OPEN

Citation: Horticulture Research (2014) 1, 6; doi:10.1038/hortres.2014.6

www.nature.com/hortres

ARTICLE

Dynamic changes in proteins during apple (*Malus* x *domestica*) fruit ripening and storage

Yun Shi[1], Li Jiang[1], Li Zhang[2], Ruoyi Kang[1] and Zhifang Yu[1]

A proteomic study, using two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight, was conducted in apple fruit (cv. 'Golden Delicious') starting at 10 days prior to harvest through 50 days in storage. Total protein was extracted using a phenol/sodium dodecyl sulfate protocol. More than 400 protein spots were detected in each gel and 55 differentially expressed proteins ($p<0.05$) were subjected to matrix-assisted laser desorption/ionization time-of-flight/time-of-flight analysis. Fifty-three of these proteins were finally identified using an apple expressed sequence tag database downloaded from Genome Database for *Rosaceae* and placed into six categories. The categories and the percentage of proteins placed in each category were stress response and defense (49.0%), energy and metabolism (34.0%), fruit ripening and senescence (5.6%), signal transduction (3.8%), cell structure (3.8%) and protein synthesis (3.8%). Proteins involved in several multiple metabolic pathways, including glycolysis, pentose–phosphate pathway, anti-oxidative systems, photosynthesis and cell wall synthesis, were downregulated, especially during the climacteric burst in respiration and during the senescent stages of fruit development. Proteins classified as allergens or involved in cell wall degradation were upregulated during the ripening process. Some protein spots exhibited a mixed pattern (increasing to maximal abundance followed by a decrease), such as 1-aminocyclopropane-1-carboxylate oxidase, *L*-ascorbate peroxidase and abscisic acid response proteins. The identification of differentially expressed proteins associated with physiological processes identified in the current study provides a baseline of information for understanding the metabolic processes and regulatory mechanisms that occur in climacteric apple fruit during ripening and senescence.

Horticulture Research (2014) **1,** 6; doi:10.1038/hortres.2014.6; Published online 22 January 2014

INTRODUCTION

Apple (*Malus domestica* L.) is one of the most widely cultivated fruits in the world for its flavor, health and nutritional value. Apple is a climacteric fruit, exhibiting a burst in respiration during ripening, and the physiology, biochemistry and molecular biology of ripening and senescence have been extensively studied.[1,2] Fruit ripening is characterized by physiological and biochemical processes, including ethylene biosynthesis, pigmentation, chlorophyll degradation, cell wall degradation, organic acid accumulation and volatile production, resulting in changes in fruit traits such as color, texture, flavor, aroma and other aspects of fruit metabolism.[3,4] These changes are associated with stages of ripening and post-harvest storage conditions. During the fruit ripening process, fruit generally, among other changes, decline in firmness, increase in flavor and undergo changes in color.[3] These complex physiological changes result from alterations in gene and protein expression that impact specific metabolic pathways.

Many 'omics' technologies, such as genomics, transcriptomics, proteomics and metabolomics, have been recently used to obtain information on global changes occurring during fruit maturation, ripening and senescence.[5] Over 150 000 expressed sequence tags (ESTs) have been collected from 'Royal Gala' apple fruit tissues.[6] The availability of the apple genome sequence has also provided a rich resource for understanding the genetic regulation of fruit ripening. Apple genes associated with cell division, flavor and aroma development, and starch metabolism during fruit development and ripening have been identified.[7] Additionally, 19 ACC synthases have been identified in the apple genome and their expression in fruit has been characterized.[8]

Comparative proteomics can be an effective tool for generating useful information regarding complex biological processes, such as fruit ripening.[5] The availability of the complete apple genome sequence can facilitate the identification of apple proteins and their putative function. Proteomic research on fruit ripening has been conducted on tomato,[9,10] strawberry,[11,12] grape,[13,14] peach,[15,16] citrus,[17] papaya,[18] mango[19] and apricot,[20] which has provided a large body of information for better understanding the process and regulation of fruit ripening and senescence.

Qin *et al.*[21] used a proteomic approach to examine the regulatory effect of reactive oxygen species (ROS) on apple fruit ripening and senescence. Results indicated that differentially expressed mitochondrial proteins were involved in the electron transport chain, tricarboxylic acid cycle, stress response and carbon metabolism. Superoxide dismutase [Mn] {SOD[Mn]} activity was reduced in response to exposure to high oxygen (100%), which was followed by an increase of damaged proteins, suggesting that ROS may regulate fruit senescence by regulating the expression of mitochondrial proteins. Zheng *et al.*[22] compared proteomic changes in apple fruit associated with ethylene treatment. However, proteomic studies of apple fruit during maturation and storage, and their association with physiological changes, have not been conducted.

In the present study, differentially expressed proteins in apple fruit during maturation and different stages of ripening were characterized. This approach was used to better understand the apple ripening process and changes during storage. This knowledge can be used to develop harvesting and post-harvest handling

[1]College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, China and [2]Suzhou Academy of Agriculture, Suzhou 215155, China.
Correspondence: ZF Yu, (yuzhifang@njau.edu.cn)
Received: 9 December 2013; accepted: 10 December 2013

practices that will ensure the delivery of high-quality apple fruit to consumers.

MATERIALS AND METHODS

Plant material

The optimal date of harvest, designated as H0, for apples (*Malus* × *domestica* Borkh. cv. Golden Delicious) grown in the Dashahe Orchard (34.52′N, 116.60′E, elevation of 30–40 m), Fengxian, Jiangsu Province, China, was 2 September 2011. This was based on fruit size, color and the historical harvest date for this cultivar. Apples used in this study were sampled 10 days (H-10) prior to H0, 5 days (H-5) prior to H0, at H0, and then at 5- or 10-day intervals during 50 days of storage (H5–H50) at 25±5 ℃ and 80–90% relative humidity. At each sampling time, three biological replicates, consisting of 20 fruits, were peeled, cut into quarters, immediately frozen in liquid nitrogen and then stored at −20 ℃ prior to protein extraction. In parallel, 10 additional fruits at each date of sampling were used to measure fruit firmness, total soluble solid content and respiratory rate. Firmness (expressed as kg cm^{-2}) was measured twice on opposite peeled sides of each fruit with a penetrometer (Xingke Instruments, Siping, China). Soluble solids were determined using a refractometer (Quanzhou Optics Instruments, Quanzhou, China) and the respiratory rate of fruits was estimated using a portable infrared CO_2 analyzer (GXH-3010E; Nuoji Instruments Inc., Changzhou, China).

Protein sample preparation

A modified phenol/sodium dodecyl sulfate (SDS) protocol[23] was used to extract protein from the fruit samples. Five grams of frozen fruit were finely ground in liquid nitrogen, suspended in 10 mL of extraction buffer (30% (w/v) sucrose, 2% (w/v) SDS, 0.1 M Tris-HCl, pH 8.0, 5% (v/v) β-mercaptoethanol), vortexed and incubated for 30 min at 4 ℃. After adding an equal volume of ice-chilled, Tris-saturated phenol (pH 7.8), the mixture was vortexed and incubated for 30 min at 4 ℃ and then centrifuged at 10 000*g* at 4 ℃ for 30 min. The upper phenol phase was collected and precipitated overnight at −20 ℃ using five times the volume of 0.1 M ammonium acetate in methanol. After centrifugation at 10 000*g* at 4 ℃ for 15 min, the supernatant was discarded and precipitated proteins were rinsed twice with ice-chilled methanol, twice with chilled 80% acetone in water and once with 100% acetone. The pellets were air dried at room temperature and dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) 3-3[(cholamidopropy) dimethylammonio-1-propanesulfonate, 1% (w/v) dithiothreitol and 0.5% (v/v) pH 4–7 immobilized pH gradient buffer) at 4 ℃. Supernatants were collected after centrifugation at 12 000*g* at 4 ℃ for 20 min and the protein concentration was determined using the Bradford method[24] with bovine serum albumin as a standard. Samples were stored at −20 ℃ until they were subjected to two-dimensional polyacrylamide gel electrophoresis (2-DE).

2-DE and gel staining

Lysis buffer was added to approximately 1.8 mg of total protein from each sample to a final volume of 360 μL and then loaded onto immobilized pH gradient strips (17 cm pH 4–7, Ready strip; Bio-Rad, Hercules, CA, USA) and rehydrated for 12 h at 20 ℃. Focusing was performed with a PROTEAN isoelectric focusing system (Bio-Rad) for a total of 60 kVh at 20 ℃ by applying the following voltages: 100 V for 1 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 4000 V for 2 h, 8000 V for 2 h and then holding at 8000 V until final volthours (60 kVh) was reached. Prior to running the second dimension, the focused strips were equilibrated for 15 min with 2% (w/v) dithiothreitol in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 20% (v/v) glycerol and 2% (w/v) SDS) followed by 15 min in the same buffer with the addition of 2.5% (w/v) iodoacetamide. The second dimension protein separation was carried out on a 12% polyacrylamide gel, using the Ettan Six vertical set (GE Healthcare, Uppsala, Sweden) with 1 W gel^{-1} for 1 h, then 15 W gel^{-1} until the bromophenol blue dye reached the bottom of the gel. The gels were stained with colloidal Coomassie brilliant blue G250.[25] Three gels, one from each biological replicate, were run from each sampling date.

