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Identification and expression analysis of genes involved in anthocyanin and proanthocyanidin biosynthesis in the fruit of blackberry

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ABSTRACT

Anthocyanin and proanthocyanin are two major secondary metabolites involved in fruit maturation. In this work, the variations of anthocyanin and proanthocyanidin content were determined during blackberry fruit development. Genes associated with biosynthesis of both two compounds were isolated. Expression patterns of these genes were also investigated by quantitative PCR throughout fruit maturation. Anthocyanins, which remained at a low concentration in the early developing stage, increased dramatically as the fruit matured. On the contrary, proanthocyanidins exhibited a continuously decreasing pattern. Transcript levels of genes specifically controlling either of these two compounds: anthocyanin synthase (*RuANS*) for anthocyanin, leucoanthocyanidin reductase (*RuLAR*) and anthocyanidin reductase (*RuANR*) for proanthocyanidin, were generally coordinated with the products' changing patterns. The expression of the *RuMYB10*, encoding a transcription factor, was also concomitant with the synthesis of anthocyanins, indicating its specificity regulation role in anthocyanin branch. The other two genes, chalcone synthase (*RuCHS*) and dihydroflavonol 4-reductase (*RuDFR*), showed cross regulation for not only the synthesis of anthocyanin and proanthocyanidin, but also other flavonoid compounds. According to the temporal expression patterns, the isolated *RuGT* gene might encode a glucosyltransferase participating in modifying metabolites besides anthocyanins. Our discoveries of the temporal gene expression and compounds accumulation will shed new light into genetic and physiological metabolisms in blackberry fruit development, thus assisting in future attempts to alter anthocyanin or PA metabolites in fruits.

1. Introduction

Fruit maturation is composed of complex metabolic changes, including primary metabolites like the major soluble sugars, organic acids, amino acids and some major secondary metabolites (e.g., flavonoids) (Fait et al., 2008). Anthocyanin and proanthocyanidin (PA) are two important products of the flavonoid pathway (Grotewold, 2006). They are key factors affecting fruit flavor and nutrition properties in persimmon, grape berries and many other fruits (Fernández et al., 2007; Hümmer and Schreier, 2008; Akagi et al., 2009). Furthermore, they are also involved in the plant resistances for both biotic and abiotic stresses (Hernández et al., 2009). Due to their biological and pharmacological importance, biochemical constitution and dynamic changes, as well as molecular biosynthesis of the two compounds have been extensively studied.

Flavonoid compounds consist of anthocyanin, PA, flavones and other products. The biosynthesis (Fig. 1), initiating from phenylalanine, is channeled into the flavonoid pathway by chalcone synthase (CHS). Further enzymatic reaction, catalyzed by dihydroflavonol reductase (DFR), allows the formation of former substrates for anthocyanin and flavan-3ols. And anthocyanidin synthase (ANS) leads to the synthesis of anthocyanidin pigments and cyanidin-derived PA. Afterwards, UDP-glucose: flavonoid 3-O-glucosyltransferase (UGT) or other glucosyltransferases (GTs) catalyze the glycosylation step to increase stability or water solubility of the anthocyanins, exhibiting a broad spectrum of color range (Grotewold, 2006; Akagi et al., 2009, 2011). In the current model, leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) provide two separate pathways for the synthesis of flavan-3ols, which are primer or extension unites for PA polymers (Dixon et al., 2005). The recent researches have demonstrated that this process is controlled by different transcription factors, including MYB, bHLH, WD40 and other proteins (Hichri et al., 2011). Generally, the MYB, bHLH and WD40 cofactors were believed to form an effective complex to specially mediate anthocyanin or PA biosynthesis (Ramsay and Glover, 2005; Hichri et al., 2011).

Blackberry (*Rubus* spp.) is an important economic and medical purpose fruit in both Europe and North America. It is an aggregate fruit with hundreds of drupelets arranged on a column of receptacle

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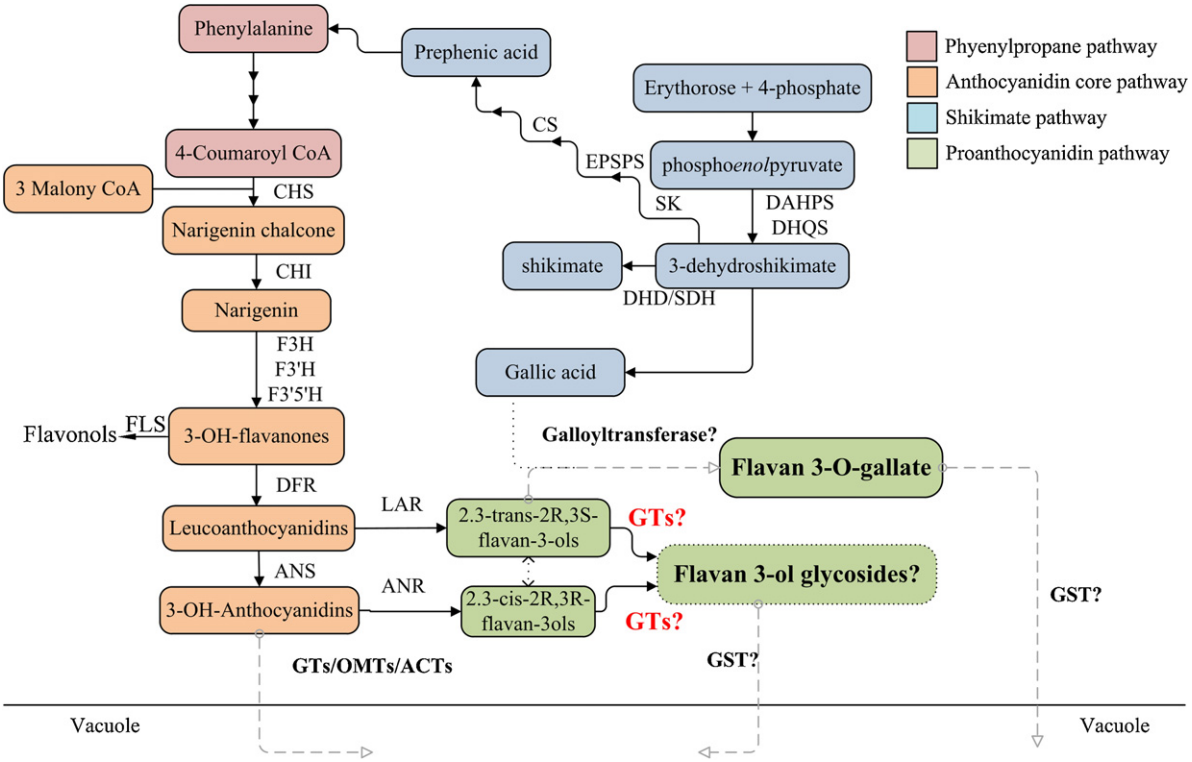

