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Research Report

Isolation and Characterization of Genes Expressed Differently in Mature Fruits of 'Redfield' and 'Greensleeves' Apples

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Abstract. Anthocyanins are responsible for most of the bright red and blue colors found in higher plants. Anthocyanin accumulation is regulated by MYB transcription factors. Some varieties of apple indicate anthocyanin accumulation with a red color in the skin and, at times, even in the cortex core, but other varieties without anthocyanin production have green-colored skin indicative of chlorophyll accumulation. To obtain gene expression profiles from the apple cultivars with different flesh and skin colors, 2 apple cultivars, the red-colored 'Redfield' and the green-colored 'Greensleeves', were used. A cDNA suppression subtractive hybridization library was established and analyzed. Six genes encoding methallothionein-like protein, chalcone isomerase, dirigent-like protein, brassinosteroid-6-oxidase, an unnamed protein product, and some unknown proteins were selected, and their expression was confirmed in the 'Redfield' cultivar through virtual northern blot analysis. In the 'Greensleeves' cultivar, 5 genes encoding gibberellins-regulated protein, a hypothetical 23.5 KD protein, anthranilate N-hydroxycinnamoyl/benzoyltransferase, and isoflavone reductase were chosen, and their upregulated transcriptional levels were identified. These selected genes were differently expressed in each apple cultivar, suggesting that these genes directly or indirectly regulate anthocyanin accumulation.

***Additional key words*:** anthocyanin, *Malus domestica*, suppression subtractive hybridization (SSH)

Introduction

The fruit forms an important component of human and animal diets and the development and ripening processes of them are unique to plants (Lelievre et al., 1997; Seymour et al., 1993). Fruit ripening involves complex biochemical and physiological changes. It is associated with color modification through alteration of accumulation of chlorophylls, carotenoids, and flavonoids; with textural modification via increased activity of cell wall degradation, with flavor and aroma modification of sugars, acids, and volatile compounds and with autocatalytic ethylene production (Lelievre et al., 1997; Seymour et al., 1993;Theologis, 1992). Fruit ripening is regulated by both internal and external factors such as ripening-induced genes, hormones, nutrients, light, and temperature (Seymour et al., 1993; Theologis, 1992). Using molecular and genetic approaches, a large number of genes expressed during fruit ripening have been identified (Giovannoni, 2001, 2004; Tanksley, 2004). With the aid of molecular and genomic techniques, a large number of genes and signal transduction pathways, which occur during this unique developmental process, have recently been uncovered in tomato and apple plants (Adams-Phillips et al., 2004; Alba et al., 2005; Goulao and Oliveira, 2007; Newcomb et al., 2006; Park et al., 2006).

Antioxidants such as flavonoids, flavonols, and flavones are key components of the health properties of fruit reducing the incidence of chronic diseases when included in a regular healthy diet (Knekt et al., 2002). Production of phytochemicals such as flavonoids and anthocyanins make fresh apples an effective dietary antioxidant supplement (Eberhardt et al., 2000). Consumption of plant-derived anthocyanin-rich foods and beverages reduces the risk of cardiovascular diseases, and it has been shown that maize kernels specially accumulating the anthocyanins protected rats against ischemia- reperfusion injury (Toufektsian et al., 2008). The red color of apple skin

is very important, and is a significant factor in determining the market value of apple. Both anthocyanin biosynthesis, which is developmentally regulated, and anthocyanin accumulation during ripening in apples are affected by UVB spectrum light and low temperature (Ubi et al., 2006).

Apples (*Malus* spp.), along with pears (*Pyrus* spp.), peaches (*Prunus* spp.), and cherries (*Prunus* spp.), belong to family Rosaceae, and apple is one of the most economically important woody plants cultured for its valuable fruit. An expressed sequence tag (EST) collection of over 150,000 has been produced from various tissues of 'Royal Gala' and 'GoldRush' apples (Newcomb et al., 2006; Park et al., 2006). Analysis of EST data revealed the presence of genes, which are expressed specifically or preferentially in fruit; these genes seem to be involved in the ripening, flavor and aroma production, and biosynthesis of color and health-related compounds (Newcomb et al., 2006; Park et al., 2006). Anthocyanins are responsible for most of the bright red and blue colors found in higher plants, and many steps and genes involved in this mechanism have been previously described (Habu et al., 1998). Previous studies have shown that the UFGT gene is critical for anthocyanin biosynthesis and that the Tow F3′H genes are regulated in the biosynthesis of anthocyanin pigments in grapes and sorghum (Kobayashi et al., 2001; Shih et al., 2006). MYB transcription factors are regulated in anthocyanin accumulation. Transgenic plants with MdMYB1 and MdMYBA, which are related to anthocyanin accumulation in apple skin, develop a red pigment in the fruits (Ban et al., 2007; Takos et al., 2006). Transgenic white-fleshed apples with overexpression of the MdMYB10 gene, which regulates flesh color, showed a red-fleshed phenotype (Espley et al., 2007).

Our previous experiments showed different expression patterns of *UFGT* and *ANS* between the 'Redfield' and 'Greensleeves' cultivars. However, the gene (ORF and promoter region) sequences were not different (data not shown). To identify the differently expressed genes in both the cultivars, we used a suppression subtractive hybridization (SSH) approach to individually identify the genes in the red and green apples. Forty-five red-induced cDNA genes and 44 green-induced cDNA genes were isolated by differential screening of the cDNA libraries. Differential expression patterns of these genes were confirmed by virtual northern analysis.

Materials and Methods

Plant Materials

'Redfield' and 'Greensleeves' apples (*Malus domestica* Borkh.) were used in this study. Plant samples were provided by the National Horticultural Research Institute, Suwon, Korea. The ripening fruits were harvested, immediately frozen in liquid nitrogen, and stored at -80℃ until use.

