milford, MA, USA) with 20 mM ammonium formate (NH₄FA), pH 9.5 as buffer A and 80% ACN/20% 20 mM NH₄FA as buffer B. The LC was performed using a gradient from 10–45% of buffer B in 30 min at a flow rate 200 μl min⁻¹. Forty-eight fractions were collected at 1 min intervals and pooled into a total of 12 fractions based on the UV absorbance at 214 nm and with multiple fraction concatenation strategy (Wang et al., 2011). This concatenation strategy is to pool disparate first dimensional hprP fractions prior to the second dimension nanoLC-MS/MS analysis without any appreciable degradation in chromatographic resolution or reduction in peptide identifications compared to the individually analyzed fractions. All of the fractions were dried and reconstituted in 150 μl of 2% ACN/0.5% NH₄FA.

The resulting 12 hprP fractions per replicate set were then used for nano liquid chromatography (LC)-MS/MS analysis in an LTQ-OrbitrapVelos (Thermo-Fisher Scientific, San Jose, CA, USA).

Raw data files for quantification acquired from the Orbitrap were converted to MGF files using Proteome Discoverer 1.3 (PD 1.3; Thermo-Fisher Scientific). Subsequent database searches were performed with Mascot Daemon (version 2.3; Matrix Science, Boston, MA, USA). Both the identifications and TMT quantitations were made against the Malus genome protein database (http://www.rosaceae.org/node/1). This analysis comprised 63 541 sequences and 23 664 265 amino acid residues (Velasco et al., 2010). The default Mascot search settings included one missed cleavage site by trypsin allowed with a fixed carbamidomethyl modification of cysteine, fixed 6-plex TMT modifications on Lys and N-terminal amine, and variable modifications of methionine oxidation, deamidation of Asn and Gln residues. The values for peptide and fragment mass tolerances were 20 ppm and 0.1 Da, respectively. To estimate the false discovery rate (FDR) as a means for verifying identifications within each replicate set, we employed the target–decoy strategy of Elias and Gygi (2007). Specifically, an automatic search of the decoy database was performed in Mascot by choosing the decoy check-box in which a random sequence of the database is generated and tested for raw spectra as well as the real database. To reduce the probability of falsely identifying any peptides, we considered only those with significant scores of ≥40 (99% confidence interval) based on the Mascot probability analysis (http://www.matrixscience.com/help/scoring_help.html#PBM). Each case required at least two unique peptide identifications, as indicated in Mascot. Those identified as belonging to the same family were grouped in our Mascot summary. Furthermore, to be considered thoroughly quantified, we required that a protein produce at least two unique peptides that generated a complete TMT 6-plex reporter ion series. The quantitative protein ratios were weighted and normalized by the median ratio with outlier removal set as automatic in Mascot for each group of experiments. The manufacturer’s recommended isotope correction factors were applied. Finally, functional annotation and classification of all identified proteins were determined according to the annotation file of the Malus genome database (http://www.rosaceae.org). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD004568.

Statistical analysis

The physiological data were analyzed by one-way ANOVA, followed by Duncan’s multiple range tests via SPSS statistical software (version 16.0; SPSS, Inc. Chicago, IL, USA). Data were presented as the means ± standard deviation of five replicate samples. To examine the quantitative precision and reproducibility of TMT, we performed careful statistical analyses to assess any internal variation between two technical replicates, as previously described (Yang et al., 2011). To associate cellular functions with the set of quantified proteins, we used in-house BLAST alignments of the filtered set protein sequences to Arabidopsis (TAIR 8.0) datasets (Sun et al., 2009). Malus proteins were functionally classified using the MapMan (3.6.0RC1:) functional classification system and k-means clustering (Thimm et al., 2004). K-means clustering was performed with the function of cluster experimental data and compared with the given annotation embedded in the MapMan program. The number of clusters was defined by the FOM (figures of merit) application. The k-means analysis was performed using Pearson correlation. The proteins in each cluster were then classified into the MapMan
Results

Fruit development of ‘Greensleeves’ apple

To examine the physiology of fruit development and sugar/acid accumulation throughout fruit development, we sampled ‘Greensleeves’ fruits at five key developmental stages. The selection of these sampling dates was based on the data shown in Supplementary Fig. S1. At the proteome level, the sampling dates correspond to the timing of major physiological events for ‘Greensleeves’ fruit (Fig. 1; Supplementary Fig. S3). The first sampling, at 16DAB, represented the period of cell division. By 41 DAB, cell division had ceased while the rate of cell expansion increased and starch began to accumulate. Sampling at 70 DAB coincided with the greatest rate of cell expansion and starch accumulation, and the onset of soluble solids accumulation. At 94 DAB, cell expansion was continuing, albeit at a reduced rate, and starch degradation was beginning while sugars continued to accumulate. The final harvest was made at 128 DAB, the stage at which starch was being rapidly converted into fruit sugars, and fruit skins were turning yellow.

Detection of proteins in developing apple fruit

Total proteins in two biological replicates at five key developmental stages (Supplementary Fig. S4) were labeled with TMT. Developmental changes in the relative abundance of any given protein were determined based on TMT 6-plex reporter ion ratios of 127/126 (41 DAB/16 DAB), 128/126 (70 DAB/16 DAB), 129/126 (94 DAB/16 DAB), 130/126 (128 DAB/16 DAB) and 131/126 (technical replicate for 16 DAB). Detailed information on the identifications and quantifications of these two independent biological replicates are shown in Supplementary Tables S1, S2. A total of 7098 proteins (Table 1) were identified, with estimated false discovery rate (FDR) values of 1.31% (Replicate 1) and 1.67% (Replicate 2). Of those, 4283 (60.3%) were present in both replicates. We quantified 6247 proteins (88%), of which 3713 (59%) overlapped between the two replicate datasets.

To assess our quantitative precision and reproducibility, we performed ANOVA with two technical replicates from the data sets. Two scatter plots of the 131/126 ratios (technical replicate for 16 DAB), 129/126 (94 DAB/16 DAB), 130/126 (128 DAB/16 DAB) and 131/126 (technical replicate for 16 DAB). Detailed information on the identifications and quantifications of these two independent biological replicates are shown in Supplementary Tables S1, S2. A total of 7098 proteins (Table 1) were identified, with estimated false discovery rate (FDR) values of 1.31% (Replicate 1) and 1.67% (Replicate 2). Of those, 4283 (60.3%) were present in both replicates. We quantified 6247 proteins (88%), of which 3713 (59%) overlapped between the two replicate datasets.

