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Differential gene expression analysis of 'Granny Smith' apple (*Malus domestica* Borkh.) during fruit skin coloration ☆

X.J. Zhang [a], L.X. Wang [a], Y.L. Liu [a], X.X. Chen [b], Y.Z. Yang [a], Z.Y. Zhao [a,c,*]

[a] College of Horticulture, Northwest A&F University, Yangling, Shaanxi 712100, PR China
[b] College of Natural Resources and Environment, Northwest A&F University, Yangling, Shaanxi 712100, PR China
[c] Apple E&T Research Centre of Shaanxi Province, Yangling, Shaanxi 712100, PR China

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ABSTRACT

'Granny Smith' apples growing under normal sunlight develop green skin, whereas the peel turns red due to anthocyanin accumulation after the removal of a bagging treatment. Two anthocyanins, Cyanidin 3-O-galactoside (cy3-gal) and Cyanidin 3-O-arabinoside (cy3-ara), were detected in the red 'Granny Smith' apple peels, and cy3-gal was determined to be chiefly responsible for the red color. The content of cy3-gal was more than 98% of the total anthocyanin in the red 'Granny Smith' peels. To better understand the molecular basis of anthocyanin biosynthesis in 'Granny Smith' apples, we performed a quantitative real-time PCR (qRT-PCR) analysis of anthocyanin biosynthetic genes (*MdCHS*, *MdF3H*, *MdDFR*, *MdANS*, *MdUFGT*, and *MdMYB1*). Our results indicate that the expression of these genes (except *MdCHS*) was associated with increased anthocyanin accumulation in the skin of 'Granny Smith' apples. Four selected genes obtained from the 'Granny Smith' skin cDNA library, phytoene synthase (PSY), WD40 repeat protein, polygalacturonase (PG), and galactosidase (GAL), were also confirmed by qRT-PCR. We found that these genes were differently expressed during 'Granny Smith' apple skin coloration, suggesting that they are directly or indirectly involved in pigment accumulation. In conclusion, anthocyanin biosynthesis in 'Granny Smith' apples is the result of interactions between multiple enzymes in the anthocyanin biosynthesis pathway, and the coloring mechanism of 'Granny Smith' apples may be similar to that of red-skinned cultivars.

1. Introduction

Apple (*Malus domestica* Borkh.) is one of the most important fruit crops in temperate regions of the world. Apple color is an important factor when considering consumer appeal, with red-colored fruits being more popular (King and Cliff, 2002). Apple skin color is thought to be determined by the interaction of anthocyanin molecules, a class of flavonoids that is responsible for the red skin color (Lancaster and Dougall, 1992), with other compounds, such as chlorophyll and carotenoids. There are more anthocyanins in red-skinned apple cultivars than in non-red-skinned cultivars (Honda et al., 2002).

In recent years, significant progress has been made in understanding the genetic regulation of anthocyanin biosynthesis in apples. The genes encoding anthocyanin biosynthesis enzymes have been isolated and studied (Honda et al., 2002; Kim et al., 2003). The transcriptional levels of these genes are positively correlated with anthocyanin accumulation (Honda et al., 2002; Ben-Yehudah et al., 2005), and the MYB-bHLH-WD40 regulatory protein complex controls their expression (Koes et al., 2005; Allan et al., 2008). In apples, MYB transcription factors (*MdMYB1*, *MdMYBA*, and *MdMYB10*) and bHLH transcription factors (*MdbHLH3* and *MdbHLH33*) have been isolated and characterized (Takos et al., 2006; Ban et al., 2007a; Espley et al., 2007). In addition to genetic factors, external factors, such as light, temperature, mineral nutrition, and orchard management practices, also affect apple anthocyanin biosynthesis, with light being the most important and essential factor (Saure, 1990), and the transcription of *MdMYB1* and several anthocyanin biosynthetic genes is regulated by light (Takos et al., 2006; Ban et al., 2007a; Ju et al., 1997; Ubi et al., 2006). Recent studies have proven that bagging treatment is an effective practice to study the effect of light on fruit anthocyanin synthesis (Ju, 1998; Zhang et al., 2011; Wei et al., 2011). It is also a useful technique for studying anthocyanin biosynthesis and gene expression in apples (Ju, 1998; Takos et al., 2006; Ban et al., 2007a).