RNA Isolation

Total RNA was isolated from the frozen samples by using a pine tree method with minor modifications. Fruit sample (3 g) was ground in liquid nitrogen and suspended in 15 mL of pre-warmed extraction buffer [2% CTAB, 2% PVP K-40, 100 mM Tris-Cl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.5 g·L^{-1} spermidine, 0.5 mM ATA, and 2% β-Me]. The mixture was incubated at 65℃ for 30 min. Then, 15 mL of chloroform/isoamyl alcohol (24:1) was added, and the mixture was centrifuged at 12,000 × g for 30 min. For chloroform/isoamyl alcohol (24:1) extraction, the supernatant was transferred to a new tube; 3.5 mL of 10 M LiCl was added to this, and the contents of the tube were mixed and precipitated overnight at 4℃. The pellet obtained after centrifugation was dissolved in 500 μL of DEPC-treated water and mixed with 500 μL of chloroform/isoamyl alcohol (24:1). Following centrifugation, the supernatant was mixed with 1 mL of EtOH and precipitated at -70℃ for 30 min; the total RNA was pelleted by centrifugation at 12,000 × g for 20 min at 4℃. The pellet was washed with 70% EtOH, air dried, and dissolved in 50 μL of DEPC-treated water. For PCR-Select cDNA subtraction, mRNA was extracted from the total RNA by using the oligotex mRNA Midi Kit (Qiagen, Valencia, CA)

Construction of Subtractive cDNA Libraries and Mirror Orientation Selection (MOS)

Suppression subtractive hybridization was performed using the PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA), according to the manufacturer's protocol. Tester and driver cDNAs were made with 3 μg of mRNA from two samples. The differentially expressed cDNAs were used to perform MOS hybridization (Denis et al., 2000). PCR products were diluted to 20-30 ng·μL^{-1}. To remove NP1 adapters, 5 μL of sample was mixed with 1 μL of *Sma*I restriction enzyme, 2 μL of 10 × *Sma*I restriction enzyme buffer, and 12 μL of H_2O. The reaction was performed at 25℃ for 1 h; then, the enzyme was inactivated by incubation at 65℃ for 30 min. One microliter of *Sma*I-digested cDNA was mixed with 1 μL of 4 × hybridization buffer [2 M NaCl, 200 mM HEPES (pH 8.3), and 0.8 mM EDTA] and 2 μL of H_2O, and incubated at 98℃ for 1.5 min in a thermal cycler, and then at 68℃ for 3 h. After hybridization, the sample was mixed with 200 μL of dilution buffer [50 mM NaCl, 20 mM HEPES (pH 8.3), and 0.2 mM EDTA] and heated in a thermal cycler at 70℃ for 7 min. One microliter of diluted cDNA was taken for MOS PCR in a total volume of 20 μL. The PCR reaction mixture contained 0.5 μL of 50 × Advantage polymerase Mix (Clontech, Mountain View, CA), 2 μL of 10 × buffer, 1 μL of NP2Rs, 10 pmol of primer

(5′-GGTCGCGGCCGAGGT), 0.5 μL of dNTPs mix (each 2.5 mM), and 15 μL of H_2O. The PCR mixture was incubated in the thermal cycler at 72℃ for 2 min for amplification. Then the program, 95℃ for 7 s, 62℃ for 20 s, and 72℃ for 2 min, was repeated. The PCR-amplified cDNAs were directly cloned into the pGEM-T easy vector (Promega, Madison, WI), and then, transformed into *Escherichia coli* Mach 1 cells.

Differential Screening of Subtracted cDNA Libraries

Individual clones obtained from 2 subtracted libraries were selected and PCR amplified using the universal primers T7 and Sp6. For blotting, 20 μL of PCR product was mixed with 10 μL of 0.3 N NaOH, 15% Ficoll, and 0.5% bromophenol blue. Then, 2 μL of each denatured sample were transferred to a Hybond-N^+ nylon membrane (GE Healthcare, Buckinghamshire, UK). The membrane was UV cross-linked (UVP, Upland, CA), and neutralized in 0.1M Tris-Cl (pH 7.4) and 2 × standard saline citrate (SSC) buffer before hybridization. The cDNA hybridization probes were prepared from the subtracted cDNAs for the removed non-cultivar specific clones, and the probes were labeled with ^{32}P-dCTP by using a PCR-Select Differential Screening Kit (Clontech, Mountain View, CA).

Virtual Northern Blot Analysis

A total of 1 μg RNA from red and green apples each was used for cDNA synthesis with the smart PCR cDNA synthesis kit (Clontech, Mountain View, CA) according to the manufacturer's protocol. The gel was blotted onto a Hybond-N^+ nylon membrane (GE Healthcare, Buckinghamshire, UK) and hybridized with ^{32}P-labeled apple cDNA probes. To confirm equal loading of cDNA, the gel was stained with ethidium bromide after electrophoresis. After hybridization, the blots were washed twice with washing buffer (0.5 × SSPE + 0.1% SDS) for 25 min. The blot was exposed to an x-ray film (Kodak XAR-5) with intensifying screen at -80℃.

cDNA Sequencing and Analysis

All cDNA sequencing was performed using T7 universal primers. All unique cDNA sequences were compared with the sequence databases by using BLASTn and BLASTx.

Results

Establishment of Two Anthocyanin-related Subtracted cDNA Libraries

To isolate a wide variety of differentially expressed genes, samples were collected from 'Redfield' and 'Greensleeves'. A total of 500 μg of RNA was used to isolate mRNA and then follow it up with PCR-Select cDNA subtraction. After MOS hybridization, many background signals were removed (Fig. 1). Four major and 2 weak bands were observed in the 'Redfield' cultivar, and 3 thick and 9 thin bands were observed in the 'Greensleeves' cultivar. The two subtracted cDNA libraries contained 386 clones in total that were individually selected. The inserts of these clones ranged from 200 to 1,000 bp in length, and most of these were between 400 and 600 bp (Fig. 2). For simplicity, the two libraries are hereafter referred to as the red and green libraries, respectively.