To assess our quantitative precision and reproducibility, we performed ANOVA with two technical replicates from the data sets. Two scatter plots of the 131/126 ratios (Supplementary Fig. S4) for each identified and quantified protein in Set 1 or 2 produced comparable results. Linear regression analysis indicated that the quantitative TMT data for the two technical replicates were highly reproducible. Randomly distributed internal errors for the Set 1 and 2 plots on the log2 scale were 0.22 and 0.31, respectively, which corresponded to an internal variation of 1.16- and 1.24-fold, respectively, thereby indicating a technical deviation. For the two biological replicates at each developmental stage, scatter plots of the 127/126, 128/126, 129/126, and 130/126 ratios for each identified and quantified protein between replicate 1 and 2 yielded comparable results (Supplementary Fig. S5). This linear regression analysis revealed a slope of ~0.97–0.98 and an R2 value of 0.916–0.962 with randomly distributed errors, again confirming that the quantitative TMT data for the two replicates were highly reproducible. The largest internal errors for four plots on the log2 scale were 0.475 for 129/126, which corresponded to an internal variation of 1.39-fold. The threshold of the deviation±0.475 at log2 (quantitative expression related to 126-tag being>1.39 or <0.72) was used for determining development-related changes in protein expression.

Dynamic reprogramming of the apple fruit proteome

We identified 7785 out of 63 541 genes expressed during fruit development, which represented 12.3% of the entire apple transcriptome. MapMan annotation was used to assign 6247 proteins quantified by TMT to functional categories. Those genes were grouped by developmental dynamics using the k-means clustering algorithm to provide a benchmark on functional categories of apple fruit developmental proteomes for future studies. In all, 79% of the quantified proteins in recognized pathways while the remainders are unassigned (Fig. S5; Supplementary Table S1). We identified eight clusters (K1–K8) with different expression patterns (Fig. 2; Supplementary Table S3). Clusters K1 and K3, both of which showed the greatest up- or down-regulation of expression during fruit development, have fewer proteins than the other clusters. Four clusters (K5–K8) do not exhibit significant changes in expression throughout fruit development, and accounted for ~44% of all quantified proteins (Fig. 2). Some of the MapMan bins showed enrichment for particular clusters of protein expression (Fig. 2). For example, those encoding enzymes for major CHO metabolism and transport are greatly enriched in K1 and K2, representing proteins that are expressed at the highest levels in mature fruit. Proteins for which expression peaked at 16 DAB (K3–K5) included those required for DNA synthesis, RNA processing and regulation, as well as ribosomal proteins involved in protein synthesis (Fig. 2). This suggests that the 16-DAB fruit undergoes substantial reprogramming as the gene expression network is being built.

| Table 1. Comparison of results for two sets of biological replicate samples |
|--------------------------|------------------|------------------|
| Summary data             | Set 1            | Set 2            |
| Total number of protein IDsa | 5351            | 6030            |
| Number of unique proteins from each setb | 1068            | 1747            |
| Combined distinct protein IDs (total/overlap) from two sets | 7098/4283 | 1747       |
| Reproducibility of protein IDs in two sets | 80.0% | 71.0%     |
| Total proteins (TMT ratio) | 4855            | 5125 (85.0%)    |
| Unique proteins with TMT ratio from each set | 1123            | 1412            |
| Combined distinct protein IDs with TMT ratio (total/overlap) from two sets | 6247/3713 | 72.4%     |
| Reproducibility of protein IDs with TMT ratio in two sets | 76.8% | 72.4%     |

aIndicates the total identified in two sets of biological replicate samples.
bDenotes the number of protein IDs exclusively identified from only one of the two sets.
Proteins involved in cell development and hormonal regulation

The dynamics associated with the accumulation of proteins involved in cell formation and hormonal regulation are of particular importance to fruit development. Whereas 132 of our examined proteins are involved in the construction of cell walls, 275 are related to internal cell development (Supplementary Table S4). Twelve clusters are identified that have different expression patterns for cell-wall and cell proteins. As shown in Fig. 3, ten proteins with peak expression in mature fruit (Cluster C1) are especially abundant in 128-DAB fruit. They included proteins related to maturation, e.g. ACD1 (MDP0000315206) for cell death, polygalacturonases (PGs) (MDP0000326734) and RD22 (MDP0000228673) for cell wall degradation, and ALB4 (MDP0000160436) for plastids (Fig. 3E). The C1 cluster also contained proteins linked to cell wall synthesis as well as cell cycling and division. Cluster C2 comprises proteins for which expression is increased as the fruit began to ripen (Fig. 3). These included lyase (MDP0000121223 and MDP0000726337) and RXF12 (MDP0000225641 and MDP0000212739), both of which are related to cell wall degradation (Fig. 3E); plus CSLG2 (MDP0000223480) and CLSG3 (MDP0000218882), for cellulose synthesis. This cluster is also enriched for proteins involved in cell wall formation (e.g. pectin esterase) and the transport of cell vesicles. Proteins with peak expression at 16 DAB (Cluster C3) included those related to the cell cycle (e.g. cyclin: MDP0000305826 and MDP0000305771; peptidylprolyl isomerase: MDP0000183239, MDP0000253995 and MDP0000239169; cyclin-dependent kinase B1:2; MDP0000265516), cell division (regulator of chromosome condensation RCC1: MDP0000282827; and PHB3: MDP0000188767), cell organization (TUB6: MDP00001323720 and MDP0000749824), cell wall proteins (LRR: MDP0000250093; RGP3: MDP0000146639; and HRGP: MDP0000453068), and xyloglucan endotransglycosylases/hydrolases (XTHs) for cell wall modifications (XTH9: MDP0000132431 and MDP0000361876; EXP: MDP0000361876). Proteins associated with the synthesis of cell wall cellulose are enriched in Cluster C4 and are highly expressed at 41 DAB. They include three mannan synthase/transferases (CSLA: MDP0000263736, MDP0000210684 and MDP0000717000) plus two cellulose synthases/transferases (CESA subfamily: MDP0000279461 and MDP0000185368).

