Process Biochemistry 50 (2015) 1774–1782

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

The effects of fruit bagging on levels of phenolic compounds and expression by anthocyanin biosynthetic and regulatory genes in red-fleshed apples

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ARTICLE INFO

Article history:
Received 11 March 2015
Received in revised form 15 June 2015
Accepted 29 June 2015
Available online 3 July 2015

Keywords:
Red-fleshed apples
Phenolics
Anthocyanins
Bagging treatment
Gene expression

ABSTRACT

Apples with red fruit flesh are desirable for breeding because they contain more health-beneficial compounds. That color trait is also considered more attractive to consumer. To obtain experimental evidence about the molecular mechanism for this pigmentation, we investigated how the practice of bagging fruit might affect levels of phenolic compounds as well as the expression of anthocyanin biosynthetic and regulatory genes. Two red-fleshed varieties from Xinjiang, P.R. China, were studied – ‘Xiahongrou’ and ‘No.1 Hongxun’. HPLC analysis showed that the contents of anthocyanins in both peel and flesh were decreased by bagging, but were increased again when fruits were re-exposed to sunlight. Except for *F3H* in ‘No.1 Hongxun’, transcription levels for most anthocyanin biosynthetic genes in the flesh were enhanced after bag removal. *MYB10* is a key transcriptional factor in the anthocyanin pathway of these two apple varieties, particularly in the flesh. These results suggest that the development of red coloring when bags are removed could be a result of light-induced coordinated expression by *MYB10* and anthocyanin biosynthetic genes in red-fleshed apples.

1. Introduction

Phenolics in fruits and vegetables have gained much attention because of their antioxidant activities and their beneficial implications for human health. This is based on research demonstrating their biological activity in preventing cancer and cardiovascular diseases [1]. Apples are one of the most important dietary sources of phenolic compounds because their consumption is widespread and they are available year-round in markets. Five major polyphenolic groups are found in various apple varieties: hydroxycinnamic acids, dihydrochalcones, flavanols, flavonols, and anthocyanins [2].

Apple color is a key factor when considering consumer appeal, with red fruits being the most popular [3]. This skin color is derived from anthocyanins that belong to a class of flavonoids. Though not as common, red flesh color is a highly desirable trait that can be found with varieties such as ‘Aerlies Red Flesh’ (syn. ‘Hidden Rose’, USA), ‘Baya Marisa’ (Germany), ‘Red Devil’ (United Kingdom), and ‘Redlove’ (Switzerland). This trait is explained by their genealogy [4]. The ancestor of many red-fleshed genotypes is the wild species *Malus niedzwetzkyana* [5], a native of Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan, Turkmenistan, and China’s Xinjiang Province [6]. These varieties have high anthocyanin contents that create a dramatic phenotype with strongly pigmented vegetative, floral, and fruit tissues [7].

Anthocyanins are synthesized from phenylalanine via the phenylpropanoid and flavonoid pathway (Fig. 1) [8]. The enzymes involved in this pathway include phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose flavonoid 3-O-glucosyl transferase (UFGT) [9]. Its three branches include flavonol synthase (FLS), which produces flavonols, as well as leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), which respectively synthesize trans- and cis-flavan-3-ols, the precursors of proanthocyanidin polymers [8]. Anthocyanin biosynthesis is positively correlated with the degree of gene expression, and is controlled at the transcriptional level [10]. Moreover, genes for anthocyanin production are regulated by the MYB-bHLH-WD40 protein complex [11]. The MYB genes (*MYB1*, *MYB10*, and *MYBA*) and the bHLH genes (*bHLH3* and *bHLH33*) have been isolated in apple and shown to induce the accumulation of anthocyanins [12]. *MYB1/A* are mainly involved in controlling the red coloration of the peel while *MYB10* is responsible for coloring of the whole fruit as well as foliage [13]. In addition to genetic components, external factors, e.g., light, temperature,

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http://dx.doi.org/10.1016/j.procbio.2015.06.024

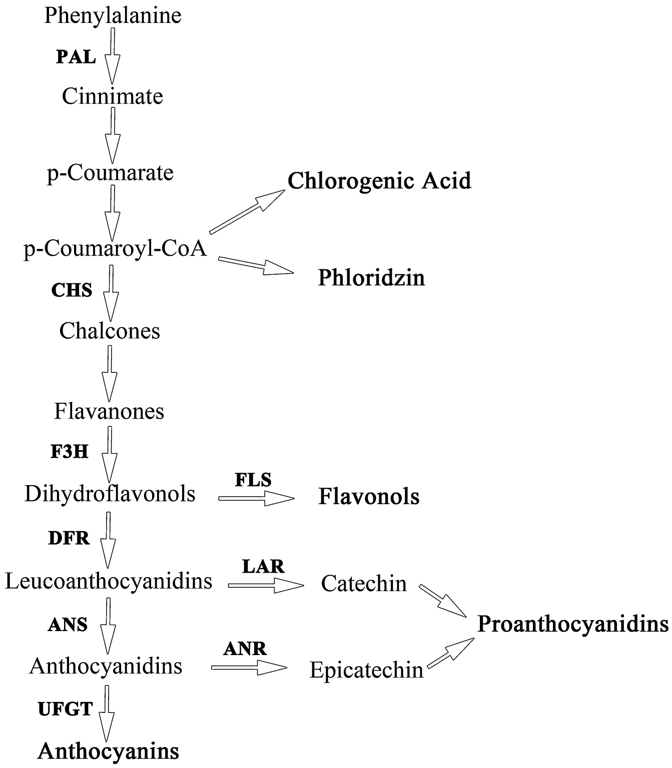

Fig. 1. Scheme of the apple phenylpropanoid pathway, showing structural genes involved in flavonoid biosynthesis. PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDP-glucose flavonoid 3-O-glucosyl transferase; FLS, flavonol synthase; LAR, leucoanthocyandin reductase; ANR, anthocyanidin reductase.

mineral nutrition, and orchard management practices, can affect apple anthocyanin biosynthesis, with light being the most essential player [3,14].