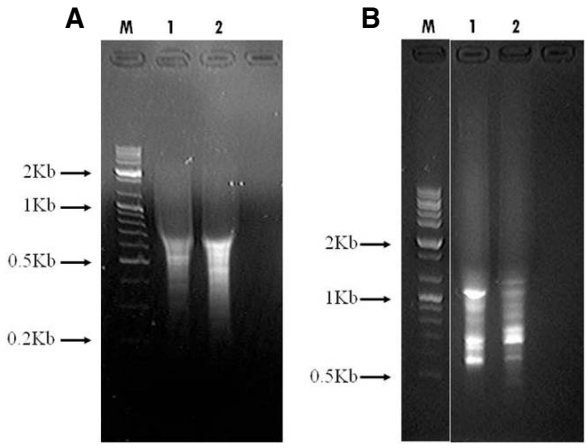

Fig. 1. SSH and MOS PCR. (A) The secondary PCR product of the subtractive 'Redfield' and 'Greensleeves' sample contains cultivar specific DNA fragment. Lane M: 1 Kb plus DNA size marker (Bioneer, Daejeon, Korea). Lane 1: Secondary product of subtracted Redfield testers contained Greensleeves drivers. Lane 2: Secondary product of subtracted Greensleeves testers contained Redfield drivers. (B) Application of the MOS hybridization to the SSH secondary PCR product. MOS hybridization was performed (A) sample and MOS-generated samples are due to the different PCR primers used. Background was removed after MOS hybridization. Lane M: 1 Kb plus DNA size marker, Lane 1: 'Redfield' MOS hybridization product after NP2Rs primers use. Lane 2: 'Greensleeves' MOS hybridization product after NP2Rs primers use.

Sequencing Analysis of Selective cDNA Clones

Initial screening of the libraries to remove false positives was performed by reverse northern blot analysis with total ^{32}P-dCTP-labeled cDNAs from the red and green apples. The findings of dot blot hybridization with 384 clones are shown in Fig. 3 (data shown for only 192 clones). Radioactive spots were excluded, and clones of different insert sizes were obtained from agarose gel electrophoresis. After dot blot hybridization screening, 89 ESTs (45 and 44 clones of different insert sizes from forward and reverse SSH libraries, respectively) were sequenced. Among the 45 clones sequenced from the red library, 42 clones were readable; these sequences represented 20 unique ESTs. Similarly, 44 clones

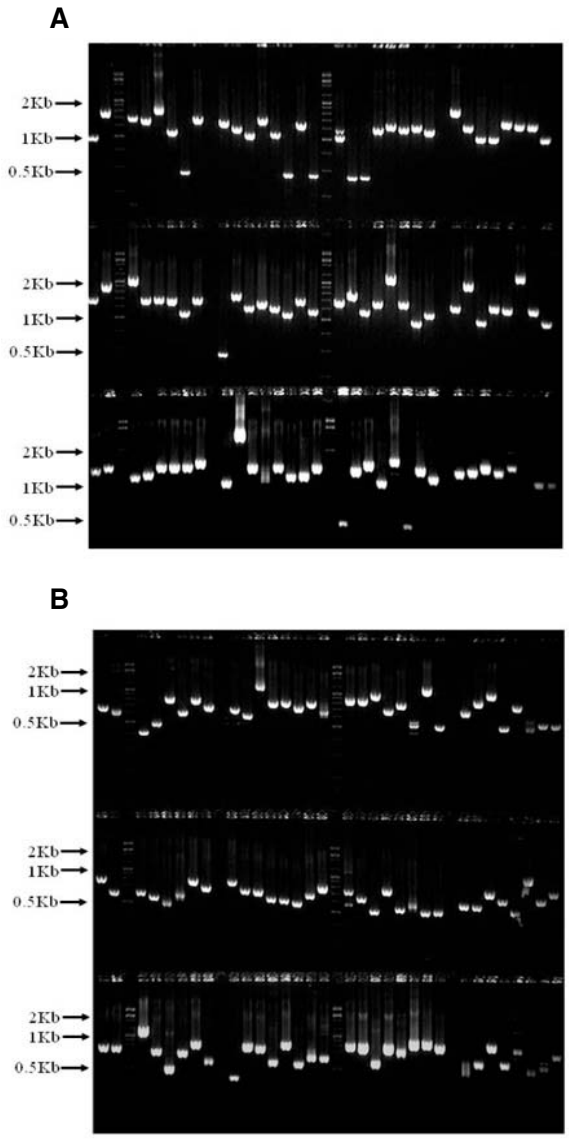

Fig. 2. PCR analysis of inserts of selected clones from the subtracted libraries. After MOS hybridization and PCR products are inserted into T-easy vector. Transformed colonies are polymerized chain reaction using nested primers. (A) Specifically expressed DNA fragments in 'Redfield' fruit are identified using PCR method. PCR primers are used in nested primers and size ranged from 400 to 1,000 bp. 250 bp clones are self ligation vector (B) Expressed fruit DNA fragments in 'Greensleeves' are performed using PCR experiment. Insert sizes are between 300 and 1,000 bp.

representing 20 unique ESTs were readable among the 44 clones sequenced from the green library. The EST sequences obtained were identified by homology searches (Blastn and Blastx) in different databases (non-redundant sequences in NCBI and EST sequences in NCBI). Eighty-nine ESTs exhibited protein homologies to previously identified or putative proteins (Tables 1 and 2). The other 6 ESTs have no significant homologies with any gene in any of the

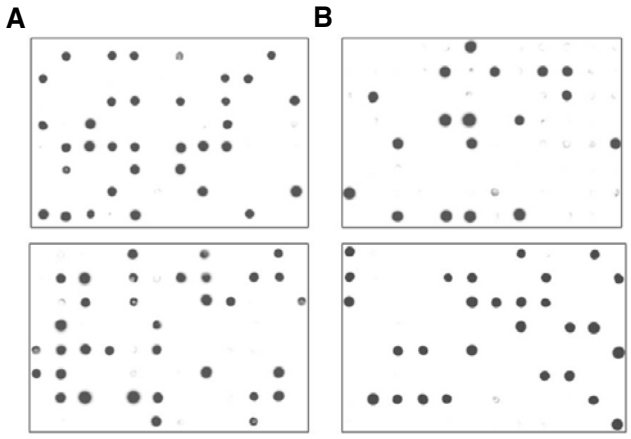

Fig. 3. Dot-blot hybridization of the 'Greensleeves' and 'Redfield' apple fruit cDNA libraries. Putative cultivar specific cDNA obtained by MOS hybridization were dot-blotted onto nylon membrane. The blots were then hybridized with total cDNA probe. (A) Subtracted Redfield fruit libraries and probes were used from Greensleeves fruit total cDNA. (B) Subtracted Greensleeves fruit libraries and Redfield fruit cDNA used as hybridization probe.

