

Microarray analysis of apple gene expression engaged in early fruit development

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Abstract To evaluate gene expressions mostly engaged in early development of apple fruit, we performed the identification of transcripts differentially expressed in young fruit by using microarrays spotted with 6,253 cDNAs collected from young and mature apple fruits of the cultivar Fuji (*Malus domestica* Borkh. cv. Fuji). A total of 3,484 cDNAs out of 6,253 were selected after quality control of microarray spots and analyzed for differential gene expression patterns between young fruit and other tissues (mature fruit, leaf and flower). Among them, 192 cDNAs

displayed a signal value higher than twofold in young fruit compared with other tissues. Blast analysis of the 192 cDNA clones identified 88 non-redundant groups encoding proteins with known function and 50 non-redundant groups with unknown function. The putative protein products were classified into the following categories: photosynthesis (16.7%), protein synthesis (12.3%), cell proliferation and differentiation (10.9%), cell enlargement (5.8%), metabolism (8.0%), stress response (7.2%), others (2.9%), and unknown functions (32.2%). Furthermore, confirming the microarray data by reverse transcription-polymerase chain reaction revealed that the wide range of transcripts differentially expressed in young fruit was expressed in other organs but not in the mature fruit. The data presented suggested that apple fruit development depends on the tight regulation of the expression of a number of genes, which are also expressed in other organs.

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Introduction

The apple (*Malus domestica*) is a member of the Rosaceae family, which is one of the most economically important woody plants cultivated for its value as fruit crops as well as for their ornamental beauty. The world's most important tree fruit crops, such as apple (*Malus domestica*), pear (*Pyrus communis*), peach (*Prunus persica*) and cherry (*Prunus avium* and *Prunus cerasus*) belong to the Rosaceae family. Fruits of these species have flesh fruits that are particularly derived

from a floral tube, which is made of morphologically leaf tissues (Dennis 1986; Rohrer et al. 1991).

The tissues that make up flesh fruits are mainly developed by cell divisions that occur relatively short period after anthesis (Biale 1964). In apples, cell divisions are completed within 3–4 weeks after full bloom and then cell expansion extends into maturation stage (Percy et al. 1996; Dong et al. 1997). It is evident that among flesh fruits the composition of edible portion is differentiated during the early period of fruit growth. During the period, proteins are chief constituents of cytoplasm of young undifferentiated cells. Carbohydrates are transported from leaves to fruits, but many of the substances found in fruits can be synthesized by fruit tissues (Piechulla et al. 1986).

Understanding of molecular events associated with early fruit development could provide valuable information for improving fruit quality. However, the majority of molecular studies have focused on floral determination and fruit ripening rather than on fruit development (Gillaspy et al. 1993). Recently, comparative analyses of expressed sequence tags from developing young fruits and of other organs identified genes linked with protective function, water flow, organic acid synthesis, sugar storage and photosynthesis in the tomato and pineapple (Lemaire-Chamley et al. 2005; Moyle et al. 2005). In the apple, over 150,000 expressed sequence tags have been collected from various tissues of fruits, focusing on the cultivar Royal gala, and predictions of protein homologues indicated the presence of many of genes involved in fruit ripening, flavor biosynthesis and color and health-related compound biosynthesis (Newcomb et al. 2006). With all these studies, to date, little has been known about the molecular events associated with early fruit development in the apple.

A cDNA microarray-based technology has been introduced to enable high throughput monitoring of gene expressions (Duggan et al. 1999). This technology has revolutionized gene expression studies by providing the means of simultaneously measuring mRNA levels of thousands of genes in complex biological samples (Cuzin 2001). Moreover, the technical merit of microarray analysis is the high sensitivities of mRNA detection (Ruan et al. 1998; Seki et al. 2001; Schena et al. 1996).

In this paper, we studied apple gene expressions by using microarray analysis to elucidate the differential gene expression profiles engaged in the control of early fruit development. With the obtained results, we will provide a practical database for comprehensive understanding of molecular events during early stages of fruit development.

Materials and methods

Plant materials and mRNA isolation

The apple (*Malus domestica* Borkh.) cultivar Fuji was used in this study. Plant samples were provided by the National Horticultural Research Institute, Suwon, Republic of Korea. Young leaves (2–3 cm in length), pre-anthesis flowers with petals just emerging from bud burst, early developing fruits (5–8 mm in length, 3 weeks after full bloom), and mature fruits (80–90 mm in length, 25 weeks after full bloom) were harvested, frozen in liquid nitrogen, and stored at -70°C . RNA was extracted from young leaves, pre-anthesis flowers, young fruits and mature fruits with a solution containing 4 M guanidine isothiocyanate and was further purified by ultracentrifugation in a 5.7 M CsCl solution (Sambrook et al. 1989). The reference sample was composed of an equal amount of all four samples. Poly (A)⁺ RNA was purified from total RNA by biotin-oilgo (dT) using polyATtract[®] mRNA isolation systems (Promega, USA).

