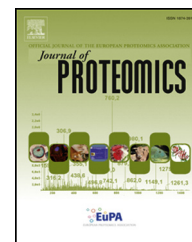


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A proteomic investigation of apple fruit during ripening and in response to ethylene treatment☆☆☆

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ABSTRACT

A proteomic approach employing a two dimensional electrophoresis (2-DE) technique with SYPRO Ruby, a fluorescent stain with improved sensitivity and quantitative accuracy, was performed to separate the total proteins from apple fruit at different stages of ripening and senescence. After imaging and statistical analyses were performed on 2340 spots, a total of 316 spots, or approximately 13.5% of the total protein population, was found to be significantly changed in this study. Of the 316 proteins, 219 spots were only present at a specific ripening stage, while 97 spots were significantly different ($p < 0.05$) throughout fruit ripening and in response to ethylene treatment. From 316 candidate spots, 221 proteins were further identified by liquid chromatography and mass spectrometry analysis with protein sequence and express sequence tag (EST) data searching. Analysis and identification of proteins revealed that apple fruit ripening is associated with increase of abundance of many proteins with functions such as ethylene production, antioxidation and redox, carbohydrate metabolism, oxidative stress, energy, and defense response. Ethylene treatment increased a group of unique proteins that were not present during normal fruit ripening and have not been previously reported. It also reduced some proteins involved in primary metabolism, including those of the last few steps of the glycolytic pathway. This study demonstrated the complexity and dynamic changes of protein profiles of apple fruit during ripening and in response to exogenous ethylene treatment. Identifying and tracking protein changes may allow us to better understand the mechanism of ripening in climacteric fruit.

Biological significance

Postharvest physiology and biochemistry has been conducted on apple fruit for many years. Ethylene plays an important role in ripening and senescence in many climacteric fruit. However, little information is available at the proteome level to investigate fruit ripening and effect of ethylene treatment. The significance of this paper is that it is the first study employing 2-DE and fluorescent dye in the investigation of the apple fruit ripening and influence of ethylene treatment. It reveals some significant biological changes in association with these events and demonstrates significant changed proteins under these conditions. Therefore, our study links the biological events with proteomic information and provides

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detailed peptide information on all identified proteins. Through the function analysis, those significantly changed proteins are also analyzed. These findings from this paper provide not only proteome information on fruit ripening, but also pave the ground for further quantitative studies using SMR to investigate certain proteins and pathways under the hypothesis involved in fruit ripening.

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1. Introduction

Apples (*Malus domestica* Borkh.) are one of the most popular fruit crops in the world, with a total production of ca. 66 million metric tonnes worldwide. In North America, the apple industry produced ca. 4.2 million tonnes of fruit in 2010. Apples have become part of our daily diet due to their nutritional value. The annual per capita consumption of apples was an estimated 7.2 kg [1]. Expanding the high-quality fruit market is key to meeting consumer demand and maintaining the apple industry. The quality of fresh fruit is measured using appearance, color, texture, flavor (taste and aroma) and nutritional value [2]. Apples are climacteric fruit that demonstrate a burst in respiration and ethylene production in association with fruit ripening after harvest, resulting in yellowing, decreased firmness, reduced acidity and formation of volatile compounds [3,4]. Developed technologies such as controlled atmosphere storage (CA) and ultra-low oxygen storage (ULO) maintain color and firmness, but are detrimental to flavor development [5]. Treatments that inhibit ripening by inhibiting ethylene biosynthesis (e.g. aminovinylglycine, AVG) [6] or action (e.g. 1-methylcyclopropene, 1-MCP) delay fruit ripening and senescence [7]. Following these treatments, fruit firmness can be maintained and volatile production partially recovered during storage, but they cannot reach the optimal levels seen in normal ripe fruit [8,9]. These results demonstrate that fruit ripening directly influences quality, and ethylene plays a very important role in the ripening process.

Molecular biological tools have been developed and used to study general plant metabolism [10]. These technologies have been applied to apple fruit to investigate and gain better understanding of ripening and senescence. Recently, molecular and genomic tools have been used to identify increasing numbers of genes involved in apple fruit ripening and senescence in relation to fruit firmness/texture [11], aroma volatile compounds [12–14], acidity [15], pigments [16], allergens [17] and other biological functions. In addition to the above identified genes, ethylene-related genes have been studied the most [14,18–21].

In comparison with other plant species, research on apple ripening has been hampered by the lack of appropriate genomic tools. Although the genome of apples has been sequenced [22], a recent public sequence databank that contains in excess of 325,020 ESTs for apple allows the identification of approximately 22,493 unigenes (NCBI: <http://www.ncbi.nlm.nih.gov/>), and offers new opportunities to identify unknown genes and proteins.

The analysis of mRNA and gene expression may not predict the exact protein concentration and activity. Therefore, proteomics has become a key tool in system biology to provide quantitative and structural information about functional

proteins and the dynamic state of the cell [23,24]. Proteomics is a systematic approach to study global changes in proteins and provides a linkage between the transcriptome and metabolome [25]. It reveals different protein expression patterns under different biological situations, such as development, stress, and postharvest treatments, and provides direct information on the proteins driving cell metabolism. Among proteomic techniques, two-dimensional electrophoresis (2-DE), a gel based technique, has been applied to resolve thousands of proteins simultaneously in order to facilitate peptide composition analysis, peptide sequencing and polypeptide identification using mass spectrometry (MS) [26]. This 2-DE-based proteomic technique has allowed the investigation of biochemical processes at the gene and protein levels; while the technique has mainly been applied to *Arabidopsis* and rice [23], it has also been used to identify proteins present in apple fruit samples with reasonable confidence [27,28]. Reports on strawberry, tomato and grapes have shown that the proteomic approach has potential to identify proteins involved in fruit ripening, the development of physiological disorders, and allergens [29–33]. These studies demonstrated that proteomics is a powerful tool that can be used to reveal the complicated protein population in specific biological samples, and the potential proteins involved in certain biological processes. Despite the current interest and application of the proteomic technique, very little data are available at the transcriptomic and proteomic levels for ripening and senescence in apple fruit.

In this study, a 2-DE based proteomic approach was applied to investigate changes in protein profiles at different stages of apple fruit ripening, and after treatment with exogenous ethylene. The objectives of this study were 1) to investigate the changes in protein profiles in association with fruit ripening stages and identify the most variable proteins during ripening and senescence; 2) to reveal the unique proteins that are affected by ethylene treatment; and 3) to establish the links between the physiological changes and the changes in protein profiles.

2. Materials and methods

2.1. Plant materials

Apples (*M. domestica* Borkh. cv. ‘Golden Delicious’) were harvested in 2005 on Oct. 04th from the Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, Kentville, Nova Scotia, Canada at the pre-climacteric stages, as determined by internal ethylene concentration and starch index. The internal ethylene concentration was $<0.1 \mu\text{L L}^{-1}$ for immature fruit, and was determined on 14 apples. A total of 120 fruit were harvested equally from 10 trees.

2.2. Exogenous ethylene treatment

Harvested apple fruit were divided into two groups. One group was treated with ethylene gas mixed with filtered air at $36 \mu\text{L L}^{-1}$ for 36 h at a flow rate of $0.41\text{--}0.5 \text{ mL s}^{-1}$, while the second group was treated with fresh filtered air at the same flow rate. After treatment, the fruit were stored in 4 L glass jars with filtered fresh air at the same flow rate at 20°C .

2.3. Biological measurements

2.3.1. Fruit respiration and ethylene production

One mL samples of internal gas were drawn into a syringe through a cannula inserted into the core cavity of each fruit. Samples were then injected into a gas chromatograph with a flame ionization detector (Carle Instruments, Anaheim, CA) equipped with a $1.9 \text{ m} \times 3.2 \text{ mm}$ (o.d.) activated alumina column with a helium carrier flow of 0.83 mL s^{-1} . Internal ethylene concentration was quantified using a known concentration of standard ethylene. Respiration of individual fruit was measured with an infrared CO_2 analyzer (ModelLi-6252, Li-COR) connected to a semi-automatic sampling device with a flow rate of 0.41 mL s^{-1} . After respiration measurements, fruit were sealed in a 4 L glass jar for 2 h, and 1 mL samples of head space were analyzed for ethylene on a gas chromatograph with a flame ionization detector (Carle Instruments, Inc., Anaheim, Calif.) as described above. Five fruit from each treatment were used for quality measurements and sampling.

2.3.2. Fruit quality

Fruit firmness was determined on opposite sides of each fruit after peel removal using a drill stand-mounted penetrometer fitted with an 11.1 mm diameter probe (Fruit texture analyzer, Guess, South Africa). Data were recorded in Newtons (N). Soluble solids were determined with a handheld temperature compensated refractometer (Atago Co., Tokyo). Titratable acidity was measured with a pooled 2 mL juice sample collected from 5 apples, and 0.1 mol L^{-1} NaOH was used to titrate the apple juice with a semi-automatic titrator (Multi-Dosimat E-415 titrator; Metrohm AG, Switzerland) to a phenolphthalein endpoint of pH 8.1. Titratable acidity was expressed as malic acid equivalents.

Based on the physiological stages indicated in Fig 1, four stages were chosen to be included in this proteomic study and fruit samples were taken at day 0, day 7, day 13 and day 21 for the control, and day 7, day 13 and day 21 for ethylene treatment. These samples are marked in the Fig. 1A. Apple peel and flesh tissues from five individual fruit were obtained at each stage as in Fig. 1A, quickly frozen in liquid nitrogen and stored at -85°C . Frozen samples were pooled and ground to a fine powder in liquid nitrogen using a stainless steel blender followed by a mortar and pestle. Ground samples were stored at -85°C until used.

2.4. Protein extraction and quantification

Protein was extracted and purified from ground samples using a developed phenol extraction followed by ammonium acetate-methanol precipitation [34]. Protein concentration in all extracts

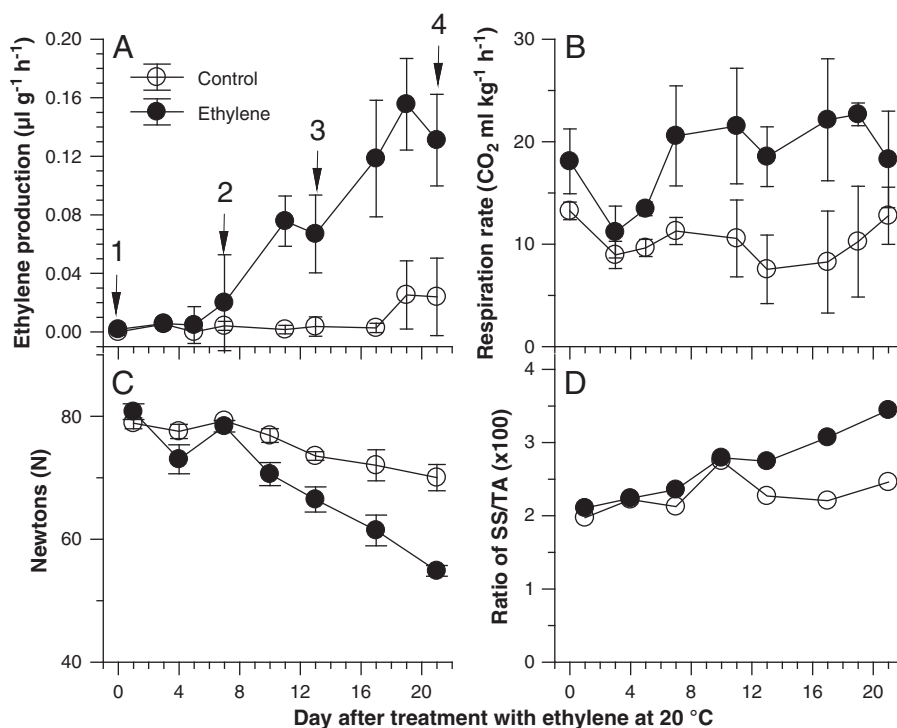


Fig. 1 – Postharvest physiological quality indices of apple fruit during ripening and after ethylene treatment. A: Ethylene production; B: respiration; C: firmness; and D: ratio of SS/TA. Arrows indicate the corresponding sampling stages. Seven stages were control (day 0), control (day 7), control (day 13), control (day 21), ethylene (day 7), ethylene (day 13) and ethylene (day 21).

was determined using the RC/DC™ protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) to remove interfering compounds, using Bovine serum albumin (BSA) as the standard for the calibration curve. Four replicated samples were collected on day 0, 7, 13 and 21 from both treatments.

2.5. 2-D electrophoresis

Extracted proteins were separated by isoelectrofocusing (IEF) on a Multiphor II system (GE Healthcare, Baie-d'Urfé, QC, Canada) using 18 cm Immobiline DryStrip gels (GE Healthcare, Baie-d'Urfé, QC, Canada) with non-linear pH gradients (pH 3–11 NL), according to the manufacturer's instructions. Protein (100.0 µg) and 10 µL of 2-D protein standard (Bio-Rad Laboratories, Hercules, CA, USA) were mixed with DeStreak Rehydration Solution (GE Healthcare, Baie-d'Urfé, QC, Canada) to a final volume of 340 µL which was loaded onto the DryStrip gel and incubated for a minimum of 10 h at room temperature. First dimension separation was conducted for 30,000 Vh at 20 °C (500 V for 1 h, 1000 V for 10 min, 2000 V for 10 min, then 3000 V for 9.66 h). After first dimension separation, strips were equilibrated with DTT equilibration buffer for 10 min, followed with equilibration in iodoacetamide buffer for 10 min at RT. Second-dimension separation was carried out on large format 12.5% acrylamide SDS-PAGE gels (24×21 cm) and run at 30 W for 30 min and 100 W for the remaining time at 15 °C on an Ettan DaltSIX multigel system (GE Healthcare, Baie-d'Urfé, QC, Canada). Resultant gels were fixed in a solution containing 50% methanol and 10% acetic acid for 30 min and then visualized by staining with fluorescent dye (SYPRO Ruby, Bio-Rad Laboratories, Hercules, CA, USA) at RT overnight. SYPRO Ruby stained gels were washed in a solution containing 10% methanol and 7% acetic acid for 30 min. Gels were scanned with a molecular imager system (PharosFX, Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Image analysis

Digital images of 2-DE gels were processed and analyzed using PDQuest 2-D Image Analysis Software (Version 8.01, Bio-Rad Laboratories, Hercules, CA, USA). Cropped images were processed with the following software settings for spot detection and background subtraction. To allow spot to spot comparisons across gels, a match set that included all gel images within the experiment was created. A standard gel (master) was generated from the image within the match set that contained the greatest number of spots without streaking or other gel distortions. The software's automatic matching tool was used to match spots across gels within match sets. All spots matched by the software program were manually verified as follows. A spot was considered to be reproducibly present/absent when it was present/

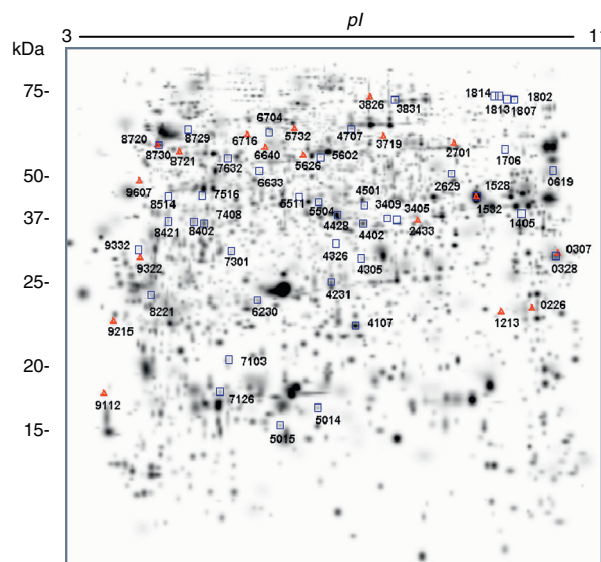


Fig. 2 – Representative 2-DE image of apple fruit proteins that changed significantly during fruit ripening and after ethylene treatment. One hundred µg of protein from fruit tissue was dissolved in DeStreak™ solution. After iso-electrofocusing, the proteins were further separated on SDS-PAGE (12.5%) polyacrylamide gels and visualized by SYPRO Ruby staining. From 97 total spots, 60 were identified by LC/MS/MS; corresponding spot numbers are listed in Table 1. Blue squares indicate increased proteins in Cluster 1. Red triangles indicate decreased proteins in Cluster 2.

absent in all four replicate gels of a given treatment. Spots that were reproducibly present in a match set member but not in the master gel were manually added to the master image. For all gels, spot quantities (total pixel intensity within spot boundaries, calculated by image analysis software for Gaussian spots) were normalized to remove variations in spot intensity caused by non-treatment effects. Normalized spot quantity was equal to raw spot quantity expressed as a percentage of the total pixel quantity of all spots in a gel. Six landmarks (2D SDS PAGE standard, Bio-Rad Laboratories, Hercules, CA, USA) were manually defined for accurate determination of Mr and pI.