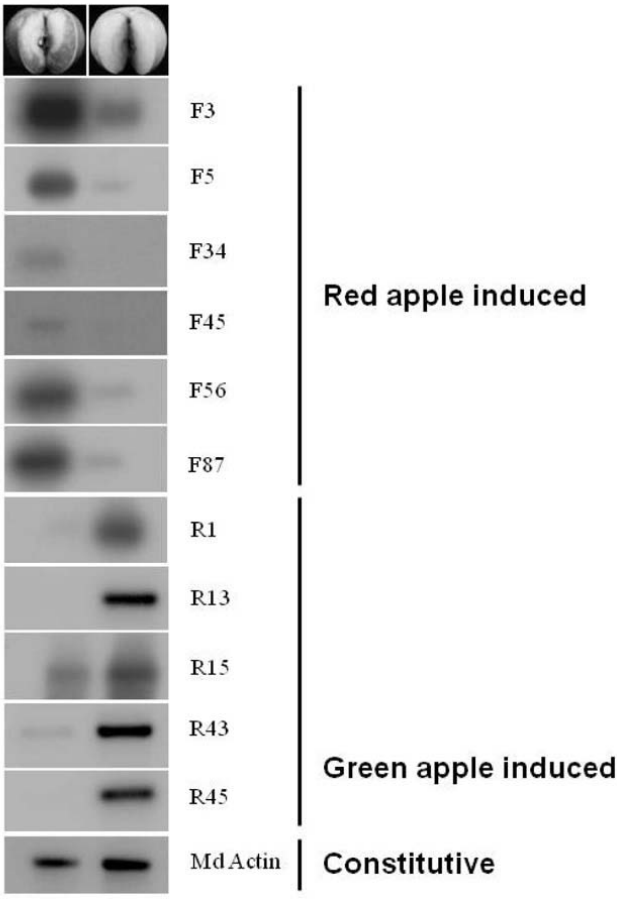

Fig. 4. Virtual Northern blot analysis of selected subtractive cDNA clones. One microgram of total cDNAs were separated by electrophoresis on a 2% agarose gel and blotted to a hybrond-N nylon membrane. To ensure equal loading of cDNA, *MdActin*, constitutively expressed gene, was used as loading control.

Table 1. Identification of EST clones from red peel and flesh apple 'Redfield'.

Clone	Length (bp)	Accession no. of matching sequence	Best e-value (BLASTx)	Source of matching sequence	Matching sequence in public database
F1	618	NP_567473	5.00E-30	*Arabidopsis thaliana*	Integral membrane family protein
F2	478	ACM62743	7.00E-50	*Garcinia mangostana*	Chalcone isomerase
F3	529	BAA96449	4.00E-06	*Pyrus pyrifolia*	Metallothionein-like protein
F4	384	NP_193297	1.00E-23	*Arabidopsis thaliana*	Integral membrane family protein
F5	514	ACM62743	9.00E-51	*Garcinia mangostana*	Chalcone isomerase
F6	828	EEF27183	2.00E-37	*Ricinus communis*	Major latex protein, putative
F7	1069	EEF50464	1.00E-101	*Ricinus communis*	Peroxisomal biogenesis factor, putative
F8	156				No significant similarity found
F9	750	ACN38271	1.00E-37	*Vitis amurensis*	Glutathione S-transferase
F10	401	BAC84694	3.00E-05	*Oryza sativa Japonica* Group	Integral membrane protein-like
F11	635	AAX20964	2.00E-84	*Malus x domestica*	PR-10 protein
F12	373	NP_193297	1.00E-13	*Arabidopsis thaliana*	Integral membrane family protein
F13	380	EEF45487	3.00E-05	*Ricinus communis*	Conserved hypothetical protein
F14	556	NP_567473	1.00E-29	*Arabidopsis thaliana*	Integral membrane family protein
F34	880	XP_002297996	3.00E-74	*Populus trichocarpa*	Predicted protein
F35	863	ACM62743	1.00E-78	*Garcinia mangostana*	Chalcone isomerase
F36	515	ACM62743	2.00E-40	*Garcinia mangostana*	Chalcone isomerase
F37	703	AAL86678	1.00E-78	*Kageneckia oblonga*	NADP dependent sorbitol 6-phosphate dehydrogenase
F38	893	BAA96449	3.00E-06	*Pyrus pyrifolia*	Metallothionein-like protein
F39	338	ABA42223	2.00E-29	*Citrus sinensis*	Glutathione S-transferase
F41	340	ACM62743	3.00E-12	*Garcinia mangostana*	Chalcone isomerase
F42	407	ABD32614	8.00E-24	*Medicago truncatula*	Peptidase T2, asparaginase 2
F43	614	NP_567473	2.00E-40	*Arabidopsis thaliana*	Integral membrane family protein
F45	770	XP_002281338	2.00E-100	*Vitis vinifera*	Brassinosteroid-6-oxidase
F46	873	AAL86680	3.00E-62	*Amelanchier alnifolia*	NADP dependent sorbitol 6-phosphate dehydrogenase
F47	536	NP_567473	7.00E-29	*Arabidopsis thaliana*	Integral membrane family protein
F48	615				No significant similarity found
F49	149				No significant similarity found
F50	537	NP_193297	5.00E-29	*Arabidopsis thaliana*	Integral membrane family protein
F51	630	BAA96449	5.00E-07	*Pyrus pyrifolia*	Metallothionein-like protein
F52	492	NP_567473	2.00E-28	*Arabidopsis thaliana*	Integral membrane family protein
F53	399	EEF45487	3.00E-05	*Ricinus communis*	Conserved hypothetical protein
F54	387	EEF45487	3.00E-05	*Ricinus communis*	Conserved hypothetical protein
F56	802	CAO44183	3.00E-109	*Vitis vinifera*	Unnamed protein product
F57	167				No significant similarity found
F58	843	ACM62743	3.00E-57	*Garcinia mangostana*	Chalcone isomerase
F59	514	ACM62743	8.00E-42	*Garcinia mangostana*	Chalcone isomerase
F61	589	NP_193297	3.00E-09	*Arabidopsis thaliana*	integral membrane family protein
F63	585	NP_567473	6.00E-41	*Arabidopsis thaliana*	Integral membrane family protein
F74	399	NP_193297	1.80E-02	*Arabidopsis thaliana*	Integral membrane family protein
F75	515	ACM62743	8.00E-42	*Garcinia mangostana*	Chalcone isomerase
F80	898	NP_567473	1.00E-19	*Arabidopsis thaliana*	Integral membrane family protein
F82	720	XP_001990516	2.00E+00	*Drosophila grimshawi*	GH18201
F87	310	ABR13280	1.00E-44	*Prunus dulcis*	Unknown protein