Construction of cDNA library and preparation of cDNA microarray

For microarray analysis, we used cDNAs isolated from two cDNA libraries that were constructed from young fruits (5–8 mm in length, 3 weeks after full bloom) and mature fruits (80–90 mm in length, 25 weeks after full bloom). Double-stranded cDNAs were synthesized from 5 µg of poly (A)⁺ RNAs. The cDNA was ligated to a Uni-ZAP XR vector (Stratagene, USA), and the ligated cDNA was packaged in vitro using a Giga-pack extract (Stratagene, USA). The phage library was converted into plasmid form by mass excision according to the procedure described by the manufacturer's protocols (Stratagene, USA). A total of 3,124 of cDNA clones and 3,129 of cDNA clones were picked at random from the cDNA libraries of young fruits and mature fruits, respectively. The inserted cDNA was amplified by PCR using T7 and T3 primer. The PCR amplification products were examined by 1% agarose gel electrophoresis, purified by using Sephadex G-50 columns, then dried and resuspended in 50% DMSO solution. The amplified cDNAs were spotted using an OmniGrid[™] Micro-arrayer (GeneMachines, San Carlos, CA, USA) onto silanized glass slides (CMT-GAPS[™], Corning, Charlotte, NC, USA). Each slide was cross-linked with 300 mJ of short wave ultraviolet (UV) irradiation (Stratalinker, Stratagene, La Jolla, CA, USA) and stored in a desiccator until use.

Preparation of fluorescent probe and hybridization

For cDNA microarray analysis, 2 µg of mRNAs from each sample (leaves, flowers, young fruits and mature fruits) and the reference sample, which was mixture of equal amount of each sample (0.5 µg of mRNAs from each sample), were reverse transcribed in the presence of Cy5-dUTP or Cy3-dUTP (NEN Life Science Products, Boston, MA, USA). The labeled cDNA were purified through a microcon-30 column (Millipore, Bedford, MA, USA) and resuspended in 80 µl of hybridization solution (3× SSC and 0.3% SDS). The mixture was denatured at 100°C for 2 min and applied to the microarray chip at 65°C for 16 h in a humidified chamber. The hybridized slide was washed in 2× SSC for 2 min, 0.1× SSC/0.1% SDS for 5 min, and 0.1× SSC for 5 min and then spin-dried prior to scanning at room temperature.

Scanning, data acquisition and data analysis

Microarray slides were scanned for Cy5 and Cy3 fluorescent signals using GenePix 4000B scanner (Axon Instruments, Union, USA) and the image files obtained were analyzed using the GenePix Pro 3.0 software package. To ensure proper quality of spots, the spot signal intensities were subjected to local background subtraction and poor quality spots (sum of median <500) were filtered from the raw data for further analysis. Data files were imported into GeneSpring 6.2 (Agilent, USA), and data for each spot were normalized by using the Lowess normalization method. To obtain differential gene expression values, fluorescence intensity ratios were calculated for two sample-reference pairs and base two logarithms were applied as described by König et al. (2004). Genes were regarded as significantly differentially expressed if $P \leq 0.05$ and fold change ≥ 2 . The cDNA microarray analysis experiments were repeated on two separate occasions in duplicate. Annotations and functional classification of genes were based on PubMed (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>), BlastX (<http://www.ncbi.nlm.nih.gov/BLAST>), Gene Ontology™ Consortium (<http://www.geneontology.org>), and other database web sites.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA (2 µg) for RT-PCR was extracted from the same materials used for microarray analyses, and then the RT product was produced by using the SuperScript™ First-strand synthesis system (Invitrogen).

The RT products were used in PCR reactions and the PCR reactions were performed as follows; denaturation at 94°C for 3 min, denaturation, annealing and extension at 94°C/30 s, 60°C/30 s, and 72°C/1 min, respectively (27 cycle), and final extension for 10 min at 72°C. Apple actin gene was used as an internal control. The final PCR products were electrophoresed on a 1% agarose gel and photographed under UV light.

