RESEARCH ARTICLE

Identification of Differentially Expressed Genes Associated with Apple Fruit Ripening and Softening by Suppression Subtractive Hybridization

Zongying Zhang1,2, Shenghui Jiang1,2, Nan Wang1,2, Min Li1,2, Xiaohao Ji1,2, Shasha Sun1,2, Jingxuan Liu1,2, Haifeng Xu1,2, Sumin Qi3, Shujing Wu1,2, Zhangjun Fei4, Shouqian Feng1,2, Xuesen Chen1,2*

1 State Key Laboratory of Crop Biology, Shandong Agricultural University, Tai’an, Shandong, China, 2 College of Horticulture Sciences, Shandong Agricultural University, Tai’an, Shandong, China, 3 College of Plant Protection, Shandong Agricultural University, Tai’an, Shandong, China, 4 Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, New York, United States of America

* chenxs@sdau.edu.cn

Abstract

Apple is one of the most economically important horticultural fruit crops worldwide. It is critical to gain insights into fruit ripening and softening to improve apple fruit quality and extend shelf life. In this study, forward and reverse suppression subtractive hybridization libraries were generated from 'Taishanzaoxia' apple fruits sampled around the ethylene climacteric to isolate ripening- and softening-related genes. A set of 648 unigenes were derived from sequence alignment and cluster assembly of 918 expressed sequence tags. According to gene ontology functional classification, 390 out of 443 unigenes (88%) were assigned to the biological process category, 356 unigenes (80%) were classified in the molecular function category, and 381 unigenes (86%) were allocated to the cellular component category. A total of 26 unigenes differentially expressed during fruit development period were analyzed by quantitative RT-PCR. These genes were involved in cell wall modification, anthocyanin biosynthesis, aroma production, stress response, metabolism, transcription, or were non-annotated. Some genes associated with cell wall modification, anthocyanin biosynthesis and aroma production were up-regulated and significantly correlated with ethylene production, suggesting that fruit texture, coloration and aroma may be regulated by ethylene in ‘Taishanzaoxia’. Some of the identified unigenes associated with fruit ripening and softening have not been characterized in public databases. The results contribute to an improved characterization of changes in gene expression during apple fruit ripening and softening.
Introduction

Apple (Malus domestica) is one of the most economically important and widely cultivated horticultural fruit crops worldwide. The fruits are rich in nutrients, non-nutrient components, polyphenols and other phytochemicals beneficial for human health [1]. However, fruit softening represents a major quality problem for apples in the marketplace. Fruit softening is undesirable as it results in decreased shelf life and lower sensory values [2]. The extent of fruit softening varies greatly among cultivars and the maintenance of fruit firmness is an important trait in apple breeding programs. Extensive research on the metabolic processes and molecular mechanisms involved in ethylene production, ethylene signaling pathway, fruit ripening and softening have been carried out, but a full understanding of these processes and the mechanisms responsible in different apple cultivars remains incomplete [3–5].

Ethylene is the dominant trigger for ripening and softening in climacteric fruit [6]. In tomato mutants, fruit ripening and softening are inhibited as a result of abnormal synthesis of ethylene [7, 8]. In apple, fruit softening coincides with the increase of endogenous ethylene production during ripening [3, 6], which can be enhanced by exogenous ethylene treatment and suppressed by inhibitors of ethylene action, such as 1-methylcyclopropene (1-MCP) [4]. There are two committed steps in the biosynthesis of ethylene, namely the formation of amino-cyclopropane-1-carboxylic acid (ACC) and its conversion to ethylene, which are encoded by the ACC synthase (ACS) and ACC oxidase (ACO) gene families, respectively [9]. Moreover, MdACS3a may determine the ethylene production and shelf life of apple fruits by acting as a switch in the transition between system-1 and system-2 ethylene synthesis [10]. Ethylene biological effects are achieved through genes in the ethylene signaling pathway, including ethylene receptors (ETRs), constitutive triple response 1 (CTR1), ethylene insensitive 2 (EIN2), EIN3/EIN3-like (EILs) and ethylene responsive factors (ERFs) [11, 12]. ERFs act by binding to the GCC-box element in promoters of genes responsive to ethylene [13]. In kiwifruit, AdEIL2 and AdEIL3 are involved in fruit ripening by activating transcription of AdACO1 [14]. In tomato, anti-sense LeERF1 fruits show a longer shelf life, suggesting that LeERF1 positively modulates fruit ripening and softening [15].

In addition to ethylene and the ethylene signaling pathway, enzymes that modify cell wall pectic and hemicellulosic polysaccharides are associated with fruit ripening and softening. Differences in the softening rates of ‘Scifresh’ and ‘Royal Gala’ may reflect cell wall structure, which is closely associated with activities of pectin methylesterase (PME) and polygalacturonase (PG) [16]. Fruit ripening and softening are closely associated with PG expression level in ‘Golden Delicious’ and ‘Fuji’ apples [17, 18]. Harb et al. suggest that higher expression levels of PG, pectin lyase, α-L-arabinofuranosidase (AF), xylanogucan endotransglycosylase 2 (XET2) and expansin 2 (EXP2) result in softening of ‘McIntosh’ apples [3]. It is suggested that AF and β-galactosidase (β-Gal) may be more closely associated with shelf life of apple than PG and PME, especially at the onset of fruit ripening and softening [19]. The above-mentioned results indicate the complexity of the regulatory mechanisms of fruit ripening and softening among different apple cultivars and the necessity for further study.

The present study focused on the apple cultivar ‘Taishanzaoxia’, which is an early-ripening cultivar with excellent fruit appearance and quality characteristics [20]. ‘Taishanzaoxia’ is extremely sensitive to ethylene and fruit firmness decreases rapidly during the late development period coincident with a burst of ethylene production, which hinders its promotion in the apple industry [12, 20]. However, these attributes make the cultivar ideal material for investigation of the mechanisms of fruit ripening and softening, especially the role of ethylene. Li et al. observe that hypersensitive ethylene signaling and MdPG1 expression lead to fruit ripening and softening in ‘Taishanzaoxia’ [12]. Although 1-MCP treatment significantly suppresses
expression of \textit{MdPG1}, fruit firmness still declines but at a slower rate [20]. Other studies of apple show that \textit{PG1}-suppressed fruits are significantly softer than \textit{ACOI}-suppressed fruits [18]. These results suggest that, in addition to \textit{MdPG1}, a suite of enzymes is required for fruit ripening and softening in ‘Taishanzaoxia’.

Subtractive suppression hybridization (SSH) is a method widely used for separation of genes differentially expressed at low levels in two closely related DNA samples [21]. Previous studies have successfully separated differentially expressed genes involved in fruit ripening and softening from apple, banana and Chinese jujube [21–23]. In the present study, forward and reverse SSH libraries were constructed to isolate up- or down-regulated genes involved in fruit ripening and softening in ‘Taishanzaoxia’. The results contribute to improved characterization of the changes in gene expression that occur during fruit ripening and softening in apple. Moreover, elucidation of the regulatory mechanisms of fruit ripening and softening in different apple cultivars is critical for germplasm research, which is beneficial for optimization of fruit quality and fruit breeding programs.