Table 2. Identification of EST clones from red peel and flesh apple 'Redfield'.

Clone	Length (bp)	Accession no. of matching sequence	Best e-value (BLASTx)	Source of matching sequence	Matching sequence in public database
R1	249	ABD33278	1.00E-09	*Medicago truncatula*	Gibberellin regulated protein
R2	437	EAQ43178	3.7	*Polaribacter sp. MED152*	Two-component system sensor histidine kinase
R3	730	EEF31838	1.00E-70	*Ricinus communis*	Isoflavone reductase, putative
R4	308	ABC47923	2.00E-16	*Malus x domestica*	Pathogenesis-related protein 5
R5	1041	BAF64462	1.00E-117	*Apple chlorotic leaf spot virus*	RNA polymerase
R6	406	EEF31838	9.00E-23	*Ricinus communis*	Isoflavone reductase, putative
R7	495	ABR13283	1.00E-28	*Prunus dulcis*	Putative lipoxigenase
R9	655	EEF31838	4.00E-54	*Ricinus communis*	Isoflavone reductase, putative
R10	505	BAG48170	3.00E-79	*Malus x domestica*	(E,E)-alpha-farnesene synthase
R11	250	ABD33278	8.00E-11	*Medicago truncatula*	Gibberellin regulated protein
R12	368	EEF30271	6.00E-11	*Ricinus communis*	Glutaredoxin
R13	395	XP_002327028	1.00E-61	*Populus trichocarpa*	Predicted protein
R14	553	ABA96258	5.40E+00	*Oryza sativa* (*japonica* cultivar-group)	Retrotransposon protein
R15	593	XP_002307937	1.00E-22	*Populus trichocarpa*	Predicted protein
R16	553	EEF31838	9.00E-43	*Ricinus communis*	Isoflavone reductase, putative
R17	395	AAX19849	1.00E-29	*Malus x domestica*	Thaumatin-like protein precursor
R18	567	ABX76301	8.00E-82	*Ageratina adenophora*	Heat shock protein 70
R20	565	EEF31838	9.00E-60	*Ricinus communis*	Isoflavone reductase, putative
R21	594	EEF31838	2.00E-44	*Ricinus communis*	Isoflavone reductase, putative
R22	363	AAX19849	1.00E-29	*Malus x domestica*	Thaumatin-like protein precursor
R24	406	EEF31838	4.00E-23	*Ricinus communis*	Isoflavone reductase, putative
R26	402	AAX19849	1.00E-34	*Malus x domestica*	Thaumatin-like protein precursor
R28	390	AAK44147	8.00E-14	*Arabidopsis thaliana*	Putative harpin-induced protein
R30	547	ABA96258	5.20E+00	*Oryza sativa* (*japonica* cultivar-group)	Retrotransposon protein, putative, Ty3-gypsy subclass
R31	552	ABR13283	2.00E-37	*Prunus dulcis*	Putative lipoxigenase
R32	687	ABA96871	1.80E+00	*Oryza sativa* (*japonica* cultivar-group)	Retrotransposon protein, putative, Ty3-gypsy subclass
R40	545	ABR13283	8.00E-59	*Prunus dulcis*	Putative lipoxigenase
R41	566	EEF31838	3.00E-45	*Ricinus communis*	Isoflavone reductase, putative
R42	692				No significant similarity found
R43	702	AAV50009	2.00E-101	*Malus x domestica*	Anthranilate N-hydroxy- cinnamoyl/benzoyltransferase
R44	439	XP_002280479	3.70E+00	*Vitis vinifera*	Hypothetical protein isoform 3
R45	657	EEF31838	2.00E-58	*Ricinus communis*	Isoflavone reductase, putative
R46	656	EEF31838	2.00E-58	*Ricinus communis*	Isoflavone reductase, putative
R47	434	EEF48844	2.00E-65	*Ricinus communis*	lipoxygenase, putative
R48	591	EEF31838	5.00E-48	*Ricinus communis*	Isoflavone reductase, putative
R49	462	ABR13283	8.00E-27	*Prunus dulcis*	Putative lipoxigenase
R50	396	AAV45140	6.50E+00	*Haloarcula marismortui* ATCC 43049	30S ribosomal protein S13P
R73	483	ABA96258	8.20E+00	*Oryza sativa* (*japonica* cultivar-group)	Retrotransposon protein, putative, Ty3-gypsy subclass
R74	507	EEF31838	6.00E-33	*Ricinus communis*	Isoflavone reductase, putative
R75	284	NP_040551	1.00E-40	Apple chlorotic leaf spot virus	Replicase
R79	639	ACE80958	1.00E-82	*Prunus dulcis x Prunus persica*	Putative allergen Pru du 2.01B
R90	322				No significant similarity found
R91	656	EEF31838	2.00E-58	*Ricinus communis*	Isoflavone reductase, putative
R93	548	ABA96258	5.20E+00	*Oryza sativa* (*japonica* cultivar-group)	Retrotransposon protein, Putative, Ty3-gypsy subclass

available public sequence databases.