Eight clusters were created due to different expression patterns for 134 hormone-related proteins (Fig. 3; Supplementary Table S5, sheet 2). Peaking in maturing fruit, the highest levels of expression are detected for proteins involved in the synthesis of ethylene (ACO1: MDP0000195885; ACO2: MDP0000200737), abscisic acid, or ABA (XDH2: MDP0000664357; NCED: MDP0000164529), jasmonic acid, or JA (LOX1/2: MDP0000450991, MDP0000211556), auxins (ILR1: MDP0000301701) and auxin signaling (NDB3: MDP0000305861; AXR1: MDP0000681336 and MDP0000773415), and gibberellins (SPINDLY: MDP0000127824). Consistent with the rapid cell division occurring in 16-DAB fruit, proteins associated with the synthesis of auxins (IAR3: MDP0000707034; ILL3: MDP0000139425) and brassinosteroids (SMT1/2: MDP0000122791 and...
MDP0000164160; DWF1: MDP0000682675) showed the highest expression, whereas the only cytokinin-related protein revealed through our proteomics approach, i.e. the cytokinin receptor HISTIDINE KINASE 3 (MDP0000155347), is also most strongly expressed in those 16-DAB fruit.

**Carbohydrate metabolism and sugar accumulation**

We selected 94 proteins that are involved in the Suc–Suc cycle, sugar transport, and starch synthesis/degradation in apple fruit (Supplementary Table S5, sheet 3). On the plasma membrane, expression of SUC4.2, which is related to post-phloem unloading, increased between 16 DAB and 128 DAB (Fig. 4). While both HT2.2 and HT4.8 showed trends similar to SUC4.2, the three sorbitol transporters (including SOT1, SOT2 and SOT4) are more abundant at 41 DAB and 128 DAB. As Sor concentration decreases in the fruit, different patterns of expression are detected for the five sorbitol dehydrogenases (SDHs). SDH2 and SDH5 expression is reduced, but SDH1 expression is increased. Within the Suc–Suc cycle, levels of NIVN5, four hexokinases (HKs), five sucrose synthases (SUSYs), five sucrose phosphate synthases (SPSs), sucrose phosphatase (SPP), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM) and UDPG-pyrophosphorylase (UGP) increased while that of the fructokinase 2 (FK2) declines. Expression for almost all of the proteins involved in starch metabolism in the plastids peaks at 70 DAB and is then maintained at high levels to fruit maturity. The same trend was found for the glucose-6-phosphate transmembrane transporter (GPT) and plastidic Glc transporter (PGLT) on the plastid membrane. On the tonoplast, expressions of tonoplast sugar transporter (TST, also called tonoplast monosaccharide transporter, TMT), EDR6, and vacuole glucose transporter (vGT) family proteins increases from 16 DAB to 128 DAB, a trend consistent with that found for Suc and Fru concentrations. By contrast, expression of VINV, located in the vacuole, is negatively correlated with the Suc concentration.

**Metabolism of organic acids and amino acids associated with glycolysis and regulation of the TCA cycle**

During fruit development, concentrations of organic acids and amino acids decreased, except for proline and methionine.
Proteomic analysis in developmental apple fruit (Li et al., 2012). Developmental progression of protein expression from 16 DAB to 128 DAB, indicated in 5-box strings. Putative protein families were obtained from the Malus genome database (Velasco et al., 2010). Viewer displays subset of primary proteins required for soluble sugar and starch metabolism and transport in apple fruit. Detailed protein and expression data are provided in Supplementary Table S5, sheet 3. Heat maps were drawn using log2-transformed TMT values. For sugar metabolism and transport: CWINV, cell wall invertase; FK, fructokinase; F6P, fructose-6-phosphate; Fru, D-fructose; Glc, D-glucose; G6P, glucose-6-phosphate; GPT, glucose-6-phosphate transmembrane transporter; HK, hexokinase; NINV, neutral invertase; PFK, phosphofructokinase; PGI, phosphoglucoisomerase; PGLT2, plastidic Glc transporter; PGM, phosphoglucomutase; SDH, sorbitol dehydrogenase; Sor, sorbitol; SOT, sorbitol transporter; SPP, sucrose-phosphate phosphatase; SPS, sucrose-phosphate synthase; Suc, sucrose; SucP, sucrose carrier or transporter; SUSY, sucrose synthase; TST, tonoplast sugar transporter; UDPG, UDP-D-glucose; vAINV, vacuolar acid invertase; vGT, vacuole glucose transporter; UGP, UDPG-pyrophosphorylase. For starch metabolism: ADG1, ADP-Glypyrophosphorylase; BMY, β-amylase; DPE, disproportionating enzyme; HGL, heteroglycan glucosidase; ISA1, alpha-amylase; LDA, limit dextrinase; PHS, α-glucan phosphorylase; pPGI, plastid phosphoglucose isomerase; PWD, phosphoglucon, waterdikinase; SBE, starch branching enzyme; SEX1, starch excess; SS1, starch synthase.

(Supplementary Table S4, sheets 3 and 4). Within the glycolysis pathway, levels of phosphofructokinases (PFKs), fructose-bisphosphate aldolases (FBAs), glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) such as GAPDH4, bisphosphoglycerate mutases (BPGMs), and enolases are much lower at 41 DAB than at 16 DAB, and are maintained at those low levels throughout the rest of fruit development. This trend is consistent with the concentrations of fructose-6-phosphate (F6P), PGA, phosphoenolpyruvate (PEP), and pyruvate measured in the fruit samples (Fig. 5). By contrast, the expression of triose-phosphate isomerases (TPIs), phosphoglycerate kinases (PGKs), three of the six GAPDHs, and two of the five pyruvate kinases (PKs) increased with fruit development. The expression of PEP carboxylase (PEPC) and three of the seven NADP-malic enzymes (NADP-MEs) peaks at 70 DAB and stays at those high levels for the remainder of fruit development. In contrast, expression for cytosolic malate dehydrogenases (MDHs) is lower at 41 DAB and 70 DAB. For pyruvate dehydrogenase (PDH), five of the nine proteins for DH.E1 (a PDH subunit) increased in abundance with fruit development, peaking at 128 DAB. By contrast, expression for four of the seven proteins for DH.E2 (another PDH subunit) is highest in 16-DAB fruit (Fig. 5). Most of the proteins detected in the TCA cycle showed increased expression with fruit development. These included six of the seven aconitases, five isocitrate dehydrogenases (ICDHs), 2-OGDH, three succinyl-CoA synthetases (SCSs), two of the four succinate dehydrogenases (SuDHs), one of the two fumarases, two of the three MDHs and three of the four malate oxidoreductases (NAD-MEs). However, levels for two SuDHs, fumarase2 and MDH1 are highest in 16-DAB fruit.