**Materials and Methods**

**Plant material and RNA isolation**

Fruits of apple ‘Taishanzaoxia’ were obtained from the Shandong Agricultural University fruit breeding orchard (36°26′N, 117°29′E) located in Tai’an, Shandong, China. The fruits were harvested at six development stages (40, 50, 60, 65, 70, 75 days after full bloom) and immediately transferred to the laboratory. Fruits that were free of mechanical injury, insects and diseases were selected for the experiment. The fruits were cut into approximately 1 cm² pieces, frozen in liquid nitrogen and stored at −80°C until use.

For each sample, 2 g of fruit tissue was quickly ground into a fine powder in liquid nitrogen with a mortar. Total RNA was isolated using the RNAprep Pure Kit (TIANGEN, Beijing, China) following the manufacturer’s protocol. One microgram of RNA was run on a 1% agarose gel to check the integrity. The concentration (ng μL⁻¹) and quality (A₂₆₀/A₂₈₀) of the total RNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

**Determination of fruit firmness and ethylene**

**Fruit firmness.** The firmness of unpeeled fruit was measured with a TA.XT plus texture analyzer (Stable Microsystems, Godalming, U.K.) with a P/2 columnar probe (2 mm diameter). The test parameter was set as, pre-test speed 2 mm s⁻¹, test speed 1 mm s⁻¹, post-test speed 10 mm s⁻¹, depth of penetration 10 mm, trigger force 10 g. The firmness was automatically obtained by Texture Exponent 32. Each fruit was punctured twice near the equator, and eight replicates were used to test the fruit firmness.

**Ethylene measurement.** Two fruits were sealed in a 1.5 L glass jar and kept at room temperature (24°C) for 6 h. Headspace samples (1 mL) were collected and analyzed with a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector. The temperatures of the separation column and detector were 70°C, 120°C respectively; nitrogen and hydrogen were used as the carrier gas at 20 mL min⁻¹, 50 mL min⁻¹. Ethylene production rate was calculated by peak area quantification. The average ethylene concentration from three jars was calculated and used in further analyses.
SSH library construction

Two SSH libraries were constructed to isolate differentially expressed genes during fruit ripening and softening. The forward library was constructed to isolate up-regulated genes with samples at 60 days after full bloom (DAFB) as the driver and samples at 70 DAFB as the tester. The reverse library was generated using reciprocal samples to isolate down-regulated genes (S1 Fig).

The SSH libraries were constructed using the PCR-Select™ cDNA Subtraction Kit (Clontech, Mountain View, CA, USA), starting with 2 mg mRNA from the tester and driver samples. The mRNA was isolated from total RNA using the FastTrack® MAG mRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA). Differentially expressed cDNA fragments digested with Rsal after a two-round PCR selection were cloned into the pMD18-T vector and transformed into Escherichia coli strain DH5a (Invitrogen). Positive clones from LB plates containing 50 mg L\(^{-1}\) ampicillin and X-Gal/IPTG were selected for PCR amplification to identify the insert sizes.

PCR amplification was performed to test the subtraction efficiency. The PCR reactions were performed in a total volume of 30 μL and included 22.4 μL sterile water, 3 μL 10× buffer, 1.2 μL of each primer (10 μM), 0.6 μL dNTPs (10 mM), 0.6 μL Taq DNA polymerase (5 U μL\(^{-1}\)) (Invitrogen) and 1 μL 10-fold diluted subtracted cDNA (2° PCR product) or unsubtracted tester control (2° PCR product). Each PCR was performed as follows: 18 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 2 min. Remove 5 μL PCR product from each reaction into a clean tube, and put the rest of the reaction back into the thermal cycler for 5 additional cycles. Repeat the step twice and then examine the 5 μL samples (removed from each reaction after 18, 23, 28 and 33 cycles) on a 1% agarose gel.

Amplification of cDNA inserts

The primers M13-47 and RV-M were used for PCR amplification of cDNA inserts from white colonies. The PCR reactions were performed in a total volume of 20 μL and included 15.2 μL sterile water, 2 μL 10× buffer, 0.5 μL of each primer (20 μM), 0.5 μL dNTPs (10 mM), 0.3 μL Taq DNA polymerase (5 U μL\(^{-1}\)) (Invitrogen) and 1 μL bacterial culture. Each PCR was performed as follows: 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 3 min, and a final extension at 72°C for 10 min. The PCR products were electrophoresed in 1% agarose gel to confirm the amplification. A subset of positive clones for which PCR products were longer than 100 bp was selected for preparation of plasmid DNA using the Plasmid DNA Extraction Kit (TIANGEN).

Sequencing and data analysis

The selected positive clones were sequenced with the universal M13 sequencing primer. The raw expressed sequence tag (EST) sequences were generated from sequencing files with the software Phred [24]. The vector, adaptor and low-quality bases were removed from raw ESTs using LUCY [25] and the resulting ESTs were assembled into unigenes using iAssembler [26] with minimum overlap of 40 bp and minimum percent identity of 97. The resulting unigene sequences were compared against the GenBank non-redundant (nr), UniProt (TrEMBL/SwissProt) and Genome Database for Rosaceae. Each unigene was annotated with Gene Ontology (GO) terms and classified into GO slim categories (http://www.geneontology.org/GO.slims.shtml).

Real-time quantitative RT-PCR analysis

Differential expression analysis of the genes was performed with quantitative real-time RT-PCR (qRT-PCR) in triplicate. First-strand cDNA was synthesized from 1 μg total RNA
using the miRcute miRNA First-strand cDNA Synthesis Kit (TIANGEN). The qRT-PCR reactions were performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), containing 1 μL cDNA (5-fold dilution), 10 μL TransStart™ Top Green qPCR SuperMix (Transgene, Beijing, China), 1 μL of each primer and 7 μL ddH2O. The qRT-PCR program was set as 94°C for 30 s, 40 cycles of 94°C for 5 s, 58°C for 15 s and 72°C for 10 s. The Actin gene served as an internal control and the relative quantification of specific mRNA levels was performed using the cycle threshold (Ct) 2\(^{-\Delta\Delta Ct}\) method [27]. Primers used in this study were designed with Primer 6 (S1 Table).

Results
Construction of SSH libraries
Ethylene production and fruit firmness were measured during fruit development period to determine sampling time points for construction of SSH libraries. Ethylene production continued to increase during fruit development period while fruit firmness declined continuously (Fig 1). The ethylene biosynthesis burst at 60–70 DAFB accompanied a dramatic decline in fruit firmness. Important gene expression and physiological changes were predicted to occur during this period. Therefore, samples at 60 DAFB and 70 DAFB were used for construction of SSH libraries to identify differentially expressed genes involved in fruit ripening and softening. The subtraction efficiency of Actin in two libraries was tested by PCR application, and it was as high as 2\(^{10}\)-fold, suggesting the subtraction was efficient (S2 Fig). The total number of clones in the forward and reverse libraries was 2600 and 2400, respectively.