'Granny Smith' apples display a green background with a pink blush in exposed areas (Reay, 1999). We found that, during the fruit ripening phase, bagged fruits that are re-exposed to sunlight accumulate

Abbreviations: CHS, chalcone synthase; F3H, flavanone 3-hydroxylase; DFR, Dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, flavonoid 3-O-glucosyltransferase; DAFB, days after full bloom; DABR, days after bag removal; qRT-PCR, quantitative real time-PCR; HPLC, high-performance liquid chromatography.

* Corresponding author at: College of Horticulture, Northwest A&F University, Yangling, Shaanxi 712100, PR China. Tel./fax: +86 29 87082922.
E-mail address: zhaozy@nwsuaf.edu.cn (Z.Y. Zhao).

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significant levels of anthocyanin in the skin (Fig. 1). Previous research commonly used red-skinned apple cultivars as experimental materials to elucidate the molecular regulation of anthocyanin biosynthesis (Takos et al., 2006; Xu et al., 2012). However, informations regarding the genetic regulation of anthocyanin biosynthesis for non-red-skinned cultivars are rare. The present study was aimed to understand the molecular mechanisms of color variability in red 'Granny Smith' apple skin, by comparison with the non-bagged fruits in terms of anthocyanin content and transcription of anthocyanin biosynthetic genes. In addition, in order to identify potential color-related genes of 'Granny Smith', a light-specific subtractive cDNA library was constructed using SSH technology (Diatchenko et al., 1996), which has been widely used to identify differentially expressed genes (Ban et al., 2007b; Han et al., 2011; Zhang et al., 2011; Zhou et al., 2012). Moreover, this cDNA library would provide a foundation for future cloning and functional analysis of genes related to 'Granny Smith' coloration.

2. Materials and methods

2.1. Plant materials

The fruits used in this experiment were obtained from the orchard of the Northwest A&F University apple experimental station in the Baishui, Shaanxi Province in 2010. For the bagging treatment, each fruit was bagged with two-layer paper bags (Hong Tai, China) on May 25th, approximately 30 days after full bloom (DAFB). The bags were 13 × 16 cm, and the inner layer was made of red paper with a wax coating, while the outside layer was taupe. The bags were removed at 165 DAFB. The fruits in the bagged group were collected at 0, 2, 4, 6, 8, 10, and 15 days after the bag removal (DABR). The control group fruits were grown under normal sunlight conditions, and they were collected at 165, 167, 169, 171, 173, 175, and 180 DAFB. At each point in time, 10 individual fruits were collected from different locations in the tree canopy. The peels, including 1 mm of cortical tissue, were carefully peeled off with a knife, frozen immediately in liquid nitrogen and stored at −80 °C until use.

2.2. Apple skin SSH library construction

Total RNA from the skin tissues of 'Granny Smith' apple was isolated using a hot borate method (Yao et al., 2005). Isolation of mRNA was performed by Oligotex® mRNA Mini Kit (QIAGEN GmbH, Germany), and cDNA was synthesized by the SMART cDNA synthesis kit (Clontech, Palo Alto, CA, USA). The SSH library of the 'Granny Smith' fruit peel was constructed using the PCR-Select™ Subtractive Hybridization kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions, with the cDNA of the peel exposed to sunlight for 0 day as the driver and the mixed peel cDNA (2, 4, 6, 8 and 10 DABR) as the tester. The second PCR products, after subtraction, were recycled and linked into the pGEM-T-Easy Vector (Promega, USA) and then were transformed into *Escherichia coli* strain TOP10 (Tiangen Biotech (Beijing) Co., LTD) and screened on the LB solid medium plate containing IPTG + X-gal + Amp at 37 °C overnight. Subsequently, recombination rate was tested by blue–white screening.