Red-fleshed apples are receiving increased attention because of their remarkable anthocyanin contents. Previous work with those types has largely focused on isolation and functional analysis of the MYB transcription factors (TFs) [7,10,15,16]. However, no reports to date have evaluated anthocyanin biosynthesis and transcriptional levels in red-fleshed apples in response to light exposure, especially in the flesh. Recent studies have shown that bagging treatment is an effective practice when investigating the influence of light on anthocyanin synthesis in fruit [17,18]. It is also a useful technique for examining anthocyanin biosynthesis and gene expression in apples [3]. Therefore, we used two red-fleshed Xinjiang varieties – 'Xiahongrou' and 'No.1 Hongxun' – to monitor how bagging might change the levels of anthocyanins and phenolic compounds as well as the expression of key anthocyanin biosynthetic and regulatory genes. We proposed that our findings would be helpful in improving our understanding of the molecular mechanism for red pigmentation in apple flesh.

2. Materials and methods

2.1. Plant materials and experimental treatments

Red-fleshed apples 'Xiahongrou' and 'No.1 Hongxun' (two Xinjiang varieties) were studied (Fig. 2). Both are within *M. niedzwetzkyana*. The 6–8-year-old trees were grafted and grown on *Malus sieversii* rootstocks, and were approximately 3.5 m tall with a central leader at a spacing of 2 m × 4 m They were cultured in an orchard at the Horticultural Experimental Station of Northwest A&F University, Yangling (34°20 N, 108°24 E), China, where they received standard horticultural treatment, including pest and disease control.

Two trees were used for each variety. 80 well-exposed fruits per tree were selected for the control group, and the rest (80–100 fruits) were bagged with light impermeable double layer (the outer layer is yellow outside and black inside, and the inner layer is red) paper bags. The bags were placed on designated young fruit approximately six weeks after bloom. Harvest time was determined by the starch index. As the past experience, all bags were removed 15 d prior to harvest at afternoon and the fruit was completely re-exposed to light for the remainder of the experimental period. The peel and flesh were separated and collected at 0, 3, 6, 9, and 15 d after the bag removal. Control samples were also collected on those days. Each treatment was repeated three times in a completely randomized design. Each point-in-time sample consisted of a total of ten fruits per replicate (five fruits per tree). All samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Analysis of phenolic compounds

Flesh and peel tissues were first ground separately to fine powders in liquid nitrogen and mixed well to ensure representative sampling of each variety for each sampling date. Their phenolic compounds were then extracted and analyzed as described by Zhang et al. [19]. Briefly, the compounds were extracted at 0 °C with 70% methanol containing 2% formic acid and analyzed using an Agilent 1200 Liquid Chromatograph equipped with a diode array detector (Agilent Technology, Palo Alto, CA, USA). An Inertsil ODS-3 column (5.0 μm particle size, 4.6 mm × 250 mm; GL Sciences Inc., Tokyo, Japan) was used in the separation, preceded by an Inertsil ODS-3 Guard Column (5.0 μm, 4.0 mm × 10.0 mm; GL Sciences Inc., Tokyo, Japan). Solvent A consisted of 10% formic acid (11.36% of 88% formic acid) in water while solvent B was 10% formic acid and 1.36% water (11.36% of 88% formic acid) in acetonitrile. The gradient followed 95% A (0 min), 85% A (25 min), 78% A (42 min), 64% A (60 min), and 95% A (65 min). Post-run time was 10 min. Simultaneous monitoring was performed at 280 nm for catechin, epicatechin, procyanidin B1, procyanidin B2, phloridzin, gallic acid, and syringic acid; at 320 nm for chlorogenic acid, caffeic acid, ρ-coumaric acid, and ferulic acid; at 365 nm for quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, quercetin-3-rutinoside, quercetin-3-xyloside, and quercetin-3-arabinoside; and at 520 nm for cyanidin-3-galactoside and cyanidin-3-glucoside. Peaks were identified by comparing retention times and UV spectra with authentic standards. Contents of individual phenolic compounds were determined based on peak areas and calibration curves derived from corresponding authentic phenolic compounds.

2.3. Expression analysis by quantitative real-time PCR

We used qRT-PCR to measure the transcript levels of key genes involved in anthocyanin biosynthesis. Total RNA was extracted from the peel and flesh samples by the Cetyltrimethyl Ammonium Bromide (CTAB) method [20]. First-strand cDNA was synthesized with a PrimeScript RT Reagent Kit (Takara Bio Inc., Tokyo, Japan) according to the manufacturer's protocol. After dilution to 150 ng/μL, quantitative real-time expression was evaluated (three technical replicates) on an iQ5.0 instrument (Bio-Rad Laboratories, Hercules, CA, USA) using a SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (Takara Bio Inc., Tokyo, Japan). The program included 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. A melting curve was generated for each sample at the end of each run to ensure the purity of the amplified products. Primers were those described by Wang et al. [12] for *Actin* (AB638619), *CHS*