Results

Analysis of differential gene expressions in early fruit development

In order to identify genes differentially expressed in young fruit, the entire sets of 6,253 apple fruit cDNA clones were spotted at a high density on the glass microscope slides. Then, hybridization experiments were performed to compare young fruits versus other organs such as leaves, flowers and mature fruits. After elimination of poor quality spots, 3,484 cDNA clones out of 6,253 were normalized and analyzed for differential expressions. The clones whose signal log ratio (base 2) that was over twofold or eightfold were counted in all three experimental combinations (young fruit versus leaf, flower or mature fruit). A signal log ratio of 1 indicates twofold in the transcript level, and -1 indicates twofold decrease. In the young fruit versus leaf hybridizations, approximately 8.8% of the spotted cDNAs showed signals over twofold stronger in young fruit, and approximately 1.4% were expressed more than eightfold higher signals in young fruit than in leaf (Table 1). In the corresponding young fruit versus flower experiments, similar comparison yielded 15.8 and 6.8%, respectively. When young fruit and mature fruit are compared, 12.8% of cDNA clones showed more than twofold and 4.3% displayed more than eightfold stronger signals in young fruit than in mature fruit.

If three organ comparisons are combined, a total of 192 cDNA clones (5.5%) were expressed twofold higher in young fruit compared with mature fruit, leaf or flower. The 192 cDNAs were then sequenced and analyzed using the BLASTX program. Multiple cDNAs representing the same gene also were identified based on sequence data of the cDNA clones, revealing that each of 192 cDNA clones were assigned to one of 88 non-redundant groups encoding proteins with known function and 50 non-redundant groups with unknown function. The putative protein products belonged to the following categories (Aharoni and O'Connell 2002; Singh et al. 2003) (Fig. 1); photosyn-

Table 1 Number of cDNA clones with young fruit-preferential expression patterns

Expression ratio ^a (log2-ratio)	Young fruit/leaf	Young fruit/flower	Young fruit/mature fruit	Young fruit/leaf, flower and mature fruit
≥1	306 (8.8%) ^b	552 (15.8%)	446 (12.8%)	192 (5.5%)
≥3	50 (1.4%)	238 (6.8%)	151 (4.3%)	35 (1%)

^a Log-ratios of 1 and 3 denote twofold and eightfold higher expression signals in transcript level

^b Percentage based on 3,484 cDNAs showing a reliable signal (sum of median >500)

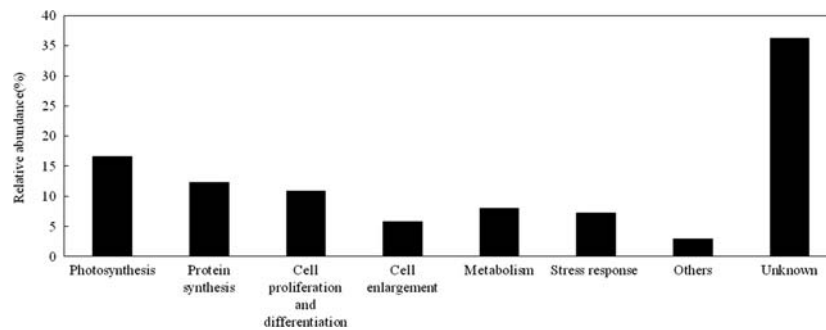


Fig. 1 Relative abundance of the putative protein products, which are highly expressed in young apples. A total of 192 cDNA clones out of the entire set of 6,253 apple cDNAs showed an expression ratios twofold higher compared with other major

thesis (16.7%), protein synthesis (12.3%), cell proliferation and differentiation (10.9%), cell enlargement (5.8%), metabolism (8.0%), stress response (7.2%), others (2.9%), and unknown functions (32.2%).

In this analysis, the majority of 88 cDNA groups with known function corresponded to the putative proteins associated with photosynthesis, protein synthesis, cell proliferation and differentiation.

Putative functions of the genes differentially expressed in young fruit

A list of the putative proteins pertaining to 88 of the differentially expressed non-redundant cDNA groups in young fruit is presented in Table 2. A total of 23 cDNA groups out of 88 were photosynthesis-specific protein homologues such as chlorophyll a/b-binding proteins, oxygen evolving enhancer proteins, photosynthetic reaction center complex proteins and cytochrome proteins. This result is consistent with the reports that developing fruits contain photosynthetically active chloroplasts (Piechulla et al. 1986, 1987). We also observed the high redundancy of sequenced clones encoding the photosynthesis-specific proteins, which resulted from high expression level in fruit and, to a lesser extent, mRNA. Our data based on the approach using microarray analyses, nevertheless, repre-

organs, such as leaves, flowers and mature fruits, were analyzed for their protein products using the BLASTX, corrected for redundancy and classified into eight protein categories

sent and provide reliable information on differential expression levels between tissues. This could be proven by the result that only small subunits of RuBPC/Oase (GeneBank no. DW248809) were expressed in young fruit based on the microarray analyses. In tomato, indeed, only small subunits of RuBPC/Oase found to be expressed in fruit, which is different from the gene expression in leaf where both small and large subunits were expressed (Sugita and Gruissem 1987).