Confirmation of Subtractive cDNA Expression Pattern by Using Virtual Northern Blot Analysis

Virtual northern analysis was performed to confirm the specifically upregulated genes from the red and green libraries by using RNA from the red and green apples. Six clones, namely, F3 (metallothionein-like protein, GenBank accession No. DT043611), F5 (chalcone isomerase, GenBank accession No. CO900583), F34 (dirigent-like protein, GenBank accession No. DR993699), F45 (brassinosteroid-6-oxidase, GenBank accession No. GO521732), F56 (an unnamed protein product, GenBank accession No. EB141366), and F87 (an unknown protein, GenBank accession No. EB157444), were selected from the red ESTs, and 5 genes, namely, R1 (gibberellin-regulated protein, GenBank accession No. CO902853), R13 (a predicted protein, GenBank accession No. EB152814), R15 (a hypothetical 23.5 KD protein, GenBank accession No. CN445418), R43 (anthranilate N-hydroxycinnamoyl/benzoyltransferase, GenBank accession No. AAV50009), and R45 (isoflavone reductase, GenBank accession No. CV658350), were selected from the green ESTs. The *MdActin* (GenBank accession No. DQ822466) gene was used as an equal loading control gene. The clones F3, F5, F56, and F87 are strongly expressed in the red cultivar, whereas F34 and F45 showed a mild expression pattern. These results indicate that the selected genes are strongly expressed in the red cultivar 'Redfield'. The genes R1, R13, R43, and R45 showed increased expression in the green cultivar, while R15 was expressed at a low level. Thus, these findings confirm that these genes are related to the cultivar-specific expression pattern.

Discussion

In the present study, the specially expressed transcription profiles of the red and green cultivars were compared by SSH analysis. Genes involved in the anthocyanin biosynthesis pathway have been studied for a long time, and it was recently reported that MYB transcription factors are related to anthocyanin accumulation in plants. Most studies have focused on the genes involved in the anthocyanin pathway obtained from simple sequence repeats and single nucleotide polymorphisms. However, we isolated cultivar-specific genes from 'Redfield' and 'Greensleeves' by using SSH, and confirmed that the selected genes were specifically expressed. 'Redfield' cultivar-specific genes were methallothionein-like protein, chalcone isomerase, dirigent-like protein, brassinosteroid-6-oxidase, and unknown proteins. 'Greensleeves' cultivar-specific genes were gibberellins-regulated protein, anthranilate N-hydroxycinnamoyl/benzoyltransferase, isoflavone reductase, and predicted proteins. Methallothionein-like proteins are studied in grapes, bananas, strawberries, and apples. Strawberries, both the wild type *Fragaria vesca* L. and the commercial type *F.* × *ananassa* Duch., showed differently patterns of expression during the ripening process. In wild type strawberry, the genes were downregulated during the ripening stage. But the commercial type strawberry was shown to have a constitutively expressed pattern. The major latex protein was upregulated in the strawberry during the ripening stage (Nam et al., 1999), but in the present study, this cultivar-specific expression pattern was not observed (data not shown). In grapes, these genes are expressed in the leaves and seeds and concomitantly expressed in the fruits (Davies and Robinson, 2000). In *Musa acuminata* (Cavendish banana), 3 homologous metallothioneins (MT) were isolated. MT3 transcript levels were high throughout the ripening Process, and MT3 expression was also greatly enhanced in response to metal stress (Liu et al., 2002). Two MT genes were isolated in apples. AMT1 and AMT2 had different expression patterns during fruit development; AMT1 expression was abundant in the flowers and during the early stages of development, and it decreased as the fruit approached maturity, while AMT2 was not detected in the flowers and young fruit and accumulated in the peel and during fruit development. Both AMT1 and AMT2 genes were upregulated during cold treatment. These results were similar to the expression pattern observed in kiwi fruits (Reid and Ross, 1997). We isolated the MT gene, which was most closely related to the MT gene of *Pyrus pyrifolia* (pear) (GenBank accession No. BAA96449) with 94% identity at the amino acid level. The AMT2 gene was expressed in the peel; therefore, we assume that this gene is related to anthocyanin accumulation. Chalcone isomerase is involved in the flavonoid biosynthetic pathway in the plants as one of the key enzymes that converts chalcone to naringenin (Bednar and Hadcock, 1988). In onions, in case of mutated chalcone isomerase (CHI) gene, the chalcone derivatives, including yellow pigments, accumulate and might be responsible for the golden color in onions (Kim et al., 2004). In *Nicotiana tabacum*, CHI expression suppressed by RNA interference (RNAi) showed the presence of white or pink petals and made yellow pollen. All transgenic plants showed up to 25% reduction in anthocyanin content compared to the wild type (Nishihara, 2005). In *Perilla frutescens* (Labiatae), differential screening was performed to isolate the differentially expressed genes from the red and green perillas, and the PfCHI1 gene was selected. PfCHI1 gene transcript levels were higher in the red than the green perillas (Yamazaki, 2008). The brassinosteroid- 6-oxidase gene was slightly expressed in the 'Redfield' cultivar. In *Vitis vinifera* (grape), transgenic plants with overexpression of the brassionsteroid-6 gene significantly promoted ripening, and treatment with

brassinazole (Brz; brassionsteroid inhibitor) reduced anthocyanin accumulation. But indole-3-acetic acid (IAA) and gibberellic acid (GA) did not affect anthocyanin accumulation (Symons et al., 2006). BRs are known to affect ethylene synthesis in some plants. Our results showed that BRs were related to ripening, which was in accordance with previous studies (Chervin et al., 2004; Yi et al., 1999). Dirigent-like protein controls the synthesis of lignans and lignins in plants. Lignan provides protection against pathogens. *Arabidopsis* spp. have 9 homologous genes; and these genes were expressed in the seed coats, flowers, stems, leaves, and roots (Kim et al., 2002). Unnamed protein products and unknown proteins have functions that are not yet characterized. The specifically expressed genes from the red colored 'Redfield' cultivar encoding MT-like protein, chalcone isomerase, dirigent-like protein, brassinosteroid-6-oxidase, and unknown proteins can be strongly related to anthocyanin accumulation.