EST sequencing and assembly
A total of 1032 clones (504 clones in forward library and 528 clones in reverse library) were selected and sequenced, with the positive rate of 89% and 88%, respectively. After removal of low-quality sequences and sequences of bacterial origin, 918 high-quality ESTs ranging between 200 and 1000 bp were obtained, with an average length of 421 bp. These ESTs were further assembled into 648 unigenes, with an average length of 437 bp, among which 67 were contigs (assembled from multiple EST sequences) and 581 were singletons (sequences that could not be assembled into a contig) (Table 1). The number of EST members in unigenes varied from one to 96, with most of the unigenes (581, 90%) present in low copy numbers. Most contigs obtained (70.1%) were composed of two ESTs (Fig 2).

Annotation and functional cataloging
The unigenes were further analyzed by searching against the National Center for Biotechnology Information non-redundant (nr) database and Genome Database for Rosaceae using the BLASTX program. A total of 443 (68.4%) unigenes showed significant similarities to known functional gene sequences in the database, and 34 showed matches against genes of unknown function or hypothetical proteins. The remaining 171 sequences did not match with any gene sequence in the database (Fig 3).

The unigenes were further annotated with GO terms and GO slim categories. Based on the GO annotations, the unigenes were classified into three ontology categories and 94 GO slims. A total of 390 unigenes were assigned to 44 GO slims in the biological process category, 356 to 25 GO slims in the molecular function category, and 381 to 25 GO slims in the cellular component category (Fig 3).

In the biological process category, ‘cellular process’ was the most prevalent (9% of sequences), followed by ‘biosynthesis process’ (7%) and ‘response to stress’ (6%). In addition, ‘fruit ripening’
Identification of Differentially Expressed Genes during Apple Fruit Ripening and Softening by SSH

(0.1%), regulation of gene expression (0.5%), secondary metabolic process (1.7%) and signal transduction (2.4%) were also identified in the biological process category. In the molecular function category, 'binding' was the most prevalent (16%) and 'hydrolase activity' (10%), 'sequence-specific DNA binding transcription factor activity' (1.1%) and 'transporter activity' (2.5%) were also identified. In the cellular component category, 'membrane' (12%) was the most dominant term, followed by 'cell wall' (3%) (Fig 4A).

Identification of fruit ripening- and softening-related genes

Although a large amount of sequence information was generated from the SSH libraries in this study, only 68 unigenes were considered to be relevant to fruit ripening and softening on the basis of GO annotations and the higher number of these genes that were sorted into different categories based on their putative functions (Table 2). The largest category, consisting of 13 (19%) genes, was associated with cell wall modification, such as XET2, XET10, pectinesterase.

Table 1. Summary statistics for the forward and reverse SSH libraries.

| Name of library | Tester DAFB | Driver DAFB | No. of clones | High quality sequence | No. of contigs | No. of singletons | No. of unigenes | Description of transcript clones |
|-----------------|-------------|-------------|---------------|-----------------------|---------------|------------------|----------------|----------------------------------|
| Forward library | 70          | 60          | 504           | 451                   | 51            | 305              | 356            | Up-regulated in flesh tissues   |
| Reverse library | 60          | 70          | 528           | 467                   | 47            | 276              | 323            | Down-regulated in flesh tissues |
| Total           | —           | —           | 1032          | 918                   | 67^a          | 581              | 648^a          | Differentially expressed in flesh tissues |

Note:
^a means 32 contigs were consisted with ESTs from both forward and reverse library.

doi:10.1371/journal.pone.0146061.t001

doi:10.1371/journal.pone.0146061.g001
(PE) and β-Gal. Twelve (18%) genes were predicted to be associated with anthocyanin biosynthesis (4CL, ANR e.g.), aroma production (FAD, LOX e.g.) and stress response. Among the differentially expressed genes, genes associated with metabolism (AM and ACO1) and transport accounted for 9% and 6% of the total, respectively. In addition, transcription factors such as ERF2, bHLH137, F-box and MADS-box were isolated from the SSH libraries, accounting for 10% of ripening- and softening-related genes.

Fig 2. Distribution of the number of EST members in each unigene.
doi:10.1371/journal.pone.0146061.g002

Fig 3. Annotation statistics for unigenes. Unigenes annotated as known proteins with E-value threshold $9 \times 10^{-6}$. Total numbers of unigenes, and unigenes unmatched in BLAST searches, without annotation and with annotation were presented. The unigenes were annotated with GO terms and grouped in three major categories: biological process, molecular function and cellular component.
doi:10.1371/journal.pone.0146061.g003
Analysis of differential expression by qRT-PCR

To validate the SSH data, 26 differentially expressed unigenes associated with cell wall modification, anthocyanin biosynthesis, aroma production, stress response, transcription, metabolism, transport, and non-annotated unigenes were analyzed by qRT-PCR (Fig 4B). Most genes...
| Unigene No. | Length (bp) | Accession No. | Annotation | Organism of Best Homology | E-Value | Source |
|------------|-------------|---------------|------------|---------------------------|---------|--------|
| UN061*     | 746         | MDP0000144790 | xyloglucan endotransglucosylase/hydrolase | Malus x domestica | 0       | FS     |
| UN088      | 499         | MDP0000320366 | probable exocyst complex component 6  | Malus x domestica | 2E-176  | RS     |
| UN203*     | 378         | MDP0000157209 | cinnamyl alcohol dehydrogenase         | Malus x domestica | 3E-115  | RS     |
| UN286      | 404         | MDP0000162904 | pectinesterase-like                     | Malus x domestica | 7E-98   | RS     |
| UN289      | 509         | MDP0000277149 | Pectate lyase precursor                 | Malus x domestica | 1E-177  | RS     |
| UN313      | 428         | MDP0000408791 | annexin D1                             | Malus x domestica | 1E-105  | RS     |
| UN357*     | 350         | MDP0000225360 | 40S ribosomal protein S14              | Malus x domestica | 2E-101  | RS     |
| UN402      | 459         | MDP0000168331 | probable exocyst complex component 6   | Malus x domestica | 6E-83   | FS     |
| UN443      | 479         | MDP0000218946 | β-1,3-galactosyltransferase 2           | Malus x domestica | 1E-115  | RS     |
| UN469      | 803         | MDP0000866084 | β-D-galactosidase                       | Malus x domestica | 0       | RS     |
| UN565*     | 500         | MDP0000320017 | xyloglucan endotransglycosylase 2       | Malus x domestica | 2E-139  | RS     |
| UN641*     | 412         | MDP0000295662 | Glucan endo-1,3-β—glucosidase precursor | Malus x domestica | 3E-103  | FS     |