Gel image acquisition and statistical analysis

Images of the stained gels were acquired with a Versdoc 3000 scanner (Bio-Rad) and image analysis was performed using PDQuest 2-D v.2 analysis software (Bio-Rad). Manual editing was performed after automated spot detection and matching of spots between different gels. Protein spots were considered valid based on their presence in at least two out of three of the 2-DE gels for each sample. Spot intensities were normalized as a percentage of the total volume in all the spots present in the whole gel. Protein percentage volume data were exported to an Excel file to calculate fold change. Only spots with at least a twofold increase/decrease in abundance and statistically significant (Student's *t*-test, $p<0.05$) were considered to be differentially expressed.[15]

Protein digestion

Differentially expressed protein spots were manually excised from 2D gels and destained in 50% (v/v) ACN and 25 mM NH_4HCO_3. Gel particles were dehydrated with 100% (v/v) ACN and vacuum-dried. Proteins were reduced in 10 mM dithiothreitol for 1 h at 56 ℃ and alkylated with 55 mM iodoacetamide for 1 h at room temperature. The gel plugs were then dehydrated with 100% (v/v) ACN again and dried under a vacuum. Proteins in the gel plugs were digested with 12.5 ng mL^{-1} trypsin (Promega, USA) in 25 mM NH_4HCO_3 overnight at 37 ℃. The resultant fragments were extracted with 67% ACN containing 0.1% trifluoroacetic acid. The pellets were air dried and stored at 20 ℃ for mass spectrometry (MS) analysis.

MS analysis and protein identification

The air-dried samples were resuspended in 0.1% trifluoroacetic acid and analyzed using a 4800 matrix-assisted laser desorption/ionization time-of-flight/time-of-flight Proteomics Analyzer (Applied Biosystems, USA), at 200 Hz laser, in a mass range 800–4000 Da of mass peaks.

GPS Explorer v. 3.5 software (Applied Biosystems) was used to conduct a combined search (MS plus MS/MS) against the apple expressed sequence tag database downloaded from the Genome Database for *Rosaceae* (GDR) (http://www.rosaceae.org/species/malus/malus_x_domestica/genome_v1.0) using an in-house Mascot search engine v2.1 (Matrix Science Ltd, London, UK) with the following settings: a missed cleavage maximum value of 2.50 ppm mass tolerance, 0.2 Da for MS/MS tolerance, a fixed modification (cysteines carbamidomethylation) and a variable modification (methionine oxidation). Only a Mascot score indicating a significant identification of 95% ($p<0.05$) was accepted. The functional annotation of the identified proteins was based on UniProt, GDR, NCBInr protein database and the literature.

RESULTS AND DISCUSSION

Physiological changes in fruit during ripening and storage

The respiratory rate, firmness and soluble solid content of apple fruit underwent significant changes during ripening (H-10–H50) and storage (Table 2a). In accordance to previous reports,[22,26] fruit exhibited a continuous decrease in firmness, and an increase in their respiratory rate (H5–H20) and soluble solid content during ripening and storage (H20), which then had a tendency to decrease upon further storage (H30–H50). A climacteric rise in respiration was observed at H20 marking the pre-climacteric to climacteric transition. The decrease of fruit firmness from 10.20 to 4.46 kg cm^{-2} occurred in parallel to changes in fruit peel color from green to yellow. Soluble solid content increased slowly up to 20 days after harvest (H20) and then subsequently declined.

Identification and differential expression of apple fruit proteins

More than 400 protein spots, ranging in molecular mass from 14–90 kDa and pH 4–7, were detected in 'Golden Delicious' apple fruit during ripening and storage (Figure 1). A total of 55 spots were differentially abundant (>two-fold) compared to the H0 stage in at least one sampling point. Among them, 53 spots were confidently identified using the GDR database, as was their subcellular location (Table 1). The 53 differentially expressed proteins are illustrated in Figure 1 and changes in protein levels, for some of the proteins, over the time course of the study (H-10–H50) are illustrated in Figure 2. Changes in the level of expression for all of the differentially expressed proteins over the time course used in this study are presented in Table 2b. The differentially expressed proteins were assigned into one of six categories based on their description in GDR, their annotation in Munich Information Center for Protein Sequences Functional Catalogue Database and published literature. The category containing most of the differentially

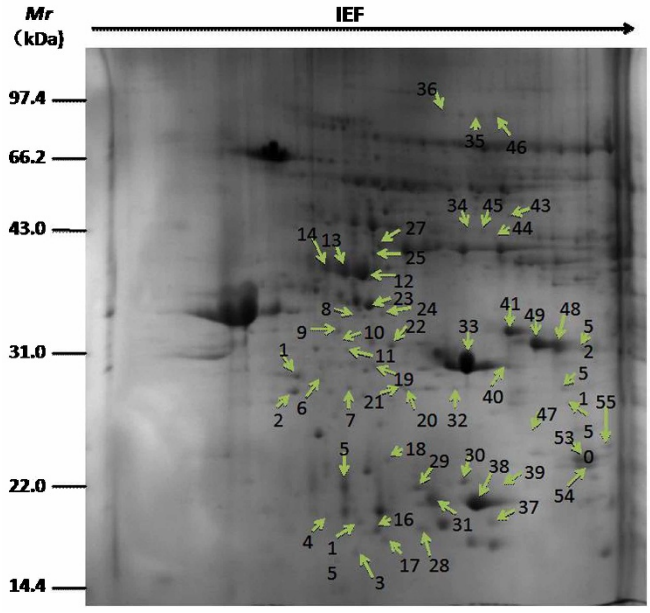


Figure 1. Identification and differential expression of apple (cv. Golden Delicious) proteins in fruit maintained at 25 ℃ and sampled during ripening and senescence. 2-DE was performed using 1.8 mg of protein, linear 17 cm IPG strips (pH 4–7) for the first dimension, and 12% SDS–PAGE gels for the second dimension electrophoresis. Gels were stained with colloidal CBB G250. Numbers with arrows indicate the differentially expressed protein spots that were identified in this study. CBB, Coomassie brilliant blue; IEF, isoelectric focusing; IPG, immobilized pH gradient; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

expressed proteins (49.0%) was defined as 'stress response and defense'. The category with the second largest percentage of proteins (34.0%) was one containing proteins involved in energy and metabolism, including carbohydrate, amino acid, nucleotide, thiamine, lipid and secondary metabolism. Three proteins (5.6%) were associated with fruit ripening and senescence; two proteins (3.8%) were associated with signal transduction; two (3.8%) with cell structure, and the remaining two (3.8%) were associated with protein synthesis. These results are illustrated in Figure 3.

Some proteins were identified in more than one spot, indicating that a small number of the differentially expressed spots were either subjected to post-translational modification or were members of multigenic protein families. Such spots included adenine phosphoribosyl transferase 1 (APR1) (two spots), 1-aminocyclopropane-1-carboxylate oxidase 1 (ACO 1) (two spots), major allergen Mal d 1 (two spots), superoxide dismutase [Cu–Zn] (Cu/Zn-SOD) (two spots) and abscisic acid response protein (three spots).

A number of proteins related to fruit respiratory pathway and quality (i.e., firmness and soluble solids) exhibited significant changes over the course of fruit maturation and ripening. Among these proteins, three (NADP-dependent malic enzyme, triosephosphate isomerase and probable 6-phosphogluconolactonase 2) are associated with fruit respiration and may have changed in abundance due to the respiratory burst at H20 (Table 2a). Five proteins (beta-galactosidase, beta-galactosidase 3, pollen-specific leucine-rich repeat extensin-like protein 3, actin-depolymerizing factor 2 and putative uncharacterized protein) are associated with cell wall metabolism and may have played a role in the observed changes in fruit firmness and three other proteins (soluble inorganic pyrophosphatase, NADP-dependent malic enzyme and triosephosphate isomerase) are putatively associated with metabolic changes associated with changes in the content of soluble solids.

Among the 53 identified proteins, 10 were proteins that were newly synthesized during the last stages of ripening and senescence (NADP-dependent malic enzyme, beta-galactosidase, beta-galactosidase 3, probable sarcosine oxidase, chavicol O-methyltransferase, 26.5 kDa heat shock protein, abscisic acid response protein, major allergen Pru ar 1, major allergen Mal d 1 and ACC oxidase 1) (Table 2b). Three proteins (oxygen-evolving enhancer protein1, probable 6-phosphogluconolactonase 2 and 17.8 kDa class I heat shock protein) disappeared during the time course of the study (Table 2b). Four proteins (NADP-dependent malic enzyme, beta-galactosidase, beta-galactosidase 3 and probable 6-phosphogluconolactonase 2) were identified potentially playing a role in changes in fruit respiration, firmness and soluble solids. As shown in Table 2b, all 53 proteins identified in this study exhibited significant differences in abundance over the time course of this study. A more detailed discussion of each protein and its putative functional role in fruit ripening and senescence follows.

Proteins related to energy and carbohydrate metabolism

The process of gluconeogenesis is essentially the reversal of the glycolysis pathway brought about by several enzymatic reactions. Cytosolic NADP-dependent malic enzyme (NADP-ME, spot 36), triosephosphate isomerase (TIM, spot 40) and a putative 6-phosphogluconolactonase 2 (6PGL 2, spot 10) were identified in this study. These proteins, as the source of pyruvate, provide energy (ATP) and reductive power (NADPH) in fruit tissue. NADP-ME, a key enzyme for malate oxidation, may promote gluconeogenesis during fruit ripening by providing a linkage between NADPH and pyruvate entering into respiratory pathways for energy production.[27] The pattern of NADP-ME expression, a respiratory related protein, mirrored the pattern of respiration in apple fruit during maturation and the ripening period. NADP-ME appeared at H15 and increased to a maximum at H20, then gradually declined at later sampling times (Table 2b). A similar relationship between cytosolic NADP-ME activity and endogenous ethylene during tomato fruit ripening was previously reported,[28] indicating that the peak of NADP-ME activity was partly consistent with the climacteric burst in respiration. These results support the premise that NADP-ME in the current study may be involved in sustaining the climacteric respiratory burst exhibited in apple during the ripening process (Table 2a).