We quantified 82 proteins associated with amino acids derived from glycolysis and the TCA cycle (Supplementary Table S4, sheets 3 and 5). We found that, after 16 DAB, concentrations of free amino acids decreased sharply except for proline and methionine (Supplementary Table S4, sheet 3). However, as fruit develops, the abundance of most proteins involved in amino acid synthesis increased while levels of precursors for amino acids decreased. These proteins include phosphoglycerate dehydrogenase and cysteine synthase (for the synthesis of Ser, Gly, Cys, and Met from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephenate dehydratase (for the synthesis of Phe, Tyr, and Trp from PGA); shikimate dehydrogenase and prephe

Downloaded from https://academic.oup.com/jxb/article-abstract/67/17/5145/2197688 by guest on 26 July 2018
free amino acids are primarily limited by the reduced supply of precursors from glycolysis and the TCA cycle. However, we also found that the abundance of some proteins peaks at 16 DAB, including S-adenosylmethionine synthetase, chorismate mutase, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, dihydrodipicolinate reductase, glutamate dehydrogenase, hydroxymethyltransferase and tryptophan synthase (Fig. 5). Almost all of these proteins are feedback-insensitive to the amino acid end-products (Pratelli and Pilot, 2014).

**Discussion**

**Apple fruit development and maturation**

The growth of apple fruit is mediated by cell division and expansion. At 16 DAB, the ‘Greensleeves’ apple fruit is undergoing rapid cell division, and this vigorous cell production ends at 41 DAB. In general, the proteins involved in cell division are highly expressed during this early developmental stage and then become much less abundant as cell production...
ceases (Malladi and Johnson, 2011). Our quantitative proteomics results revealed this pattern of expression for more than 1156 proteins involved in DNA synthesis, RNA processes and regulation and protein synthesis. As fruit development continues, coordinated changes in the expression of core cell-cycle genes occur in a manner similar to that described by Malladi and Johnson (2011).

Several B-type cyclin-dependent kinases (CDKs) are positively associated with cell production during apple fruit development, but the abundance of two cyclin-dependent kinase F1 (CDKF1) proteins are negatively correlated with cell division. In addition, two homologous proteins of AUXIN RESPONSE FACTOR in apple – ARF17 and ARF117 – are most highly expressed in flesh tissues undergoing rapid cell division. Other research has shown that ARFI06 mRNA, expressed during the cell division and expansion phases of apple fruit development, is co-localized to a major fruit-size QTL (Devoghalare et al., 2012). However, in Arabidopsis, TONSKOKU is expressed in the S-phase of the cell cycle and positively regulates cell-cycle progression (Suzuki et al., 2005) whereas its homolog in apple is negatively associated with cell division.

Subsequent developmental stages feature post-mitotic cell expansion, a process that continues until maturity and contributes to the majority of fruit growth and the increase in size (Malladi and Johnson, 2011). This cell enlargement primarily occurs due to dramatic increases in the vacuolar compartment and cell vacuolation. During this process, the pro-vacuoles gradually fuse to form the central vacuole, which can occupy up to 90% of the flesh cell volume (Zhang et al., 2014). In Arabidopsis, RabGTPases are required for vacuole fusion (Woollard and Moore, 2008). Three homologs of AtRabG3 showed increased expression during cell expansion in apple. Furthermore, most of the proteins involved in cell vesicle transport exhibited coordinated changes in cell expansion, while expression also peaked at 16 DAB for two identified homologs of VTII11, which is critical to the homotypic fusion of both storage and lytic vacuoles in Arabidopsis (Zheng et al., 2014). Therefore, these results suggest that, within apple flesh cells, the formation and growth of the central vacuole is correlated with pro-vacuoles, although this is accomplished by a different controlling mechanism.

The cell wall must not only maintain structural integrity during cell growth, but also allow for cells to expand. In young fruits that are undergoing rapid cell division (16 DAB), expression peaks concurrently for most cell wall proteins, as well as those involved in cell wall modifications and the synthesis of their precursors. Proteins involved in mechanisms for cell wall loosening primarily contain expansins, XTHs and endoglucanases, as well as those for pectin modifications (Bashline et al., 2014). Although we found high levels of expression for XTH9, plus one expansin and some PGs in 16-DAB fruit, most of the proteins related to cell wall modifications and pectin esterases (e.g. endotransglucosylase and pectin methylesterase, or PME) were more highly expressed in the flesh during cell expansion. Because PME activity in apple increases during early fruit growth but then decreases towards ripening and softening (Goulao et al., 2007; Segonne et al., 2014), these PME family members might play an important role in regulating cell wall loosening and cell expansion in apple. Although expression levels for most of the proteins related to cell wall degradation were highest at 16 DAB, two PGs and RD22 showed sharply increased expression in 128-DAB fruit. A role for PG1 in the loss of intercellular adhesion and softening has already been confirmed in apple (Atkinson et al., 2012; Longhi et al., 2013).

Hormones are key regulators of fruit development and ripening, with ethylene controlling many processes during fruit ripening (Eccher et al., 2014). Its increased biosynthesis is coupled with a rapid increase in the respiration rate. Ethylene is synthesized from S-adenosyl-L-methionine (SAM) by two key enzymes, 1-aminoacyclopropane-1-carboxylate synthase (ACS) and 1-aminoacyclopropane-1-carboxylate oxidase (ACO), both of which are encoded by multigene families with complex expression patterns. Although we did not monitor ACS expression, we did identify three ACO proteins with high expression at 128 DAB. In particular, ACO1 and ACO2 are positively correlated with ethylene synthesis and fruit maturaion (Wang et al., 2009). Additionally, high levels of Met, the precursor of SAM (Ravanel et al., 1998), during fruit ripening (Supplementary Table S5) may be linked to increased levels of ethylene biosynthesis. Other hormones (e.g. ABA, JA) also play important positive or negative roles, either alone or as part of the crosstalk with ethylene (Eccher et al., 2014). We found that both xyloglucan endotransglucosylases/hydrolases and NCED, two key enzymes for ABA biosynthesis, peaked in their expression in 128-DAB fruit, which is consistent with a previous report of high ABA synthesis during fruit ripening (Setha et al., 2004).