2.7. MS analysis and protein identification

Selected spots were excised from 2-DE gels (ExQuest spot cutter, Bio-Rad Laboratories, Hercules, CA, USA) under ultraviolet light. Spots were reduced with DTT to break disulfide linkages,

Fig. 3 – Hierarchical clustering analysis of 97 protein spots ($p < 0.05$) in apple fruit that changed significantly during ripening and after ethylene treatment. The spot number is indicated on the right side of the plot. Two main clusters were formed: Cluster 1 shows the spots that were absent at day 0 and early ripening stages, and then increased during fruit ripening and in response to ethylene treatment; Cluster 2 reveals the spots which decreased during fruit ripening and after ethylene treatment. All data were transformed in $\log(x + 1)$ of the abundance. Increasing intensities of red or green color indicate differentially up- or down-regulated proteins compared with the control at day 0, respectively. Gray color indicates the absent proteins at the corresponding stages. Identified proteins are also marked on Fig. 2 and listed in Table 1.

alkylated with iodoacetamide, and then digested with trypsin (sequencing grade, Promega, www.promega.com). The resultant peptides were extracted in washes of ammonium

bicarbonate solution, acetonitrile, and 1.0% formic acid. Extraction solvent was removed under vacuum and the peptides were re-suspended in 30 μ L of 5% MeOH, 0.5% formic acid. HPLC was

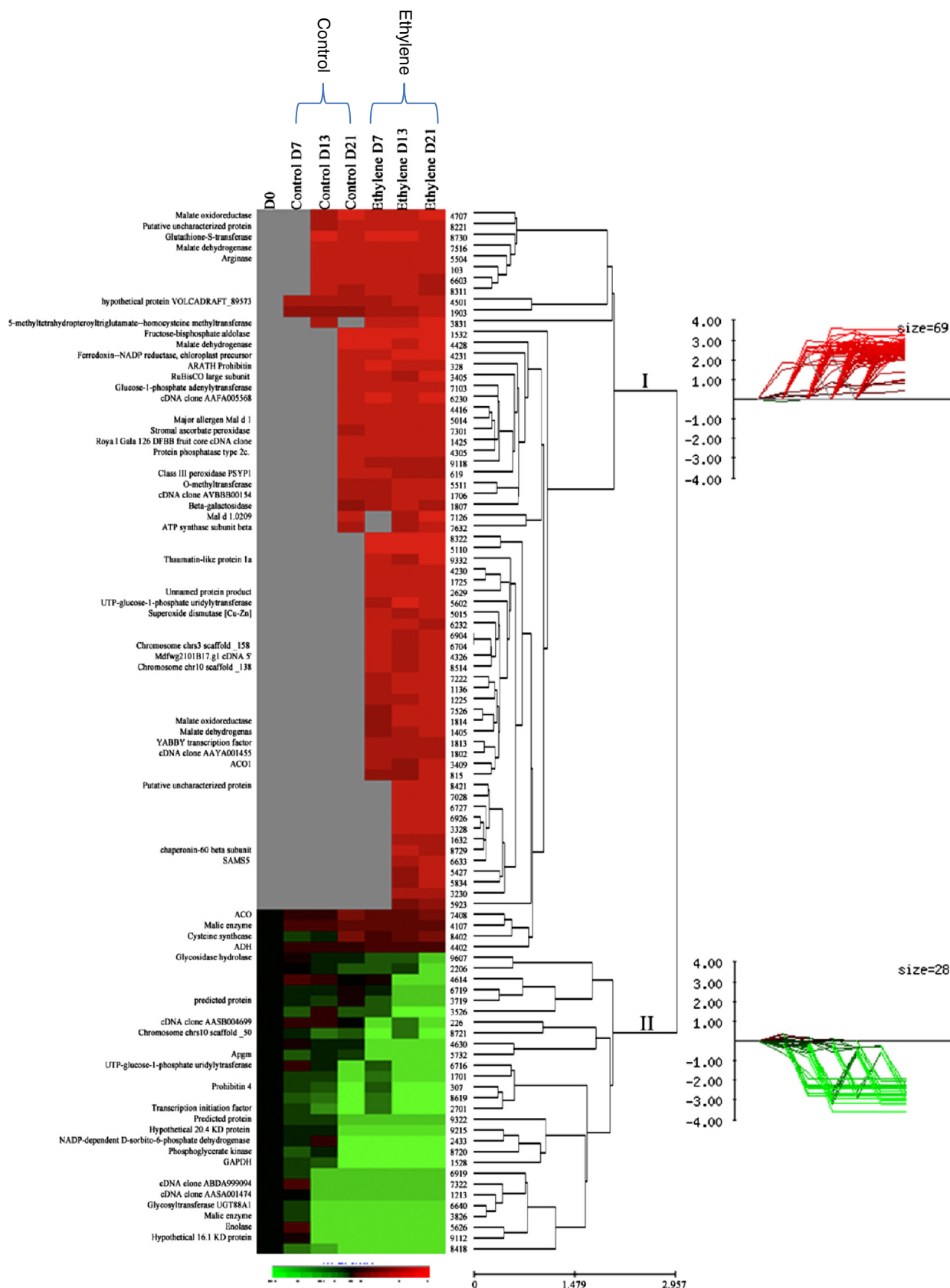
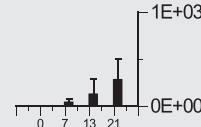
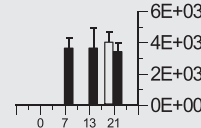
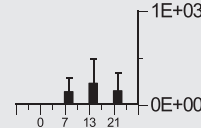
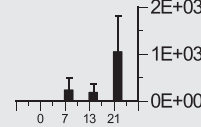
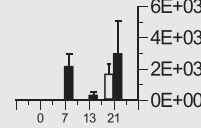
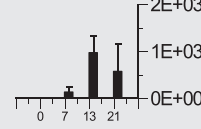
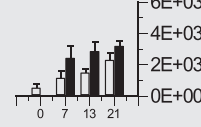
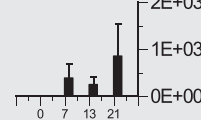
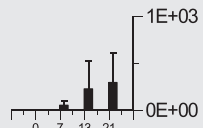
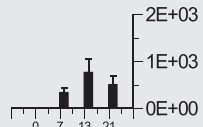
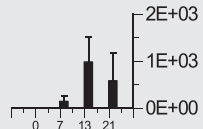
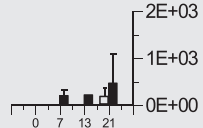
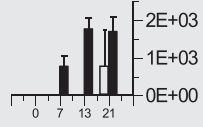
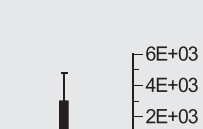
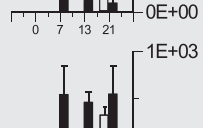
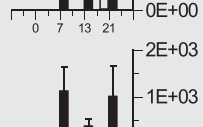


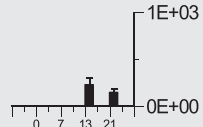
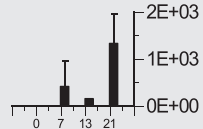
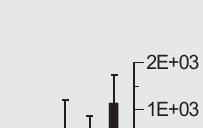
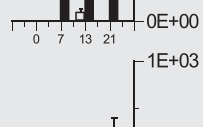
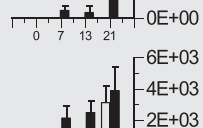
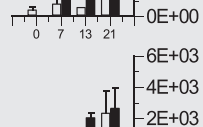
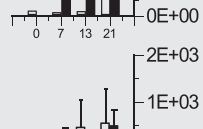
Table 1 – Proteins in apple tissue that changed significantly during ripening and in response to ethylene treatment. Protein spots excised from gels stained with SYPRO Ruby were digested with trypsin and identified following liquid chromatography and mass spectrometry analysis (LC/MS/MS).

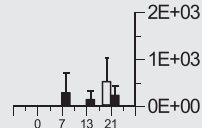
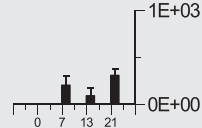
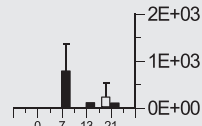
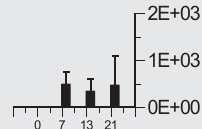
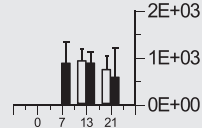
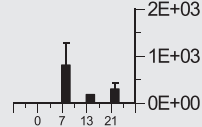
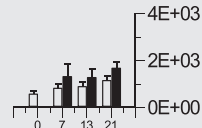
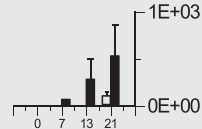
Spot	Protein name	Organism	Genbank/EST accession	Mascot score	Match% Cov. ^a	Mr. (kDa)		pI		Local ^d	Protein abundance ^e
						Exp. ^b	Ther. ^c	Exp. ^b	Ther. ^c		
Cluster no. 1 (increased abundance)											
Carbon and energy metabolism											
1405	Malate dehydrogenase	Vitis vinifera	A7QZG8	417	23	35.19	37.6	8.32	8.12	Cyto	
1532	Fructose-bisphosphate aldolase	Fragaria ananassa	Q9FUG7	301	20	39.79	38.51	7.11	6.93	Cyto	
1807	Beta-galactosidase	Pyrus communis	Q68UW0	127	3	76.13	80.41	7.93	5.89	Cyto	
3405	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Gaultheria shallon	gi 6561345	48	4	34.91	48.59	6.39	6.14	Chlo	
4428	Malate dehydrogenase	Vitis vinifera	A3DSX0	765	65	35.07	35.59	5.97	6.01	Cyto	
5602	UTP-glucose-1-phosphate uridylyltransferase	Pyrus pyrifolia	O64459	151	5	52.65	51.85	5.85	5.99	Cyto	
4107	Malic enzyme, putative	Ricinus communis	gi 255546341	108	5	65.4	22.65	5.98	6.0	Cyto	
4707	Malate oxidoreductase (malic enzyme)	Arabidopsis thaliana	gi 46600240	75	10	63.05	64.28	6.07	5.57	Cyto	

1814	Malate oxidoreductase (malic enzyme)	<i>Arabidopsis thaliana</i>	O82191	108	6	77.82	64.28	7.58	6.32	Cyto	
7103	Glucose-1-phosphate adenylyltransferase	<i>Vitis vinifera</i>	A7Q2V5	211	14	56.4	55.953	- ^f	8.44	Cyto	
7516	Malate dehydrogenase	<i>Malus domestica</i>	A3DSX0	369	21	40.26	35.59	5.0	6.01	Cyto	
7632	ATP synthase subunit beta	<i>Physocarpus opulifolius</i>	Q1PGD5	714	33	52.25	51.78	5.2	5.27	Chlo Plas Thyl	
4231	Ferredoxin-NADP reductase, chloroplast precursor (FNR) gb AAA21758.1 ferredoxin NADP+ reductase precursor	<i>Malus domestica</i>	gi 226749466	243	24	31.42	29.22	6.09	9.52	Chlo	
328	Allergens and defense ARATH Prohibitin	<i>Arabidopsis thaliana</i>	O04331	131	12	30.82	30.40	-	6.99	Mito Nucl PM VM	
5014	Major allergen Mal d 1	<i>Malus domestica</i>	Q40280	121	18	16.56	17.54	5.83	5.62	Cyto	
6230	Royal Gala apple skin peel, tree-ripened fruit 150 DAFB cDNA clone AAFA005568	<i>Malus domestica</i>	gi 91066390	106	16	26.85	12.28	5.41	6.84		
7126	Mal d 1.0209	<i>Malus domestica</i>	A0AUB1	80	8	26.47	17.52	4.98	5.46	Cyto	

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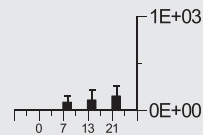
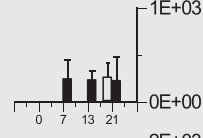
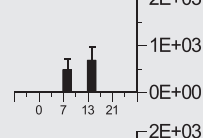
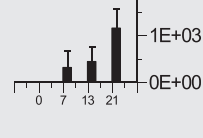
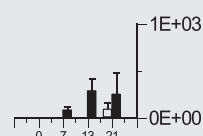
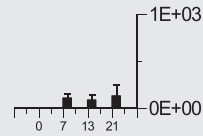
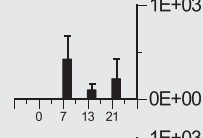
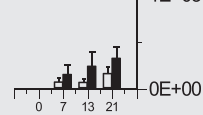
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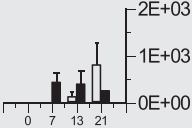
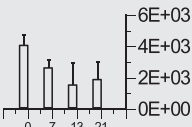
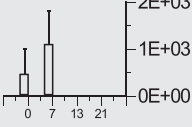
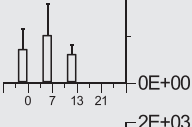
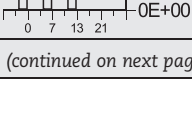
Spot	Protein name	Organism	Genbank/EST accession	Mascot score	Match% Cov. ^a	Mr. (kDa)		pI		Local ^d	Protein abundance ^e
						Exp. ^b	Ther. ^c	Exp. ^b	Ther. ^c		
8729	Chaperonin-60 beta subunit	<i>Solanum tuberosum</i>	gi 1762130	115	5	62.83	63.27	4.87	5.72	Cyto	
9332	Thaumatococcus-like protein 1a	<i>Malus domestica</i>	Q9FSG7	119	9	31.41	25.69	–	4.98	Extr	
3831	Amino acid metabolism 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	<i>Ricinus communis</i>	gi 255569484	108	2	76.04	84.89	6.38	6.09	Cyto	
6633	S-Adenosylmethionine synthetase 5	<i>Vitis vinifera</i>	A7Q0V4	177	8	48.2	42.79	5.42	5.57	Cyto	
3409	1-aminocyclopropane-1-carboxylate oxidase 1	<i>Malus domestica</i>	Q00985	325	36	34.71	35.41	6.33	5.24	CW	
7408	1-aminocyclopropane-1-carboxylate oxidase	<i>Malus domestica</i>	Q00985	232	42	34.13	35.41	5.02	5.24	CW	
8402	Cysteine synthase	<i>Vitis vinifera</i>	A5AFH5	196	13	34.29	34.37	4.93	5.39	Chlo Mito	
5504	Arginase	<i>Arabidopsis thaliana</i>	gi 15236640	268	15	37.74	37.54	5.84	6.11	Chlo	

Nucleotide metabolism										
6704	Chromosome chrs3 scaffold _158	<i>Vitis vinifera</i>	A7QSD0	129	9	61.66	58.01	5.49	7.54	
8514	Chromosome chr10 scaffold _138	<i>Vitis vinifera</i>	A7QPL9	323	20	39.84	31.86	4.7	5.68	
Redox										
619	Class III peroxidase PSYP1	<i>Pinus sylvestris</i>	Q9FYS6	94	4	48.28	39.62	–	5.82	
7301	Stromal ascorbate peroxidase	<i>Retama reatam</i>	Q8H1K8	235	16	31.27	39.63	5.23	7.02	Chlo Cyto 
8730	Glutathione-S-transferase	<i>Arabidopsis thaliana</i>	P42760	92	9	56.74	23.48	4.62	5.82	Cyto 
5015	SODCP_MEDSA Superoxide dismutase [Cu–Zn], chloroplast precursor	<i>Medicago sativa</i>	gi 12230569	180	30	14.65	20.82	5.57	6.02	Chlo Plas 
Secondary metabolism										
4402	Alcohol dehydrogenase	<i>Solanum tuberosum</i>	gi 13549123	98	4	30.76	30.46	6.96	7.66	Cyto 
5511	O-methyltransferase	<i>Pyrus pyrifolia</i>	Q9ST37	221	14	39.6	42.28	5.7	5.66	