TIM is involved in glycolysis, catalyzing the interconversion of dihydroxyacetone phosphate with glyceraldehyde 3-phosphate, which finally favors glyceraldehyde 3-phosphate, resulting in the formation of pyruvate. TIM, a key enzyme in sugar metabolism, was reactivated by glutathione and involved in the redox regulation by glutathionylation.[29] In the current study, TIM concentration increased during apple fruit maturation, which suggests that it would promote respiration and catabolic metabolism, such as redox regulation and the accumulation of energy. The decrease in TIM observed after harvest occurred in parallel with the reduction in antioxidant enzymes and total soluble solid content (Table 2a). This relationship has also been reported in other fruit tissues.[13,14,22]

The pentose–phosphate pathway plays an important role in generating NADPH and in oxidative stress response.[30] 6PGL 2, which is involved in the pentose–phosphate pathway and Entner–Doudoroff pathway, catalyzes the oxidative reaction of 6-phosphate gluconolactone into glucose 6-phosphate. In the current study, 6PGL 2 significantly decreased to 0.18-fold at H30, relative to the level present at H0 (optimal harvest time), and disappeared during subsequent sampling times (Table 2b). This suggests that antioxidant enzymes were also downregulated during ripening and storage, especially after the climacteric peak (H15–H20). In general, antioxidant capacity would have been weakened due to the reduced pool of NADPH which could protect against oxidative stress.[31]

Table 1. Identification of 53 differentially expressed protein spots in the apple proteome during ripening and senescence as determined by 2-DE analysis, MALDI-TOF-TOF, and MASCOT analysis.

Spot no. [a]	Protein name [b]	GDR accession no. [c]	M ascot score [d]	Matched % Cov. [e]	Matched peptides [f]	Theo Mr(kDa) /pI [g]	Obse Mr(kDa) /pI	Subcellular location [h]
Energy and Metabolism								
Energy (gluconeogenesis, photosynthesis)								
Gluconeogenesis								
36	NADP-dependent malic enzyme(NADP-ME)	MDP0000268037	49	1	1	83.34/5.81	80.00/6.00	Cytoplasm
40	Triosephosphate isomerase (TIM)	MDP0000694943	431	32	7	27.49/5.76	29.97/6.30	Cytoplasm
Photosynthesis								
8	Oxygen-evolving enhancer protein1 (OEE1)	MDP0000248920	444	38	8	35.29/6.09	34.19/5.51	Chloroplast
Metabolism (carbohydrate, amino acid, nucleotide, thiamine, lipid and secondary)								
Carbohydrate metabolism								
10	Probable 6-phosphogluconolactonase 2 (6PGL 2)	MDP0000223309	109	7	5	34.08/5.12	31.73/5.43	Chloroplast
19	Soluble inorganic pyrophosphatase(PPase)	MDP0000281233	407	11	6	64.36/5.35	29.79/5.62	Cytoplasm.
43	Putative uncharacterized protein	MDP0000127723	328	19	4	40.73/5.71	49.93/6.31	Nuclear
35	Beta-galactosidase	MDP0000416548	425	14	10	81.52/5.69	79.87/6.07	Apoplast
46	Beta-galactosidase 3	MDP0000030527	66	1	1	95.63/8.34	79.73/6.15	Apoplast
Amino acid metabolism								
20	L-asparaginase 1	MDP0000658649	334	17	5	34.63/5.21	27.93/5.79	Endoplasmic reticulum
27	Cysteine synthase	MDP0000325786	340	22	7	41.19/7.60	41.35/5.66	Chloroplast stroma Chromoplast.
34	Probable sarcosine oxidase	MDP0000686885	577	23	7	44.10/5.59	44.26/6.09	Cytoplasm
44	Isovaleryl-CoA dehydrogenase 2 (IVD 2)	MDP0000225981	408	25	8	58.20/6.11	42.73/6.24	Mitochondrion
Nucleotide metabolism								
2	Adenine phosphoribosyltransferase 1 (APRT 1)	MDP0000121897	625	76	7	13.00/5.03	27.84/5.17	Chloroplast Cytoplasm
6	UMP/CMP kinase (UMP/CMPK)	MDP0000240174	320	21	7	28.49/5.69	28.59/5.32	Cytoplasm
7	Adenine phosphoribosyltransferase 1 (APRT 1)	MDP0000137185	616	42	8	27.00/8.40	28.00/5.48	Chloroplast Cytoplasm
Thiamine metabolism								
24	Thiazole biosynthetic enzyme (THI1)	MDP0000206098	714	27	9	39.30/5.68	34.48/5.67	Chloroplast
Lipid metabolism								
25	Epoxide hydrolase 2 (EH 2)	MDP0000161121	349	16	8	35.34/5.20	40.38/5.61	Cytoplasm Peroxisome
Secondary metabolism								
45	Chavicol O-methyltransferase (CVOMT 1)	MDP0000219062	314	21	5	42.82/5.89	43.53/6.17	Cytoplasm
Stress response and defense								
1	Ferritin-3	MDP0000189389	296	14	4	35.46/5.69	28.93/5.18	Chloroplast
3	Thioredoxin H-type (Trx-H)	MDP0000415439	435	43	7	13.88/5.39	16.30/5.51	Cytoplasm
5	MLP-like protein 328	MDP0000132530	132	22	4	17.83/5.13	21.60/5.45	Cytoplasm
16	Superoxide dismutase [Cu-Zn]	MDP0000258717	380	23	3	22.80/6.09	18.01/5.60	Chloroplast
17	Glycine-rich RNA-binding protein (GRP1A)	MDP0000797759	412	52	8	16.88/5.61	17.17/5.68	Nucleus.
21	26.5 kDa heat shock protein	MDP0000435717	364	16	6	37.21/9.34	28.17/5.76	Mitochondrion
22	L-ascorbate peroxidase 2	MDP0000210077	625	20	8	37.95/5.71	31.71/5.73	Cytoplasm
23	Putative lactoylglutathione lyase	MDP0000319112	401	29	8	39.97/6.40	35.32/5.60	Cytoplasm
28	Superoxide dismutase [Cu-Zn]	MDP0000250286	517	29	4	22.28/6.28	18.30/5.83	Chloroplast.
33	Abscisic acid stress ripening protein homolog	MDP0000253074	211	11	1	25.72/6.01	30.64/6.05	Nuclear
37	Superoxide dismutase [Cu-Zn] 1	MDP0000201158	173	42	2	80.44/7.88	18.62/6.11	Cytoplasm.
39	17.8 kDa class I heat shock protein	MDP0000791550	261	31	5	17.52/5.99	21.42/6.27	Cytoplasm.
41	Abscisic acid response protein	MDP0000868045	414	31	4	19.86/8.61	33.55/6.29	Nuclear
47	Outer membrane lipoprotein blc (temperature-induced lipocalin)	MDP0000148808	145	20	6	21.47/6.18	24.96/6.40	Cell outer membrane
49	Abscisic acid response protein	MDP0000868045	257	17	3	19.86/8.62	32.46/6.43	Nuclear
50	Superoxide dismutase [Mn]	MDP0000187714	444	13	7	60.57/8.51	27.00/6.60	Mitochondrion matrix.
51	Glutathione S-transferase	MDP0000096349	632	57	9	24.03/6.17	28.13/6.58	Cytoplasm
52	Abscisic acid response protein	MDP0000868045	397	31	4	19.86/8.68	31.09/6.65	Nuclear
53	universal stress protein (USP) family protein	MDP0000639609	130	10	1	19.81/6.30	23.34/6.68	Cytoplasm
55	Peroxiredoxin-2F, mitochondrial (PRXIIF)	MDP0000258515	161	15	2	21.61/8.77	23.90/6.85	Mitochondrion matrix
4	Major allergen Pru ar 1	MDP0000288293	340	33	5	17.61/5.10	19.28/5.35	Cytoplasm
15	Major allergen Mal d 1	MDP0000216907	444	38	5	17.52/5.54	18.47/5.53	Cytoplasm
18	MLP-like protein 329	MDP0000277802	383	31	4	17.83/5.41	23.27/5.68	Cytoplasm
31	Major allergen Mal d 1	MDP0000942516	566	28	6	17.53/5.62	20.27/5.82	Cytoplasm
38	Major allergen Mal d 1	MDP0000942516	592	52	9	17.53/5.62	20.68/6.10	Cytoplasm
54	Thaumatin-like protein 1a	MDP0000223025	85	1	2	12.26/5.71	22.79/6.68	Extracellular

Continued

Table 1. Continued

Spot no. [a]	Protein name [b]	GDR accession no. [c]	M ascot score [d]	Matched % Cov. [e]	Matched peptides [f]	Theo Mr(kDa) /pI [g]	Obse Mr(kDa) /pI	Subcellular location [h]
Ripening and senescence								
12	1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1)	MDP0000195885	617	39	9	35.56/5.24	38.74/5.53	Cytoplasm
13	1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1)	MDP0000195885	547	42	9	35.56/5.24	39.08/5.44	Cytoplasm
14	1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1)	MDP0000195885	593	42	9	35.56/5.25	39.72/5.76	Cytoplasm
Signal transduction								
9	14-3-3 protein 7	MDP0000270640	207	13	4	30.54/5.31	32.77/5.42	Cytoplasm
48	GTP-binding nuclear protein Ran-3	MDP0000130864	66	8	2	28.54/6.14	31.30/6.53	Cytoplasm
Cell structure								
11	Pollen-specific leucine-rich repeat extensin-like protein 3 (AtPEX3/Pollen-specific LRR)	MDP0000158152	45	2	1	60.12/6.37	31.19/5.47	Cell wall
30	Actin-depolymerizing factor 2 (ADF 2)	MDP0000245712	277	27	4	23.07/6.84	21.90/5.99	Extracellular
Protein synthesis								
29	Eukaryotic translation initiation factor 5A-2	MDP0000201872	308	42	6	17.76/5.46	21.48/5.81	Cytoplasm
32	Proteasome subunit beta type-1	MDP0000193666	55	4	1	34.19/8.07	28.22/6.02	Cytoplasm Nucleus

[a] Numbering corresponds to the 2-DE gel in Fig.1.
[b] Names of the proteins obtained via the MASCOT software v2.1 against the apple EST database from Genome Database for Rosaceae (GDR) (http://www.rosaceae.org/species/malus/malus_x_domestica/genome_v1.0)
[c] Accession number from the apple EST database from GDR.
[d] Total ion score for the entire protein and for ions complemented by 100% of the confidence index (C.I).
[e] Percent sequence coverage.
[f] Numbers of matched peptides.
[g] Theoretical molecular mass (Mr) and isoelectric point (pI) of the homologous protein available at the apple EST database, observed molecular mass (Mr) and isoelectric point (pI) estimated in comparison to the 2-DE gel with marker proteins.
[h] Subcellular location of each spot is based on the Uniprot Database.