During the early developmental stages of apple fruits, we found differential expressions of the genes that encode protein synthesis, cell proliferation, differentiation and enlargement-related proteins. Seventeen cDNA groups corresponded to ribosomal proteins, including 60 S acidic ribosomal protein P3 (DW248932) that is apparently limited to plants (Szick et al. 1998). Fifteen cDNA groups encoded the putative proteins involved in cell proliferation and differentiation with transcriptional, post-transcriptional and cell cycle-dependent activity. Eight cDNA groups that are involved in cell enlargement included a putative expansin (DW248979), which has the capacity to induce extensibility of plant cell walls (Kende et al. 2004). A cDNA group encoding brassinosteroid-regulated protein, BRU1 (DW248986) has been reported to play a casual role in cell elongation (Zurek and Clouse 1994). Another one cDNA group encoding a γ -tonoplast intrinsic protein (γ -TIP) (DW248835), represents

Table 2 Genes with young fruit-preferential expression patterns

Clone ID	Genbank accession number ^a	Log2-ratio ^b Young fruit/Others	Putative identity ^c	Identity (%)	Number of clones ^d
Photosynthesis					
F1-23-50	DW248936	6.3	Chlorophyll a/b binding protein	91	13
F1-16-79	DW248822	4.6	Chlorophyll a/b binding protein of LHCII type I(CAB)	91	2
F1-19-62	DW248931	4.8	Chlorophyll a-b binding protein CP24 10A	69	2
F1-10-39	DW248806	4.3	Chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit	90	7
F4-21-62	DW248895	4.8	23 kDa oxygen evolving protein of photosystem II	66	8
F4-1-69	DW248964	4.6	Cytochrome b6-f complex subunit 4	97	1
F4-19-44	DW248976	2.9	Cytochrome P450 77A3	70	1
F1-27-38	DW248841	2.9	Light harvesting chlorophyll a /b-binding protein Lhcb1*2-2	100	1
F1-11-54	DW248913	5.1	Light-harvesting chlorophyll a/b-binding protein (LHCP) precursor	96	4
F1-13-60	DW248918	5.2	Photosystem I reaction center subunit II	97	2
F1-15-56	DW248920	3.3	Photosystem I reaction center subunit III	81	2
F1-27-91	DW248942	4.3	Photosystem I reaction center subunit V	75	1
F1-13-62	DW248919	5.1	Photosystem I reaction centre subunit IV/PsaE	94	1
F1-16-10	DW248922	2.9	Photosystem I reaction centre subunit N	91	1
F1-23-77	DW248839	6.9	Photosystem II	45	4
F4-18-53	DW248889	3.4	Photosystem II 10 kDa polypeptide	62	1
F1-32-35	DW248854	2.7	Photosystem II 10 kDa polypeptide precursor	71	3
F4-8-2	DW248987	4.5	Plastocyanin	59	1
F1-7-79	DW248960	4.7	Putative 33kDa oxygen evolving protein of photosystem II	83	1
F1-10-64	DW248809	4.7	Ribulose-1,5-bisphosphate carboxylase small subunit	99	3
F1-11-78	DW248914	3.0	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase	88	2
F1-28-83	DW248844	2.3	Rubisco activase	82	1
F1-27-73	DW248941	3.9	Type I (26 kDa) CP29 polypeptide	80	2
Protein synthesis					
F1-3-36	DW248944	7.3	40S Ribosomal protein S10	77	1
F1-11-82	DW248915	3.1	40S Ribosomal protein S17	83	1
F1-26-86	DW248938	4.3	50S Ribosomal protein L13	74	1
F1-20-69	DW248932	2.1	60S Acidic ribosomal protein P3	69	1
F1-27-42	DW248939	1.7	60S Ribosomal protein L12	95	1
F1-31-53	DW248948	3.3	60S Ribosomal protein L24	77	1
F4-5-32	DW248982	3.3	60S Ribosomal protein L26	57	1
F1-17-58	DW248827	1.9	60S Ribosomal protein L27A	89	2
F1-18-42	DW248830	2.6	Plastid ribosomal protein L19	64	1
F5-676	DW248993	1.6	Ribosomal protein L17	100	1
F1-3-39	DW248945	2.6	Ribosomal protein L21	94	1
F4-12-85	DW248966	3.5	Ribosomal protein L27a	83	2
F1-8-78	DW248962	2.7	Ribosomal protein L27-homolg	81	1
F1-18-46	DW248929	2.2	Ribosomal protein L37	85	1
F1-7-44	DW248959	2.0	Ribosomal protein S27	80	1
F4-18-73	DW248975	2.0	Ribosomal protein S5	94	1
F4-21-33	DW248978	4.0	Ribosomal RNA small subunit methyltransferase C	92	1
Cell proliferation and differentiation					
F4-16-91	DW248973	2.9	DNA polymerase III subunit tau	72	1
F1-22-86	DW248836	1.8	F-box protein family	43	1
F1-7-26	DW248957	3.9	Histone H3	87	1
F1-6-66	DW248955	4.3	Histone H3.2	100	2
F1-32-91	DW248950	3.5	Homeodomain-leucine zipper protein 56	41	1
F1-10-62	DW248808	2.7	Kinesin motor protein (Kin2)	72	1
F1-27-61	DW248940	1.2	Oligouridylate binding protein-like protein	72	1
F4-14-64	DW248969	6.0	Putative copia-like polypeptide	61	1
F4-5-74	DW248984	4.7	Retrotransposon protein	35	1
F1-20-89	DW248933	3.1	Ribonuclease inhibitor	70	1
F1-6-75	DW248956	2.4	RNA-binding protein	57	1
F1-31-46	DW248947	2.9	RNA-binding protein precursor	85	1
F4-16-80	DW248878	5.1	RNA-directed DNA polymerase	55	1
F1-6-26	DW248954	2.5	Ubiquitin extension protein	77	2
F1-10-58	DW248911	1.9	VIP3 protein	65	1