Gibberellin-regulated protein, anthranilate N-hydroxycinnamoyl, isoflavone reductase, and 2 unknown proteins showed a 'Greensleeves' cultivar-specific gene expression pattern. Light and sucrose trigger anthocyanin accumulation. In *Arabidopsis* spp., sucrose but neither glucose nor fructose increased the expression of anthocyanin biosynthesis genes and triggered anthocyanin synthesis. The relationship between anthocyanin accumulation and various hormones and sucrose was investigated in *Arabidopsis* spp.; abscisic acid and jasmonate showed synergic effect, but gibberellin treatments repressed anthocyanin biosynthesis (Loreti et al., 2008; Solfanelli et al., 2006; Ubi et al., 2006). Therefore, it is suspected that gibberellin-regulated proteins could suppress anthocyanin accumulation, and particularly be expressed in the 'Greensleeves' cultivar. Anthocyanins are known to be antioxidants for humans, but in grape leaves, they are defense-related genes that become highly expressed after infection with pathogens. Transcription levels of the PR-2, PR-3, and PR-4 genes increased, and the transcript levels of the chalcone synthase (CHS) and CHI genes also upregulated after pathogen infection. The role of anthocyanin in plants is to help resist pathogenic infections (Kortekamp, 2006). Therefore, anthocyanin is a very important compound in plants; if a plant does not accumulate anthocyanin, then they accumulate phytoalexin, another compound important in plant defense (Wu and VanEtten, 2004). Isoflavone formation, catalyzed by isoflavone reductase (IFR), is an intermediate step in the biosynthesis of these compounds. Following the inactivation of *Pisum sativum* L. (pea) IFR genes by RNAi knock-out, the resulting transgenic plants exhibited repressed phytoalexin synthesis and were attacked easily by pathogens (Kortekamp, 2006). We analyzed the transcript levels of anthocyanin synthesis-related genes in the 'Redfield' and 'Greensleeves' cultivars, and found that no anthocyanin synthase genes were expressed in the 'Greensleeves' cultivar (data not shown), which did not accumulate anthocyanins but produced phytoalexin. Anthranilate N-hydroxycinnamoyl/benzoyltransferase (HBCT) catalyzes phytoalexin biosynthesis. In suspension-cultured carnation cells, the expression pattern of HBCT activity showed rapid upregulation within 30 min, followed by a slow decrease (Yang et al., 1998). The HBCT and IFR genes played a role in protection from pathogens by activating phytoalexin synthesis. The functions of the predicted and hypothetical 23.5 KD proteins were not discovered. The cloned and expressed genes from the green-colored cultivar 'Greensleeves', gibberellins-regulated protein, anthranilate N-hydroxycinnamoyl/benzoyltransferase, isoflavone reductase, and predicted proteins, could be involved in anthocyanin biosynthesis, accumulation, or suppression.

Though we could not confirm the role of expressed genes through studies of transgenic plants, the red- and green-colored apple cultivar-specific genes selected from cDNA SSH analysis are likely to be strongly related with anthocyanin biosynthesis or suppression.

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Literature Cited

Adams-Phillips, L., C. Barry, and J. Giovannoni. 2004. Signal transduction systems regulating fruit ripening. Trends Plant Sci. 9:331-338.

Alba, R., P. Payton, Z. Fei, R. McQuinn, P. Debbie, G.B. Martin, S.D. Tanksley, and J.J. Giovannoni. 2005. Transcriptome and selected metabolites analyses reveal multiple points of ethylene control during tomato fruit development. Plant Cell 17:2954-2965.

Ban, Y., C. Honda, Y. Hatsuyama, M. Igarashi, and H. Bessho. 2007. Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. Plant Cell Physiol. 48:958-970.

Bednar, R.A. and G.C. Hadcock. 1988. Purification and characterization of chalcone isomerase from soybeans. J. Biol. Chem. 263: 9582-9588.

Chervin, C., A. El-Kereamy, J.P. Roustan, L. Latche, J. Lamon, and M. Bouzayen. 2004. Ethylene seems required for the berry development and ripening in grape, a non-climacteric fruit. Plant Sci. 167:1301-1305.

Davies, C. and S.P. Robinson. 2000. Differential screening indicates a dramatic change in mRNA profiles during grape berry ripening. cloning and characterization of cDNA encoding putative cell wall and stress response proteins. Plant Physiol. 122:803-812.

Eberhardt, M.V., C.Y. Lee, and R.H. Liu. 2000. Antioxidant activity of fresh apples. Nature 405:903-904.

Espley, R., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and A.C. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor. MdMYB10. Plant J. 49:414-427.