In plants, photoassimilate partitioning between sucrose and starch involves inorganic pyrophosphatase (PPase, spot 19), which hydrolyzes pyrophosphate (PPi) to phosphate (Pi), which affects the efficiency of sucrose synthase especially in the absence of PPi.[32] In our study, the 3.22-fold downregulation of PPase in the latter stages of sampling (H40–H50) would have resulted in a decrease in PPi and thus the synthesis of sucrose. The decreased content of oxygen-evolving enhancer protein 1 (OEE1, spot 8), associated with photosynthesis II, has been reported in other fruits.[9,20] The decrease in OEE1 would have promoted the degradation of chlorophyll and the transition of chloroplasts to chromoplasts.[10] The reduced levels of OEE1 in apple tissues coincided with the loss of green color in apple fruit (Table 2a).

Proteins related to cell wall metabolism

The reduction in fruit firmness that occurs during ripening is partially regulated by the activity of cell wall-degrading enzymes, which lead to biochemical and structural alterations in cell walls. The enzymes related to cell wall degradation in our study included

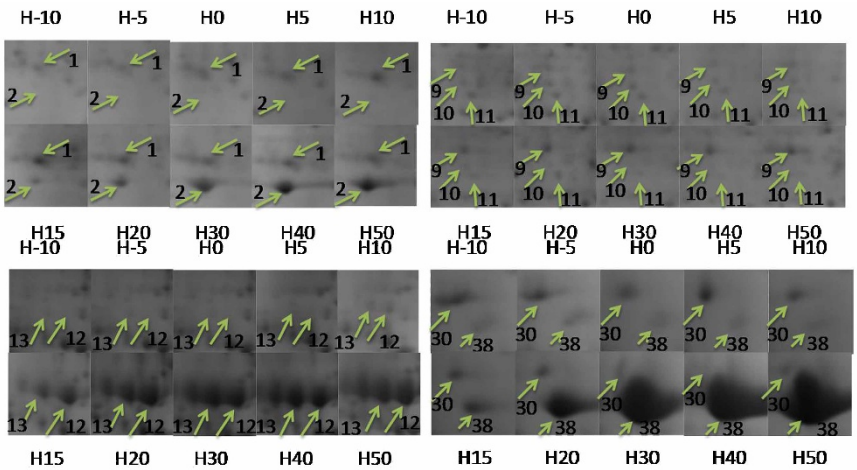


Figure 2. Close-up views of a selected sample of differentially abundant proteins marked in Figure 1. Different ripening stages are displayed above and below the protein images. H-10 is 10 days before the optimal harvest date (H0) and H5–H50 are days of storage at 25 ℃ after H0. Arrows and numbers indicate the spots with differential protein expression.

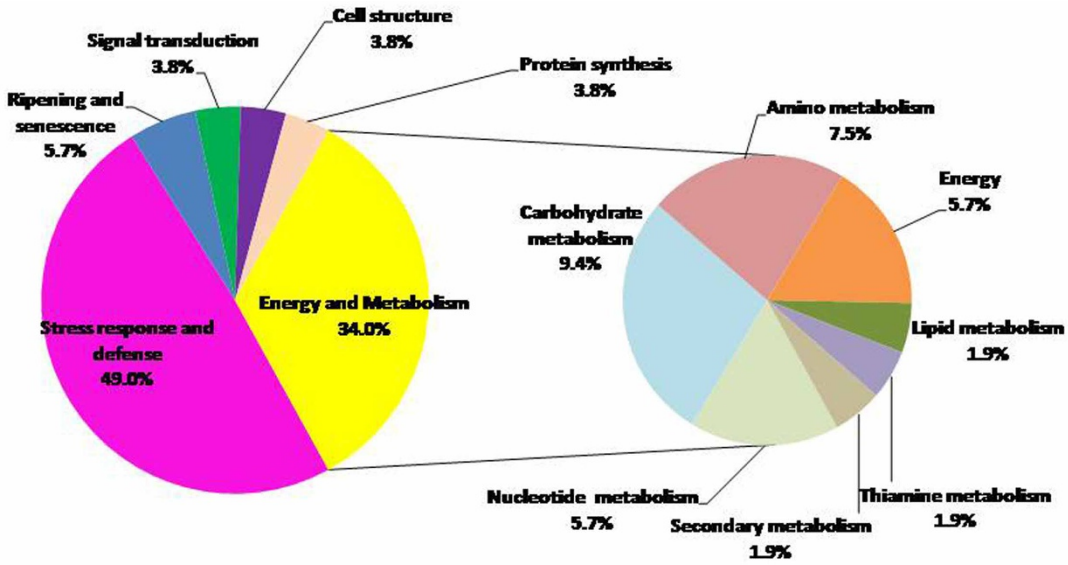


Figure 3. Classification of differentially expressed proteins identified in apple fruits at different ripening stages into functional categories. The classification is based on protein descriptions in the GDR, protein annotations in MIPS Functional Catalogue Database and published literature. MIPS, Munich Information Center for Protein Sequences.

beta-galactosidase (spot 35) and beta-galactosidase 3 (spot 46) (Table 2b). The presence of both enzymes appeared at H15 and significantly increased during and after the climacteric burst (Table 2a). This pattern of expression coincided with the loss in fruit firmness observed in the latter stages of fruit ripening (Table 2a). Similar observations were previously reported in apple,[33] tomato[34] and kiwi fruit.[35] Suppression of these two enzymes during the early ripening stage could retard fruit softening and/or extend fruit shelf-life, which has been demonstrated by genetic engineering with anti-sense genes.[36]

Proteins involved in cell wall synthesis were greatly downregulated during the storage period, including a pollen-specific leucine-rich repeat extensin-like protein 3 (pollen-specific LRR, spot 11), actin-depolymerizing factor 2 (ADF 2, spot 30) and a putative uncharacterized protein (spot 43), with ADF 2 showing the greatest reduction (Table 2b). ADF is involved in the stabilization of the actin cytoskeleton.[37] The decreased levels of ADF 2 may cause an instability in the cytoskeleton during fruit ripening, thus contributing to reduction in firmness (Table 2a).

Proteins related to ethylene biosynthesis

Ethylene plays a critical role in the ripening process of climacteric fruits, where an ethylene burst during fruit ripening is followed by a respiratory peak.[38] Ethylene, which is regulated by two main enzymes: ACO and synthase, affects the transcription and translation of many ripening-related genes.[39] ACO 1 (spots 12, 13 and 14) significantly increased during the early stages of ripening and reached a maximum at H15 (Table 2b), indicating that ACO 1 increased the endogenous level of ethylene, which then led to the rise in the rate of respiration. Among the three ACO proteins that were identified, the most interesting one is spot 12, since it represents a newly accumulated protein. It is possible that these three proteins may be divided into two types, with one functioning in ethylene biosynthesis system I and the other functioning in system II. An increase in ACO 1 during ripening was also reported in peach fruits.[40] The ACO gene has been previously studied in apple[41] and reported to be a key factor in ripening processes in peach, such as ethylene biosynthesis, pigmentation, cell wall metabolism, carbohydrate metabolism and signal transduction.[42] Further studies are needed, however, in order to fully understand the role of ethylene-related gene synthesis on the genetic basis of the ripening process in climacteric fruits.