Fig. 1. The proposed pathway of anthocyanin and proanthocyanidin biosynthesis in plants. Abbreviations are as follows: chalcone synthase (CHS); flavanone 3-hydroxylase (F3H); flavonoid 3′-hydroxylase (F3′H); flavonoid 3′5′-hydroxylase (F3′5′H); dihydroflavonol 4-reductase (DFR); anthocyanidin synthase (ANS); flavonol synthase (FLS); UDP-glucose: flavonoid 3-O-glucosyltransferase (GTs); anthocyanidin reductase (ANR); leucoanthocyanidin reductase (LAR); glutathione S-transferase (GST); O-methyltransferases (OMTs); acyltransferases (ACTs); 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS); 3-dehydroquinate synthase (DHQS); 3-dehydroquinate dehydratase/shikimate 5-dehydrogenase (DHD/SDH); shikimate kinase (SK); 5-enolpyruvylshikimate 3-phosphte synthase (EPSPS); chorismate synthase (CS).

tissues. In the recent studies, extracts of the fruit exhibited effective health-promoting properties due to the presence of rich polyphenol compounds (Dai et al., 2007; Cuevas-Rodriguez et al., 2010a; Esselen et al., 2011). Detailed composition of these compounds was illustrated by works of Cuevas-Rodriguez et al. (2010b), Fan-Chiang and Wrolstad (2005) and Mertz et al. (2007). Ripening physiology of the developing fruits, including soluble solids content, titratable acidity, anthocyanin content as well as ethylene changes were also uncovered (Perkins-Veazie et al., 2000). During the procession of blackberry maturation, three distinct color changes are undergone: initially green, the full redness and last blackness. However, molecular discoveries involving this procession are still lacking.

This work presented (1) the isolation of six key structural genes controlling the anthocyanin and PA synthesis branch of the flavonoid biosynthesis pathway, as well as the regulator gene *RuMYB10*, from the fruits of blackberry, (2) the study of expression patterns for these genes during the development of fruit from green to bright black, and in parallel, (3) the accumulation of anthocyanin and PAs. The understanding of their temporal regulation will be important for future attempts to alter anthocyanin or PA in fruits.

2. Materials and methods

2.1. Plant materials

The leaves and fruits of the blackberry cultivar 'Arapoho', growing under standard cultural conditions (see supplementary material S1) in Sichuan, south western part of China, were harvested in 2010 and 2011, respectively (Wang et al., 2008). Young leaves, 5 days after unfolding, were collected and kept at −20 °C for further use. Nonterminal fruits, at different maturation stages as described by Perkins-Veazie et al. (2000): green (14 days post anthesis, 14 dPA), green/red (21 dPA), red (35 dPA), red/black (42 dPA) and shiny black (49 dPA) were sampled. Approximately 20 berries from at least five potted plant bunches were collected for each stage. All fruits were frozen in liquid nitrogen upon harvesting in the field and stored at −80 °C until analyzed.

2.2. Isolation of DNA and RNA

Genomic DNA was isolated from blackberry leaves with the method described for raspberry (*Rubus ideaus*) by Lodhi et al. (1994). Fruits at different developmental stages were used for total RNA isolation, using the protocol adopted by Jones et al. (1997). Integrity and purity of the total RNA were verified on 1% (w/v) ethidium bromide-stained agarose gel in addition to the absorbance spectrum at wavelength from 220 to 300 nm by utilizing Nanodrop 1000 (Thermo Scientific, USA).

2.3. PCR primer design and gene isolation

Before the study was conducted, public resources concerning blackberry fruits were first utilized to explore genomic information of the 7 genes. The EST library (Lewers et al., 2008) and the sequence galleries from raspberry EST unigene assembly version 4 (Jung et al., 2008) were directly download and deposited locally to construct a 'combined library'. Gene sequences, *CHS* (GenBank ID: 60280214), *ANS* (GenBank ID: 4588782), *DFR* (GenBank ID: 4588780), *ANR* (GenBank ID: 73655644), *GT* (GenBank ID: 117938437), *LAR* (GenBank ID: 73655703), *RiMYB10* (GenBank ID: 161878916) and *β-actin* (GenBank ID: 353259714) for