2.3. Sequencing and BLAST analysis

White colonies were isolated on selective media, Luria-Bertani ampicillin (LB-Amp) solid medium, and these clones were grown overnight in 100 μL LB-Amp medium at 37 °C, respectively. Two μL of bacterium culture for each colony was used to amplify the inserts with SP6 and T7 primers. The cDNA fragments were sequenced by Invitrogen Trading Co. (Shanghai). All the inserted sequences were queried for similarity in the NCBI database using the BLASTX sequence comparison software at the website (http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Anthocyanin extraction and measurement

For the high-performance liquid chromatography (HPLC) analysis, the anthocyanins were extracted from 1 g of finely ground plant material in 1 mL 1% (v/v) HCl-methanol for 24 h at 4 °C on a rotating wheel in the dark. The samples were clarified by centrifugation at 13,000 ×*g* for 15 min at 4 °C, and then 1.5 mL of supernatant was transferred to an autosampler vial. The analysis was conducted using an HPLC PDA (Waters, USA), which was equipped with a model 1525 binary solvent delivery system (Waters, USA), an on-line degasser and a 2707 autosampler (Waters, USA). The data acquisition and processing were performed with Breeze software. For all the samples, the anthocyanin separations were performed using a 250 × 4.6 mm i.d., 5 μm C18 column (Diamonsil, China). Solvent A was 10% (v/v) formic acid, and solvent B was methanol. The gradient of solvent B was as follows: 0 min, 17%; 1 min, 17%; 10 min, 35%; 20 min, 37%; and 25 min, 100%. The gradient was run at a flow rate of 1 mL · min^{-1} at a column temperature of 40 °C, and a 5 μL sample was injected. Absorbance was measured at 530 nm. The standards were Cyanidin 3-O-galactoside, Cyanidin 3-O-glucoside (Sigma, USA), Cyanidin 3-O-arabinoside, and Cyanidin 3-O-rutinoside (Polyphenols Laboratories, Hanaveien). Anthocyanins were expressed as mg/100 g fresh wight (FW).

2.5. Analysis of gene expression profile by qRT-PCR

Isolation of total RNA from the pericarp of 'Granny Smith' apples and first-strand cDNA synthesis were performed as described above. The transcript levels of *MdCHS*, *MdF3H*, *MdDFR*, *MdANS*, *MdUFGT*, *MdMYB1*, and four selected genes were analyzed by qRT-PCR using SYBR green master mix (Takara, Japan) and an IQ5 real-time PCR cycler (Bio-Rad Laboratories, USA) in a reaction volume of 25 μL. All the gene-specific primers were designed using the Primer Premier 5.0 program (Supplementary

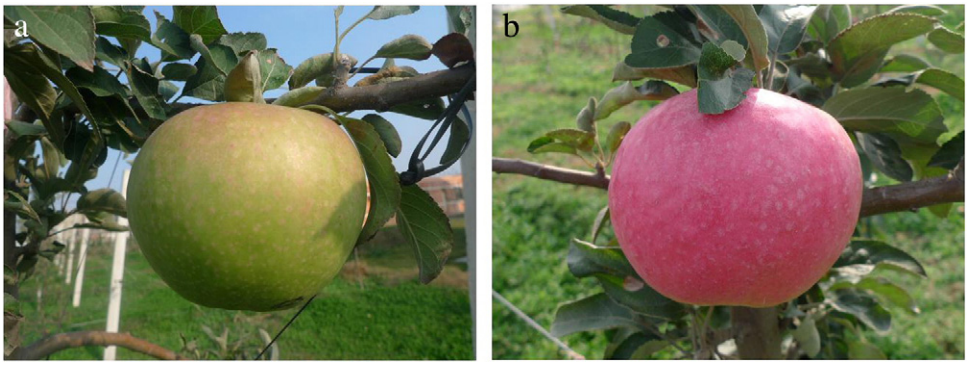

Fig. 1. The 'Granny Smith' apple. a. Control fruit (175 DAFB); b. bagged fruit (10 DABR).