**Anthocyanin biosynthesis**

| Unigene No. | Length (bp) | Accession No. | Annotation                          | Organism of Best Homology | E-Value | Source |
|------------|-------------|---------------|-------------------------------------|---------------------------|---------|--------|
| UN011      | 531         | MDP0000195254 | snakin-1                            | Malus x domestica         | 1E-149  | FS     |
| UN015*     | 834         | MDP0000221213 | anthocyanin reductase-like          | Malus x domestica         | 0       | RS     |
| UN043      | 779         | MDP0000478473 | cytochrome P450 716B2-like          | Malus x domestica         | 0       | FS     |
| UN054      | 880         | MDP0000321833 | calreticulin-3-like                 | Malus x domestica         | 0       | FS     |
| UN368      | 700         | MDP0000248148 | glucosyltransferase                 | Malus x domestica         | 0       | FS     |
| UN422      | 751         | MDP0000593031 | UDP-glycosyltransferase 87A2-like    | Malus x domestica         | 0       | FS     |
| UN459      | 363         | MDP0000371499 | isoflavone reductase-like protein 6  | Malus x domestica         | 5E-31   | FS     |
| UN481      | 493         | MDP0000219282 | glycosyltransferase UGT88A1          | Malus x domestica         | 1E-44   | FS     |
| UN499      | 318         | MDP0000523205 | naringenin,2-oxoglutarate 3-dioxygenase | Malus x domestica         | 3E-180  | FS     |
| UN504*     | 393         | MDP0000229348 | cinnamoyl acid hydroxylase          | Malus x domestica         | 3E-131  | FS     |
| UN511      | 879         | MDP0000568913 | antranilate N-hydroxycinnamoyl/     | Malus x domestica         | 0       | FS     |
| UN514*     | 391         | MDP0000277093 | 4-coumarate-CoA ligase              | Malus x domestica         | 2E-27   | FS     |

**Aroma production**

| Unigene No. | Length (bp) | Accession No. | Annotation                          | Organism of Best Homology | E-Value | Source |
|------------|-------------|---------------|-------------------------------------|---------------------------|---------|--------|
| UN046*     | 400         | MDP0000284275 | fatty acid desaturase 2             | Malus x domestica         | 9E-147  | FS     |
| UN114      | 222         | MDP0000806502 | 4-hydroxyphenylpyruvate dioxygenase  | Malus x domestica         | 1E-104  | RS     |
| UN118*     | 650         | MDP0000184619 | GDSL esterase/lipase                | Malus x domestica         | 0       | RS     |
| UN244*     | 278         | MDP0000753947 | lipoxigenase                        | Malus x domestica         | 5E-95   | FS     |
| UN316*     | 557         | MDP0000233141 | acetyl-CoA carboxylase               | Malus x domestica         | 2E-111  | RS     |
| UN346      | 416         | MDP0000412863 | probable ribose-5-phosphate isomerase | Malus x domestica         | 5E-62   | FS     |
| UN393      | 557         | MDP0000169311 | linoleate 13S-lipoxygenase 2—1       | Malus x domestica         | 7E-136  | FS     |
| UN414      | 443         | MDP0000298065 | aspartate aminotransferase 2         | Malus x domestica         | 3E-103  | FS     |
| UN474*     | 496         | MDP000027939 | O-methyltransferase                  | Malus x domestica         | 2E-151  | FS     |
| UN510*     | 231         | MDP0000129670 | fatty acid desaturase 5             | Malus x domestica         | 8E-07   | FS     |
| UN536      | 522         | MDP0000298646 | cytochrome P450                      | Malus x domestica         | 0       | FS     |
| UN599      | 822         | XM_008369286.1 | DNA-(apurinic or apyridimidic site) lyase | Malus x domestica         | 0       | FS     |

**Stress response**

| Unigene No. | Length (bp) | Accession No. | Annotation                          | Organism of Best Homology | E-Value | Source |
|------------|-------------|---------------|-------------------------------------|---------------------------|---------|--------|
| UN029      | 410         | MDP0000867730 | high molecular weight heat shock protein | Malus x domestica         | 7E-21   | FS     |
| UN044      | 821         | MDP0000207137 | glutathione peroxidase 8             | Malus x domestica         | 0       | RS     |
| UN055      | 468         | MDP0000128468 | abscisic stress ripening protein     | Malus x domestica         | 4E-69   | RS     |

(Continued)
(25, 96%) were up-regulated during fruit development period. Only one MADS-box gene showed a down-regulated expression pattern, which accounted for 80% of the total expression during 40–60 DAFB and was negatively correlated with ethylene production. The expression of genes associated with cell wall modification (XET2, XET10, β-Glu, β-Gal and CAD), anthocyanin biosynthesis (4CL and bHLH) and aroma production (FAD2 and O-methyltransferase) was significantly up-regulated during 60–70 DAFB and showed a significant positive

| Unigene No. | Length (bp) | Accession No. | Annotation | Organism of Best Homology | E-Value | Source |
|-------------|-------------|---------------|------------|---------------------------|---------|--------|
| UN112*      | 307         | MDP0000764121 | cysteine protease | Malus x domestica          | 9E-174  | RS     |
| UN137       | 363         | MDP000198482  | glyceraldehyde-3-phosphate dehydrogenase | Malus x domestica | 4E-53   | RS     |
| UN180       | 346         | MDP000150587  | putative aconitase | Malus x domestica | 0       | RS     |
| UN254       | 520         | MDP000625137  | protein disulfide isomerase | Malus x domestica | 3E-150  | RS     |
| UN403*      | 479         | MDP000260947  | aldehyde dehydrogenase | Malus x domestica | 0       | FS     |
| UN523       | 434         | MDP000413395  | NADH-cytochrome b5 reductase-like protein | Malus x domestica | 2E-104  | FS     |
| UN567       | 533         | MDP000120022  | Acid phosphatase 1 precursor | Malus x domestica | 1E-180  | FS     |
| UN586       | 412         | MDP000519575  | peroxiredoxin | Malus x domestica | 0       | FS     |
| UN609       | 798         | MDP000239328  | methylmalonate-semialdehyde dehydrogenase | Malus x domestica | 0       | FS     |

Table 2. (Continued)

| UniGene No. | Length (bp) | Accession No. | Annotation | Organism of Best Homology | E-Value | Source |
|-------------|-------------|---------------|------------|---------------------------|---------|--------|
| UN041       | 584         | MDP000295589  | ARF domain class transcription factor | Malus x domestica | 0       | FS     |
| UN212       | 410         | MDP000534977  | TCP domain class transcription factor | Malus x domestica | 1E-133  | RS     |
| UN386       | 856         | MDP000262032  | NAC domain class transcription factor | Malus x domestica | 0       | FS     |
| UN490*      | 444         | MDP000517257  | ethylene response factor 2 | Malus x domestica | 9E-70   | FS     |
| UN496*      | 666         | MDP00013331   | MADS-box protein | Malus x domestica | 0       | FS     |
| UN575       | 521         | MDP000269701  | DNA-directed RNA polymerase subunit | Malus x domestica | 6E-176  | FS     |
| UN596*      | 482         | MDP000261131  | F-box family protein | Malus x domestica | 6E-142  | FS     |
| UN600*      | 493         | MDP000410728  | transcription factor bHLH137 | Malus x domestica | 2E-58   | FS     |