Table 2 continued

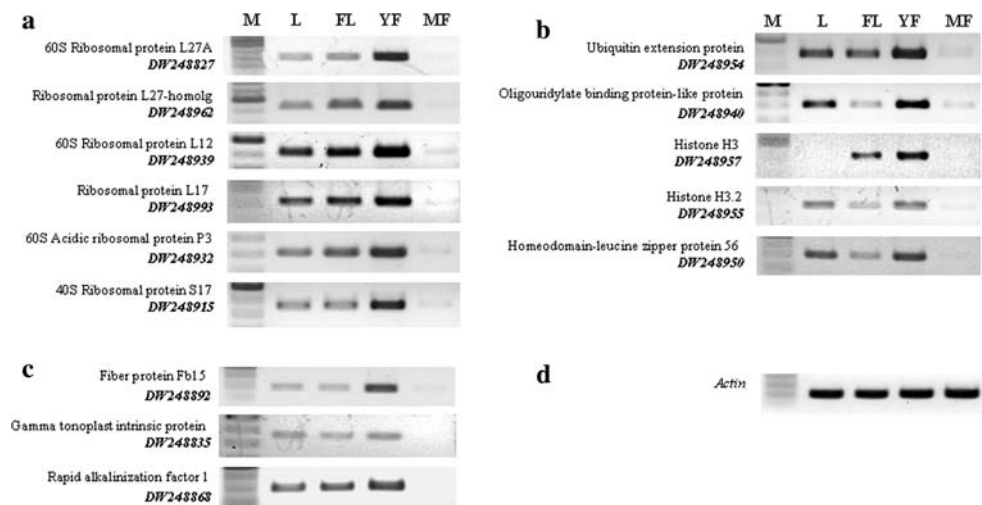
Clone ID	Genbank accession number ^a	Log2-ratio ^b Young fruit/Others	Putative identity ^c	Identity (%)	Number of clones ^d
Cell enlargement					
F1-22-68	DW248835	3.1	Gamma tonoplast intrinsic protein	96	2
F4-21-56	DW248979	4.1	Expansin	86	1
F4-13-16	DW248868	2.1	Rapid alkalization factor 1	62	1
F4-16-2	DW248971	2.5	Pollen Ole e 1 allergen and extensin	66	1
F4-21-21	DW248893	3.5	Outward rectifying potassium channel	86	1
F4-21-9	DW248980	3.0	Mitochondrial import receptor subunit TOM7-2	60	1
F4-7-16	DW248986	2.7	Brassinosteroid-regulated protein (BRU1)	79	1
F4-20-32	DW248892	1.6	Fiber protein Fb15	75	1
Metabolism					
F1-27-44	DW248842	3.8	(S)-2-hydroxy-acid oxidase	84	1
F4-5-61	DW248983	5.5	3-ketoacyl-CoA synthase	83	1
F4-16-82	DW248972	2.6	Anthocyanidin-3-glucosyltransferase-like	41	1
F1-19-32	DW248930	1.9	Cobalamine-independent methionine synthase	95	1
F1-1-32	DW248803	3.9	CP12 protein	56	4
F1-3-44	DW248946	2.8	Geranylgeranyl reductase	91	1
F5-77	DW248994	2.9	Glutamate-1-semialdehyde 2,1-aminomutase (GSA)	87	1
F1-17-26	DW248823	2.1	NADH-ubiquinone oxidoreductase 18 kDa subunit	91	1
F4-17-44	DW248881	3.6	Phosphatidate cytidyltransferase	75	1
F4-8-64	DW248988	2.8	Phosphoglycerate kinase	79	2
F1-17-16	DW248927	2.0	Polyphenol oxidase	43	1
Stress response					
F1-16-47	DW248924	2.5	Cytosolic ascorbate peroxidase	81	2
F1-5-46	DW248952	3.7	Cytosolic class I small heat shock protein 1B	70	1
F1-10-73	DW248912	3.5	Cytosolic class II lowmolecular weight heat shock protein	77	1
F1-21-71	DW248833	6.8	Ferredoxin	60	1
F4-13-14	DW248967	2.5	Ferredoxin reductase	57	1
F1-13-47	DW248917	1.6	Major allergen and lipid transfer protein Mal d 3	100	2
F1-32-90	DW248949	3.4	Nonspecific lipid-transfer protein 2 (LTP 2)	80	1
F1-16-91	DW248926	1.9	Peroxioredoxin	95	1
F5-146	DW248910	1.5	Serpin-like protein	63	1
F1-30-7	DW248852	4.9	Ultraviolet-B-repressible protein	50	1
Others					
F1-11-66	DW248811	2.1	Pollen coat-like protein	61	2
F4-11-57	DW248965	7.1	Putative senescence-associated protein	74	1
F4-14-34	DW248968	5.7	Senescence-associated protein	72	1
F4-4-71	DW248981	2.3	Late gene regulator	91	1