Giovannoni, J. 2001. Molecular biology of fruit maturation and ripening. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52:725-749.
Giovannoni, J. 2004. Genetic regulation of fruit development and ripening. Plant Cell 16:S170-S180.
Goulao, L.F. and C.M. Oliveira. 2007. Molecular identification of novel differentially expressed mRNAs up-regulated during ripening of apples. Plant Sci. 172:306-318.
Habu, Y., Y. Hisatomi, and S. Lida. 1998. Molecular characterization of the mutable flaked allele for flower variegation in the common morning glory. Plant J. 16:371-376.
Kim, M.K., J.H. Jeon, L.B. Davin, and N.G. Lewis. 2002. Monolignol radical-radical coupling networks in western red cedar and *Arabidopsis* and their evolutionary implications. Phytochemistry 61:311-322.
Kim, S., R. Jones, K.S. Yoo, and L.M. Pike. 2004. Gold color in onions (*Allium cepa*): A natural mutation of the chalcone isomerase gene resulting in a premature stop codon. Mol. Gen. Genomics 272:411-419.
Knekt, P., J. Kumpulainen, J.H. Rissanen, M. Heliovaara, A. Reunanen, T. Hakulinen, and A. Aromaa. 2002. Flavonoid intake and risk of chronic diseases. Amer. J. Cli. Nutr. 76:560-568.
Kobayashi, S., M. Ishimaru, C.K. Ding, H. Yakushiji, and N. Goto. 2001. Comparison of UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT) gene sequences between white grapes (*Vitis vinifera*) and their sports with red skin. Plant Sci. 160:543-550.
Kortekamp, A. 2006. Expression analysis of defence-related genes in grapevine leaves after inoculation with a host and a non-host pathogen. Plant Physiol. Biochem. 44:58-67.
Lelievre, J.M., A. Latche, B. Jones, M. Bouzayen, and J.C. Pech. 1997. Ethylene and fruit ripening. Physiol. Plant 101:727-739.
Liu, P., C.J. Goh, C.S. Loh, and E.C. Pua. 2002. Differential expression and characterization of three methallothionein-like genes in Cavendish banana (*Musa acuminate*). Physiol. Plant 114:241-250.
Loreti, E., G. Povero, G. Novi, C. Solfanelli, A. Alpi, and P. Perata. 2008. Gibberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in *Arabidopsis*. New Phytologist 179:1004-1016.
Nam, Y.W., L. Tichit, M. Leperlier, B. Cuerq, I. Marty, and J.M. Leliever. 1999. Isolation and charaterization of mRNAs differentially expressed during ripening of wild strawberry (*Fragaria vesca* L.) fruits. Plant Mol. Biol. 39:629-636.
Newcomb, R.D., R.N. Crowhurst, A.P. Gleave, E.H.A. Rikkerink, A.C. Allan, L.L. Beuning, J.H. Bowen, E. Gera, K.R. Jamieson, B.J. Janssen, W.A. Laing, S. McArtney, B. Nain, G.S. Ross, K.C. Snowden, E.J.F. Souleyre, E.F. Walton, and Y.K. Yauk. 2006. Analysis of expressed sequence tags from apple. Plant Physiol. 141:147-166.
Nisahiro, M., T. Nakatsuka, and S. Yamamura. 2005. Flavonoid component and flower color change in transgenic tobacco plants by suppression of chalcone isomerase gene. FEBS Lett. 579:6074-6078.
Park, S., N. Sugimoto, M.D. Larson, R. Beaudry, and S. van Nocke. 2006. Identification of genes with potential roles in apple fruit development and biochemistry through large-scale statistical analysis of expressed sequence tags. Plant Physiol. 141:811-824.
Reid, S.J. and G.S. Ross. 1997. Up-regulation of two cDNA clones encoding methallothionein-like proteins in apple fruit during cool storage. Physiol. Plant 100:183-189.
Seymour, G.B., J.E. Taylor, and G.A. Tucker. 1993. Biochemistry of fruit ripening. Chapman & Hall, London.
Shih, C.H., I.K. Chu, W.K. Yip, and C. Lo. 2006. Differential expression of two flavonoid 3'-hydroxylase cDNAs involved in biosynthesis of anthocyanins pigments and 3-deoxyanthocyanidin phytoalexins in sorghum. Plant Cell Physiol. 47:1412-1419.
Solfanelli, C., A. Poggi, E. Loreti, A. Alpi, and P. Perata. 2006. Sucrose-specific induction of the anthocyanins biosynthesis pathway in Arabidopsis. Plant Physiol. 140:637-646.
Symons, G.M., C. Davies, Y. Shavrukov, I.B. Dry, and J.B. Reid. 2006. Grapes on steroids. Brassinosteroids are involved in grape berry ripening. Plant Physiol. 140:150-158.
Takos, A., F.W. Jaffe, S.R. Jacob, J. Bogs, S.P. Robison, and A.R. Walker. 2006. Light-induced expression of a MYB gene regulates anthocyanins biosynthesis in red apples. Plant Physiol. 142:1216-1232.
Tanksley, S.D. 2004. The genetic, developmental and molecular bases of fruit size and shape variation in tomato. Plant Cell 16:S181-S189.
Theologis, A. 1992. One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. Cell 70:81-184.
Toufektsian, M.C., M.D. Lorgeril, N. Nagy, P. Salen, M.B. Donati, L. Giordano, H.P. Mock, S. Peterek, A. Matro, K. Petroni, R. Pilu, D. Rotilio, C. Tonelli, J.D. Leiris, F. Boucher, and C. Martin. 2008. Chronic dietary intake of Plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. J. Nutr. 138:747-752.
Ubi, B.E., C. Honda, H. Bessho, S. Kondo, M. Wada, S. Kobayashi, and T. Moriguchi. 2006. Expression analysis of anthocyanin biosynthetic genes in apple skin: Effect of UV-B and temperature. Plant Sci. 170:571-578.
Wu, Q. and H.D. VanEtten. 2004. Introduction of Plant and Fungal Genes intp Pea (*Pisum sativum* L.) Hairy Roots Reduces Their Ability to Produce Pisatin and Affects Their Response to a Fungal Pathogen. MPMI 17:798-804.
Yamazaki, M., M. Shibata, Y. Nishiyama, K. Springob, M. Kitayama, N. Shimada, T. Aoki, S.I. Ayabe, and K. Saito. 2008. Differential gene expression profiles of red and green forms of Perilla frutescens leading to comprehensive identification of anthocyanins biosynthetic genes. FEBS J. 275:3494-3502.
Yang, Q., B. Grimming, and U. Matern. 1998. Anthranilate N-hydroxycinnamoyl/benzoyltansferase gene from carnation : rapid elicitation of transcription and promoter analysis. Plant Mol. Biol. 38:1201-1214.
Yi, H.C., S. Joo, K.H. Nam, B.G. Kang, and W.T. Kim. 1999. Auxin and brassinosteroid differentially regulated the expression of three members of the 1-aminocyclopropane-1-carboxylate synthesis gene family in mung bean (*Vigna radiate* L.). Plant Mol. Biol. 41:443-454.