Regulatory gene network for carbohydrate and sugar accumulation in developing apple fruit

As a sink organ, apple fruit obtains Sor and Suc via phloem transport from source leaves (Li et al., 2012). However, this process can be affected by the unloading activity of the fruit. For example, levels of SUC4s, key proteins involved in Suc post-phloem unloading in apple (Wei et al., 2014), increased with fruit development and sugar accumulation. Although we did not detect CWINV (which is involved in apoplastic unloading of Suc), perhaps because of its low enzyme activity, it was shown previously that two hexose transporters, HT2.2 and HT4.8, transfer Glc and/or Fru into the cytosol of parenchyma cells (Wei et al., 2014). By contrast, we found that levels of both SOT1 and SOT2, two key transporters associated with Sor post-phloem unloading (Wei et al., 2014), were obviously unchanged when Sor accumulation began to decline with fruit development.

In parenchyma cells, Sor and Suc are rapidly utilized to decrease their concentrations, thereby promoting phloem unloading. In particular, Sor, after being taken up into the cytosol of parenchyma cells by SOT, is mainly converted to Fru by SDH. As Sor concentrations decrease with fruit development, enzyme activity, mRNA expression (Li et al., 2012), and abundance of the SDH2 and SDH5 proteins decrease while SDH1 tends to increase. Imported Suc can be
converted to Fru and Glc/UDPG by NINV and SUSY. Of the three NINV proteins, NINV5 shows increased expression along with SUSY over time, with their activities being highest in young fruit (Li et al., 2012). These results suggest that SDH1 plays a key role in establishing sink strength for Sor, while NINV5 is important for Suc unloading in apple. From an evolutionary perspective, the peak accumulation of soluble sugars in mature fruit helps attract animals for seed dispersal. Before the fruit matures, however, excess carbon is primarily stored as tasteless starch in the plastids (Li et al., 2012), which may be beneficial in protecting the fruit from animal predation. At 41 DAB, starch begins to accumulate, and increased expression of glucose-6-phosphate transmembrane transporter (GTP) on the plastid membrane facilitates the import of G6P into the plastids as a precursor for starch synthesis. Meanwhile, several enzymes involved in starch formation, i.e. starch branching enzyme (SBE), starch synthase (SS) and ADP glucose pyrophosphorylase, are co-expressed with the GTP proteins, and levels for most of them peak in abundance at 70 DAB. This suggests that starch synthesis in apple fruit is achieved via the coordinated expression of those proteins. However, the expression of these proteins does not decline from 94 to 128 DAB when starch degradation occurs, possibly because a decrease in substrate availability leads to reduced starch synthesis. It is interesting that most of the proteins involved in starch degradation (e.g. β-amyloses BMY and AMY, α-amyrase ISA, starch excess SEX and disproportionating enzyme DPE) are co-expressed with those related to synthesis, but their expression levels are not at a maximum when starch levels are declining, with the exception of ISA2, which shows peak expression at 128 DAB. One possible explanation is that the starch degradation pathway is more active in the early phase of the fruit maturation process than in the later phase, as suggested by Janssen et al. (2008).

The central vacuole contains almost all of the stored Fru, Glc and Suc (Yamaki, 1984). During fruit development, excess soluble sugars are imported into the vacuole for accumulation from the cytosol via tonoplast carrier proteins (Wei et al., 2014). This serves several functions: (i) to increase turgor and promote cell expansion, (ii) to store sugars in case of future carbon shortages, and (iii) to ensure mature fruit being sufficiently sweet and attractive to animals for seed dispersal. The rapid accumulation of Fru during fruit cell expansion results from the coordinated actions of the following three factors. First, an adequate supply of Fru must be generated from Sor and Sucrose. Although the activities of SDH, INVs and SUSY decrease with fruit development (Li et al., 2012), most Sor is converted to Fru and is found in only low concentrations in the fruit. Second, decreased expression of FK2 protein (a putative high-Km FK member) indicates that less Fru is metabolized with fruit development and more is available for accumulation. Finally, upregulation of TST proteins on the tonoplast suggests that active transport of Fru from the cytosol into the vacuole is enhanced during fruit development (Wei et al., 2014). Carrier proteins encoded by TSTs move Glc, Fru and Suc into the vacuole (Schulz et al., 2011). Expression of the two TST1/2 proteins in apple follows a trend similar to that of Fru concentrations, implying that both proteins are involved in transporting Fru into the vacuole.

AtERD6 and AtESLI (or AtEDR6L-3) are highly homologous to MdEDR6 members (Wei et al., 2014) and serve as vacuolar exporters of Glc (Klemens et al., 2014). Expression of three MdEDR proteins is highest in mature fruit. This protein expression pattern is positively correlated with Fru and Suc concentrations, similar to what is reported for mRNA expression (Wei et al., 2014). Assuming the substrate for EDR6 in apple is specific to Glc, we would expect that a low Glc concentration in the vacuole would be related to high EDR6 expression in mature fruit. The most likely reason for the low accumulation of Glc in apple fruit is the enhanced expression of HK and PGI proteins with fruit development, which diverts the Glc generated from starch breakdown for Suc synthesis. Thus, Glc does not show accumulation while Glc flux is increased from starch degradation in mature fruit.

Continued Suc accumulation during the late stage of fruit development coincides with increased TST1/2 protein expression. There are two sources of Suc for accumulation: either originating from sucrose transported into the fruit or being synthesized from Sor or starch within the fruit. When apple cortex tissue is incubated with radioactive Sor, Suc is produced (Beruter and Kalberer, 1983). In mature fruit, more Glc is generated as a result of starch degradation, but Glc level does not change while Suc level increases, suggesting that the Glc derived from starch breakdown is largely used for Suc synthesis. This is consistent with the increased expression of HK, SPS and UGP proteins in mature fruit. As AIVIN protein expression and enzyme activity are low in mature fruit (Li et al., 2012), Suc hydrolysis in the vacuole is expected to be minimal.