^a The accession number registered in GenBank^b Averaged log2-ratio of young fruit versus leaf, flower and mature fruit^c Functional annotation of gene with maximum homology is obtained using BLASTX program^d Number of cDNA clones

the plant aquaporin families, which are characteristics of channel proteins that facilitate the transportation of water across cell membranes (Maurel and Chrespeels 2001). Eleven cDNA groups out of 88 corresponded to the putative proteins involved in the metabolism of growth regulators, polyphenols and other small compounds. Other ten cDNA groups were homologues to the putative proteins that have stress response functions. These proteins include heat shock proteins, nonspecific lipid transfer proteins, and oxygen stress-responding proteins.

As shown in Fig. 2, RT-PCR analysis of 14 genes that selected according to their microarray expression profiles and putative functions in early fruit growth confirmed the corresponding microarray results. Most of the genes encoded putative proteins related to synthesis of protein building blocks, cell division and enlargement. All the selected genes showed strong expression in the young fruit, but also they were detected in leaf and flower. Since gene specific primers are used for RT-PCR, there should be no chance of cross hybridization between different members.

Fig. 2 RT-PCR analysis of 14 cDNAs related to protein biosynthesis (a), cell proliferation and differentiation (b) and cell enlargement (c). Apple *actin* gene was used as an internal control (d). First strand cDNAs were prepared from the total RNA from young leaves *L*, mature flower at pre-anthesis *FL*, young fruit *YF* and mature fruit *MF*



Genes expressed at higher levels in young fruit compared with mature fruit

We further found a distinct temporal expression profile in young fruit compared with mature fruit, where genes showed fold changes greater than eight in young fruit compared with mature fruit (Table 3). Most gene species down regulated during fruit maturation were genes encoding photosynthesis-specific proteins with 16 putative protein products identified. Five genes related to protein synthesis and 11 genes related to cell proliferation and differentiation were down regulated during fruit maturation. In addition, we observed an abundant expression of outward rectifying potassium channel (DW248893) gene in young fruit, which has been thought to act as a driving force for turgor and K^+ accumulation in the process of cell enlargement (Arend et al. 2005). The 13 genes encoding proteins related to metabolism, stress response and senescence were also found to be subjected to down-regulation by fruit maturation. These results suggest that the acquisition of the flesh fruit trait depends on the tight temporal regulation of a number of genes, which are also expressed in other organs.