Proteins related to stress response and defense

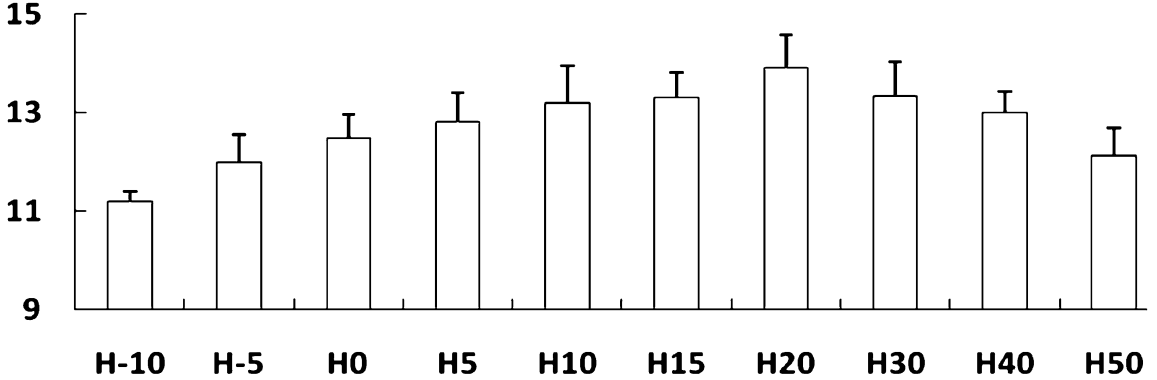
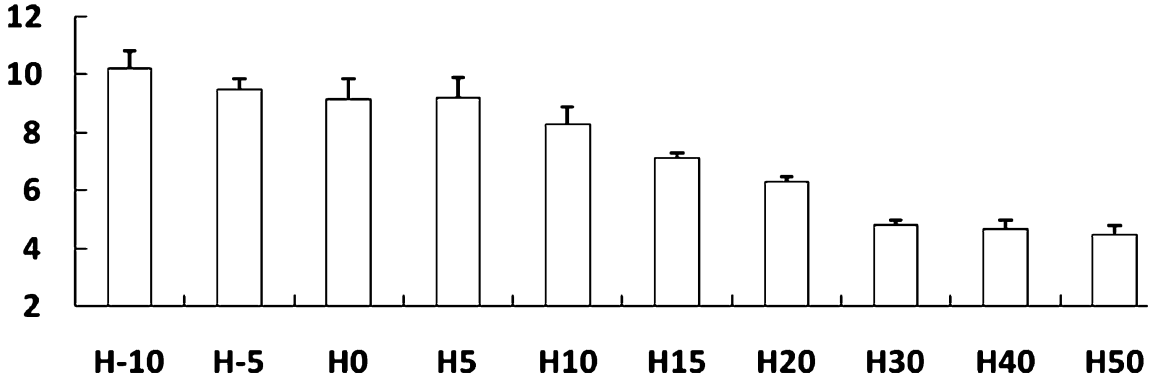
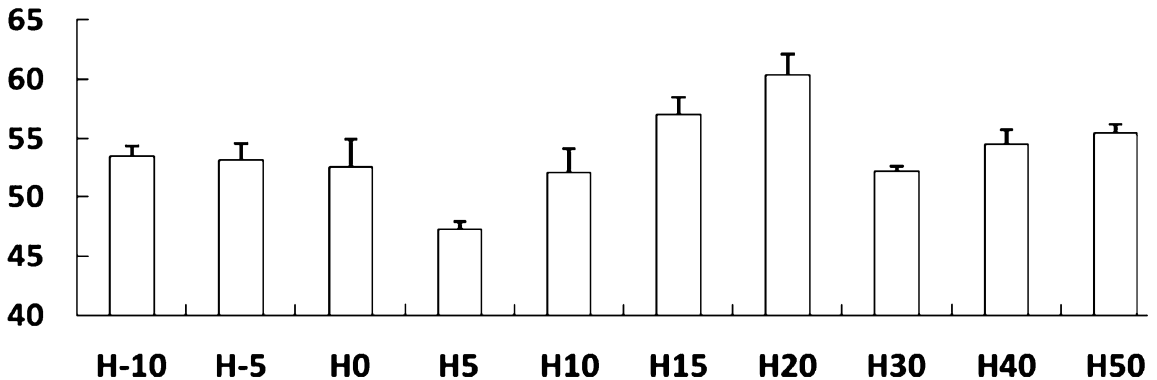
Fruit ripening and senescence have been considered as oxidative processes.[43] In the current study, several anti-oxidative enzymes were identified and their differential expressions appeared be associated with both ripening and senescence. Anti-oxidative enzymes identified include: Cu/Zn-SOD (spots 16, 28 and 37); peroxiredoxin-2F (PRXIIF, spot 55); Mn-SOD (spot 50); thioredoxin H-type (trx-H, spot 3) and ferritin-3 (spot 1). Among them, Mn-SOD (spot 50) levels increased during ripening (Table 2b). High levels of Mn-SOD activity were reported to be associated with the green to red transition of pepper fruit.[44] Levels of Cu/Zn-SOD (spots 16, 28 and 37) and peroxiredoxin-2F (PRXIIF, spot 55) decreased more than two-fold during the later stages of fruit senescence (at H30–H50) (Table 2b). Although SOD activity has been reported to increase during apple fruit senescence,[45] our results indicated that the total amount of SOD proteins decreased. Therefore, further research on SOD activity and abundance is warranted.

Trx-H levels, a disulfide oxidoreductase associated with oxidative stress response, were significantly elevated during the latter part of ripening and senescence (H20–H50) (Table 2b). The *trx-H* gene was also reported to be significantly upregulated in *Arabidopsis* during senescence.[46] Ferritin-3 abundance exhibited a pattern similar to respiration, increasing by 2.66-fold (relative to H0) at H20, and then gradually decreasing in later time periods (Table 2b). These results were similar to a study in peach fruit,[47] suggesting that the respiratory burst has a significant influence on the regulation of the antioxidant system in apple fruits.

L-ascorbate peroxidase (APX, spot 22), glutathione S-transferase (GST, spot 51) and lactoylglutathione lyase (LGL, spot 23), all of which are involved in the ascorbate-glutathione cycle, exhibited a tendency to increase during apple ripening and senescence (Table 2b). APX is considered to play the major role in scavenging ROS and protecting cells from various stresses.[48] In our study, APX was elevated 2.12-fold at H30 relative to levels at H0 and then subsequently decreased at later time points. This expression pattern was in good accordance with a report of abundance in tomato.[10] GST can play a significant role in scavenging cytotoxic

Table 2. The dynamic changes in apple fruits of different ripening stages

(a) Ripening stage (fruit color), respiration, firmness and soluble sugar content of fruits used in the proteomic analysis. Fruits were sampled prior to optimal harvest (H-10 and H-5), optimal harvest (H0) and different time points (H5–H50) after optimal harvest as fruit were stored at 25℃ and 90% relative humidity

No.	Quality and respiration index	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
1	Soluble solids content (%)										
2	Firmness (kg cm^{-2})										
3	Respiratory rate (CO_2 mg kg^{-1} h^{-1})										

Continued

Table 2. Continued

(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
Energy and metabolism												
Energy (gluconeogenesis, photosynthesis)												
Gluconeogenesis												
36	NADP-dependent malic enzyme (NADP-ME)		0.024	0.012	0	*	*	*	*			
40	Triosephosphate isomerase (TIM)		0.16	0.08	0	0.43	0.48	0.23	0.25			
Photosynthesis												
8	Oxygen-evolving enhancer protein 1 (OEE1)		0.086	0.043	0	2.98	0.45	0.32	0.15	0	0	
Metabolism (carbohydrate, amino acid, nucleotide and thiamine, lipid and secondary)												
Carbohydrate metabolism												
10	Probable 6-phosphogluconolactonase 2 (6PGL 2)		0.024	0.012	0	0.18	0	0				

Continued

Table 2. Continued

(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
19	Soluble inorganic pyrophosphatase (PPase)											
43	Putative uncharacterized protein											
35	Beta-galactosidase											
46	Beta-galactosidase 3											
Amino-acid metabolism												
20	*L*-asparaginase 1											

Continued

Table 2. Continued

(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
												
27	Cysteine synthase											
34	Probable sarcosine oxidase											
44	Isovaleryl-CoA dehydrogenase 2 (IVD 2)											
Nucleotide metabolism												
2	Adenine phosphoribosyltransferase 1 (APRT 1)											
6	UMP/CMP kinase (UMP/CMPK)											

Continued

Table 2. Continued

(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
7	Adenine phosphoribosyltransferase 1 (APRT 1)		0.06	0.03	0					2.64		2.64
Thiamine metabolism												
24	Thiazole biosynthetic enzyme (THI1)		0.08	0.04	0					2.96	2.02	3.02
Lipid metabolism												
25	Epoxide hydrolase 2 (EH 2)		0.08	0.04	0				0.38	0.35	0.35	0.44
Secondary metabolism												
45	Chavicol *O*-methyltransferase (CVOMT 1)		0.046	0.023	0		*	*	*	*	*	*
Stress response and defense												
1	Ferritin-3		0.2	0.1	0				2.66			

H-10 H-5 H0 H5 H10 H15 H20 H30 H40 H50

Continued

Table 2. Continued

(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
3	Thioredoxin H-type (Trx-H)			2.17					2.58	3.46	3.71	5.44
5	MLP-like protein 328								0.49	0.46		0.41
16	Superoxide dismutase [Cu–Zn]									0.49	0.43	
17	Glycine-rich RNA-binding protein (GRP1A)		3.83									
21	26.5-kDa heat shock protein						*	*`	*	*`	*	*`

Continued

Table 2. Continued

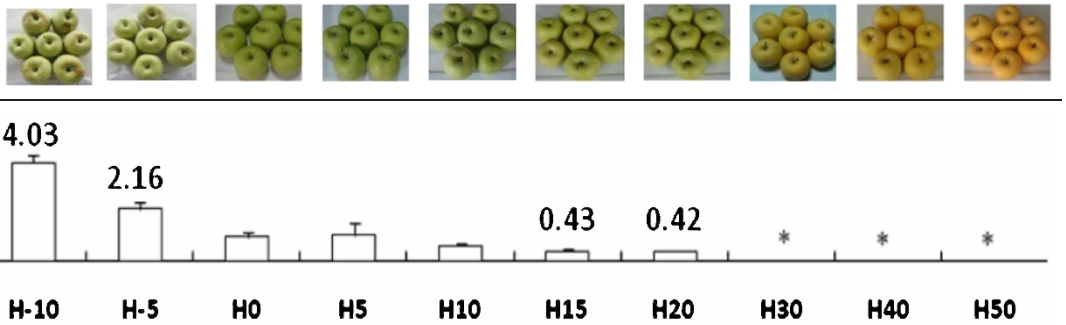
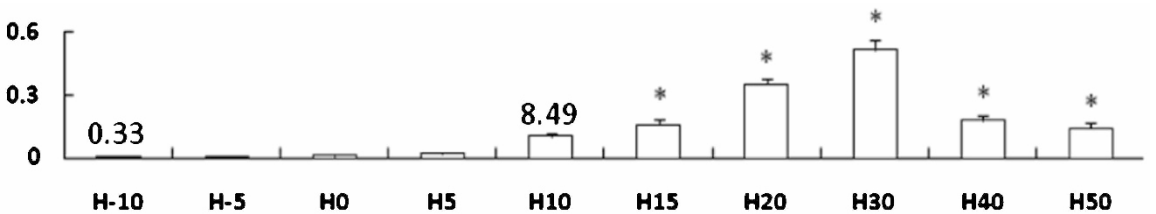
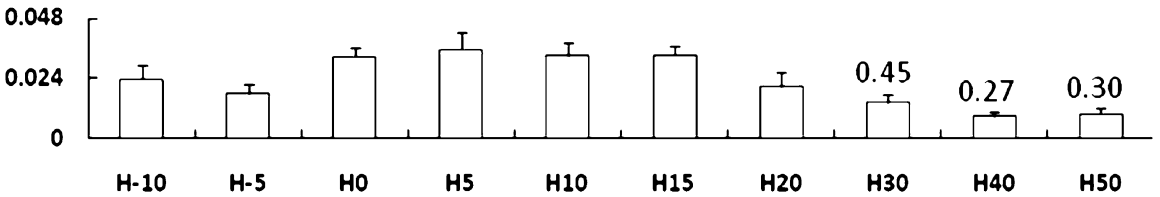
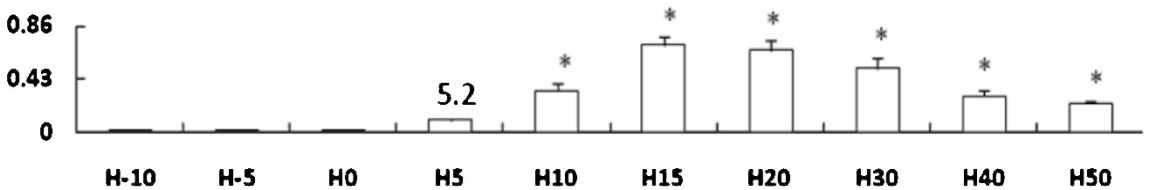
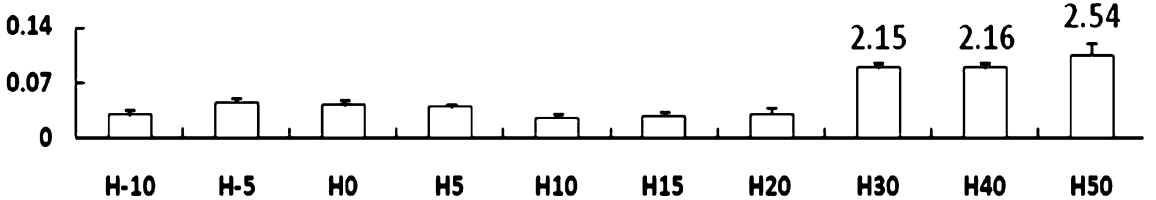
(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
22	*L*-ascorbate peroxidase 2		0.16	0.08	0					2.12		
23	Putative lactoylglutathione lyase		0.36	0.18	0	0.28	0.45					
28	Superoxide dismutase [Cu–Zn]		0.12	0.06	0				0.50	0.36	0.21	0.37
33	Abscisic acid stress ripening protein homolog		0.2	0.1	0					0.32	0.33	0.30
37	Superoxide dismutase [Cu–Zn] 1		0.12	0.06	0					0.49	0.45	0.38