Metabolism

| UniGene No. | Length (bp) | Accession No. | Annotation | Organism of Best Homology | E-Value | Source |
|-------------|-------------|---------------|------------|---------------------------|---------|--------|
| UN018*      | 326         | MDP000195885  | 1-aminocyclopropane-1-carboxylate oxidase 1 | Malus x domestica | 3E-158  | FS     |
| UN030       | 541         | MDP000793077  | cobalamin-independent methionine synthase | Malus x domestica | 5E-115  | FS     |
| UN070       | 816         | MDP000661864  | phosphoenolpyruvate carboxylase kinase | Malus x domestica | 0       | RS     |
| UN166       | 514         | MDP000293776  | phosphofructokinase beta subunit | Malus x domestica | 0       | RS     |
| UN437*      | 490         | MDP000196961  | β-amylase | Malus x domestica | 1E-140  | FS     |
| UN542       | 284         | MDP000221561  | malic enzyme | Malus x domestica | 2E-153  | FS     |

Transport

| UniGene No. | Length (bp) | Accession No. | Annotation | Organism of Best Homology | E-Value | Source |
|-------------|-------------|---------------|------------|---------------------------|---------|--------|
| UN199*      | 325         | MDP000281884  | sugar transporter | Malus x domestica | 2E-23   | RS     |
| UN302       | 695         | MDP000312731  | thioredoxin domain-containing protein 9 | Malus x domestica | 0       | RS     |
| UN306       | 570         | MDP000767304  | ADP-ribosylation factor | Malus x domestica | 0       | RS     |
| UN309       | 499         | MDP000181026  | Endoplasmic reticulum vesicle transporter protein | Malus x domestica | 5E-47   | RS     |

Not annotated

| UniGene No. | Length (bp) | Accession No. | Annotation | Organism of Best Homology | E-Value | Source |
|-------------|-------------|---------------|------------|---------------------------|---------|--------|
| UN376*      | 414         | MDP000223932  | hypothetical protein | Malus x domestica | 2E-144  | FS     |
| UN525*      | 400         | MDP000233440  | uncharacterized protein | Malus x domestica | 6E-86   | FS     |

Note: * means unigenes chosen for qRT-PCR analysis.

doi:10.1371/journal.pone.0146061.002
correlation with ethylene production. Moreover, the expression of ACO1, ERF2 and UN525 was also significantly correlated with ethylene production (Table 3). Although it was not significantly correlated with ethylene production, they were all up-regulated during 60–70 DAFB, such as LOX, AM and GDSL esterase/lipase et al (Fig 4B).

Discussion

Crispness and a sweet-tart flavor in apples are sought-after consumer traits, with texture being a principal quality attribute [3]. Cultivars such as ‘Fuji’ are widely cultivated around the world because of their excellent fresh quality and longer shelf life. A burst of ethylene production occurs during late development period of ‘Taishanzaoxia’ fruit, and 1-MCP treatment significantly inhibits ethylene production, which indicates that ‘Taishanzaoxia’ is extremely sensitive to ethylene [12, 20]. Based on previous studies, we propose that apple cultivars can be divided into three types according to sensitivity to ethylene: ethylene-insensitive (‘Fuji’ e.g.), ethylene-intermediate (‘Golden Delicious’ e.g.) and ethylene-sensitive (‘Taishanzaoxia’, ‘Jonagold’ e.g.) [28]. The burst of ethylene production has negative effects on fruit quality but provides a suitable model for investigation of fruit ripening and softening processes dependent on ethylene.

Ethylene biosynthesis and signal transduction

MdACS1 is linked to fruit internal ethylene concentration (IEC) with the MdACS1-1 allele linked to high IEC [5]. We found that the allelotype of ‘Taishanzaoxia’ was MdACS1-1/-1, which was consistent with elevated expression of MdACS1 and ethylene burst during the late development period (data not shown). Previous studies have shown that MdACO1 independently affects the internal ethylene concentration and MdACO1-suppressed apple fruits produce no detectable increase in endogenous ethylene concentration [6, 29]. ERFs represent the last step in the ethylene signaling pathway, and the increased expression of MdERF2 in ripening fruits is repressed by 1-MCP treatment [30]. In this study, ACO1 and ERF2 were isolated from the forward library and were up-regulated during fruit development period and significantly correlated with ethylene production (Fig 4B, Table 3). Consistent with the present results, the elevated expression of ACO1 and ERF2 is accompanied by increased ethylene production, which suggests that both ACO1 and ERF2 play an important role in regulation of fruit ripening and softening through ethylene biosynthesis and signal transduction.

Cell wall modification

Fruit softening is probably caused by the cumulative effect of a range of modifications occurring in the network of polymers that make up the primary cell wall [31]. In the present study, six unigenes associated with cell wall modification were identified, namely XET2, XET10, PE, β-Glu, β-Gal and CAD. XET is thought to play a key role in fruit softening through disassembly of xylloglucan and preparation for further modification by other enzymes [32]. PE catalyzes the hydrolytic de-esterification of pectins causing pectic chain esterification, which is further hydrolyzed to pectate by polygalacturonase [33]. β-Glu is likely to target the glucan backbone of xylloglucan, the hemicellulosic polysaccharide that bridges cellulose microfibrils [34]. A decrease in the galactose content of the cell wall is associated with an increase of β-Gal activity during fruit ripening [35]. CAD is a key enzyme involved in lignin biosynthesis, which is an important component of secondary cell walls and provides essential strength, hydrophobicity and resistance to the harsh external environment to the cell wall [36]. Expression of the five genes (XET2, XET10, β-Glu, β-Gal and CAD) was significantly correlated with fruit firmness and ethylene production, suggesting that fruit softening was closely associated with elevated expression of these ethylene-regulated genes. In the soft/crisp strains of Malus sieversii f.
niedzwetzkyana, fruit softening in the early and late development periods may result from differential expression of XET gene family members dependent on or independent of ethylene [37]. Previous studies show that the transcripts of XET2 and XET10 are the most abundant in ripe fruits [29], which is consistent with the current results. However, the roles of other XET family members dependent on or independent of ethylene in fruit ripening and softening are unknown. Therefore, an important focus of future research will be to determine the roles of ethylene or other upstream regulators in regulating XET family members and other cell-wall-modifying genes during fruit ripening and softening.