Discussion

In this study, we performed a broad analysis of differential gene expressions in developing apple fruits by using a cDNA microarray. A total of 192 cDNA clones showed relatively high expression over twofold during the early stage of fruit development. Since microarray cDNAs were randomly chosen, it is highly probable that very redundant cDNA clones showed strong expression level. Thus, 192 cDNAs were subjected to

correction for redundancy and assigned to 88 non-redundant cDNA groups with known function and 50 non-redundant cDNA groups with unknown function. A large percentage (16.7%) of cDNA groups were identified as genes encoding photosynthetic specific-proteins, which provide direct evidence for the previous reports that these genes have the strongest expression level during the early stage of fruit development (Piechulla et al. 1986; Sugita and Gruissem 1987; Wanner and Gruissem 1991). Detailed sequence analysis has demonstrated that the photosynthetic genes were light harvesting chlorophyll a/b-binding proteins (DW248841, DW248913), oxygen evolving enhancer protein family (DW248806, DW248895, DW248960), photosynthetic reaction center complex proteins (DW248918, DW248920, DW248942, DW248919, DW248922), cytochrome proteins (DW248964, DW248976), a small subunit of RuBPC/Oase (DW248809), and RuBPC/Oase activase (DW248914). In case of tomato fruit, similar group of genes are also expressed in early development stage, most notably between 7 and 14 days after pollination (Piechulla et al. 1986; Sugita and Gruissem 1987; Wanner and Gruissem 1991; Gillaspay et al. 1993).

A total of 39 cDNA groups out of 88 non-redundant groups encoded to protein biosynthesis, cell division and enlargement-related proteins in the early stage of fruit development. Cell proliferation and differentiation in fruit tissues, coordinated with protein synthesis, contribute by far the most to the enlargement of fruit cells (Gillaspay et al. 1993). Based on our microarray data, we have performed RT-PCR for gene expression analysis of protein biosynthesis, cell division and enlargement-related genes. Several genes related to small and large subunits of ribosomal proteins showed high expression in young fruit. It was previously re-

Table 3 Genes with expressions higher than eightfold in young fruit compared with mature fruit

Functional category	Number of genes	Description (GeneBank number ^a)
Photosynthesis	16	Type I (26 kDa) CP29 polypeptide (DW248941), Ribulose-1,5-bisphosphate carboxylase small subunit(DW248809), Plastocyanin(DW248987), Photosystem II 10 kDa polypeptide(DW248889), Photosystem II(DW248839), Photosystem I reaction centre subunit IV/PsaE(DW248919), Photosystem I reaction center subunit II(DW248918), Light-harvesting chlorophyll a/b-binding protein (LHCP) precursor(DW248913), Cytochrome P450 77A3(DW248976), Cytochrome b6-f complex subunit 4(DW248964), Putative 33 kDa oxygen evolving protein of photosystem II(DW248960), Chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit(DW248806), Chlorophyll a-b binding protein CP24 10A(DW248931), Chlorophyll a/b-binding protein(DW248936), Chlorophyll a/b binding protein of LHCII type I(CAB)(DW248822), 23 kDa oxygen evolving protein of photosystem II(DW248895)
Protein synthesis	5	Ribosomal RNA small subunit methyltransferase C(DW248978), Ribosomal protein L27a(DW248966), Plastid ribosomal protein L19(DW248830), 50S Ribosomal protein L13(DW248938), 40S Ribosomal protein S10(DW248944)
Cell proliferation and differentiation	11	Ubiquitin extension protein(DW248954), RNA-directed DNA polymerase(DW248878), RNA-binding protein precursor(DW248947), Ribonuclease inhibitor(DW248933), Retrotransposon protein(DW248984), Putative copia-like polyprotein(DW248969), Kinesin motor protein (Kin2)(DW248808), Homeodomain-leucine zipper protein 56(DW248950), Histone H3.2(DW248955), Histone H3(DW248957), DNA polymerase III subunit tau(DW248973)
Cell enlargement	1	Outward rectifying potassium channel (DW248893)
Metabolism	7	Phosphoglycerate kinase(DW248988), Phosphatidate cytidyltransferase(DW248881), Geranylgeranyl reductase(DW248946), CP12 protein(DW248803), Anthocyanidin-3-glucosyltransferase-like(DW248972), 3-ketoacyl-CoA synthase(DW248983), (S)-2-hydroxy-acid oxidase(DW248842)
Stress response	5	Ultraviolet-B-repressible protein(DW248852), Nonspecific lipid-transfer protein 2 (LTP 2)(DW248949), Ferredoxin(DW248833), Cytosolic class II low molecular weight heat shock protein(DW248912), Cytosolic class I small heat shock protein 1B(DW248952)
Others	1	Senescence-associated protein(DW248968)