Continued

Table 2. Continued

(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
39	17.8-kDa class I heat shock protein											
41	Abscisic acid response protein											
47	Outer membrane lipoprotein blc (temperature-induced lipocalin)											
49	Abscisic acid response protein											
50	Superoxide dismutase [Mn]											

Continued

Table 2. Continued

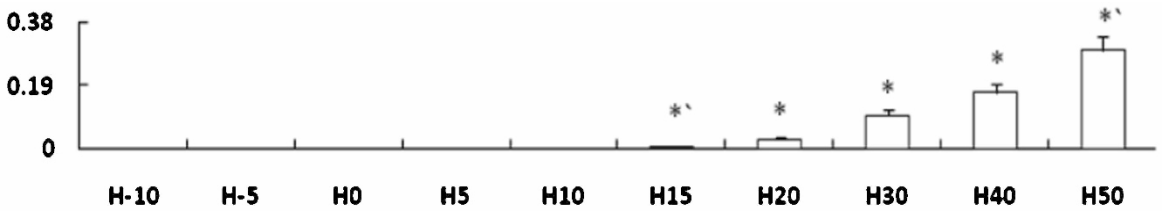
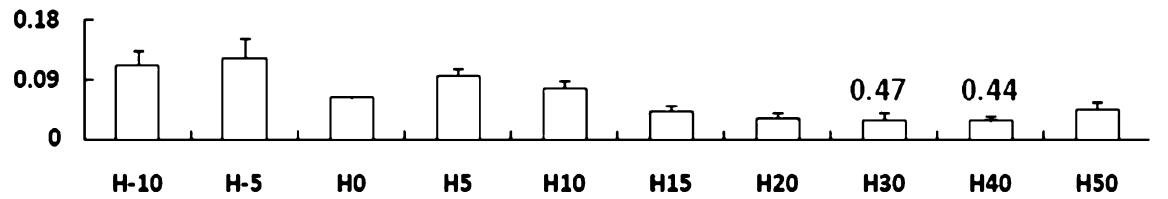
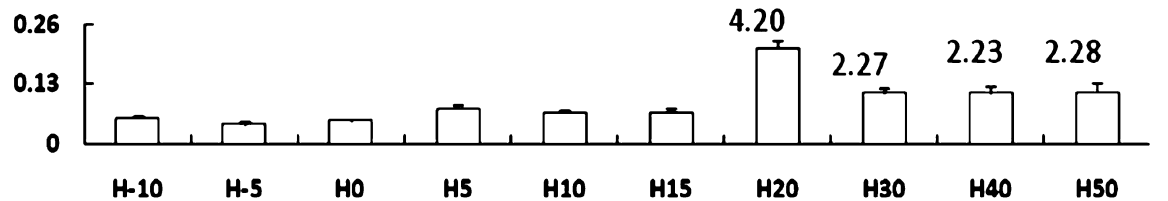
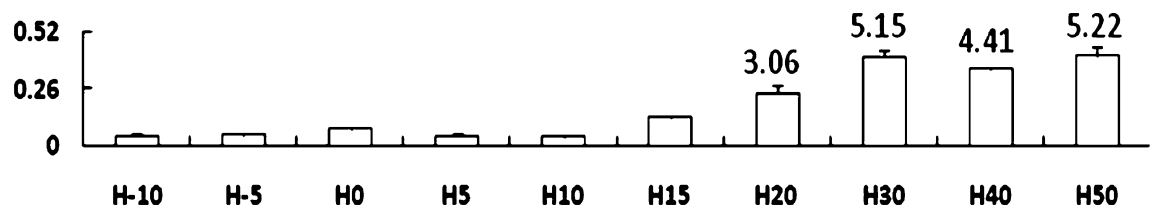
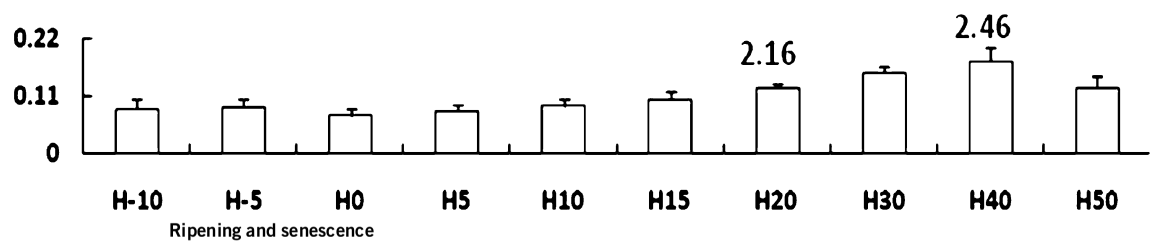
(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
51	Glutathione *S*-transferase						2.09	4.4	7.7	*	*	*
52	Abscisic acid response protein						*	*	*	*	*	*
53	Universal stress protein (USP) family protein							2.22	2.31	2.60	3.19	2.64
55	Peroxiredoxin-2F, mitochondrial (PRXIIF)								0.46	0.37	0.30	0.23
4	Major allergen Pru ar 1								*	*	*	*

Continued

Table 2. Continued

(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10 H-5 H0 H5 H10 H15 H20 H30 H40 H50
15	Major allergen Mal d 1		
18	MLP-like protein 329		
31	Major allergen Mal d 1		
38	Major allergen Mal d 1		
54	Thaumatin-like protein 1a		

Continued

Table 2. Continued

(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
12	1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1)		0.38 0.19 0			*	*	*	*	*	*	*
13	1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1)		0.24 0.12 0	0.31	0.27		3.69	*	4.65	3.15	3.32	2.91
14	1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1)		0.12 0.06 0					7.57	5.45	5.52	3.83	3.20
Signal transduction												
9	14-3-3 protein 7		0.1 0.05 0						2.56	2.99	3.16	3.37
48	GTP-binding nuclear protein Ran-3		0.34 0.17 0				2.21	3.52	5.08	4.6	3.5	4.2

H-10 H-5 H0 H5 H10 H15 H20 H30 H40 H50

Continued

Table 2. Continued

(b) Changes in protein levels, as a percentage of the total volume of each protein across all samples, of 53 differentially expressed apple fruit proteins. Numbers above the bars indicate fold-change relative to the level of expression at H0

Spot no.	Protein name	Spots % volume variations[a] ($p<0.05$)	H-10	H-5	H0	H5	H10	H15	H20	H30	H40	H50
Cell structure												
11	Pollen-specific leucine-rich repeat extensin-like protein 3 (AtPEX3/Pollen-specific LRR)										0.48	0.41
30	Actin-depolymerizing factor 2 (ADF 2)									0.37	0.15	0.19
Protein synthesis												
29	Eukaryotic translation initiation factor 5A-2							0.50	0.49	0.48	0.27	0.32
32	Proteasome subunit beta type-1		2.83	2.56					8.62	*	*`	*

[a] *x* axis denotes the ripening stages and *y* axis denotes the relative levels of protein abundance (normalized volume of spots); values are expressed as the mean of three replications±standard deviation.

* Differentially expressed fold (compared to H0) is greater than 10-fold.

and genotoxic compounds.[48] The abundance of GST, relative to H0, was significantly elevated 4.39-, 7.73-, 19.72-, 18.4- and 26.57-fold at H15, 20, 30, 40 and 50, respectively (Table 2b). This pattern of accumulation was similar to reports on mango mesocarp[19] and peach[49] at the proteomic level, and pear fruit[50] at the transcriptomic level.

In our study, the majority of anti-oxidative enzymes began to decline at or after H20, indicating that the climacteric rise in the respiration rate may significantly contribute to increased levels of oxidative stress. Other studies have also indicated that the ability of ROS scavenging compounds appears to decrease during the latter stages of apple ripening and as a result, may play a major role in inducing senescence.[48,51] Based on the data obtained in the present study, SOD may have a great effect on the oxidative-redox system in apple during ripening and senescence.