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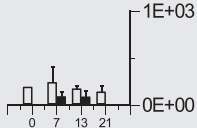
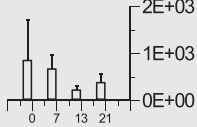
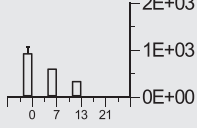
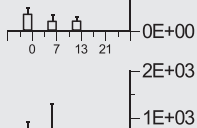
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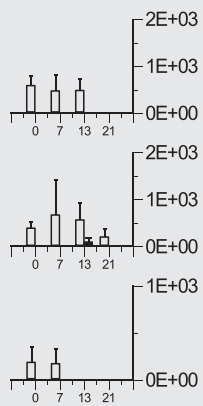
Spot	Protein name	Organism	Genbank/EST accession	Mascot score	Match% Cov. ^a	Mr. (kDa)		pI		Local ^d	Protein abundance ^e
						Exp. ^b	Ther. ^c	Exp. ^b	Ther. ^c		
Protein synthesis, storage and transport											
1813	YABBY transcription factor	<i>Lilium longiflorum</i>	gi 145105281	59	14	77.84	21.89	7.72	9.23	Nucl	
4305	PROTEIN PHOSPHATASE TYPE 2C.	<i>Malus domestica</i>	gi 51293112	169	12	30.59	27.68	6.14	7.05	Cyto	
8421	Putative uncharacterized protein	<i>Oryza sativa</i>	A2YZ57	238	15	34.34	24.06	4.71	4.78	Micr	
2629	Unnamed protein product	<i>Vitis vinifera</i>	gi 297744298	117	4	47.47	46.8	6.82	8.54		
Miscellaneous											
1706	040223AVBB001542CT (AVBB) Royal Gala young shoot cDNA clone AVBBB00154, mRNA sequence	<i>Malus domestica</i>	gi 149780208	350	16	55.44	64.34	7.88	9.21		
1802	010611AAYA001455HT (AAYA) Royal Gala 126 DAFB fruit cDNA clone AAYA001455, mRNA sequence	<i>Malus domestica</i>	gi 91026481	94	8	76.05	26.18	8.13	6.67		
4326	Mdfwg2101B17.g1 Apple_EST_Mdfwg cDNA 5' similar to ref NP_181684.1 esterase D-related	<i>Malus domestica</i>	gi 226745144	96	12	32.01	26.74	5.96	5.97		
4501	Hypothetical protein VOLCADRAFT_89573	<i>Volvox carteri f. nagariensis</i>	gi 302835439		3	36.34	82.62	6.16	4.55		

8221	Putative uncharacterized protein	<i>Oryza sativa</i>	A2X1A0	132	11	27.26	23.42	4.55	5.08		
Cluster no. 2 (decreased abundance) Carbon and energy metabolism											
1528	Glyceraldehyde 3-phosphate dehydrogenase	<i>Daucus carota</i>	gi 51703306	117	13	39.76	36.7	7.1	7.05	Cyto	
2433	NADP-dependent D-sorbito-6-phosphate dehydrogenase	<i>Malus domestica</i>	gi 54686942	267	29	34.44	24.61	6.54	6.15	Cyto	
3826	Malic enzyme, putative	<i>Ricinus communis</i>	gi 255546341	216	6	77.34	65.47	6.2	5.98	Cyto	
5626	Enolase	<i>Ricinus communis</i>	ENO_RICCO	261	15	53.12	48.11	5.73	5.56	Cyto	
5732	Apgm	<i>Malus domestica</i>	gi 4165550	219	8	62.91	60.93	5.67	5.44	Cyto	
6640	Glycosyltransferase UGT88A1	<i>Malus pumila</i>	gi 165909411	226	9	55.88	54.02	5.46	5.67	Cyto	
6716	UTP-glucose-1-phosphate uridylyltransferase	<i>Pyrus pyrifolia</i>	O64459	220	5	60.71	51.4	5.33	5.99	Cyto	
8720	Phosphoglycerate kinase, putative	<i>Ricinus communis</i>	gi 223547261	133	17	56.67	50.1	4.61	8.74	Cyto	

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Table 1 (continued)

Spot	Protein name	Organism	Genbank/EST accession	Mascot score	Match% Cov. ^a	Mr. (kDa)		pI		Local ^d	Protein abundance ^e
						Exp. ^b	Ther. ^c	Exp. ^b	Ther. ^c		
9607	Glycosidase hydrolase, weak Alpha amylase catalytic region domain	<i>Ralstonia solanacearum</i>	gi 299075270	54	1	45.00	129.56	–	6.03	Plas	
8721	Nucleotide metabolism Chromosome chrs10 scaffold _50	<i>Vitis vinifera</i>	A7Q4R4	320	38	54.33	50.04	4.79	4.76		
307	Stress and defense Prohibitin 4	<i>Arabidopsis thaliana</i>	gi 15232129	58	7	31.07	30.62	–	6.93	Chlo Mito CW PM VM	
3719	Predicted protein	<i>Populus trichocarpa</i>	gi 224132234	173	8	60.07	60.93	6.3	6.20	Cyto	
2701	Protein synthesis, storage and transport Transcription initiation factor	<i>Rabidopsis lyrata</i>	gi 297851212	90	2	57.51	79.97	6.84	9.01	Nucl	
9322	Miscellaneous Predicted protein	<i>Micromonas pusilla</i>	gi 226454765	72	3	30.65	85.67	–	5.39		
9112	Hypothetical 16.1 kDa protein	<i>Malus domestica</i>	gi 50891266	83	14	18.25	102.68	–	7.33		

9215	Q9SVQ7 Hypothetical 20.4 kDa protein	<i>Malus domestica</i>	gi 46990116	130	10	25.06	22.25	–	4.76	
226	Royal Gala 10 DAFB fruit cDNA clone AASB004699	<i>Malus domestica</i>	gi 48269420	106	21	26.12	22.12	–	9.41	
1213	Royal Gala 10 DAFB fruit cDNA clone AASA001474	<i>Malus domestica</i>	gi 48267080	189	18	25.83	23.31	7.76	7.89	

^a %Cov: percent of coverage.
^b Exp: experimental value.
^c Theo: theoretical value found at NCBI.
^d Cellular location, Chlo, chloroplast; Cyto, cytoplasm; CW, cell wall; Extr, extracellular; Micr, microtubule; Mito, mitochondria; Nucl, nucleus; Plas, plastid; PM, plasma membrane; Thyl, thylakoid; VM, vacuolar membrane.
^e Protein abundance (mean ± SD, n=4). Open bars and black bars indicate control and ethylene treated, respectively.
^f Out of range of land-markers.

performed on an LC Packings Ultimate nano-flow system (Dionex, Sunnyvale, CA, USA). Samples (7 μ L) were injected directly onto a monolithic reversed phase capillary column, 0.100 $\times$ 150 mm Chromolith CapRod RP-C₁₈ endcapped (Merck KgaA, 64271 Darmstadt, Germany). The flow rate after splitting was 180 nL min⁻¹ and the sample was sprayed through a distal coated fused silica emitter tip, 75 μ m ID with 15 μ m ID tip (New Objective, Woburn, MA, USA). Mass spectrometry was performed on a hybrid triple quadrupole linear ion trap (QTRAP4000, LC/MS/MS, Applied Biosystems, Foster City, CA, USA) equipped with a nanospray ion source. Spectra were acquired as follows using Information Dependent Acquisition mode in the Analyst 1.5.2 software. For each MS scan, 400–1400, the four most intense ions were selected for an Enhanced Resolution (ER) scan in order to determine their charge states. Enhanced Product Ion scans, 100 to 1700, were initiated by the presence of doubly and triply charged ions with intensities exceeding 1×10^5 counts. Former target ions were excluded for 30 s after four occurrences. The ion spray voltage was 2100 V, the curtain gas was set to 20 (arbitrary units) and the declustering potential was 75 V. MS/MS peak lists were generated for database searching using MASCOT version 2.3 or using ProID (Applied Biosystems, Foster City, CA, USA) software. Product ion scans for precursors within a 1.2 Da range were summed if collected within 30 IDA cycles of each other. All spectra were centroided and de-isotoped.

The raw MS/MS data were searched against NCBI *viridiplantae* entries, 19,981,550 sequences, updated Aug. 27th 2012 (NIH, Bethesda, MD, USA) and SwissProt database, 536,789 sequences, updated Aug. 07th 2012 (Sprot version 57.15) using the MASCOT algorithm (Version 2.20, Matrix Science, London, UK). The MS/MS data were also used to search against the Apple EST database, 1,947,072 entries, updated Aug. 27th 2012 (NIH, Bethesda, MD, USA). The MS and MS/MS mass tolerance were 1.2 and 0.8 Da respectively, and one missed cleavage was allowed. Carboxamidomethyl cysteins and oxidized methionine were set as fixed and variable modifications, respectively. Proteins with significant peptide matches were selected for error-tolerant searching. The data were also searched against the SwissProt database to ensure no peptides from trypsin or keratin were present. False positive peptide identification rates were also calculated using the decoy option provided by MASCOT and estimated as below 3.5%. Peptide ion scores greater than 45 indicate identity or extensive homology ($p < 0.05$) and are referred to as significant hits. Peptides below the significance threshold were only reported where other significant hits to the same protein were present. The protein identification was based on at least two matched peptides.

2.8. Statistical analysis

Experimental design and data analyses were conducted using R program (v2.6.2, www.r-project.com), SAS (v9.1.3, SAS Institute Inc., USA) and GenStat Version 8.1 (VSN International Ltd., 2005, Herts, UK). ANOVA was conducted on all spots from the dataset with a p -value of 0.05 as the cut-off. Hierarchical clustering analysis of spots was conducted using PermutMatrix software [35]. It allowed for clustering spots according to their abundance profile. Pearson's distance and Ward's algorithm were used for data aggregation.

3. Results

3.1. Physiological characteristics of fruit ripening and senescence

In order to link changes in the apple proteome to fruit physiology, biological measurements (fruit respiration, ethylene production, firmness, soluble solids and titratable acidity) were made during apple fruit ripening and in response to ethylene treatment (Fig. 1A–D). Low internal ethylene concentration ($< 0.1 \mu\text{L L}^{-1}$) confirmed that fruit were harvested at the pre-climacteric stage. At 20 °C, typical climacteric changes in ethylene production and respiration were shown in the control treatment after 21 days (Fig. 1A and B, respectively). Fruit firmness declined gradually from 80 to 71 N (Fig. 1C), while the ratio of SS/TA increased slightly from 1.96 to 2.3 after 21 days (Fig. 1D). The ethylene treatment significantly accelerated fruit ripening with typical climacteric changes in ethylene production and respiration at 7 days after treatment (Fig. 1A and B). Fruit firmness significantly declined to 54.8 N after 13 days at 20 °C (Fig. 1C), while the SS/TA ratio increased from 1.96 to 3.4 at 21 days (Fig. 1D).

3.2. Protein profiles during fruit ripening and with ethylene treatment

Total protein yields were compared between climacteric fruit ripening and ethylene treatment. No significant difference in apple fruit protein content was found among the ripening stages or between the control and ethylene treatments (Table S1).

2-DE analysis of protein extracted from apple tissue showed distinct spots from 10 to 250 kDa with pI between 3 and 11 with low background. We detected 2304 protein spots on 2-D gels resolved from apple tissues from all stages in the pH range of 3–11 (Fig. 2). Protein profiles at different ripening stages and after ethylene treatment are also shown in Fig. S1A–G and Table S1.

In this study, two approaches were undertaken to sort significant changes in protein profiles of apple fruit. The first approach was to identify the significantly changed spots using ANOVA ($p < 0.05$). During ANOVA analysis, spots which presented in only one stage were omitted to minimize the interference of missing values. From this analysis, 97 spots showed significant changes in this study. Based on the changes in spot quantity, two significant clusters were created based on the statistical analysis of the trends of changes to these spots, which comprised 69 and 28 spots, respectively. Cluster 1 showed the increasing trend of spots which were mainly absent at day 0 and early stages of ripening, but increased during fruit ripening and after ethylene (Fig. 3). It also contained four spots which were present at day 0, which were the most abundant proteins during fruit ripening and enhanced with ethylene treatment (Fig. 3). Cluster 2 illustrated the spots that were present at day 0, but significantly decreased during fruit ripening and after ethylene treatment (Fig. 3). Among these, the quantitative changes of those identified proteins are also presented in Table 1. These results clearly demonstrate a dynamic change in protein population from the time the fruit was harvested through ripening and in response to ethylene treatment. The second approach was to find the spots which were uniquely

present at a specific stage during ripening and in response to ethylene treatment. From this approach, 221 spots were sorted and found to be present in 4 specific stages of fruit ripening and after ethylene treatment.

3.3. Protein identification by LC/MS/MS

3.3.1. Identification of proteins that changed significantly during fruit ripening and in response to ethylene treatment

ANOVA analysis revealed that there were 97 spots that changed significantly during ripening and after ethylene treatment. Further analysis of these proteins using hierarchical clustering analysis resulted in two clusters showing dynamic changes of protein abundance (Fig. 3). The first cluster presented proteins showing an increased trend. From 69 spots, 42 proteins were identified in this group (Table 1, Fig. 2 and Fig. 3). This cluster also included 4 proteins that were in relatively high quantity at day 0 and increased during fruit ripening and with ethylene treatment. These proteins were identified as malic enzyme, alcohol dehydrogenase, 1-aminocyclopropane-1-carboxylate oxidase (isomers), and cysteine synthase. The second cluster presented the group of 28 proteins, which were decreased during this study (Table 1). Among these, 18 proteins were identified. The quantitative changes of protein abundance are also shown in Table 1, Fig. 2 and Fig. 3, demonstrating their relationships to fruit ripening and in response to ethylene treatment. Identified protein sequence information is also included in Table S1.

In addition to ANOVA on significant changed spots, gel analysis also showed the spots uniquely present at different ripening stages. These spots were also excised and identified by LC/MS/MS. From 219 total spots, 161 spots were identified (Tables S3 and S4), and marked on the corresponding images (Fig. S1A–G). At day 0, 30 spots were uniquely presented, but were absent in later stages. Of these 30 spots, 24 were identified. During fruit ripening, another 56 spots were present only at day 7, 13, or 21; among them, 49 were identified (Table S3). Ethylene treatment resulted in a group of unique proteins (133) present only at day 7, 13, or 21. From these proteins, 88 spots were identified (Table S4).

3.3.2. Protein identification and functional classifications

Based on the identifications of the most significantly changed proteins, dynamic protein networks during fruit ripening and after ethylene treatment were studied using GO annotation terms and results from this study. In order to obtain a better understanding of the protein functions, we focused on the proteins that changed significantly over the course of the study. Among the 42 increased proteins present in Cluster 1, 15 molecular functions and 15 biological processes were classified. The oxidation–reduction process, defense response, oxidative stress response, metabolic process and carbohydrate metabolism were classified as the top five biological processes (Fig. S2). The 18 decreased proteins identified in Cluster 2 were also grouped into 15 molecular functions and 15 biological processes. Among them, the oxidation–reduction process, metabolic process, malate metabolism, glycolysis, and phosphorylation plus translational initiation were revealed as the top five biological processes (Fig. S2).

4. Discussion

4.1. Proteins that increased significantly during apple fruit ripening and in response to ethylene treatment

Ethylene and ethylene-induced ripening processes have been studied intensively in many fruits, especially climacteric fruit which show a significant burst of respiration and ethylene production during fruit ripening [36]. Treatment with ethylene induces ethylene production and triggers downstream metabolism, leading to changes in various fruit quality attributes [14]. Little information is available at the proteome level on the proteins changed significantly during ripening and in response to ethylene treatment.