^a The accession number registered in GenBank

ported that mutations of ribosomal protein genes affect embryo viability or plant development in plants (Van Lijsebettens et al. 1994; Tsugeki et al. 1996; Ito et al. 2000). In addition, a positive correlation was reported between the expression level of ribosomal protein genes and cell differentiation (Joanin et al. 1993). We also observed differential expressions of the genes that encode histone (DW248957, DW248955), ubiquitin extension (DW248954), leucine zipper protein (DW248950) and oligouridylylate binding protein (DW248940), which are associated with cell proliferation and differentiation during fruit growth. The replication-dependent expressions of histones have been extensively studied and their mRNA accumulates have been used as a marker for cell division (Koning et al. 1991; Dong et al. 1998). The strong expression of ubiquitin extension gene in young fruits is consistent with its role as the G1/S phase specific gene and its enhanced expression in rapidly dividing tobacco cells (Callis et al. 1990). The tonoplast protein, γ -TIP (DW248835), has been used as a useful qualitative marker for expanding tissues in pears and peaches (Ludevid et al. 1992). In the case of pears and peaches, however, the γ -TIP mRNA accumulation has not been detected at the early stage of fruit development. Here

we report that, unlike the pear and peach, the apple accumulates γ -TIP mRNAs in the early developing fruits. In addition, the clone that is the homologue of fiber protein (DW248892) putatively related cell wall biosynthesis (John and Crow 1992).

Many of the stress-related genes expressed in young apples encode proteins known to protect fruit cells from environmental stresses. Heat shock proteins (DW248952, DW248912) are known as a family of proteins that function as molecular chaperones by preventing protein aggregation and also known to be involved in drought responses (Campalans et al. 2001). Nonspecific lipid transfer protein (DW248949) has been identified as major allergens of Rosaceae fruits and known to participate in defense reactions against insects and animals (Pastorello et al. 2001; Gao et al. 2005).

Most of the fruits developed from genocidium contain ovarian tissues and at least one carpel (Gillaspay et al. 1993). In nearly all Rosaceae, however, fruits developed from organs other than genocidium are called “pome” (Rohrer et al. 1991). There have been many studies on morphological relationship between pome fruits and other reproductive or vegetative organs, such as inflorescences, flowers and leaves. A

conspicuous feature is the floral tube that consists of fused and enlarged bases of the sepals, petals and stamens that come from morphologically leaf tissue. Majority of pome's flesh develops from the floral tube, and the cells of the flesh are thin-walled and parenchymatous with considerable intercellular space. In apples, the fused carpels develop rapidly to make up the bulk of fruit and these carpels contain most of the chloroplasts that give the developing fruit its green appearance. Indeed, most fruit cells have functional chloroplasts during the early fruit development stages. The process of molecular events during apple fruit development is not, however, described yet. The recent large-scale apple EST sequencing project, focusing on cultivate Gala, have provide over 150,000 ESTs, among them a set of 42,938 non-redundant sequences comprising 17,460 tentative contigs and 25,478 singletons, representing approximately one-half the expressed genes from apple (Newcomb et al. 2006). Identification through new genes and functional analysis of the EST sequencing data will allows discovery of temporal and spatial gene regulation during fruit development, but it should still take time to complete the identification.

In the present study, we produced 6,253 ESTs from apple fruits of the cultivar Fuji and selected 192 cDNAs that display differential expression profile between young fruit and other organs or mature fruit in ripening phase on the microarray analysis basis. A high of selected cDNAs showed no homology (32.2%) with known function based upon pre-existing database and the remaining cDNAs represented putative photosynthesis-specific proteins, proteins related to biosynthesis of protein building blocks, cell division and enlargement, metabolism and stress response. More interesting feature is that transcripts analyzed by RT-PCR are expressed in fruit but also detected in leaf and flower. This indicate that the majority of the early growing fruit-related genes reflect either ontogenic relationships or processes common to fruit and other organs. On the other hand, the selected genes were hardly detected in mature apple fruit and this reflects tight regulation of temporal expression of the gene during fruit development. Our results are consistent with the results from the previous study (Lemarie-Chamley et al. 2005), where the comparative transcriptome analyses of developing fruits and other organs in tomato failed to identify large sets of genes specifically expressed in fruit and, instead, showed the coordinated temporal and spatial expression in the fruit tissues of genes that also participate in other developmental programs and in other plant tissues.

In this paper, analysis of differential expression profiles using the microarray method identified a

number of genes represent an initial molecular description of early fruit development in apples. This information will be useful for understanding fruit development in apples, but also in other fruit crops.

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