Several low-molecular-weight heat shock proteins were differentially expressed during apple ripening. A 26.5-kDa mitochondrial heat shock protein (spot 21) appeared at H10 and increased during subsequent time points, and the level of a 17.8-kDa class I cytoplasmic heat shock protein (spot 39) was initially high at pre-harvest (H-10), slowly decreased during subsequent time periods and disappeared at H30 (Table 2b). In general, heat shock proteins act as molecular chaperones and are induced by heat shock and other environmental and developmental conditions in fruit, where they are believed to a play a protective role against biotic and abiotic stress.[52] A small heat shock protein 21 expressed in transgenic tomato protects photosynthesis II from oxidative stress during fruit maturation.[53] These results suggested that heat-shock proteins may play an important role in protecting fruit from oxidative stress during ripening and senescence.

Apples, and other species in the *Rosaceae* family, often contain compounds that are allergenic to humans. The allergic reaction presents itself as IgE-mediated symptoms occurring mainly at the mucosa of lips, tongue and throat after ingestion of apples and other *Rosaceous* fruits. In apple, the major apple allergen is the Bet v 1 homolog protein Mal d 1.[54] In the present study, several allergens exhibited a strong increase in expression during the process of fruit ripening and senescence (Table 2b). These include: major allergen Mal d 1 (spots 15, 31 and 38), major allergen Pru ar 1 (spot 4) thaumatin-like protein 1a (spot 54) and MLP-like protein 329 (spot 18). Previous studies have indicated that both Mal d 1 and thaumatin-like protein in apple fruit significantly increase during storage.[55,56] Our results confirm these reports. The level for all of the allergenic proteins in apple identified in the current study significantly increased during fruit ripening, indicating that apple allergens may be ripening-induced proteins. Additional information regarding the regulation of apple allergen gene expression by ethylene and the ethylene inhibitor, 1-MCP, could assist in developing approaches to more effectively regulate fruit ripening.[57]

Abscisic acid stress ripening protein homolog (ASR, spot 33), a low-molecular-weight protein with strong hydrophillicity, is associated with plant developmental processes. ASR has been demonstrated to reduce oxidative stress induced by H_2O_2 and effectively act as an ROS scavenger.[58] In the current study, the pattern of ASR expression was similar to a previous report in peach where it increased prior to the climacteric burst and then decreased,[59] allowing for a large accumulation of ROS after the climacteric. An abscisic acid response protein (spots 41, 49 and 52), which responds to the stress-related hormone, ABA, increases more than 10-fold (relative to H0) during ripening (Table 2b). Very little information is available pertaining to the role of this protein in fruit development and as a result, further research is warranted.

Proteins related to other metabolic processes

Amino acids are building blocks for proteins and can be converted into other substrates such as glucose, fatty acids, purines and pyrimidines. Both sarcosine oxidase (spot 34) and isovaleryl-CoA dehydrogenase 2 (IVD 2, spot 44) are flavoproteins. They are involved not only in amino-acid synthesis and degradation, but also in the oxidation reduction and electron transport chain of flavin adenine dinucletide and ubiquinone.[60,61] *L*-asparaginase (spot 20) and cysteine synthase (spot 27) play a pivotal role in nitrogen metabolism.[62,63] All four of these proteins increased during fruit ripening (Table 2b), indicating that they may play a significant role in protein metabolism. Two of the proteins identified in the current study, eukaryotic translation initiation factor 5A-2 (eIf-5A2, spot 29) and proteasome subunit beta type-1 (spot 32), exhibited distinct patterns of differential expression (Table 2b). The eukaryotic translation initiation factor 5A-2 gradually increased from H-10–H10 and then decreased in H15–H50, while the proteasome subunit beta type-1 protein was very low in H-10–H15 and then significantly increased from H20 to H50.

Differentially expressed proteins identified in the current study related to other metabolic processes included: thiazole biosynthetic enzyme (THI1, spot 24); epoxide hydrolase 2 (EH, spot 25) and chavicol O-methyltransferase (CVOMT1, spot 45). CVOMT1 appeared at H10 and increased thereafter, while THI1 was present throughout the time course, but gradually increased from H15 to H50 (Table 2b). In contrast, EH gradually decreased from H0 to H50 (Table 2b). EH and CVOMT1 both affect aromatic compound metabolism[64,65] and CVOMT1 may be involved in ethylene biosynthesis.[65] The 14-3-3 protein (spot 9), a key regulator of signal transduction proteins such as receptors and protein kinases, was strongly upregulated strongly during ripening, indicating that signal transduction was enhanced following the climacteric.

Traditionally, factors such as fruit taste, firmness, color and aroma, serve as indices of fruit ripeness and quality. The proteomic profiling conducted in the present study provides an opportunity for better understanding the metabolic processes that are involved in apple fruit ripening and their regulation. Future studies should focus on specific proteins identified in this study, and their coding genes, in order to determine a clear linkage to the biological and physiological processes that occur during fruit ripening. Proteomic information when combined with genomic, transcriptomic and metabolomic data could provide a comprehensive knowledge of apple fruit ripening and lead to the development of new approaches, which aim to improve fruit quality during prolonged periods of storage.

CONCLUSION

Proteomic profiling of apple fruit (cv. 'Golden delicious') ripening and senescence was performed in order to identify specific proteins and changes in their abundance, which were associated with physiological and quality changes of apple. A total of 53 proteins were confidently identified from the >400 protein spots obtained. The majority of the identified proteins were related to stress response and defense, energy and metabolism, cell wall metabolism and ethylene biosynthesis. Results confirmed that ethylene biosynthesis enzymes (ACOs) increased earlier than respiratory-related enzymes (NADP-ME) and indicated that the respiratory burst (climacteric) induced an imbalance between energy metabolism and ROS metabolism, as indicated by an increase in the abundance of oxidation-related proteins and a decrease in the level of anti-oxidative proteins, and accelerated apple fruit ripening.

Data on the proteomic changes that occur during the ripening and senescence of apple fruits provide evidence for the role of specific proteins in the physiology and quality of apple fruit. These results could help us to better understand the processes involved in the ripening and senescence of apple fruits.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This research was financially supported by the Priority Academic Program Development of Jiangsu Higher Education Institution.