4.1.1. Ethylene production and perception

ACS and ACO, two key enzymes catalyzing the last steps of the biosynthetic pathway of ethylene, have been isolated and extensively studied in apple fruit [19]. One ACO isomer (spot 3409) was identified in Cluster 1, demonstrating a clear induction by ethylene treatment, while another ACO isomer (spot 7408) was identified as one of the high abundance proteins increased in late ripening and further enhanced by ethylene treatment. We also identified S-adenosylmethionine synthetase (SAM synthetase, spot 6633), homocysteine methyltransferase (spot 3831) and o-methyltransferase (spot 5511) present in Cluster 1 as increased proteins by ethylene treatment. In addition to ethylene biosynthesis, SAM is also involved in biosynthesis of polyamine and many methylation reactions. The possible role of S-AdoMet as the methylation donor on modification of nucleic acids, proteins and lipids is well known. The increase of SAM synthetase during fruit ripening was also reported in proteomic studies in cherry tomato fruit [37] and peach [38]. These identifications provided proteomic evidence that ethylene plays an important role in methionine metabolism and regulation of fruit ripening. A transcription factor, YABBY2 (spot 1813) was also found after ethylene treatment. The transcript of YABBY2 was reported to be highly abundant from breaker to red stage in tomato fruit [39]. Also, a few proteins in relation to ubiquitin–proteasome pathways in ethylene response, such as SMT3 (spot 8036; day 7), ubiquitin protein ligase (spot 3126; day 14), GTP binding proteins (spot 3227; day 13 and 4207; day 7), and an ethylene receptor protein (spot 2322; Day 21), with a proteasome 27 kDa subunit (spot 5325; day 7), 20S proteasome beta-subunit and proteasome alpha subunit (spot 2214; day 21 and 8236; day 7) were uniquely present at certain stages after ethylene treatment (Table S2). The identification of these ubiquitin enzymes and proteasomes indicates the possible protein degradation mechanisms are initiated by fruit ripening and ethylene treatment.

4.1.2. Proteomic changes in the antioxidant–redox mechanism during apple fruit ripening and in response to ethylene treatment

One of the most significant functional classifications of identified proteins in this study is in relation to the oxidation–reduction process and the response to oxidative stress. In Cluster 1, class III peroxidase (spot 619), superoxidase dismutase (SOD, spot 5015), ascorbate peroxidase (spot 7301) and glutathione transferase (GSTs, spot 8730) were identified and showed an increasing trend in late ripening stages and the

ethylene treatment. In addition, other proteins related to oxidative stress, ascorbate and glutathione metabolism, peroxidase, and polyphenol oxidase were also identified at various stages of fruit ripening and after ethylene treatment (Tables S1 and S2). The involvement of antioxidant-redox state in fruit ripening and senescence has been proposed as a responsive system of oxidative stress [40]. These enzymes have great potential to remove the free radical and hydrogen peroxide in apples is in association with respiration burst. In apples, both SOD and CAT enzyme activity seem to be closely related to the onset of ethylene production and show different patterns dependent on the cultivar [41]. However, their response as defense response seemed to be fruit maturity related [42]. In our study, catalase (CAT, spot 1410) was identified as a consistent protein (data not shown). As one of the most efficient enzymes to remove H_2O_2 through a dismutation without reductant, CAT plays an important role in fruit development and ripening. Proteomic data on CATs and APXs have been reported in grape berries during ripening, and showed that the two isomers of CATs decreased, while one APX increased and another decreased [43]. Plant GSTs conjugate GSH into a number of herbicides, leading to their detoxification and herbicide metabolism and selectivity in crops and weeds [44]. In addition to the detoxification of herbicides, GST functions as a facilitator of transport between cellular compartments and other secondary metabolisms such as glutathionylation, followed by rapid processing to introduce sulfur into metabolites, and transient conjugation to facilitate isomerisation reactions [45]. The exact role of GST in apple fruit is not clear; however, it has been reported that the gene expression of GST, a homologue of phi-class glutathione S-transferase, showed the greatest increase (ca. 9000 fold) in Japanese pear during ripening [46]. In strawberry fruit, GST gene expression was inhibited by 1-MCP treatment [47]. Both SOD and GST have been identified as ripening-induced proteins in mango fruit [48]. These results indicate that GST and the oxidative-redox system play an important role in fruit ripening. The identification of these proteins in relation to hydrogen peroxide generation and scavenger systems provides further proteomic evidence that oxidative stress response may be involved in fruit ripening and ethylene response. Whether these changes are one of the initiators of fruit ripening, or the consequences of fruit ripening and ethylene treatment remain to be tested.

4.1.3. Allergens and defense response proteins occurring during apple fruit ripening and in response to ethylene treatment
Allergens have been recognized as defense-associated proteins in response to biotic and abiotic stresses in apple fruit [17]. In this study, we identified a group of allergens in apple fruit which are mainly present as a result of ethylene treatment. In Cluster 1, two major allergens, d1 and an isomer d1.029, were identified as up-regulated proteins (spot 5014 and 7126), as well as the thaumatin-like protein (spot 9332). It was reported that gene expression of a protein sharing 90% sequence homology with Mal d1 was up-regulated in response to ethylene [49]. Those results are also in agreement with the report that Mal d1 apple allergens are enhanced by fruit ripening and under the regulation of ethylene [50]. The identification of the thaumatin-like protein revealed that it is up-regulated by ethylene treatment. These proteomic results confirmed the findings at the transcript level that

allergens are responsive to fruit ripening and ethylene treatment. The results also indicate that fruit ripening, and especially ethylene treatment, play an important role in formation and regulation of fruit allergens [51]. Ethylene treatment also induced cyclophilin (spot 115), four other major allergens (spot 4129, 6126, 5204 and 6127), and pathogenesis-related protein 8 (spot 9338) at different stages (Table S4). The successful identification of many allergens in apple fruit during ripening and ethylene treatment provides additional peptide sequence information for future quantitative proteomic analysis of apple allergens using a selective reaction monitoring (SRM) approach in order to investigate the effect of postharvest handlings on apple fruit allergens.

4.1.4. Proteins associated with primary and secondary metabolisms in response to ethylene treatment

Organic acids, predominately malic acid and citric acid, are the most significant acids determining the eating quality in fruits during ripening. Malic acid is one of the most dominant acids in apples and showed significant changes in correlation with fruit respiration and ethylene production. We identified two malate dehydrogenases (spot 1405 and 7516) as well as two malate oxidoreductases (spot 1814 and 4707) in Cluster 1 with a significantly increasing trend. Among them, only spots 1405 and 1814 were induced by ethylene. A malic enzyme (spot 4107) was also identified as one of the most abundant proteins in this cluster. Malic enzyme catalyzes the reversible conversion between malate and pyruvate, and is potentially involved in both malate synthesis and degradation. The identification of these proteins not only confirmed the results of decreased titratable acidity, but further indicated that malate metabolism plays an important role in the supply of energy and substrates for many secondary metabolisms in apple fruit during ripening and in response to ethylene treatment. In addition, proteins associated with carbohydrate metabolisms, electron transport/energy production and photosynthetic apparatus were also identified. Fructose-bisphosphate aldolase (spot 1532), ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (spot 3405), ferredoxin-NADP reductase (spot 4231) and glucose-1-phosphate adenylyltransferase (spot 7103) were increased in late ripening and ethylene-treated fruit. An isomer of UTP-glucose-1-phosphate uridylyltransferase (spot 5602) was induced by ethylene treatment. ATP synthase beta subunits (spot 7632) were also identified as increased proteins in Cluster 1. Together with other ATP synthases identified in various stages during ripening (Table S3, spots 6629 and 7626) indicated greater energy (ATP) generation by the synthesis of ATP from ADP during fruit ripening to meet the climacteric respiration. A beta-galactosidase (β -Gal, spot 1807) was also identified in Cluster 1 as result of ethylene treatment, which coincided with a decrease in fruit firmness. Apple fruit softening is highly dependent on ethylene [52] and gene expression levels of β -Gal have been associated with the softening pattern in many apple cultivars [53,11]. The identification of β -Gal confirmed that its regulation is ethylene-dependent [54].

Among the proteins characterized at different stages in response to ethylene treatment, many proteins involved in primary processes, such as glycolysis, gluconeogenesis, C-compounds and carbohydrate metabolisms, were also identified (Table S2).

4.2. Proteins that decreased significantly during fruit ripening and in response to ethylene treatment

Identification of decreased proteins in Cluster 2 also provides clues for in-depth understanding of the dynamic nature of fruit ripening and the effect of ethylene treatment. It reveals the shift of metabolic processes from growth to ripening. Among the 18 identified proteins, the identification of glyceraldehyde-3-phosphate dehydrogenase-like protein (spot 1528), NADP-dependent D-sorbitol-6-phosphate dehydrogenase (spot 2433), enolase (spot 5626), apgm (spot 5732), UTP-glucose-1-phosphate uridylyltransferase (spot 6717), and phosphoglycerate kinase (spot 8720) as decreased proteins by ethylene treatment revealed that carbon fixation, the dehydration step in the glycolysis pathway, and carbohydrate metabolism are decreased. Apgm protein is responsible for maintaining the equilibrium between glucose-1-phosphate and glucose-6-phosphate. It was reported that apgm was involved in the regulation of carbon partitioning between glycolysis and carbohydrate polymerization in the development of apple fruit [55]. Its decrease indicated the accumulation of glucose-6-phosphate, which is one of the important substrates of carbohydrate metabolism. The decrease in abundance of phosphoglycerate kinase, apgm, and enolase has also been found in ripe mango fruit as compared with unripe fruit, which indicated a slower carbon flow through the glycolysis pathway during fruit ripening [48]. In grape berries, the down-regulation of these proteins was also reported, implying a reduced energy conserving reaction in glycolysis [43]. It appears that ethylene treatment influences specific steps in the glycolysis pathway to regulate the requirement of energy and substrates. NADP-dependent D-sorbitol-6-phosphate dehydrogenase (spot 2433) was also decreased. S6PDH is responsible for synthesis of sorbitol. Sorbitol is transported from leaves to fruit with sucrose via phloem. In transgenic apple plants, altered levels of S6PDH resulted in changes in the sugar-acid balance and starch accumulation in fruit [56]. Glycosyltransferase (spot 6640), which is responsible for various catalytic processes in plants, and plays an important role in both “housekeeping” and in buffering the impact of biological and abiotic challenges [57], was also decreased by ethylene treatment. The identification of decreased proteins (Cluster 2), in conjunction with the identification of proteins present only at day 0 (Table S2) indicate that pathways involved in the transition from maturation to fruit ripening, such as photosynthesis, carbohydrate metabolism, and glycolysis, are influenced by ethylene.

5. Concluding remarks

This is the first proteomic approach to study apple fruit tissues at different stages of ripening and in association with ethylene treatment. Qualitative and quantitative analysis of 2-DE spots combined with LC/MS/MS identification revealed some important details of the protein population and provided a deeper insight into understanding of ripening and senescence processes of this fruit. A comparative analysis of proteomic 2-DE maps at the different stages in fruit ripening and after ethylene treatment yields important qualitative and quantitative information of fruit ripening and its regulation and response to ethylene treatment.

The identification of significantly changed proteins indicates a dynamic network of proteins in the regulation of fruit ripening and in response to ethylene treatment. In addition, ethylene treatment induced a group of unique proteins that were not present during normal fruit ripening and had not been reported before. The proteins identified in this study strongly suggest that the antioxidant–redox system, ethylene biosynthesis, defense mechanisms, and primary and secondary metabolisms are dominant metabolic activities during apple fruit ripening. Further study including targeted quantitative proteomic approaches will be developed and applied to investigate the targeted proteins responsible for fruit ripening and senescence.

Abbreviations

CA	controlled atmosphere
LC-MS/MS	liquid chromatography–tandem mass spectrometry
NL	non-linear
Mr.	molecular weight
pI	iso-electric point
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
ASB-14	amidodisulfobetaine-14
IPG	immobilized pH gradient
2-DE	2-dimensional electrophoresis

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.02.006>.

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Supplementary figure legends

Figure S1. 2-DE analysis of apple fruit protein extracts from stage specifically abundant proteins of apple fruit during ripening and senescence. 100 µg of protein from fruit tissue was dissolved in DeStreak™ solution. After iso-electrofocusing, the proteins were further separated on SDS-PAGE (12.5%) polyacrylamide gels and visualized by Sypro Ruby staining. A: CT day 0; B: CT day 7; C: CT day 13; D: CT day 21; E: ET day 7; F: ET day 13 and G: ET day 21. Squares indicate proteins identified by LC/MS/MS with corresponding numbers listed in Table S2 and S3.

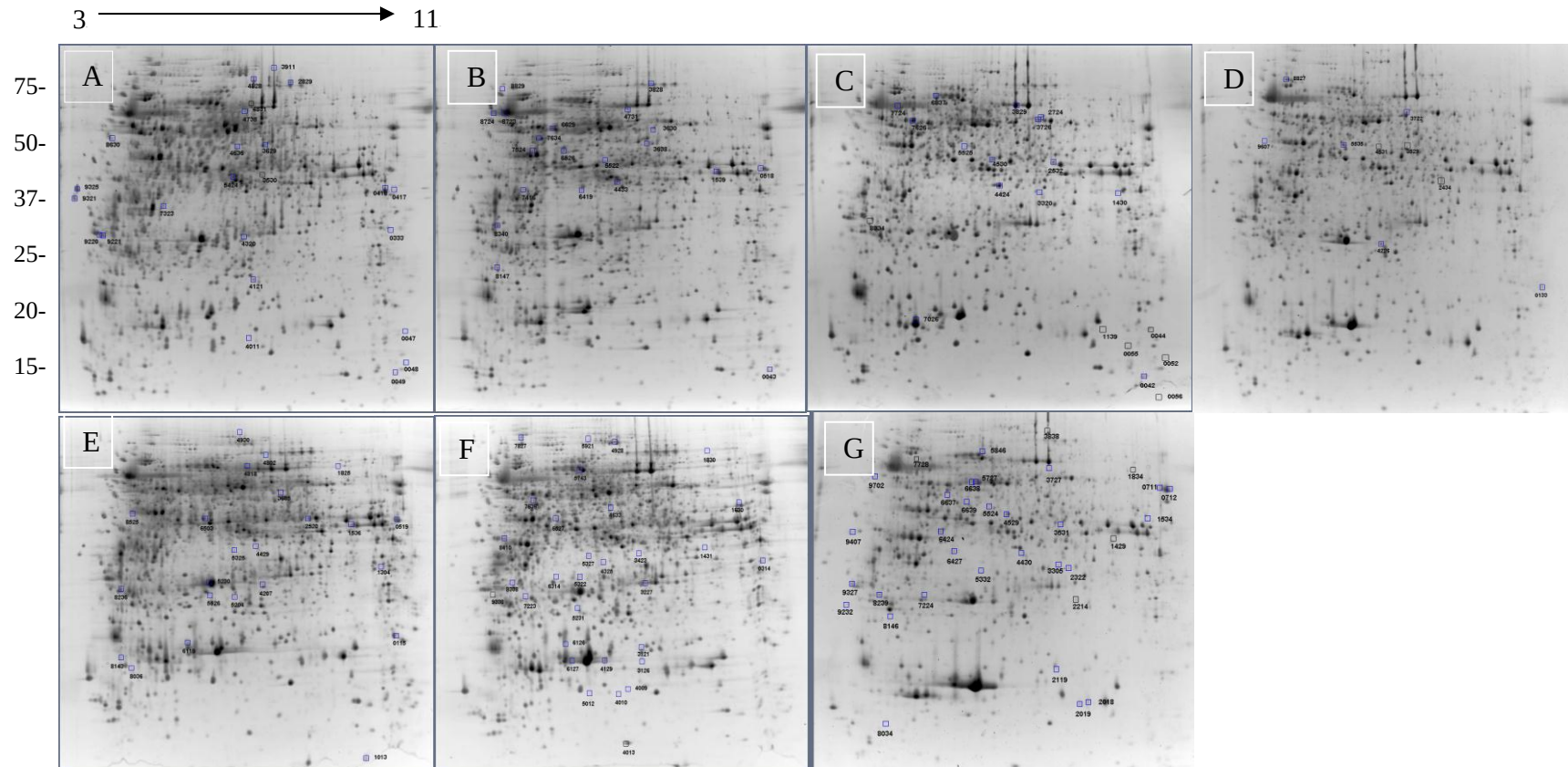


Table S1. Summary of the protein extraction and spot detection in apple fruit proteomic analysis.