**Anthocyanin biosynthesis**

In this study, SSH libraries were constructed to identify differentially expressed genes involved in fruit ripening and softening, especially softening-related genes. Interestingly, genes associated with anthocyanin biosynthesis and aroma production were identified from the libraries. Anthocyanins are water-soluble flavonoid pigments that are important contributors of the red skin coloration of apple fruits [38]. The regulation of anthocyanin biosynthesis is mainly at the

---

**Table 3. Gene expression statistics during the fruit development period.**

| Gene name          | Source | The expression during 40-50 DAFB (%) | The total expression during 40-75 DAFB (%) | Correlation with ethylene |
|--------------------|--------|--------------------------------------|------------------------------------------|---------------------------|
| XET2               | FS     | 3                                    | 97                                       | 0.780*                    |
| XET10              | FS     | 30                                   | 70                                       | 0.896*                    |
| PE                 | RS     | 40                                   | 60                                       | 0.710                     |
| β-Glu              | FS     | 2                                    | 98                                       | 0.949**                   |
| β-Gal              | FS     | 7                                    | 93                                       | 0.812*                    |
| CAD                | RS     | 24                                   | 76                                       | 0.863*                    |
| 4CL                | FS     | 26                                   | 74                                       | 0.822*                    |
| ANR                | RS     | 42                                   | 58                                       | -0.026                    |
| C4H                | FS     | 37                                   | 63                                       | 0.511                     |
| bHLH137            | FS     | 40                                   | 60                                       | 0.921**                   |
| FAD2               | FS     | 22                                   | 78                                       | 0.962**                   |
| FAD5               | FS     | 10                                   | 90                                       | -0.008                    |
| acetyl-CoA carboxylase | RS   | 36                                   | 64                                       | 0.172                     |
| LOX                | FS     | 32                                   | 68                                       | 0.654                     |
| O-methyltransferase | FS   | 0                                    | 100                                      | 0.909*                    |
| GDSL esterase/lipase | RS | 35                                   | 65                                       | 0.552                     |
| cysteine protease  | RS     | 46                                   | 54                                       | 0.151                     |
| aldehyde dehydrogenase | FS | 32                                   | 68                                       | 0.258                     |
| AM                 | FS     | 30                                   | 70                                       | 0.757                     |
| ACO1               | FS     | 0                                    | 100                                      | 0.917**                   |
| ERF2               | FS     | 34                                   | 66                                       | 0.753                     |
| MADS-box           | FS     | 80                                   | 20                                       | -0.849*                   |
| F-box              | FS     | 39                                   | 61                                       | 0.702                     |
| sugar transporter  | RS     | 26                                   | 74                                       | 0.632                     |
| UN376              | FS     | 42                                   | 58                                       | 0.198                     |
| UN525              | FS     | 23                                   | 77                                       | 0.869*                    |

Note:
* means the significant level of 5% and
** means the significant level of 1%.

doi:10.1371/journal.pone.0146061.t003
level of transcriptional regulation of structural genes by transcription factors [39]. The transcription factors that control anthocyanin biosynthesis include MYB, bHLH and WD40 [40]. We isolated a novel transcription factor, bHLH137, from the forward library. In addition, structural genes such as C4H and 4CL were also identified from the forward library. These genes showed an up-regulated expression pattern, which was consistent with their positively regulatory roles in the anthocyanin biosynthesis pathway. In the reverse library, ANR was observed to be down-regulated during 60–70 DAFB. ANR catalyzes reduction of anthocyanidins to form flavan-3-ols, thus the present findings are consistent with its negative role in the anthocyanin biosynthesis pathway [38]. Correlation analysis showed that only 4CL and bHLH137 were significantly correlated with ethylene concentration, which suggested that ethylene played an important role in anthocyanin biosynthesis in ‘Taishanzaoxia’. This observation verified the results of a previous study that accumulation of anthocyanidin was dependent on ethylene in ‘Taishanzaoxia’ [41]. Although MYB is thought to play an important role in anthocyanin biosynthesis, it was not isolated from the present libraries. MYB may be involved in anthocyanidin biosynthesis through other regulatory pathways, such as light and temperature [42]. Efficient induction of anthocyanin biosynthesis in transient assays by MdMYB10 is dependent on the co-expression of two distinct bHLH proteins from apple, MdbHLH3 and MdbHLH33 [43]. Therefore, further investigations are needed to explain the interaction of bHLH137 with MYB and the role of bHLH137 in regulating the expression of C4H, 4CL and ANR.

Aroma production

In apple, the typical aroma compounds are fruity esters that develop during ripening with the maximum endogenous ester concentration occurring at the climacteric peak [44]. These compounds can be broadly separated into straight- and branched-chain esters. Straight-chain esters are synthesized from fatty acids via the lipoxygenase (LOX) pathway, whereas branched-chain esters are produced from the metabolism of branched-chain amino acids such as isoleucine [45]. Fatty acids are important precursors in the formation of the characteristic aroma in tomato, apple, kiwifruit and pear [46]. FAD is involved in biosynthesis of straight-chain esters by mediating accumulation of linoleic acid and linolenic acid as substrates [47]. Linoleic acid and linolenic acid are converted to hydroperoxide fatty acids via LOX activity [48]. Acetyl CoA, FAD2, FAD5 and LOX were isolated from the SSH libraries and the elevated expression of these genes was consistent with the maximum concentration of esters during the late fruit development period in ‘Taishanzaoxia’ [49]. In addition, GDSL esterase/lipase and O-methyltransferase were also identified from the libraries. FAD2 and O-methyltransferase were up-regulated and significantly correlated with ethylene production. The identification of these aroma-related genes supported the previous hypothesis that aroma production may be regulated by ethylene in ‘Taishanzaoxia’ [50].

Stress response

Fruit ripening and softening have been considered a form of stress for fruits [23]. A suite of unigenes coding for stress-related proteins was identified from the SSH libraries, such as cysteine protease and aldehyde dehydrogenase. The cysteine protease is involved in apoptosis, biotic and abiotic stress, whereas aldehyde dehydrogenase is involved in acetaldehyde detoxification, participation in intermediary metabolism, protection from osmotic stress and generation of NAD(P)H [51, 52]. The elevated expression of these genes verified that they may be involved in fruit ripening and softening as a stress response.
Metabolism, transport and transcription

In addition, AM and sugar transporter also showed an increasing expression pattern during 40–75 DAFB, suggesting that both genes may play an important role in fruit ripening and softening. In addition, we identified unigenes similar to F-box and MADS-box. In Arabidopsis, F-box proteins direct the ubiquitination and subsequent degradation of positive regulators of ethylene action by incorporation into SCF E3 complexes and interaction with EIN3/EIL transcription factors [53]. F-box may be involved in fruit ripening and softening by regulation of the ethylene signaling pathway. MADS-box is thought to play an important role in regulation of fruit ripening and softening because MADS-RIN, a member of the MADS-box family of transcription regulators, is an essential regulator of fruit ripening, acting as an upstream regulatory cascade of ethylene, which interacts with promoters of genes involved in fruit ripening and softening, such as ACS, PG2A, EXP1, ethylene receptor NR, E4 and E8 [54]. Interestingly, MADS-box was down-regulated and showed a significantly negative correlation with ethylene, and thus may be a negative regulator in initiating the ethylene burst. It would be of interest in future studies to determine the roles of F-box and MADS-box interaction with ethylene signaling elements and ripening- and softening-related genes.

This study also identified a large number of genes did not match any sequences currently available in public databases or show homologies to known sequences with unknown functions, such as UN376 and UN525, suggesting that the mechanism of fruit ripening and softening is complex and much remains unknown. Overall, the present study provides valuable information on the isolation and monitoring of genes differentially expressed at lower levels during apple fruit ripening and softening, and especially indicates that fruit texture, coloration and aroma may be regulated by ethylene in 'Taishanzaoxia'. The information is a valuable foundation for subsequent research to elucidate the ethylene-dependent regulatory network of fruit texture, coloration and aroma to gain a better understanding of biochemical events that occur during fruit ripening and softening.