REFERENCES

1 Park S, Sugimoto N, Larson MD, Beaudry R, van Nocker S. Identification of genes with potential roles in apple fruit development and biochemistry through large-scale statistical analysis of expressed sequence tags. *Plant Physiol* 2006; **141**: 811–824.
2 Dandekar AM, Teo G, Defilippi BG *et al*. Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit. *Transgenic Res* 2004; **13**: 373–384.
3 Song J, Bangerth F. The effect of harvest date on aroma compound production from 'Golden Delicious' apple fruit and relationship to respiration and ethylene production. *Postharvest Biol Technol* 1996; **8**: 259–269.
4 Lee YP, Yu GH, Seo YS *et al*. Microarray analysis of apple gene expression engaged in early fruit development. *Plant Cell Rep* 2007; **26**: 917–926.
5 Palma JM, Corpas FJ, del Río LA. Proteomics as an approach to the understanding of the molecular physiology of fruit development and ripening. *J Proteomics* 2011; **74**: 1230–1243.
6 Newcomb RD, Crowhurst RN, Gleave AP *et al*. Analyses of expressed sequence tags from apple. *Plant Physiol* 2006; **141**: 147–166.
7 Janssen B, Thodey K, Schaffer R *et al*. Global gene expression analysis of apple fruit development from the floral bud to ripe fruit. *BMC Plant Biol* 2008; **8**: 16.
8 Li T, Tan D, Yang X, Wang A. Exploring the apple genome reveals six ACC synthase genes expressed during fruit ripening. *Sci Hort* 2013; **157**: 119–123.
9 Faurobert M, Mihr C, Bertin N *et al*. Major proteome variations associated with cherry tomato pericarp development and ripening. *Plant Physiol* 2007; **143**: 1327–1346.
10 Rocco M, D'Ambrosio C, Arena S, Faurobert M, Scaloni A, Marra M. Proteomic analysis of tomato fruits from two ecotypes during ripening. *Proteomics* 2006; **6**: 3781–3791.
11 Bianco L, Lopez L, Scalone AG *et al*. Strawberry proteome characterization and its regulation during fruit ripening and in different genotypes. *J Proteomics* 2009; **72**: 586–607.
12 Aragüez I, Cruz-Rus E, ÁngelBotella M, Medina-Escobar N, Valpuesta V. Proteomic analysis of strawberry achenes reveals active synthesis and recycling of *L*-ascorbic acid. *J Proteomics* 2013; **83**: 160–179.
13 Giribaldi M, Perugini I, Sauvage FX, Schubert A. Analysis of protein changes during grape berry ripening by 2-DE and MALDI-TOF. *Proteomics* 2007; **7**: 3154–3170.
14 Zhang J, Ma H, Feng J, Zeng L, Wang Z, Chen S. Grape berry plasma membrane proteome analysis and its differential expression during ripening. *J Exp Botany* 2008; **59**: 2979–2990.
15 Zhang L, Yu Z, Jiang L, Jiang J, Luo H, Fu L. Effect of post-harvest heat treatment on proteome change of peach fruit during ripening. *J Proteomics* 2011; **74**: 1135–1149.
16 Liu J, Sui Y, Wisniewski M *et al*. Effect of heat treatment on inhibition of *Monilinia fructicola* and induction of disease resistance in peach fruit. *Postharvest Biol Technol* 2012; **65**: 61–68.
17 Muccilli V, Licciardello C, Fontanini D *et al*. Proteome analysis of *Citrus sinensis* L. (Osbeck) flesh at ripening time. *J Proteomics* 2009; **73**: 134–152.
18 Nogueira SB, Labate CA, Gozzo FC, Pilau EJ, Lajolo FM, Oliveira do Nascimento JR. Proteomic analysis of papaya fruit ripening using 2DE-DIGE. *J Proteomics* 2012; **75**: 1428–1439.
19 Andrade JdM, Toledo TT, Nogueira SB, Cordenunsi BR, Lajolo FM, do Nascimento JRO. 2D-DIGE analysis of mango (*Mangifera indica* L.) fruit reveals major proteomic changes associated with ripening. *J Proteomics* 2012; **75**: 3331–33341.
20 D'Ambrosio C, Arena S, Rocco M *et al*. Proteomic analysis of apricot fruit during ripening. *J Proteomics* 2012; **78**: 37–57.
21 Qin G, Wang Q, Liu J, Li B, Tian S. Proteomic analysis of changes in mitochondrial protein expression during fruit senescence. *Proteomics* 2009; **9**: 4241–4253.
22 Zheng Q, Song J, Campbell-Palmer L *et al*. A proteomic investigation of apple fruit during ripening and in response to ethylene treatment. *J Proteomics* 2013; **93**: 276–294.
23 Wang W, Scali M, Vignani R *et al*. Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. *Electrophoresis* 2003; **24**: 2369–2375.
24 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–254.
25 Barraclough D, Obenland D, Laing W, Carroll T. A general method for two-dimensional protein electrophoresis of fruit samples. *Postharvest Biol Technol* 2004; **32**: 175–181.
26 Bobelyn E, Serban AS, Nicu M, Lammertyn J, Nicolai BM, Saeys W. Postharvest quality of apple predicted by NIR-spectroscopy: study of the effect of biological variability on spectra and model performance. *Postharvest Biol Technol* 2010; **55**: 133–143.
27 Bolton MD. Primary metabolism and plant defense-fuel for the fire. *Mol Plant Microbe Interact* 2009; **22**: 487–497.
28 Goodenough P, Prosser I, Young K. NADP-linked malic enzyme and malate metabolism in ageing tomato fruit. *Phytochemistry* 1985; **24**: 1157–1162.
29 Ito H, Iwabuchi M, Ogawa Ki. The sugar-metabolic enzymes aldolase and triose-phosphate isomerase are targets of glutathionylation in *Arabidopsis thaliana*: detection using biotinylated glutathione. *Plant Cell Physiol* 2003; **44**: 655–660.
30 Adyanthaya I, Kwon YI, Apostolidis E, Shetty K. Apple postharvest preservation is linked to phenolic content and superoxide dismutase activity. *J Food Biochem* 2009; **33**: 535–556.
31 León AM, Palma JM, Corpas FJ *et al*. Antioxidative enzymes in cultivars of pepper plants with different sensitivity to cadmium. *Plant Physiol Biochem* 2002; **40**: 813–820.
32 Sonnewald U. Expression of *E. coli* inorganic pyrophosphatase in transgenic plants alters photoassimilate partitioning. *Plant J* 1992; **2**: 571–581.
33 Ross GS, Wegrzyn T, MacRae EA, Redgwell RJ. Apple β-galactosidase (activity against cell wall polysaccharides and characterization of a related cDNA clone). *Plant Physiol* 1994; **106**: 521–528.
34 Wallner SJ, Walker JE. Glycosidases in cell wall-degrading extracts of ripening tomato fruits. *Plant Physiol* 1975; **55**: 94–98.
35 Bonghi C, Pagni S, Vidrih R, Ramina A, Tonutti P. Cell wall hydrolases and amylase in kiwifruit softening. *Postharvest Biol Technol* 1996; **9**: 19–29.
36 Brummell DA, Harpster MH. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. In: *Plant Cell Walls*. Dordrecht: Springer; 2001. p311–340.
37 Clément M, Ketelaar T, Rodiuc N *et al*. Actin-depolymerizing factor2-mediated actin dynamics are essential for root-knot nematode infection of *Arabidopsis*. *Plant Cell Online* 2009; **21**: 2963–2979.
38 Alexander L, Grierson D. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *J Exp Botany* 2002; **53**: 2039–2055.
39 Pech JC, Bouzayen M, Latché A. Climacteric fruit ripening: ethylene-dependent and independent regulation of ripening pathways in melon fruit. *Plant Sci* 2008; **175**: 114–120.
40 Ruperti B, Bonghi C, Rasori A, Ramina A, Tonutti P. Characterization and expression of two members of the Peach 1-aminocyclopropane-1-carboxylate oxidase gene family. *Physiol Plant* 2001; **111**: 336–344.
41 Shaw JF, Chou YS, Chang RC, Yang SF. Characterization of the ferrous ion binding sites of apple 1-aminocyclopropane-1-carboxylate oxidase by site-directed mutagenesis. *Biochem Biophys Res Commun* 1996; **225**: 697–700.
42 Tonutti P, Bonghi C, Ruperti B, Tornielli GB, Ramina A. Ethylene evolution and 1-aminocyclopropane-1-carboxylate oxidase gene expression during early development and ripening of peach fruit. *J Am Soc Hort Sci* 1997; **122**: 642–647.
43 Peroni LA, Ferreira RR, Figueira A, Machado MA, Stach-Machado DR. Expression profile of oxidative and antioxidative stress enzymes based on ESTs approach of citrus. *Genet Mol Biol* 2007; **30**: 872–880.
44 Jiménez A, Gómez JM, Navarro E, Sevilla F. Changes in the antioxidative systems in mitochondria during ripening of pepper fruits. *Plant Physiol Biochem* 2002; **40**: 515–520.
45 Du Z, Bramlage WJ. Superoxide dismutase activities in senescing apple fruit (*Malus domestica* Borkh.). *J Food Sci* 1994; **59**: 581–584.
46 Laloi C, Mestres-Ortega D, Marco Y, Meyer Y, Reichheld JP. The *Arabidopsis* cytosolic thioredoxin h5 gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor. *Plant Physiol* 2004; **134**: 1006–1016.
47 Hu H, Liu Y, Shi GL *et al*. Proteomic analysis of peach endocarp and mesocarp during early fruit development. *Physiol Plant* 2011; **142**: 390–406.
48 Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 2010; **48**: 909–930.
49 Nilo P R, Campos-Vargas R, Orellana A. Assessment of *Prunus persica* fruit softening using a proteomics approach. *J Proteomics* 2012; **75**: 1618–1638.
50 Nishitani C, Shimizu T, Fujii H *et al*. Oligoarray analysis of gene expression in ripening Japanese pear fruit. *Sci Hort* 2010; **124**: 195–203.
51 Kan J, Wang HM, Jin CH. Changes of reactive oxygen species and related enzymes in mitochondrial respiration during storage of harvested peach fruits. *Agric Sci China* 2011; **10**: 149–158.
52 Aghdam MS, Sevillano L, Flores FB, Bodbodak S. Heat shock proteins as biochemical markers for postharvest chilling stress in fruits and vegetables. *Sci Hort* 2013; **160**: 54–64.
53 Neta-Sharir I, Isaacson T, Lurie S, Weiss D. Dual role for tomato heat shock protein 21: protecting photosystem II from oxidative stress and promoting color changes during fruit maturation. *Plant Cell Online* 2005; **17**: 1829–1838.

54 van Ree R, Fernández-Rivas M, Cuevas M, Aalberse RC. Pollen-related allergy to peach and apple: an important role for profilin. *J Allergy Clin Immunol* 1995; **95**: 726–734.
55 Matthes A, Schmitz-Eiberger M. Apple (*Malus domestica* L. Borkh.) allergen Mal d 1: effect of cultivar, cultivation system, and storage conditions. *J Agric Food Chem* 2009; **57**: 10548–10553.
56 Botton A, Lezzer P, Dorigoni A, Barcaccia G, Ruperti B, Ramina A. Genetic and environmental factors affecting allergen-related gene expression in apple fruit (*Malus domestica* L. Borkh). *J Agric Food Chem* 2008; **56**: 6707–6716.
57 Yang X, Song J, Campbell-Palmer L, Walker B, Zhang Z. Allergen related gene expression in apple fruit is differentially controlled by ethylene during ripening. *Postharvest Biol Technol* 2012; **63**: 40–49.
58 Kim IS, Kim YS, Yoon HS. Rice ASR1 protein with reactive oxygen species scavenging and chaperone-like activities enhances acquired tolerance to abiotic stresses in *Saccharomyces cerevisiae*. *Mol Cells* 2012; **33**: 285–293.
59 Nilo R, Saffie C, Lilley K *et al*. Proteomic analysis of peach fruit mesocarp softening and chilling injury using difference gel electrophoresis (DIGE). *BMC Genomics* 2010; **11**: 43.
60 Trickey P, Wagner MA, Jorns MS, Mathews FS. Monomeric sarcosine oxidase: structure of a covalently flavinylated amine oxidizing enzyme. *Structure* 1999; **7**: 331–345.
61 Araújo WL, Ishizaki K, Nunes-Nesi A *et al*. Identification of the 2-hydroxyglutarate and isovaleryl-CoA dehydrogenases as alternative electron donors linking lysine catabolism to the electron transport chain of *Arabidopsis mitochondria*. *Plant Cell Online* 2010; **22**: 1549–1563.
62 Warrilow AG, Hawkesford MJ. Separation, subcellular location and influence of sulphur nutrition on isoforms of cysteine synthase in spinach. *J Exp Botany* 1998; **49**: 1625–1636.
63 Dickson JM, Vincze E, Grant MR *et al*. Molecular cloning of the gene encoding developing seed *L*-asparaginase from Lupinus angustifolius. *Plant Mol Biol* 1992; **20**: 333–336.
64 Schöttler M, Boland W. Biosynthesis of dodecano-4-lactone in ripening fruits: crucial role of an epoxide-hydrolase in enantioselective generation of aroma components of the nectarine (*Prunus persica* var. nucipersica) and the strawberry (*Fragaria ananassa*). *Helv Chim Acta* 1996; **79**: 1488–1496.
65 Gang DR, Lavid N, Zubieta C *et al*. Characterization of phenylpropene *O*-methyltransferases from sweet basil facile change of substrate specificity and convergent evolution within a plant *O*-methyltransferase family. *Plant Cell Online* 2002; **14**: 505–519.