Treatment	Protein yield (mg/g FW) ^a	Resolved spots ^b	Differential spots Significantly changed spots ^c	Spot detected uniquely ^d	Spots identified by LC/MS-MS ^e
Control d0	0.47±0.026	1302±21.0		30	24
Control d7	0.42±0.033	1467±59.1	194	22	20
Control d13	0.41±0.055	1409±57.7	197	25	20
Control d21	0.37±0.064	1234±41.1	238	9	9
Ethylene d7	0.43±0.055	1609±132.0	197	48	23
Ethylene d13	0.39±0.067	1678±86.5	217	44	31
Ethylene d21	0.44±0.056	1356±88.7	240	41	34

^a: Mean ±SD of four biological replicates.

^b: Mean ±SD of four replicates gels.

^c: Number of significantly variable spots, using control of d0 as reference with Student t-test at 99 % significance.

^d: Spots present only at this stage in the experiment.

^e: Identified spots are listed in Table S3.

Table S2. Proteins in apple fruit tissue that changed significantly during ripening and in response to ethylene treatment. Protein spots excised from gels stained with SYPRO Ruby were digested with trypsin and identified following liquid chromatography and mass spectrometry analysis (LC/MS/MS).

Spot	Protein name		Genbank/EST accession	Mascot score	Match% Cov.	Mr. (kDa)		pI		Local ^d	Matched sequences ^e
						Exp. ^b	Ther ^c .	Exp. ^b	Ther ^c .		
<u>Cluster No. 1 (increased abundance)</u>											
Carbon and energy metabolism											
1405	Malate dehydrogenase	<i>Vitis vinifera</i>	A7QZG8	417	23	35.19	37.6	8.32	8.12	Cyto	IQNGGTEVVEAK LLGVTMLDVVR DDLNFNINAGIVK AGAGSATLSMAYAAVK ANTFVAEVLGLDPR TGAEIYPLGPLNEYER
1532	Fructose-bisphosphate aldolase	<i>Fragaria ananassa</i>	Q9FUG7	301	20	39.79	38.51	7.11	6.93	Cyto	LGEGAAESLHVVDYKY GILAADESTGTIGK ASPEVIAEYTVR GILAADESTGTIGKR YAIICQENGLVPIVEPEIL VDGPHSIDK
1807	Beta-galactosidase	<i>Pyrus communis</i>	Q68UW0	127	3	76.13	80.41	7.93	5.89	Cyto	LVQQAGLFLVNLRL STPEMWPDLIQK
3405	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	<i>Gaultheria shallon</i>	gi 6561345	48	4	34.91	48.59	6.39	6.14	Chlo	IPTAYVKTFQHPPHGIQ VER TFQHPPHGIQVER
4428	Malate dehydrogenase	<i>Vitis vinifera</i>	A3DSX0	765	65	35.07	35.59	5.97	6.01	Cyto	ALGQVSR IVQGLSIDEFSRK LNVQVSDVK EFAPSIPEK KKLDATAEELSEEK LSSALSAASSACDHIR

											VLVTGAAGQIGYALVPM IAR MELVDAAFPLLK IVQGLSIDEFSR MELVDAAFPLLK KLDATEEELSEEK ELVADDAWLNGEFISTV QQR NVIIWGNHSSSQYPDVN HATVK VLVVANPANTNALILK ELVADDAWLNGEFISTV QQR
5602	UTP-glucose-1-phosphate uridylyltransferase	<i>Pyrus pyrifolia</i>	O64459	151	5	52.65	51.85	5.85	5.99	Cyto	LVVEDFSPLPSK VLQLETAAGAAIR
4107	Malic enzyme, putative	<i>Ricinus communis</i>	gi 255546341	108	5	65.4	22.65	5.98	6.0	Cyto	SIQVIVVTDGER AIKPTVLIGSSGVGR
4707	Malate oxidoreductase	<i>Arabidops is thaliana</i>	gi 46600240	75	10	63.05	64.28	6.07	5.57	Cyto	ALGQVSER IVQGLSIDEFSR
1814	(malic enzyme) Malate oxidoreductase (malic enzyme)	<i>Arabidops is thaliana</i>	O82191	108	6	77.82	64.28	7.58	6.32	Cyto	YMALTELQER LLNDEFYIGLR AIKPTVLIGSSGVGR
7103	Glucose-1-phosphate adenylyltransferase	<i>Vitis vinifera</i>	A7Q2V5	211	14	56.4	55.953	- ^f	8.44	Cyto	ETDADITVAALPm DEKR SVLGIIILGGGAGTR IINSDNVQEAAR IYVLTQFNSASLNR
7516	Malate dehydrogenase	<i>Malus doemstica</i>	A3DSX0	369	21	40.26	35.59	5.0	6.01	Cyto	VLVTGAAGQIGYALV PMIAR MELVDAAFPLLK VLVVANPANTNALILK RLNVQVSDVK

7632	ATP synthase subunit beta	<i>Physocarpus opulifolius</i>	Q1PGD5	714	33	52.25	51.78	5.2	5.27	Chlo Plas Thyl	IVQGLSIDEFSR ITFGEGSQYGYVK MPNIYNALVVR FVQAGSEVSALLGR VGLTALTMAEYFR TVLIMELINNIK MPSAVGYQPTLSTEMGT LQER VALVYGQMNEPPGAR IAQIIGPVLDAFPPGK IFNVLGEPVDNLGPVDTR GIYPAVDPLDSTSTMLQ PR
4231	Ferredoxin--NADP reductase, chloroplast precursor (FNR) gb AAA21758.1 ferredoxin NADP+ reductase precursor	<i>Malus domestica</i>	gi 226749466	243	24	31.42	29.22	6.09	9.52	Chlo	EGQSIGVIPDGIDK LYSIASSALGDFGDSK LVYTNDAGEIVK DPNATIIMLGTGTGIAPF R
Allergens and defense											
328	ARATH Prohibitin	<i>Arabidopsis thaliana</i>	O04331	131	12	30.82	30.40	-	6.99	Mito Nucl PM VM Cyto	DLQMVNLTLR AVVAQFNADQLLTERP HVSALVR
5014	Major allergen Mal d 1	<i>Malus domestica</i>	Q40280	121	18	16.56	17.54	5.83	5.62	Cyto	KAFVLDADNLIPK HAEILEGDGGPGTIKK
6230	Royal Gala apple skin peel, tree-ripened fruit 150 DAFB cDNA clone AAFA005568	<i>Malus domestica</i>	gi 91066390	106	16	26.85	12.28	5.41	6.84		TGGLGGGYGDTNAYSG EGR
7126	Mal d 1.0209	<i>Malus domestica</i>	A0AUB1	80	8	26.47	17.52	4.98	5.46	Cyto	AFVLDADNLIPK

8729	Chaperonin-60 beta subunit	<i>Solanum tuberosum</i>	gi 1762130	115	5	62.83	63.27	4.87	5.72	Cyto	VVAAGANPVLITR DATTIVGDGSTQEAVN KR
9332	Thaumatococcal protein 1a	<i>Malus domestica</i>	Q9FSG7	119	9	31.41	25.69	-	4.98	Extr	SACLAFGDSK SVDAPSPWSGR
Amino acid metabolism											
3831	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	<i>Ricinus communis</i>	gi 255569484	108	2	76.04	84.89	6.38	6.09	Cyto	GVTAYGFDLVR YLFAGVVDGR
6633	S-Adenosylmethionine synthetase 5	<i>Vitis vinifera</i>	A7Q0V4	177	8	48.2	42.79	5.42	5.57	Cyto	TNMVMVFGEITTK TIGFVSDDVGLDADNC K
3409	1-aminocyclopropane-1-carboxylate oxidase 1	<i>Malus domestica</i>	Q00985.1	325	36	34.71	35.41	6.33	5.24	CW	FKEMVAAK AHSDAGGIILLFQDDK KTEDAPTYPK HLPSSNISEIPDLEEEYR K LLDLLCENLGLEK MSIASFYNPGRNDSFISPA PAVLEKK
7408	1-aminocyclopropane-1-carboxylate oxidase	<i>Malus domestica</i>	Gi 2385371	232	42	34.13	35.41	5.02	5.24	CW	HLPSSNISEIPDLEEEYR KTMKEFAVELEK LAEKLLDLLCENLGLEK GYLKKVFYGSKGPNFG TK AHSDAGGIILLFQDDK VIAQSDGTRMSIASFYNPGRNDSFISPA PAVLEKK FVFDDYMKLYSGLKFQ AK
8402	Cysteine synthase	<i>Vitis</i>	A5AFH5	196	13	34.29	34.37	4.93	5.39	Chlo	LIITMPASMSLER

		<i>vinifera</i>								Mito	LYGVEPVESAVLSGGK
5504	Arginase	<i>Arabidopsis thaliana</i>	gi 15236640	268	15	37.74	37.54	5.84	6.11	Chlo	LIVVVFPSFGER VLTDVGDVPVQEIR LMNVISESVK IMEGGYAR FGVEQYEMR DTVDGMTAMVAAK
Nucleotide metabolism											
6704	Chromosome chrs3 scaffold _158	<i>Vitis vinifera</i>	A7QSD0	129	9	61.66	58.01	5.49	7.54		CMALSTVVVFGDSK ASFAGDLNFGYK
8514	Chromosome chr10 scaffold _138	<i>Vitis vinifera</i>	A7QPL9	323	20	39.84	31.86	4.7	5.68		IGGVMIMGDR VCAELNVDGLR LCLLNVIDPK VCAELNVDGLRGDIVT NR INMVDLPLGATEDR
Redox											
619	Class III peroxidase PSYP1	<i>Pinus sylvestris</i>	Q9FYS6	94	4	48.28	39.62	-	5.82		MGQLDVLTGSQGEIR
7301	Stromal ascorbate peroxidase	<i>Retama reatam</i>	Q8H1K8	235	16	31.27	39.63	5.23	7.02	Chlo Cyto	EDIKELLK HAANAGLVNALK MGLNDKEIVALSGAHT LGR DGP GAPGGQSWTAQW LK
8730	Glutathione-S-transferase	<i>Arabidopsis thaliana</i>	P42760	92	9	56.74	23.48	4.62	5.82	Cyto	VLIALHEK AITQIAHEFSDK
5015	SODCP_MEDSA Superoxide dismutase [Cu-Zn], chloroplast precursor	<i>Medicago sativa</i>	gi 12230569	180	30	14.65	20.82	5.57	6.02	Chlo Plas	HAGDLGNIIADANGVA EATIVDNQIPLTGPNSVI GRALVVHELEDDLK LACGVVGLTPV
Secondary metabolism											

4402	Alcohol dehydrogenase	<i>Solanum tuberosum</i>	gi 13549123	98	4	30.76	30.46	6.96	7.66	Cyto	VVMVTGASSGIGR
5511	O-methyltransferase	<i>Pyrus pyrifolia</i>	Q9ST37	221	14	39.6	42.28	5.7	5.66		FGNLFNEAMEADSKLIGR VVVEECGGVFEGLK FGNLFNEAMEADSK SIETQLMFDMLMMSILP GKER
Protein synthesis, storage and transport											
1813	YABBY transcription factor	<i>Lilium longiflorum</i>	gi 145105281	59	14	77.84	21.89	7.72	9.23	Nucl	LPSAYNRFMK AAKPDIPHREAFSMAAK
4305	PROTEIN PHOSPHATASE TYPE 2C	<i>Malus domestica</i>	gi 51293112	169	12	30.59	27.68	6.14	7.05	Cyto	VT DANILEK HLTEEAVK LVVANVGDSR
8421	Putative uncharacterized protein	<i>Oryza sativa</i>	A2YZ57	238	15	34.34	24.06	4.71	4.78	Micr	ATTETVDALR ATNIDDVDKTMDEINEQ TENMK LYEQQIEQLGNFQLR LNETLEMLEK DTLPDDILAPVLK VDIFAQAR
2629	Unnamed protein product	<i>Vitis vinifera</i>	gi 297744298	117	4	47.47	46.8	6.82	8.54		
Miscellaneous											
1706	040223AVBB001542CT (AVBB) Royal Gala young shoot Malus x domestica cDNA clone AVBBB00154,	<i>Malus domestica</i>	gi 149780208	350	16	55.44	64.34	7.88	9.21		NVLVGDFMPSVK DGSASYLIPLQK GETDFGLTK IASDTTGLQAK ENAGSTLSFDSVK LNPPVPLQFAR VFDEPDTFKPDR YDSITGSGTSITALEK

mRNA sequence											
1802	010611AAYA0014 55HT (AAYA) Royal Gala 126 DAFB fruit cortex Malus x domestica cDNA clone AAYA001455, mRNA sequence	<i>Malus domestica</i>	gi 91026481	94	8	76.05	26.18	8.13	6.67		TAVFNTAK TSQVQMKPVYSR
4326	Mdfwg2101B17.g1 Apple_EST_Mdfw g cDNA 5'	<i>Malus domestica</i>	gi 226745144	96	12	32.01	26.74	5.96	5.97		MYDYVVK AFSNYLGGNK FNDVSGTILIDQ
4501	Hypothetical protein VOLCADRAFT_8 9573	<i>Volvox carteri f. nagariensis</i>	gi 302835439	54	3	36.34	82.62	6.16	4.55		QSVAEATAAAAAR APAGELSEAAKEAR
8221	Putative uncharacterized protein	<i>Orzya sativa</i>	A2X1A0	132	11	27.26	23.42	4.55	5.08		EIAAYDIQTTR CGVCQIGVIGQK
<u>Cluster No.2 (decreased abundance)</u>											
Carbon and energy metabolism											
1528	Glyceraldehyde 3-phosphate dehydrogenase	<i>Daucus carota</i>	gi 51703306	117	13	39.76	36.7	7.1	7.05	Cyto	DAPMFVVGVNEK AASFNIIPSSTGAAK LTGMSFR AGIALNDNFVK
2433	NADP-dependent D-sorbito-6-phosphate		gi 54686942	267	29	34.44	24.61	6.54	6.15	Cyto	SREELFITTK ETASLLGEDK ADVGEALAEAFK

	dehydrogenase										SIGLSNYELFLTR HGVLPHTAHTPLGGAA ANK
3826	Malic enzyme, putative	<i>Ricinus communis</i>	gi 255546341	216	6	77.34	65.47	6.2	5.98	Cyto	SIQVIVVTDGER LLNDEFYIGLR AIKPTVLIGSSGVGR
5626	Enolase	<i>Ricinus communis</i>	ENO_RICCO	261	15	53.12	48.11	5.73	5.56	Cyto	YGQDATNVGDEGGF APNIQENK VVIGMDVAASEFYGS DK VNQIGSVTESIEAVR IEEELGAEAVYAGAK
5732	Apgm	<i>Malus domestica</i>	gi 4165550	219	8	62.91	60.93	5.67	5.44	Cyto	LDQLLLLLLK ALEYADFDKFDK YAGMLQYDGELK TSGEYLTYNQVR
6640	Glycosyltransferase UGT88A1	<i>Malus pumila</i>	gi 165909411	226	9	55.88	54.02	5.46	5.67	Cyto	SALQEISK EiangleASGQR GVLPEGFLER DEEGGFVSGEEVER
6716	UTP-glucose-1- phosphate uridylyltransferase	<i>Pyrus pyrifolia</i>	O64459	220	5	60.71	51.84	5.33	5.99	Cyto	VLQLETAAGAAIR LVVEDFSPLPSK
8720	Phosphoglycerate kinase, putative	<i>Ricinus communis</i>	gi 223547261	133	17	56.67	50.1	4.61	8.74	Cyto	SVGDLTAADLK FSLAPLVPR LSELLGIQVVK RPFAAIVGGSK IGVIESLLEK GVSLLLPTDVVIADK
9607	Glycosidase hydrolase, weak Alpha amylase catalytic region domain	<i>Ralstonia solanacea rum</i>	gi 299075270	54	1	45.00	129.56	-	6.03	Plas	VATDAPLR DAFDTFRAELEK