**Metabolism of organic acids related to glycolysis and the TCA cycle in apple fruit**

The balance between sugars and organic acids is largely responsible for the flavor of apple. Metabolism of the latter is closely related to respiratory metabolism, involving both glycolysis and the TCA cycle, which is where amino acids are derived via interaction with nitrogen metabolism (Sweetlove et al., 2010; Pratelli and Pilot, 2014). As F6P concentrations decreases with fruit development, levels of PGA, PEP and pyruvate also decline. Although the expression of phosphofructokinase (PFK), a kinase that phosphorlates F6P during glycolysis (Fernie et al., 2004), is not noticeably changed, we found here that three of the eight detected FBAs are down-regulated while three others are up-regulated in the mature fruit. The expression of most proteins, including GAPDH, PGK, PK and pyruvate dehydrogenase E1 (DH.E1), increases with fruit development while that of BPGM and enolase decreases as the supply of substrate decreases in the glycolysis pathway. Considering that previous studies have demonstrated the important role of cytosolic BPGM (but not FPK) in controlling glycolysis within heterotrophic tissues (Westram et al., 2002; Fernie et al., 2004), diminished BPGM expression in mature apple fruit can result in a decrease in glycolysis activity via PEP level as found in grape (Martinez-Esteso et al., 2013).
Malate, the main organic acid in apple fruit, decreases in abundance during the maturation process, similar to the pattern seen with other organic acids (Zhang et al., 2010). It accumulates in the vacuole after originating from one of the two pathways: either converted via oxaloacetate (OAA) from PEP in the cytosol or produced through the TCA cycle in the mitochondria (Etienne et al., 2013). In the cytosol, two enzymes – PEPC (irreversibly) and PK (reversibly) – are co-expressed, which enhances their profiles. Cytosolic NAD-dependent malate dehydrogenase catalyzes the reversible conversion of OAA into malate and is the most likely route for its synthesis (Yao et al., 2011; Etienne et al., 2013). However, we detected five cMDHs with different protein profiles. Whereas cMDH2, -3, and -5 are highly expressed in mature fruit and are negatively related to the malate concentration, expression of the cMDH1 protein decreased over time. In addition, three of the seven NADP-dependent malic enzymes (NADP-cMEs), which reversibly convert malate into pyruvate in the cytosol, increased in their levels with fruit development while other enzymes within that family remained unchanged. These results confirmed that, in the cytosol, malate synthesis is controlled through the combined activities of cMDH1 and NADP-cME. In the mitochondria, the TCA cycle allows pyruvate to be oxidized into CO₂, with co-enzymes being reduced through a series of conversions between organic acids that include malate and citrate (Etienne et al., 2013). This process accommodates the demand for intermediates and ATP needed for fruit development.

As fruit development shifts more towards cell expansion in the mid-to-late season, respiration slows down along with a decrease in the concentrations of pyruvate and organic acids. The TCA cycle begins with the condensation of OAA and acetyl-CoA, the latter input allowing the TCA cycle to maintain a cyclic flux mode without a net synthesis of cycle intermediates. Enzymes that directly control citrate synthesis include the mitochondrial citrate synthase (CS) while citrate degradation involves the mitochondrial aconitase and NAD-dependent ICDH. This CS activity is positively correlated with citrate accumulation in citrus (Sadka et al., 2001) and strawberry (Iannetta et al., 2004), which is consistent with our finding that protein expression of two CSs decreases along with citrate concentrations as apple fruit continued to mature. In contrast, levels of aconitase and ICDH proteins peak in the mature fruit, possibly reflecting an overall reduction in citrate levels. In the fruit mitochondria, malate can be oxidized either into OAA by mitochondrial NAD-dependent malate dehydrogenase (NAD-mtMDH) (Sweetman et al., 2009), which feeds the cycle, or into pyruvate by mitochondrial NAD-dependent malic enzyme (NAD-mtME), with expression profiles that increase over time and interrupt the cycle. These two competing metabolic pathways influence fruit acidity in different ways. While malate oxidation by NAD-mtMDH leads mainly to citrate production, hence affecting the malate:citrate ratio in fruit cells, malate oxidation by NAD-mtME leads to diminished acidity because organic acids must be imported into the mitochondria to compensate for the loss of malate (Etienne et al., 2013). We noted here that the levels of mitochondria dicarboxylate/tricarboxylate carriers were elevated in 70-DAB fruit. The enhanced mtME and mtMDH expression and decreased acidity found in mature fruit (Fig. 5) supports the proposition that malate and citrate metabolism in the mitochondria depends upon NAD-mtMDH and NAD-mtME activity (Etienne et al., 2013). However, NAD-mtMDH appears to be involved in malate degradation when grape berries are ripening (Sweetman et al., 2009; Martínez-Esteso et al., 2011).

Just like sugars, most of the malate and citrate in apple fruit is found in the vacuole (Yamaki, 1984). Transport of malate into the vacuole is via facilitated diffusion and the malate is retained in the vacuole via acid trapping (Etienne et al., 2013). An aluminum-activated malate transporter is thought to be responsible for vacuolar malate transport in apple based on co-localization with the QTL for apple acidity (Bai et al., 2012; Khan et al., 2013). Although we did not detect ALMT or the tonoplast malate transporter, expression increased over time for most proteins associated with the vacuole, e.g. H⁺-ATPase (V-ATPase), which regulates the transfer of malate/citrate into the vacuoles via an electric potential and a pH gradient (Etienne et al., 2013).