Conclusions

We identified differentially expressed genes involved in fruit ripening and softening in 'Taishanzaoxia' apple. Twenty-six genes associated with cell wall modification, anthocyanin biosynthesis, aroma production, stress response, metabolism, and transcription as well as non-annotated genes are indicated to play important roles in fruit ripening and softening. 'Taishanzaoxia' apple is extremely sensitive to ethylene and fruit texture, coloration and aroma may be all regulated by ethylene. The present findings provide novel insights into the ethylene-dependent regulatory mechanism of fruit ripening and softening, and further enrich our knowledge of the factors that contribute to fruit quality.

Supporting Information

S1 Fig. Flow chart summarizing the construction of SSH libraries and data analysis procedures.
(TIF)

S2 Fig. Analysis of subtraction efficiency by PCR amplification.
(TIF)

S1 Table. Primers used for qRT-PCR.
(XLSX)
Acknowledgments

We appreciate the assistance of Chuanzeng Wang, Jing Zhang, Yanting Wang, Rui Zhang, Ling Su, Jiyun Wu, Xuefang Zhao, Changzhi Qu, Yanmin Zhang and Xiaoliu Chen in setting up the experiment.

Author Contributions

Conceived and designed the experiments: ZYZ XSC. Performed the experiments: ZYZ SHJ NW JXL DYW HFX. Analyzed the data: ML XHJ SSS SMQ ZJF. Contributed reagents/materials/analysis tools: ZYZ NW SJW ZJF XSC. Wrote the paper: ZYZ SJW ZJF SQF XSC.

References

1. Gerhauser C (2008) Cancer chemopreventive potential of apples, apple juice, and apple components. Planta Medica 74: 1608–1624. doi:10.1055/s-0028-1088300 PMID: 18855307
2. Zhu Y, Barratt B H (2008) Md-ACS1 and Md-ACO1 genotyping of apple (Malus x domestica Borkh.) breeding parents and suitability for marker-assisted selection. Tree Genetics & Genomes 4: 555–562.
3. Harb J, Gapper N E, Giovannoni J J, Watkins C B (2012) The role of ethylene and cold temperature in the regulation of the apple POLYGALACTURONASE1 gene and fruit softening. Plant Physiology 153: 294–305. doi:10.1104/pp.109.151092 PMID: 20237022
4. Tacken Emma, Ireland Hilary, Gunaseelan Kularajathevan, Karunairetnam Sakuntala, Wang Daisy, Schultz Keith, et al. (2010) The role of ethylene and cold temperature in the regulation of the apple POLYGALACTURONASE1 gene and fruit softening. Plant Physiology 153: 294–305. doi:10.1104/pp.109.151092 PMID: 20237022
5. Oraguzie N C, Volz R K, Whitworth C J, Bassett H C M, Hall A J, Gardiner S E (2007) Influence of Md-ACS1 allelotype and harvest season within an apple germplasm collection on fruit softening during cold air storage. Postharvest Biology & Technology 44: 212–219.
6. Costa F, Stella S, Van De Weg W E, Guerra W, Cecchinel M, Dallavia J, et al. (2005) Role of the genes Md-ACO1 and md-acs1 in ethylene production and shelf life of apple (Malus domestica Borkh.). Euphytica 141: 181–190.
7. Thompson A J, Tor M, Barry C S, Vrebalov J, Orfila C, Jarvis M C, et al. (1999) Molecular and genetic characterization of a novel pleiotropic tomato-ripening mutant. Plant Biology 120: 383–389. PMID: 10364389
8. Manning K, Tor M, Poole M, Hong Y, Thompson A J, King G J, et al. (2006) A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nature Genetics 38: 948–952. PMID: 16832354
9. Bleeker A B, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annual Review of Cell and Developmental Biology 16: 1–18. PMID: 11031228
10. Wang A, Yamakake J, Kudo H, Waka a Y, Hatsuyama Y, Igarashi M, et al. (2009) Null Mutation of the MdACSS Gene, Coding for a Ripening-Specific 1-Aminocyclopropane-1-Carboxylate Synthase, Leads to Long Shelf Life in Apple Fruit. Plant Physiology 151: 391–399. doi:10.1104/pp.109.135822 PMID: 19587104
11. Bleeker A B (1999) Ethylene perception and signaling: an evolutionary perspective. Trends in Plant Science 4: 269–274. PMID: 10407443
12. Li M, Zhang Y, Zhang Z, Ji X, Zhang R, Liu D, et al. (2013) Hypersensitive ethylene signaling and ZMDPG1 expression lead to fruit softening and dehiscence. PLoS One 8: e68745. doi:10.1371/journal.pone.0068745 PMID: 23527016
13. Chen Y F, Etheridge N, Schaller G E (2005) Ethylene signal transduction. Annals of Botany 95: 901–915. PMID: 15753119
14. Yin X R, Allan A C, Chen K S, Ferguson I B (2010) Kiwifruit EIL and ERF Genes Involved in Regulating Fruit Ripening. Plant Physiology 153: 1280–1292. doi:10.1104/pp.110.157081 PMID: 20457803
15. Li Y C, Zhu B Z, Xu W T, Zhu H L, Chen A J, Xie Y H, et al. (2007) LeERF1 positively modulated ethylene triple response on etiolated seedling, plant development and fruit ripening and softening in tomato. Plant Cell Report 26: 1999–2008.

16. Ng Jovyn KT, Roswitha S, Paul W S, Hallett I C, Miriam I H, Roneel P, et al. (2013) Cell wall structures leading to cultivar differences in softening rates develop early during apple (Malus domestica) fruit growth. BMC Plant Biology 13: 183. doi: 10.1186/1471-2229-13-183 PMID: 24252512

17. Wakasa Y, Kudo H, Ishikawa R, Akada S, Senda M, Niizeki M, et al. (2006) Low expression of an endo-polygalacturonase gene in apple fruit with long-term storage potential. Postharvest Biology & Technology 39: 193–198.

18. Atkinson R G, Sutherland P W, Johnston S L, Gunaseelan K, Hallett I C, Mitra D, et al. (2012) Down-regulation of POLYGALACTURONASE1 alters firmness, tensile strength and water loss in apple (Malus domestica) fruit. BMC Plant Biology 12: 129. doi: 10.1186/1471-2229-12-129 PMID: 22856470

19. Wei J, Ma F, Shi S, Qi X, Zhu X, Yuan J (2010) Changes and postharvest regulation of activity and gene expression of enzymes related to cell wall degradation in ripening apple fruit. Postharvest Biology & Technology 56: 147–154.

20. Liu M Y, Wu L, Liu J, Fang L, Song Y, Cui M, et al. (2012) The regulation of 1-methylcyclopropene on softening and expression of relevant genes in Taishanzhaoxia’apple. Acta Physiologiae Sinica 39: 845–852.