Nucleotide metabolism

8721	Chromosome chr10 scaffold _50	<i>Vitis vinifera</i>	A7Q4R4	320	38	54.33	50.04	4.79	4.76		LHFFMVGFAPLTSR LAVNLIPFPR FPGQLNSDLRVSEQFT AFR KLAVNLIPFPR STKEVDEQINVQNK GHYTEGAELIDSVLD VVR MMLTFSVFPSPK GHYTEGAELIDSVLD VVRK LTFSVFPSPKREILHIQ GGQCGNQIGAKEVDE QINVQNK LHFFMVGFAPLTSR AVLMDLEPGTMDSVR NSSYFVEWIPNNVK ASTFIGNSTSIQEFR
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Stress and defense

307	Prohibitin 4	<i>Arabidops is thaliana</i>	gi 15232129	58	7	31.07	30.62	-	6.93	Chlo Mito CW PM VM Cyto	DLQMVNLTLR VLPSIGNEVLK
3719	Predicted protein	<i>Populus trichocarp a</i>	gi 224132234	173	8	60.07	60.93	6.3	6.20		ELAVSIEGK TFSYAGFEQQPK GGADQFIEEAER NSTVVAGGGAIDMEI SR

Protein synthesis, storage and transport

2701	Transcription initiation factor	<i>rabidopsis lyrata subsp. lyrata</i>	gi 297851212	90	2	57.51	79.97	6.84	9.01	Nucl	MDLSIVK KLSGEIGGTQFGK
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Miscellaneous										
9322	Predicted protein	<i>Micromonas pusilla</i>	gi 226454765	72	3	30.65	85.67	-	5.39	VAVVAVGVK AGFGGKGGGGEGGA SASK PLGTLEVLLVEAK
9112	Hypothetical 16.1 KD protein	<i>Malus domestica</i>	gi 50891266	83	14	18.25	102.68	-	7.33	SVLDAFFLGK LVEEFQEDVLER
9215	Q9SVQ7 Hypothetical 20.4 KD protein	<i>Malus domestica</i>	gi 46990116	130	10	25.06	22.25	-	4.76	SVLDAFFLGK LVEEFQEDVLER
226	Royal Gala 10 DAFB fruit cDNA clone AASB004699	<i>Malus domestica</i>	gi 48269420	106	21	26.12	22.12	-	9.41	IEVEYYGSPVGLK TIANISSPDASSFLVQP YDK
1213	Royal Gala 10 DAFB fruit cDNA clone AASA001474	<i>Malus domestica</i>	gi 48267080	189	18	25.83	23.31	7.76	7.89	ELAENANGASPVK AMAGGYVGQTIFPR DISELNLAK

^a%Cov: Percent of coverage;

^bExp: experimental value;

^cTheo: theoretical value found at NCBI;

^d: Cellular location, Chlo, chloroplast; Cyto, cytoplasm; CW, cell wall; Extr, extracellular; Micr, microtubule; Mito, mitochondria; Nucl, nucleus; Plas, plastid; PM, plasma membrane; Thyl, thylakoid; VM, vacuolar membrane.

^e: Sequences matched with protein or EST databases with confidence score >95% from MASCOT.

^f: out of range of land-markers.

Table S3. Proteins in apple fruit tissue that changed significantly at different stages during ripening. Protein spots excised from gels stained with SYPRO Ruby were digested with trypsin and identified following liquid chromatography and mass spectrometry analysis (LC/MS/MS).

Spot	Protein name	Organism	Genbank/EST accession	Mascot score	Match% Cov. ^a	Mr. (kDa)		pI		Local ^d	Matched sequences ^e
						Exp. ^b	Ther. ^c	Exp. ^b	Ther. ^c		
CT Day0											
Carbon and energy metabolism											
333	Mdlv3_4029o09.y 1 Mdlv3 cDNA clone Mdlv3_4029o09 5' similar to TR:O82670 O82670 BETA- GALACTOSIDAS E	<i>Malus domestica</i>	gi 54621384	128	15	28.45	16.87	- ^f	4.89	Cyto	ISVDIPVTFASGK AGAGITGPVQLK
418	Pectin methylesterase isoform 1	<i>Fragaria x ananassa</i>	gi 62288364	126	/9	34.8	24.63	-	8.44	CW	DITFQNTAGPSK VGSDLSAFYR
3530	Glyceraldehyde-3- phosphate dehydrogenase, cytosolic	<i>Antirrhinum majus</i>	gi 120666	178	5	36.18	36.77	6.25	8.3	Cyto	KVIISAPSK DAPMFVVG VNEK AASFNIIPSSTGAAK AGIALNDNFVK
3911	NADP-malic enzyme 1; malate dehydrogenase (oxaloacetate- decarboxylating)	<i>Arabidopsis thaliana</i>	gi 15225262	217	7	84.15	64.52	6.33	6.32	Cyto	GLAFTEK YMALTELQER LLNDEFYIGLR AIKPTVLIGSSGVGR
4828	Ribulose bisphosphate carboxylase large	<i>Adoxa moschatelli na</i>	gi 131899	130	7	78.68	51.70	6.06	6.13	Chlo	DTDILAAFR TFQGPPHGIQVER EITLGFVDLLR

4831	chain (RuBisCO large subunit) Predicted protein	<i>Populus trichocarpa</i>	gi 224142207	93	4	66.71	65.39	6.08	6.32	Cyto	GLLPPATISQQLQEK SIQVIVVTDGER
5424	Malate dehydrogenase, cytoplasmic	<i>Mesembrya nthemum crystallinum</i>	gi 12229778	144	10	35.82	35.82	5.60	6.00	Cyto	VLVVANPANTNALI LKEFAPSIPEK LNVQVSDVK
Nucleotide metabolism											
8630	Q9STA6 RAD23 PROTEIN.	<i>Malus domestica</i>	gi 46423877	73	20	49.83	23.31	4.55	6.09	Nucl	LINEPVEGGEGNLLEQ LGAAVPQAVTVTPEER LEAMGFDR
Nitrogen metabolic process											
4636	Beta-ureidopropionase, putative	<i>Ricinus communis</i>	gi 255584039	145	6	47.27	47.09	5.94	5.98	Cyto	KYNMVIISPILER NAAIANSYFVGSINR
Allergens and defense											
417	Prohibitin	<i>Nicotiana benthamiana</i>	gi 71370259	117	11	34.67	31.83	-	9.49	Memb	TLGENYNER AVVAQYNASQLITQR EFTAAIEAK
2829	Mdfrt3029i17.y1 Mdfrt <i>Malus x domestica</i> cDNA clone Mdfrt3029i17 5' similar to TR:O50000 O50000 ABSCISIC STRESS RIPENING PROTEIN HOMOLOG.	<i>Malus domestica</i>	gi 48737626	110	16	79.68	20.12	6.49	5.95		YSETTAAYGSTTTTHES ELDYK EEEEAYGK
9220	Hypothetical	<i>Vitis vinifera</i>	gi 147799045	87	2	27.54	113.14	-	8.72		SIEALM RDADAK

protein											VAPLIIGR
Redox											
3629	Monodehydroascorbate reductase	<i>Camellia sinensis</i>	gi 198400317	131	5	29.9	25.3	6.41	6.39		YVILGGGVVSAGYAAR AYLFPESPAR
Protein synthesis, storage and transport											
47	Tubulin beta-1 chain (Beta-1-tubulin)	<i>Pisum sativum</i>	gi 267072	107	15	18.6	51.10	5.52	4.14	Cyto Micr	AVLMDLEPGTMDSIR. FPGQLNSDLR YLTASAMFR
48	Unknown	<i>Solanum tuberosum</i>	gi 76573307	158	23	16.6	14.84	4.68	9.90	Ribo	VSVLNDALK FLIVMQKHGYIGEF EYVDDHR
9221	Elongation factor 1B ALPHA-SUBUNIT	<i>Malus domestica</i>	gi 53858643	239	81	27.69	63.90	-	4.47	Nucl	AITFSDLYTDAGLK ALDEFLAGK LTLDDIK VYAAVLEKPAGSYAN VSK
Miscellaneous											
49	Hypothetical 13.9 kD protein	<i>Malus domestica</i>	gi 52861726	109	16	10.81	130.11	-	8.91		XYIQHPPTVEK YGSTYYK` QLLEENK
4011	020801AVBC009537HT (AVBC) Royal Gala young shoot cDNA clone AVBC009537, mRNA sequence	<i>Malus domestica</i>	gi 91020678	87	24	14.29	21.31	6.11	10.24		TPGPGAQSALR IEDVTPIPTDSTR ALHLLSGQNCLS LCLSWIML
4121	TR:Q9SSL1 Q9SSL1 F15H11.6 PROTEIN	<i>Malus domestica</i>	gi 46990701	110	14	23.57	18.16	6.08	8.67		SSDPYVVIK MNLEGVPSGTIV
4320	Royal Gala young shoot cDNA clone AVBBB00578	<i>Malus domestica</i>	gi 116981253	222	11	28.04	38.64	6.02	8.42		IVTVGIVGGSDL TLIGTQSLK TVGHTVTSPEDT
4738	Unnamed protein product	<i>Vitis vinifera</i>	gi 157348972	123	4	39.8	36.5	7.96	6.68		MLIQMVDTELEI AMVELEGAPFK

7323	Thiazole biosynthetic enzyme	<i>Pseudotsuga menziesii</i>	gi 56481797	119	7	31.07	36.63	6.13	9.23		HAALFTSTIMSK LFNAVA AEDLI
9321	M9 xylem cDNA clone AAZA003716.	<i>Malus domestica</i>	gi 48284054	514	60	30.21	20.76	-	4.14		TEEATVEEPVAA ESEAAATTEEPK ETTPPV EAEIEKI EAPVPVEVETK AAEAAEVEESTV EAEEP K EVVTAEPVAVEL TVESPAAEEATT K TVEAAAAEEAT K EVVTAEPVAVD VAEAAEVEEPTV EAEEP K
9325	Royal Gala phloem cDNA clone AAOA009563	<i>Malus domestica</i>	gi 48125635	205	34	32.07	23.26	-	4.22		
<u>CT Day 7</u>											
Carbon and energy metabolism											
43	TR:Q9SUU5 Q9SUU5 Ubiquinol-cytochrom C reductase like protein	<i>Malus domestica</i>	gi 48693418	65	11	11.16	23.16	-	7.15	Memb Mito	EALGALPLYQR SYLQEM LALVK
1539	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (Fragment)	<i>Hordeum vulgare</i>	gi 16226113	80	11	36.28	13.87	7.43	8.78	Cyto	VLPELNGK LTGMAFR
3638	GDP-D-mannose-4, 6-dehydratase (MUR1)	<i>Arabidopsis thaliana</i>	gi 6562268	53	2	47.34	42.03	6.35	5.75	Cyto	LFLGNLQASR
4433	Cytosolic malate dehydrogenase	<i>Malus domestica</i>	gi 78216493	207	13	34.2	35.97	5.99	6.01	Cyto	MELVDAAFP LL VLVVANPANTN

6629	ATP synthase subunit beta, chloroplastic	<i>Raphanus sativus</i>	ATPB_RAPS A	333	18	54.88	53.79	5.64	5.68	Chlo Plas Thyl	EFAPSIPEK LNVQVSDVK AVAMSATEGLK SAPAFIQLDTK LSIFETGIK IGLFGGAGVGK VGLTALTMAEY FVQAGSEVSALI MPSAVGYQPTL LQER
7524	Actin	<i>Pyrus communis</i>	gi 60650116	236	14	37.75	38.54	5.11	5.47	Cyto	AGFAGDDAPR DAYVGDEAQSK DLTDALMK GYMFTTTAER EITALAPSSMK LADAVGLTLGPI TNSDAGDGTTL GYISPQFVTNPE VGAATETELEDI LGADIVQK
8723	RuBisCO large subunit-binding protein subunit alpha, chloroplastic	<i>Pisum sativum</i>	RUBA_PEA	295	10	58.4	61.99	4.61	5.15	Cyto	
Amino acid metabolism											
4731	Methylenetetrahydr ofolate reductase, putative, expressed	<i>Oryza sativa</i>	gi 108711743	166	6	50.7	64.66	4.92	5.75		SNGIQNVLAL R GFLTIN SQPAVN AYLEFFCSK
Nucleotide metabolism											
5522	Unknown	<i>Populus trichocarpa</i>	gi 118481558	125	23	38.3	34.27	8.3	5.78		VGDEV YGDINEK SLGADLAIDYTK LPEK ILIIGGTGYIGK FFPSEFGNDVDR
6419	Isoflavone reductase homolog Bet v 6.0101	<i>Betula pendula</i>	gi 4731376	85	8	33.00	33.13	5.56	7.82		
7634	26S protease regulatory subunit	<i>Solanum tuberosum</i>	gi 1709798	76	7	25.0	46.67	5.54	5.50	Cyto Nucl	GVLLYGPPGTGI VVGSEFVQK

6B homolog										Prot	FDAQTGADR
Redox and defense											
8147	Peroxiredoxin	<i>Phaseolus vulgaris</i>	gi 11558242	98	8	52.2	28.77	5.18	5.17	Chlo PM	SYDVLIPDQGIA GLFIIDK
8829	CPHSC70-1 (chloroplast heat shock protein 70-1); (ATP binding)	<i>Arabidopsis thaliana</i>	gi 15233779	244	5	73.4	76.57	4.65	5.07	Chlo Plas	QAVVNPENTFFS QFAAEEISAQVL IAGLEVLR IINEPTAASLAYC IPAVQELVR
Protein synthesis, storage and transport											
7416	hypothetical protein CHLNCDRAFT_30464, partial	<i>Chlorella variabilis</i>	gi 307109575	83	25	33.13	15.57	4.89	10.59		EGQMEAVKVM STHAMGEAMAC AMGAMNRR
8340	14-3-3 family protein	<i>Malus domestica</i>	gi 55375985	218	11	28.79	29.79	4.59	4.75	CW Chlo Mito Nucl PM ER	TVDVEELTVEEF NLLSVAYK EAAESTLLAYK
8724	Protein disulfide isomerase homolog	<i>Malus domestica</i>	gi 51293408	247	31	62.19	25.23	4.56	5.17		VVVADNIQEYIK LAPILDEVAVSY FDATSN DVPSDF SEPIPEPNNEPVK
Miscellaneous											
518	020801AVBC010494HT (AVBC) Royal Gala young shoot cDNA clone AVBC010494, mRNA sequence	<i>Malus domestica</i>	gi 48419840	99	10	39.45	20.00	-	9.38		VNTIITASR IGATSDLQASK
3630	Hypothetical protein VITISV_035302	<i>Vitis vinifera</i>	gi 147771818	67	2	52.88	84.88	6.4	8.75		ISKGALVVMK FGMQGSKLR

3828	Royal Gala 126 DAFB fruit core cDNA clone AAXA003420	<i>Malus domestica</i>	gi 91024911	110	10	74.71	21.42	6.38	5.26		GVTAYGFDLVR IIEVNALAK
6526	Uncharacterized protein	<i>Arabidopsis thaliana</i>	gi 15239473	63	2	44.37	88.77	5.39	5.32		SGSGK GISNGEGGK SNGGATESS
<u>CT day 13</u>											
Carbon and energy metabolism											
2532	Fructose-biphosphate aldolase	<i>Sesuvium portulacastrum</i>	gi 196051521	129	7	35.5	38.68	4.62	6.49	Cyto	GILAADESTGTIO FASINVENVESN
3726	Ribulose 1,5-bisphosphate carboxylase large subunit	<i>Biebersteini a orphanidis</i>	gi 3929535	88	4	57.94	49.14	6.43	5.98	Chlo	KAGVMDYK EITLGFVDLLR
3829	NADP-malic enzyme 1; malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+)	<i>Arabidopsis thaliana</i>	gi 15225262	250	8	64.75	64.53	6.23	6.32	Cyto	YMALTELQER LLNDEFYIGLR IWLVDSK AIKPTVLIGSSGV LPRPKDIVK
4530	Cytosolic phosphoglycerate kinase	<i>Pisum sativum</i>	gi 9230771	375	18	38.14	42.26	5.97	5.73	Cyto	LVAQIPEGGVLL YLKPSVAGFLM DYLVGAVSNPK GVSLLLPTDVVI GVTTIIGGGDSV
7626	ATP synthase subunit beta, mitochondrial precursor	<i>Nicotiana plumbaginifolia</i>	gi 114421	532	22	53.71	59.93	5.09	5.95	Mito	LVLEVAQ HLGENMVRTI AMDGTEGLVR VLNTGSP ITVPV EAPAFVEQATEC ILVTGIKVVDLL IGLFGGAGVGK AHGGFSVFAGV