In conclusion, we have presented a comprehensive analysis of the proteome of apple during fruit development. The resulting extensive list of proteins complements those previously obtained through gel-based proteomic methods, providing better proteome coverage, high reproducibility and fewer internal variations. This will serve as a platform for further examination of the functions of these proteins and subsequent manipulation of fruit quality traits. It will also generate opportunities to use a systems biology approach towards understanding the complex process of apple fruit development. This study has provided a comprehensive view of the major pathways required for apple fruit development, and new insights into processes underlying apple fruit development and key fruit quality traits. In particular, the findings of this study help elucidate the metabolism and storage of sugars and organic acids (mainly malate) and energy-related pathways (respiration and glycolysis), as well as other aspects (of fruit development and metabolism) that could not be systematically analyzed otherwise (e.g. regulation of metabolism for proteins, nucleotides, polyphenols and aroma). As we found that the expression of some proteins undergoes major changes at specific developmental stages, they might be used as novel protein biomarkers for delineating apple fruit development and quality.

Supplementary data

Supplementary data are available at JXB online.

**Figure S1.** Changes in fresh weight, starch concentrations and soluble solids content (SSC) during ‘Greensleeves’ apple fruit development.

**Figure S2.** Schematic diagram for the workflow conducted in this study.

**Figure S3.** Images from SDS gel electrophoresis of apple fruit proteins used in the present proteomics study.

**Figure S4.** Comparison of log₂ TMT ratios for 3713 proteins identified in biological replicate Sets 1 and 2.
Figure S5. Comparison of log2 TMT ratios for 3713 proteins identified in two sets of technical replicates.

Figure S6. Functional categories for 6247 quantified proteins.

Table S1. Proteins identified from two biological replicate datasets.

Table S2. Proteins quantified from two biological replicate datasets. Sheet 1, all quantified proteins from Set1; Sheet 2, all quantified proteins from Set2; Sheet 3, all quantified proteins from the two sets, showing relative levels of expression; Sheet 4, results of MapMan bins of all quantified proteins from the two sets, as well as relative.

Table S3. The 7098 proteins quantified in the present study for developing apple fruit.

Table S4. Changes in metabolite concentrations in developing apple fruit.

Table S5. Details of proteins presented in Figs 3–5.

Acknowledgements

This work was supported in part by the Program for the National Natural Science Foundation of China (No.31372038), New Century Excellent Talents in University (No. NCET-12-0474), the Project of Shaanxi Province Youth Science and Technology New Star (No. 014KJXX-43) and Cornell Agricultural Experiment Station. The authors would like to thank Richard Raba for maintaining the plants. The Agilent GC/MS system used in this work was generously donated by Dr David Zimerman, Cornell Pomology.

References

Atkinson RG, Sutherland PW, Johnston SL, Gunaseelan K, Hallett IC, Mitra D, Brummell DA, Schröder R, Johnston JW, Schaffer RJ. 2012. Down-regulation of POLYGALACTURONASE1 alters firmness, tensile strength and water loss in apple (Malus × domestica) fruit. BMC Plant Biology 12, 129.

Bai Y, Dougherty L, Li M, Fazio G, Cheng L, Xu K. 2012. A natural mutation-led truncation in one of the two aluminum-activated malate transporters in apple provides insight into the central metabolism changes driving grape berry development and fruit set, and fruit growth in apple (Malus × domestica). Journal of the American Society for Horticultural Science 137, 115–123.

Bashline L, Lei L, Li S, Gu Y. 2014. Cell wall, cytoskeleton, and cell expansion in higher plants. Molecular Plant 7, 586–600.

Beruter J, Kalberer PP. 1983. The uptake of sorbitol by apple fruit tissue. Zeitschrift für Pflanzenphysiologie 110, 113–125.

Bielieski RL. 1969. Accumulation and translocation of sorbitol in apple phloem. Australian Journal of Biological Sciences 22, 611–620.

Cheng L, Raba R. 2009. Accumulation of macro- and micronutrients and nitrogen demand-supply relationship of ‘Gaia’/M.26 trees grown in sand culture. Journal of the American Society for Horticultural Science 134, 7–13.

Cheng L, Zhou R, Reidel EJ, Sharkey TD, Dandekar AM. 2005. Antisense inhibition of sorbitol synthesis leads to up-regulation of starch synthesis without altering CO₂ assimilation in apple leaves. Planta 220, 767–776.

Devoghelae F, Doucen T, Guittion B, et al. 2012. A genomics approach to understanding the role of auxin in apple (Malus × domestica) fruit size control. BMC Plant Biology 12, 7.

Eccher G, Ferrero S, Populin F, Colombo L, Botton A. 2014. Apple (Malus × domestica L. Borkh.) as an emerging model for fruit development. Plant Biosystem 148, 157–168.

Elia JS, Gygi SP. 2007. Target-decoy strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nature Methods 4, 207–214.

Elsharkawy I, Liang D, Xu K. 2015. Transcriptome analysis of an apple (Malus × domestica) yellow fruit somatic mutation identifies a gene network module highly associated with anthocyanin and epigenetic regulation. Journal of Experimental Botany 47, 218–225.

Etienne A, Génaud M, Lobit P, Mbéguié-A-Mbéguié D, Bugaud C. 2013. What controls fleshy fruit acidity? A review of malate and citrate accumulation in fruit cells. Journal of Experimental Botany 64, 1451–1469.

Ferrero S, Carretero-Paulet L, Mendes MA, Botton A, Eccher G, Masiero S, Colombo L. 2015. Transcriptomic signatures in seeds of apple (Malus domestica L. Borkh.) during fruitlet abscission. Plos One 10, e0120503.

Fernie AR, Carrari F, Sweetlove LJ. 2004. Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. Current Opinion in Plant Biology 7, 254–261.

Goffinet MC, Robinson TL, Lakso AN. 1995. A comparison of ‘Empire’ apple fruit size and anatomy in unthinned and hand-thinned trees. Journal of Horticultural Science 70, 375–387.

Goulao LF, João S, Sousa I, Oliveira CM. 2007. Patterns of enzymatic activity of cell wall modifying enzymes during growth and opening of apples. Postharvest Biology and Technology 3, 307–318.

Iannetta PPM, Escobar NM, Ross HA, Souleyre EJF, Hancock RD, Witte CP, Davies HV. 2004. Identification, cloning and expression analysis of strawberry (Fragaria×ananassa) mitochondrial citrate synthase and mitochondrial malate dehydrogenase. Physiologia Plantarum 121, 15–26.

Janssen BJ, Thodey K, Schaffer RJ, et al. 2008. Global gene expression analysis of apple fruit development from the floral bud to ripe fruit. BMC Plant Biology 8, 16.