21. Lin X G, Yao L X, Li Y Y, Wu R L, Pang X M (2013) Identification of genes associated with fruit ripening in Ziziphus jujuba using suppression subtractive hybridization approach. Acta Physiologiae Plantarum 35: 1997–2008.

22. Han S E, Lee H E, Heo S, Sun S K, Kim W T, Kim D (2011) Isolation and Characterization of Genes Expressed Differently in Mature Fruits of Redfield’and Greensleeves’Apples. Horticulture, Environment, and Biotechnology 52: 413–421.

23. Manrique-Trujillo S M, Ramirez-Lopez A C, Ibarra-Laclette E, Gomez-Lim M A, et al (2007) Identification of genes differentially expressed during ripening of banana. Journal of Plant Physiology 164: 1037–1050. PMID: 16934912

24. Ewing B, Hillier L D, Wendl M C, Green P (1998) Base-calling of automated sequencer traces using phred. I. Accuracy Assessment. Genome Research 8: 175–185. PMID: 9521921

25. Chou H, Holmes M (2001) DNA sequence quality trimming and vector removal. Bioinformatics 17: 1093–1104. PMID: 11751217

26. Yi Z, Zhao L, Gao J, Fei Z (2011) iAssembler: a package for de novo assembly of Roche-454/Sanger transcriptome sequences. BMC Bioinformatics 12: 453. doi: 10.1186/1471-2105-12-453 PMID: 22111509

27. Livak K J, Schmittgen T D (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2–ΔΔCT Method. Methods 25: 402–408. PMID: 11846609

28. Chen X S, Song J, Gao L P, Ji X H, Zhang Y, Mao Z Q, et al. (2014) Developing mechanism of fruits texture in ‘Jonagold’apple and its crisp flesh sport. Scienitia Agricultura Sinica 47: 727–735.

29. Atkinson R G, Johnston S L, Yauk Y K, Sharma N N, Schröder R. (2009) Analysis of xyloglucan endotransglucosylase/hydrolase (XTH) gene families in kiwifruit and apple. Postharvest Biology & Technology 51: 149–157.

30. Wang A, Tan D, Takahashi A, Li T Z, Harada T (2007) MdERFs, two ethylene-response factors involved in apple fruit ripening. Journal of Experimental Botany 58: 3743–3748. PMID: 18057044

31. Payasi A, Mishra N N, Chaves A L, Singh R (2009) Biochemistry of fruit softening: an overview. Physiology & Molecular Biology of Plants 15: 103–113.

32. Munoz-Bertomeu J, Miedes E, Lorenes E P (2013) Expression of xyloglucan endotransglucosylase/hydrolase (XTH) genes and XET activity in ethylene treated apple and tomato fruits. Journal of Plant Physiology 170: 1194–1201. doi: 10.1016/j.jplph.2013.03.015 PMID: 23628624

33. Guo S, Sun H, Zhang H, Liu J, Ren Y, Gong G (2015) Comparative Transcriptome Analysis of Cultivated and Wild Watermelon during Fruit Development. Plos One 10: e130267.

34. Choudhury S R, Roy S, Singh SK, Sengupta DN (2010) Molecular characterization and differential expression of β-1,3 glucanase during ripening in banana fruit in response to ethylene, auxin, ABA, wounding, cold and light—dark cycles. Plant Cell Reports 29: 813–828. doi: 10.1007/s00299-010-0866-0 PMID: 20467747

35. Ross G S, Wegrzyn T, MacRae E A, Redgwell R J (1994) Apple beta-galactosidase activity against cell wall polysaccharides and characterization of a related cDNA clone. Plant Physiology 106: 521–526. PMID: 7991682

36. Cao J Q, Li B, Yang Y, Hun W R, Wang Y J, Fan L (2014) Advanced progress in the cinnamyl alcohol dehydrogenase gene involved in lignin biosynthesis. Molecular Plant Breeding 12: 1034–1043.
37. Zhang R, Zhang Z Y, Gao L P, Ji X H, Mao Z Q, Xu H F, et al. (2015) The study on the molecular mechanism controlling differences in fruit texture formation of apple soft/crisp strains. Scientia Agricultura Sinica 48: 3676–3688.

38. Wang L, Zhang X, Liu Y, Shi X, Wang Y, Zhang C, et al. (2013) The effect of fruit bagging on the color, phenolic compounds and expression of the anthocyanin biosynthetic and regulatory genes on the’-Granny Smith’apples. European Food Research & Technology 237: 875–885.

39. Palapol Y, Ketsa S, Lin-Wang K, Ferguson I B, Allan A C (2009) A MYB transcription factor regulates anthocyanin biosynthesis in mangosteen (Garcinia mangostana L.) fruit during ripening. Planta 229: 1323–1334. doi: 10.1007/s00425-009-0917-3 PMID: 19306102

40. Koes R E, Verweij C W, Quattrocchio F M (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends in Plant Science 10: 236–242. PMID: 15882656

41. Liu J, Wei J L, Liu M Y, Song Y, Feng S Q, Wang C Z, et al. (2012) The relationships between the enzyme activity of anthocyanin biosynthesis, ethylene release and anthocyanin accumulation in fruits of precocious apple cultivars. Acta Horticulturae Sinica 39: 1235–1242.

42. Ubi B, Honda C, Bessho H, Kondo S, Wada M, Kobayashi S T (2006) Expression analysis of anthocyanin biosynthetic genes in apple skin: Effect of UV-B and temperature. Plant Science 170: 571–578.

43. Espley R V, Hellens R P, Putterill J, Stevenson D E, Kutty Amma S, Allan A C (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant Journal 49: 414–427. PMID: 17181777

44. Defilippi B G, Danekar A M, Kader A A (2005) Relationship of ethylene biosynthesis to volatile production, related enzymes, and precursor availability in apple appearance and flesh tissues. Journal of Agricultural and Food Chemistry 53: 3133–3141. PMID: 15826070

45. Souleyre E J F, Greenwood D R, Friel E N, Karunairetnam S, Newcomb R D (2005) An alcohol acyl transferase from apple (cv. Royal Gala), MpAAT1, produces esters involved in apple fruit flavor. Febs Journal 272: 3132–3144. PMID: 15955071

46. Schwab W, Davidovich-Rikanati R, Lewinsohn E (2008) Biosynthesis of plant-derived flavor compounds. Plant Journal 54: 712–732. doi: 10.1111/j.1365-313X.2008.03446.x PMID: 18476874

47. Fong W P, Cheng C H, Tang W K. (2006) Antiquitin, a relatively unexplored member in the superfamily of aldehyde dehydrogenases with diversified physiological functions. Cellular Molecular Life Science 63: 2881–2885.

48. Gagne J M, Smalle J, Gingerich D J, Walker J M, Yoo S D, Yanagisawa S, et al. (2004) Arabidopsis EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proceedings of the National Academy of Sciences of the United States of America 101: 6803–6808. PMID: 15090654

49. Catherine M, Julia V, Petra T, James J G (2011) The tomato MADS-box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NON-RIPEINING-dependent manner. Plant Physiology 157: 1568–1579. doi: 10.1104/pp.111.181107 PMID: 21941001