											EMIESGVIK IPSAV GYQPTLA GGLQER
Nitrogen metabolic process											
3320	Mdfrt3114m21.y1 Mdfrt cDNA clone Mdfrt3114m21 5' similar to TR:O64999 O64999 PUTATIVE HIGH AFFINITY NITRATE TRANSPORTER	<i>Malus domestica</i>	gi 55699477	24	8	29.05	18.74	6.35	9.59	Memb	ANLCYRAS RHHAK
Redox and defense											
56	Similar to TR:O81187 O81187, Glutaredoxin	<i>Malus domestica</i>	gi 46607077	145	25	7.97	17.65	-	7.87	Cyto	AQELVSSNSVVV QLLTQLGVTYK LVPLLTQAGAV
4424	Calcium ion binding / calcium- dependent phospholipid binding	<i>Arabidopsis thaliana</i>	gi 15238320	63	2	33.93	36.35	6.07	5.76	Apop Cyto Memb Nucl Thyl	ELSSDFER LLLPLVSTFR
7724	Predicted protein	<i>Populus trichocarpa</i>	gi 224073124	280	8	61.49	64.79	4.93	5.73	Apop Chlo Cyto Mito Nucl PM	VVAAGANPVLIT GYISPYFVTDSE NLIEAAEQDYER NAGVNGSVVSE
Protein synthesis, storage and transport											
42	Mddb5017e24.y1 Mddb cDNA clone	<i>Malus domestica</i>	gi 51241082	41	16	16.48	21.41	-	9.00	Ribo	ILIDLRFQNSVSE QPSLGTKAAIFF

	Mddb5017e24 5' similar to SW:RS23_FRAAN P46297 40S RIBOSOMAL PROTEIN S23											
52	Unknown	<i>Populus trichocarpa</i>	gi 118481493	232	35	52.9	12.50	5.71	9.68	Cyto	LILLSN NCPPLRKSEI EYYAMLAK VSCLSIIDAG DSDIHK FWVLDNGTIAA DKTLTPNIR	
55	Similar to ref NP_175831.1 protein translation factor Sui1 homolog-related	<i>Arabidopsis thaliana</i>	gi 226783057	86	11	15.79	25.46	-	7.96	Nucl		
8334	14-3-3 protein 2	<i>Hevea brasiliensis</i>	gi 326694865	267	17	29.52	29.93	4.59	4.76	CW Chlo Mito Nucl PM	YEEMVEFMEK TVDVEELTVEEF NLLSVAYK TAGER DSTLIMQLLR	
Secondary metabolism												
6837	Polyphenol oxidase precursor	<i>Prunus armeniaca</i>	gi 51292771	185	16	67.54	20.81	5.35	9.03	Chlo Plas Thyl	VSNSPITIGGFK DIEFESNEAVK FPATFDSK	
Miscellaneous												
44	Similar to TR:Q9ZRD7 Q9ZRD7 GMFP7	<i>Malus domestica</i>	gi 46992507	110	11	16.35	19.82	-	9.89		QFQTVEIK LTVVGYVDPGK	
1139	MYC binding protein MM-1	<i>Malus domestica</i>	gi 46595151	270	18	18.13	19.68	7.35	8.48		LSPEQLK SNFDQLVEVASH SIADEAGAVLQA TNIMFLGDGR TDPNQNTGIVIQ	
1430	Royal Gala young shoot cDNA clone	<i>Malus domestica</i>	gi 48419840	97	23	33.03	20.45	8.05	9.38			

AVBC010494											VNTIITASR IGATSDLQASK TFTTAET <u>ML</u> NA Oxidation (M) NIAGFNPETTLV
2724	Royal Gala 126 DAFB fruit cortex cDNA clone AAYA004541	<i>Malus domestica</i>	gi 48280956	89	15	60.44	21.48	6.49	8.98		
5525	021119ABMA0017 35HT (ABMA) M9 phloem cDNA clone ABMA001735,	<i>Malus domestica</i>	gi 91043310	124	17	45.23	24.17	5.65	9.12		SGEGIEQLAPSE EPTK LGFMMSGFVK GLVVPVVR
7026	Predicted protein	<i>Physcomitre lla patens</i>	gi 168009365	55	4	17.26	20.9	5.14	10.91		LFMEWVNKSNK AGPSILHP NASLPKEK
<u>CT Day 21</u>											
Carbon and energy metabolism											
3529	Formate dehydrogenase, precursor, mitochondrial	<i>Malus domestica</i>	gi 54623434	178	25	41.35	25.42	6.24	8.51	Mito	IDPELEKQTGAK FEEDLDAMLPK GEEFPAQNYIVK
3722	Atp1 (mitochondrion)	<i>Malus domestica</i>	gi 401661928	295	11	58.67	55.60	6.21	6.23	Mito	VVSVGDGIAR VVDGLGVPIDG GIRPAINVGLSV QPQYAPLPIEK SVKPELLQSLLE LVYEANPLSFLV K
4531	Fructose- biphosphatase-like protein	<i>Arabidopsis thaliana</i>	gi 71824233	176	16	41.04	23.33	5.96	6.64	Cyto	DAPKPLDIVSNE VATDAPLR DAFDTFRAELEK
9607	Glycosidase hydrolase, weak Alpha amylase catalytic region domain	<i>Ralstonia solanacearu m</i>	gi 299075270	54	10	45.00	129.56	-	6.03	Plas	
Amino acid metabolism											
5535	Hypothetical	<i>Vitis</i>	gi 147771009	104	5	42.12	45.92	5.57	5.74		LLSSTEPTLR

	protein	<i>vinifera</i>									ASSIVISGTDLIR
	Nucleotide metabolism										
8827	ER-binding protein	<i>Malus pumila</i>	gi 57639078	776	23	74.45	73.75	4.83	5.14	Cyto Nucl	VFSPEEVSAMIL IKDAVVTVPAYI DAGVIAGLNVA AAAIAYGLDK II VEIESLFDGVDF ARFEELNND LFI QIDEIVLVGGST DILLLDVAPLTL GVMTK FDLTGIPPAPR EVEAVCNPII SA
	Redox and defense										
4226	Dehydroascorbate reductase	<i>Malus x domestica</i>	gi 110083901	140	15	26.06	23.61	5.94	6.18	Apop Chlo Mito PM Vacu	ILGSFVTFL K DPGDGSE QALL VTAADLSLAPK YTELLFSR
	Miscellaneous										
133	Unnamed protein product	<i>Vitis vinifera</i>	gi 157342485	103	8	56.0	48.4	-	4.41		VTSNALY LINRLDSTRG
2434	Dessication-related protein, putative; 70055-71849	<i>Arabidopsis thaliana</i>	gi 12323093	105	5	33.99	32.95	6.63	8.27		LVAGLLGVESG IR GMLYARAAH

^a%Cov: Percent of coverage;

^bExp: experimental value;

^cTheo: theoretical value found at NCBI;

^d: Cellular location, Apop, apoplast; Chlo, chloroplast; Cyto, cytoplasm; CW, cell wall; ER, endoplasmic reticulum; Extr, extracellular; Memb, Membrane; Micr, microtubule; Mito, mitochondria; Nucl, nucleus; Plas, plastid; PM, plasma membrane; Prot, Proteasome; Ribo, ribosome; Thyl, thylakoid; Vacu, vacuole; VM, vacuolar membrane.

^e: Sequences matched with protein or EST databases with confidence score >95% from MASCOT.

^f: out of range of land-markers.

Table S4. Proteins in apple fruit tissue that changed significantly at different stages after ethylene treatment. Protein spots excised from gels stained with SYPRO Ruby were digested with trypsin and identified following liquid chromatography and mass spectrometry analysis (LC/MS/MS).

Spot	Protein name	Organism	Genbank/EST accession	Mascot score	Match% Cov. ^a	Mr. (kDa)		pI		Local ^d	Matched sequences ^e
						Exp. ^b	Ther. ^c	Exp. ^b	Ther. ^c		
<u>Ethylene Day 7</u>											
Carbon and energy metabolism											
519	Peroxisomal (S)-2-hydroxy-acid oxidase OS	<i>Spinacia oleracea</i>	GOX_SPIOL	114	5	38.98	40.31	- ^f	9.16	Pero	AIALTVDTPR IPVFLDGGVR
1536	Binding / catalytic/ coenzyme binding	<i>Arabidopsis thaliana</i>	gi 18399328	84	23	36.15	14.84	7.3	9.90	Mito	YLVQQLAK ANVVINLIGR
2520	Cystolic glyceraldehyde-3-phosphate dehydrogenase	<i>Arabidopsis thaliana</i>	gi 166706	145	14	17.5	37.07	5.06	6.34	Cyto	IGI NGFGR TLLFGEKPVTVFGIR VPTVDVSVVDLTVR AGIALSDK FVK
4207	GTP-binding nuclear protein Ran1	<i>Solanum lycopersicum</i>	gi 585777	105	13	27.32	25.5	6.11	6.25	PM Memb	LVIVGDGGTGK HLTGEFEK NLQYYEISAK
4802	Cytosolic phosphoglycerate kinase	<i>Pisum sativum</i>	gi 9230771	272	14	73.79	42.26	6.14	5.73	Cyto	LVAQIPEGGVLLLENVR ELDYLVGAVSNPK FAAGTEAIAK GVTTIIGGGDSVAAVEK
4930	Aconitase	<i>Prunus avium</i>	gi 34851120	96	2	91.41	99.4	5.88	6.01	Cyto Mito	ILLESAIR VALIESYLR
5230	Triosephosphate isomerase, chloroplast precursor	<i>Fragaria ananassa</i>	gi 226770755	226	21	27.6	25.35	5.57	5.99	Chlo	TFDVCFQQLK IIYGGSVNGGNSAELAK EDIDGFLVGGASLK GPEFATIVNAVTA
6503	Phosphoglycerate	<i>Musa</i>	gi 102140037	520	22	17.5	50.16	5.06	8.74	Chlo	SVGDLTAADLK

	kinase, chloroplast, putative	<i>acuminata</i>										LAALADLYVNDAFGTA HR RPFAAIVGGSK IGVIESLLEK GVSLLLPTDVVIADK TFNDALDTTKTIIWNGP MGV _{FEFDK} GVTTIIGG GDSVAAVEK SIEALM RDADAK VAPLIIG R
Amino acid metabolism												
1825	Predicted protein	<i>Chlamydomonas reinhardtii</i>	gi 159465763	50	3	66.70	58.32	6.55	6.20	Chlo Mito		TTESRHAALVVDVLGR HAALVVDVLGR
Nucleotide metabolism												
3635	Vacuolar protein sorting-associated protein VPS4, putative	<i>Ricinus communis</i>	gi 223536871	355	22	51.87	48.4.	6.29	6.99	Vacu		AGLNSAIIR QALQEAVILPVK FPQFFTGK AFLLYGPPGTGK AVATEADSTFFSISSDL VSK LVSNFLQMAR VLVLAATNTPYALDQAI R
4818	T-complex protein 1 subunit zeta	<i>Zea mays</i>	gi 195647166	277	5	66.74	59.56	5.96	6.23	Cyto		VMLVGGAGDLK LNPNAEVLNK EMQIQNPTAIMIAR GIDPPSLDLLAR YTFVENVK GPNDHTIAQIK
Allergen and defense												
5204	Major allergen	<i>Malus domestica</i>	gi 886683	87	5	35.82	17.60	8.35	5.86	Extr		AFVLDADNL IPK LIESYLK

Redox and defense											
4429	Short chain dehydrogenase reductase	<i>Solanum tuerrosum</i>	gi 50346893	86	6	32.55	29.23	6.05	5.77	Cyto PM	VALVTGGATGIGESIVR
5226	Dehydroascorbate reductase	<i>Malus domestica</i>	gi 110083901	67	5	44.45	23.62	5.87	6.18	Apop Chlo Mito PM Vacu	VTAADLSLAPK
Protein synthesis, storage and transport											
115	Cyclophilin	<i>Phaseolus vulgaris</i>	gi 829119	132	16	21.55	18.37	-	8.36	Chlo Plas	VFFDMTIGGQPAGR
5325	Proteasome 27kD subunit	<i>Eptatretus burgeri</i>	gi 53867158	208	19	32.02	27.53	5.83	5.60	Cyto Nucl Prot	HVVFGQVVEGLDVVK VFQIEYAAK AVDNSGTVIGIK VPDELLEEK DGVVLGVEK
6119	Eukaryotic translation initiation factor 5A-1	<i>Medicago sativa</i>	IF5A1_MED SA	148	18	20.16	17.87	5.36	5.48	Cyto	TYPQQAGTIR NGYIVIK LPTDDSLLTQIK
8036	Ubiquitin-like protein SMT3	<i>Cannabis sativa</i>	gi 55859483	149	7	15.57	39.6	4.71	8.72	Cyto Nucl	GQDGNEVF FR QSVEFNSIAFL FDGR
8143	Ribosomal protein L12	<i>Arabidopsis thaliana</i>	gi 468773	69	12	51.4	19.74	6.56	5.51	Chlo Plas	IGSEIS SLTLEEARIL VDYLQDK
8236	Proteasome subunit alpha type-5 (20S proteasome alpha subunit E)	<i>Glycine max</i>	gi 12229923	130	5	29.9	26.13	6.41	4.70	Cyto Nucl Prot	LFQVEYAIEAIK ITSPLLE PSSVEK
Photosynthesis											
8525	Magnesium-chelatase subunit chlI, chloroplastic	<i>Nicotiana tabacum</i>	gi 3334149	276	17	35.82	46.88	8.35	6.64	Chlo Plas	IGGVMIMGDR SLVDLLPEIK INMVDLPLGATEDR EGISISHPAR

											FILIGSGNP EEGELRPQLLDR FGMHAQVGTVR
Miscellaneous											
1013	Hypothetical protein VOLCADRAFT_106641	<i>Volvox carteri f. nagariensis</i>	gi 302847042	47	1	10.58	29.60	7.25	6.69		AAAAGAGDGD TIR TGAGAGAPAGMEEAAK R
1304	TR:O22707 O22707 F8A5.21 PROTEIN.	<i>Malus domestica</i>	gi 46614012	90	29	29.65	22.82	8.45	6.34	Chlo PM	ELGIGIVAYSPLGR LTPEEMAELESYASADA VK
<u>Ethylene Day 13</u>											
Carbon and energy metabolism											
1431	Mdfwg2099M01.g 1 Apple_EST_Mdfw g cDNA 5'	<i>Malus domestica</i>	gi 226747786	108	11	33.03	21.76	8.05	6.88	CW	TVSEAVAA APEK TNIMFLGDGR
3227	GTP-binding nuclear protein Ran1	<i>Solanum lycopersicum</i>	gi 585777	105	8	28.35	25.57	6.30	6.25	PM Memb	LVIVGDGGTGK
4633	NADP-dependent isocitrate dehydrogenase	<i>Prunus persica</i>	gi 15982950	119	6	47.64	46.95	5.92	6.54	Cyto Mito	VANPIVEMDGD EMT R AFAEASMTTAYEK
4928	Cytosolic aconitase	<i>Pyrus pyrifolia</i>	gi 304442678	406	8	85.42	98.49	5.97	5.91	Cyto	ILLES AIR SANAVQANMDLEFQR TSLAPGSGVVTK YLLNSGLQK DFNSYGSR LLNGEVGPK LFVFDAATR GPMLLGVK