Khan S, Beekwilder J, Schaart A, Mumm R, Soriano J, Jacobsen E. 2013. Differences in acidity of apples are probably mainly caused by a malic acid transporter gene on LG16. Tree Genetics and Genomes 9, 475–487.

Khan AM, Olsen KM, Sovero V, Kushad MM, Kornan BS. 2014. Fruit quality traits have played critical roles in domestication of the apple. Plant Genome 7, 3.

Klemens PAW, Patzke K, Trentmann O, Poschet G, Böttner M, Schulz A, Martin I, Hedrich R, Neuhaus HE. 2014. Overexpression of a proton-coupled vacuolar glucose exporter impairs freezing tolerance and seed germination. New Phytologist 202, 188–197.

Legay S, Guerriero G, Deleruelle A, Lateur M, Evers D, André CM, Hausman JF. 2015. Apple russetting as seen through the RNA-seq lens: strong alterations in the exocarp cell wall. Plant Molecular Biology 88, 21–40.

Li M, Feng F, Cheng L. 2012. Expression patterns of genes involved in sugar metabolism and accumulation during apple fruit development. Plos One 7, e33085.

Longhi S, Hamblin MT, Trainotti L, Peace CP, Velasco R, Costa F. 2013. A candidate gene based approach validates Md-PG1 as the main responsible for a QTL impacting fruit texture in apple (Malus × domestica Borkh.). BMC Plant Biology 13, 37.

Malladi A, Hirst PM. 2010. Increase in fruit size of a spontaneous mutant of ‘Gaia’ apple (Malus × domestica Borkh.), is facilitated by altered cell production and enhanced cell size. Journal of Experimental Botany 61, 3003–3013.

Malladi A, Johnson JK. 2011. Expression profiling of cell cycle genes reveals key facilitators of cell production during carpel development, fruit set, and fruit growth in apple (Malus × domestica Borkh.). Journal of Experimental Botany 62, 205–219.

Martinez-Esteso MO, Villela-Antón MT, Pedreño MÁ, Valero ML, Bru-Martinez R. 2013. iTRAQ-based protein profiling provides insights into the cecal metabolism changes driving grape berry development and ripening. BMC Plant Biology 13, 167.

Megger DA, Pott LL, Ahrens M, Padden J, Bracht T, Kuhlmann K, Eisenacher M, Meyer HE, Sitek B. 2014. Comparison of label-free and label-based strategies for proteome analysis of hepatoma cell lines. Biochimica et Biophysica Acta – Proteins and Proteomics 1844, 967–976.

Nguyen-Quoc B, Foyer CH. 2001. A role for ‘ futile cycles involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. Journal of Experimental Botany 52, 881–889.

Pratelli R, Pilot G. 2014. Regulation of amino acid metabolic enzymes and transporters in plants. Journal of Experimental Botany 65, 5535–5556.

Ravanel S, Gakiere B, Job D, Douce R. 1998. The specific features of methionine biosynthesis and metabolism in plants. Proceedings of The National Academy of Sciences 1, 7805–7812.
Wang P, Sun X, Xie Y, Li M, Chen W, Zhang S, Liang D, Ma F. 2014. Melatonin regulates proteomic changes during leaf senescence in Malus hupehensis. Journal of Pineal Research 67, 291–307.

Wei X, Liu F, Chen C, Ma F, Li M. 2014. The Malus domestica sugar transporter gene family: identifications based on genome and expression profiling related to the accumulation of fruit sugars. Frontiers in Plant Science 5, 669.

Westram A, Lloyd JR, Roessner U, Riesmeier JW, Kossmann J. 2002. Increases of 3-phosphoglyceric acid in potato plants through antisense reduction of cytoplasmic phosphoglycerate mutase impairs photosynthesis and growth, but does not increase starch contents. Plant Cell and Environment 25, 1133–1143.

Woollard AA, Moore I. 2008. The functions of RabGTPases in plant membrane traffic. Current Opinion in Plant Biology 11, 610–619.

Xia G, Cheng L, Lakso AN, Goffinet M. 2009. Effects of nitrogen supply on source-sink balance and fruit size of ‘Gala’ apple trees. Journal of the American Society for Horticultural Science 134, 126–133.

Yamaguchi H, Kanayama Y, Yamaki S. 1994. Purification and properties of NAD-dependent sorbitol dehydrogenase from apple fruit. Plant Cell and Physiology 35, 887–892.

Yamaki S. 1984. Isolation of vacuoles from immature apple fruit flesh and compartmentation of sugars, organic acids, phenolic compounds and amino acids. Plant Cell and Physiology 25, 151–166.

Yang Y, Xu Q, Owsiany K, Zhang S, Thannhauser TW, Li L. 2011. Evaluation of different multidimensional LC-MS/MS pipelines for isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis of potato tubers in response to cold storage. Journal of Proteome Research 10, 4647–4660.

Yao Y, Li M, Zhai H, You C, Hao Y. 2011. Isolation and characterization of an apple cytosolic malate dehydrogenase gene reveal its function in malate synthesis. Journal of Plant Physiology 168, 474–480.

Zhang C, Hicks GR, Raikhel NV. 2014. Plant vacuole morphology and vacuolar trafficking. Frontiers in Plant Science 5, 476.

Zhang Y, Li P, Cheng L. 2010. Developmental changes of carbohydrates, organic acids, amino acids, and phenolic compounds in ‘Honeycrisp’ apple flesh. Food Chemistry 123, 1013–1018.

Zheng J, Han SW, Rodriguez-Welsh MF, Rojas-Pierce M. 2014. Homotypic vacuole fusion requires VTI11 and is regulated by phosphoinositides. Molecular Plant 7, 1026–1040.

Zheng Q, Song J, Campbell-Palmer L, Thompson K, Li L, Walker B, Cuic Y, Lib X. 2013. A proteomic investigation of apple fruit during ripening and in response to ethylene treatment. Journal of Proteomics 93, 276–294.

Zhou L, Adams RM, Karuna C, Hurst GB, Hettich RL, Chongle P. 2012. Systematic comparison of label-free, metabolic labeling, and isobaric chemical labeling for quantitative proteomics on LTQ Orbitrap Velos. Journal of Proteome Research, 11, 1582–1590.