7636	Succinyl CoA ligase β subunit	<i>Lycopersicon esculentum</i>	gi 27803873	176	9	50.52	45.16	5.12	5.86	Mito	MLGQILVTK VVDGLAPK LNFDDNAAYR AILVNIFGGIMK
Amino acid metabolism											
4013	Cysteine protease inhibitor	<i>Pyrus communis</i>	gi 2317876	69	17	6.77	10.7	6.09	5.67	Chlo Mito	VLDDKVQVVSGTMHYL K VQVVSGTMHYLK
5322	β -cyanoalanine synthase 1	<i>Malus domestica</i>	gi 93359259	85	8	29.46	41.09	5.62	7.6	Mito	TQVSQLIGRTPIVYLNK DRPALSMINDAEEK
Lipid metabolism											
1630	3-Ketoacyl-CoA thiolase B; acetyl-CoA C-acyltransferase	<i>Mangifera indica</i>	gi 1129145	310	10	48.65	45.74	8.14	8.48	Chlo Mito Nucl Pero VM	FKDEIIPVATK KDGSTTAGNSSQVSDGA GAVLLMK DGSTTAGNSSQVSDGAG AVLLMK GLPILGVFR
Allergen and defense											
4009	Nucleoside diphosphate kinase B (NDP kinase B) (NDK B)	<i>Flaveria bidentis</i>	gi 1346675	82	10	17.5	16.19	5.06	6.43	Apop Chlo Pero PM Vacu	IIGATNPAESAPGTIR LLTVDQAFAE
4129	Major allergen mal d1	<i>Malus domestica</i>	gi 4590376	285	42	18.33	17.69	5.84	5.77	Extr	GVYTFENEYTSEIPPPR AFVLDADNLIPK QAEILEGNNGPGTIK ITFGEQSQYGYVK LVACGSGSTIK
5012	Nucleoside diphosphate kinase B; Short=NDP kinase	<i>Flaveria bidentis</i>	gi 1346675	80	11	13.43	16.19	5.75	6.43	Apop Chlo Pero PM	IIGATNPAESAPGTIR

										Vacu	
6126	Major allergen	<i>Malus domestica</i>	gi 886683	192	18	21.2	17.65	5.47	5.86	Extr	QAEILEGNNGPGTIK KITFGEQSQYGYVK ITFGEQSQYGYVK
6127	Major allergen Mal d 1 (Allergen Mal d I)	<i>Malus domestica</i>	gi 1346478	240	32	17.86	17.69	5.67	5.54	Extr	AFVLDADNLIPK QAEILEGNNGPGTIK ITFGEQSQYGYVK LVACGSGSTIK
9338	Pathogenesis- related protein 8	<i>Malus domestica</i>	gi 83853955	100	6	28.4	32.13	-	4.75	Extr	SQVLPTIK FYDNGYSASIK
Protein synthesis, storage and transport											
3126	Protein binding / ubiquitin-protein ligase	<i>Arabidopsis thaliana</i>	gi 18409633	146	13	16.69	17.63	6.20	6.23	Cyto	TLGSGGSSVVVPR LLEELER
7827	Transitional endoplasmic reticulum ATPase	<i>Arabidopsis thaliana</i>	gi 11265361	92	5	85.27	94.35	4.96	5.37	Cyto Nucl	LAGESESNLR IVSQLLTLMDGLK EIDIGVPDEIGR
Secondary metabolism											
3423	Putative short- chain type alcohol dehydrogenase	<i>Solanum tuberosum</i>	gi 13549123	102	4	33.4	30.76	6.36	6.96	Cyto PM	VVMVTGASSGIGR
5743	Polyphenol oxidase precursor	<i>Prunus armeniaca</i>	gi 3282505	345	9	65.21	67.43	5.62	6.39	Chlo Plas Thyl	MYLYFYER IDENLAIMYR EKVDEEELLIK FDVFINDDAESLSR VSNSPITIGGFK
Miscellaneous											
314	Predicted protein	<i>Hordeum vulgare subsp.</i>	gi 326498579	101	7	30.68	26.43	-	9.27		TAESEEYER AFEEAELPR

1830	010812AASB0024 19HT (AASB) Royal Gala 10 DAFB fruit cDNA clone AASB002419, mRNA sequence	<i>Vulgare Malus domestica</i>	gi 48270991	243	22	77.27	25.18	6.99	5.58	FSEIMDR VEVENSGVSK IESVINSTD IVALEQGIK QSLAAAVESNLK
3121	Mdfrfu3513I12.g1 Apple_EST_Mdfrf u cDNA 5' similar to gb AAP03422.1 unknown protein (<i>Oryza sativa</i>)	<i>Malus domestica</i>	gi 226779885	109	12	21.04	18.91	6.23	7.03	SPAGITER MVGMVSI GDVVR
4010	AU223568 Apple shoot cDNA library cDNA clone S0389 5'	<i>Malus domestica</i>	gi 15184592	275	32	13.32	20.39	6.01	6.95	GLVGDHIS FINVDRPFAEK KIIGATNP AESAPGTIR IIGATNP AESAPGTIR GDYAIEIGR NIIHGSD SAEGAR AMSMNLLR SYVDMST VDADTSSK GGYFLEAP VSGSK KPAEDGQL VILAAGEK SFYLGQVG NGAK
4325	Mdstw6027P20.g1 Apple_EST_Mdst w cDNA 5' similar to ref NP_566768.1 gamma hydroxybutyrate dehydrogenase (<i>Arabidopsis thaliana</i>)	<i>Malus domestica</i>	gi 226743647	299	28	31.34	24.25	5.85	5.75	AMSMNLLR SYVDMST VDADTSSK GGYFLEAP VSGSK KPAEDGQL VILAAGEK SFYLGQVG NGAK
5231	010604AAXA0 06441HT (AAXA) Royal Gala 126 DAFB	<i>Malus domestica</i>	gi 48279046	249	23	25.40	22.44	5.60	5.61	LAEEILK GVQSVEG VEAK GGSPYGAG TYAGDGTR QPSDLELE TASHQGK

	fruit core cDNA clone AAXA006441									
5327	Mdfw2038f23.y1 Mdfw cDNA clone Mdfw2038f23 5' similar to TR:Q9SG13 Q9SG13 PUTATIVE 6-PHOSPHOGLUCONOLACTONASE	Malus domestica	gi 46990309	81	12	32.02	15.85	5.68	9.10	FQTEEEVAVR YTADLSAK
5921	001204AAOA007815HT (AAOA) Royal Gala phloem cDNA clone AAOA007815, mRNA sequence	Malus domestica	gi 48128363	122	11	84.92	21.64	5.70	7.88	DSDADLNFPTR MVELMGTPEQIAK
6314	Oxidoreductase NAD-binding domain-containing protein	Arabidopsis thaliana	gi 71817113	111	19	29.14	24.98	5.35	5.3	GAVVELSQVMGK IEPPENYPTVLIFATGSGI SPIR
6527	020814AARA009837HT (AARA) Royal Gala partially senescing leaf cDNA clone AARA009837, mRNA sequence	Malus domestica	gi 48262837	120	10	43.05	24.05	5.37	5.46	AGADVTVASVEK VQLDDKVVTSR
7223	Similar to TR: Q43670 Dormancy	Malus domestica	gi 49634991	134	20	26.77	16.97	4.96	4.80	FQVFLSDVFK LAAFLEEINK

8338	related protein Putative mismatch binding protein Mus3 (ISS)	<i>Ostreococcus tauri</i>	gi 308812812	53	2	28.76	65.91	4.8	5.96		AWYTAEV RMDINVD
8410	Putative copia-like transposable element	<i>Malus domestica</i>	gi 51293866	143	15	34.65	23.01	4.75	9.62		QENSALT TTLDK LNETLE MLEK ATTETV DALR
Ethylene Day 21											
Carbon and energy metabolism											
711	Pectin methylesterase isoform 1	<i>Fragaria ananassa</i>	gi 62288364	211	2	56.4	24.63	-	8.44	CW	DITFQNTAGPSK VGSDL SAFYR TDPNQNTGIVIQK
1429	Malate dehydrogenase, cytosolic, putative	<i>Arabidopsis thaliana</i>	gi 15219721	217	14	35.34	35.89	6.26	6.11	Cyto	VLVTGAAGQIGYALVP MIAR MELIDAAFP LLK VLVVANPANTNALI LK
3531	Aldolase, cytosolic	<i>Fragaria ananassa</i>	gi 10645188	147	7	37.59	38.90	6.44	6.93	Cyto	GILAAD ESTGTIGK ASPEVIAEYTVR
3727	PREDICTED: NADP-dependent malic enzyme-like	<i>Glycine max</i>	gi 356558908	135	4	61.95	65.55	6.36	5.75	Cyto	QYQVPLQK VLEVLK SIQVIVVTDGER
3838	Malic enzyme, putative	<i>Ricinus communis</i>	gi 255546341	107	4	88.58	65.48	6.34	5.98	Cyto	SIQVIVVTDGER AIKPTVLIGSSGVGR
4529	Putative hydroxyproline rich glycoprotein	<i>Malus domestica</i>	gi 55857606	195	21	43.73	22.61	5.89	6.23	CW Extr	EGLGSYVR RDDDENYPVSGEHETK KDEEEEGGSGGFGDYL K
5727	UTP-glucose-1- phosphate uridylyltransferase	<i>Pyrus pyrifolia</i>	UGPA_PYRP Y	421	13	53.18	51.98	5.61	5.99	Cyto	SDVASLSQISENEK LVVEDFSPLPSK LDLLLSQGK GGTLISYEGR MEIIPNPK

6424	Malate dehydrogenase precursor	<i>Medicago sativa</i>	gi 3282505	165	4/12	34.25	43.4	5.22	8.11	Cyto	LEIPDNAVIANK GVDVVVIPAGVPR LFGVSTLDVVR IQNAGTEVVEAK AGAGSATLSMAYAAAR
6639	Unknown	<i>Zea mays</i>	gi 223975935	80	5	46.35	43.23	5.49	5.21	Apop CW Chlo Memb Mito Nucl Thyl Cyto	FSLAPLVGR ELDYLVGAVSSPK
7728	Mdlr7010A23.g1 Apple_EST_Mdlr cDNA similar to sp P34105 MAOX_POPTR NADP-DEPENDENT MALIC ENZYME (NADP-ME) emb CAA39690.1	<i>Malus domestica</i>	gi 71814647	194	15	66.1	26.06	5.06	5.43	Cyto	GLLPPATASQQQLQER YMALTELQER TVQVIVVTDGER
8034	Profilin-3	<i>Malus domestica</i>	PROF3_MALDO	167	24	10.91	14.06	4.62	4.67	Cyto	DYMVIQGEPPGAVIRFD QPGTLAPTGLFLGGTK
8146	Phosphoribosyl transferase	<i>Arabidopsis thaliana</i>	gi 15235709	102	6	24.35	20.33	4.75	5.83	Cyto	DTIDLFVER GFLFGPPIALAIGAK
Amino acid metabolism											
6638	EDA9 (embryo sac development arrest 9); NAD binding / amino acid binding / binding / catalytic/ cofactor binding / oxidoreductase,	<i>Arabidopsis thaliana</i>	gi 15235282	98	23	55.56	63.57	5.53	6.16	Chlo Memb Mito	YVGVS LVGK LAVQLVAGGSGVK

	acting on the CH-OH group of donors, NAD or NADP as acceptor											
Nucleotide metabolism												
3305	D111/G-patch domain-containing protein	Arabidopsis thaliana	gi 15230903	61	7	30.81	30.93	6.38	5.07	Nucl	QSGSGRAEPVTMDIR AGVGR	
Lipid metabolism												
6427	S-Malonyltransferase [acyl-carrier protein]	Brassica napus	gi 226776058	147	17	32.19	19.57	5.38	4.95	Cyto	GEAMQAAADAANK SAMVSVIGLDSK LAETQIR	
9407	Plastid-lipid associated protein PAP3	Brassica rapa	gi 14248552	62	4	34.47	39.27	-	4.35	Plas	GDGGLFVL A REGSSLLEL	
Redox and defense												
4430	PDX1 (Pyridoxin biosynthesis 1.3)	Arabidopsis thaliana	gi 15240972	98	11	32.59	33.48	6.04	5.79	Cyto PM	GEAGTGNIIIEAVR NMDDDEVFTFAK LAAPYDLVMQTK	
2322	Ethylene receptor	Malus domestica	gi 60281001	50	5	29.9	23.27	6.58	7.71	ER Memb	HVRMLTHEPR Oxidation (M)	
5332	Ascorbate peroxidase	Pimpinella brachycarpa	gi 6651272	59	22	29.7	27.90	5.67	5.69	Cyto Chlo	TGGPFGTMR DIVTLS GGHTLGR	
Protein synthesis, storage and transport												
712	Elongation factor 1-alpha (EF-1-alpha)	Triticum aestivum	gi 399414	211	13	60.65	49.48	-	9.2	Nucl	YYCTVIDAPGHR YEEIVK IGGIGTVPVGRVETGVI KPGMVVTFGP TGLTTEVK QTVAVGVK	

2214	20S proteasome beta subunit 5	<i>Prunus persica</i>	gi 16588840	96	6	27.26	21.88	6.58	6.08	Cyto Nucl Prot VM	YDMSVEEAAELAR
2119	Cyclophilin-like protein	<i>Triticum aestivum</i>	gi 37788308	107	9	20.32	26.06	-	9.4	Chlo Plas	VYFDISIGNPVGK DFMIQGGDFDK
8239	26S proteasome regulatory particle non-ATPase subunit 12	<i>Camellia sinensis</i>	gi 113196591	78	8	27.96	31.24	4.57	4.95	Cyto Nucl Prot	IEDQDA FERDFFQLKP
9332	Elongation factor 1-delta	<i>Pimpinella brachycarpa</i>	EF1D_PIMB R	100	14	31.41	24.59	-	4.41	Nucl	ASAAEDDDDDVDLFG EETEEKK LVAVGYGK
Secondary metabolism											
5846	Polyphenol oxidase precursor	<i>Prunus armeniaca</i>	gi 3282505	188	6	74.02	67.63	5.71	6.39	Chlo Plas Thyl	IDENLAIMYR FDVFINDDAESLSR VNSPITIGGFK
Miscellaneous											
1534	Predicted protein	<i>Micromonas sp. RCC299</i>	gi 255086487	48	2	43.42	87.90	8.46	4.77		LAATEAAAK TAAIEAAKAK
1834	Predicted protein	<i>Physcomitrella patens</i>	gi 168017610	157	5	67.44	63.60	6.8	7.21		YLALDEADR MLDMGFEPQIR TGVTPILVATDVAAR
2018	110 kDa 4SNC-Tudor domain protein	<i>Pisum sativum</i>	gi 13111324	96	3	77.41	59.63	7.19	6.55		DVEIEVETVDR FYVQTVGDQK
2019	PREDICTED: uncharacterized acetyltransferase At3g50280-like	<i>Brachypodium distachyon</i>	gi 357125920	53	4	14.74	53.97	6.66	8.42		LASDATTTTLR. FDGKMSAFPGR
5524	Hypothetical protein OsI_02577	<i>Oryza sativa</i>	gi 218188534	47	5	45.55	33.65	5.75	8.86	Chlo PM	LRAAIPVR LATGEPLR

6637	Os11g0696200	<i>Oryza sativa</i>	gi 115486733	138	11	46.85	49.28	5.27	5.39	LVVKPDMLFGK GELDDTAAFK VVLDCATADPDGR
7224	ARG10	<i>Vigna radiata</i>	gi 2970051	121	5	20.3	25.75	5.48	5.62	QQYGLAKS ANEVILVIEAYK
9327	Conserved hypothetical protein	<i>Ricinus communis</i>	gi 255550093	65	3	30.12	57.09	-	7.87	MNETNNTK LATGEPLR
9702	Predicted protein	<i>Physcomitre lla patens</i>	gi 168067984	159	13	56.84	21.59	4.54	4.36	SPASDTYVIFGEAK DIELVMTQAGVSR

^a%Cov: Percent of coverage;

^bExp: experimental value;

^cTheo: theoretical value found at NCBI;

^d: Cellular location, Apop, apoplast; Chlo, chloroplast; Cyto, cytoplasm; CW, cell wall; ER, endoplasmic reticulum; Extr, extracellular; Memb, Membrane; Micr, microtubule; Mito, mitochondria; Nucl, nucleus; Pero, peroxisome; Plas, plastid; PM, plasma membrane; Prot, Proteasome; Ribo, ribosome; Thyl, thylakoid; Vacu, vacuole; VM, vacuolar membrane.

^e: Sequences matched with protein or EST databases with confidence score >95% from MASCOT.

^f: out of range of land-markers.

Figure S2. Assignment of the identified spots into putative functional group categories. Proteins were categorized into 15 biological processes according to the Gene Ontology annotation (<http://www.ebi.ac.uk/QuickGO/>). The protein counts indicate the number of protein hits that match each function category. A: Molecular functions; B: Biological process.

