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Research article

Effect of calcium on strawberry fruit flavonoid pathway gene expression and anthocyanin accumulation

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ABSTRACT

Two diploid woodland strawberry (*Fragaria vesca*) inbred lines, Ruegen F7-4 (red fruit-bearing) and YW5AF7 (yellow fruit-bearing) were used to study the regulation of anthocyanin biosynthesis in fruit. Ruegen F7-4 fruit had similar total phenolics and anthocyanin contents to commercial octoploid (*F.* × *ananassa*) cultivar Seascape, while YW5AF7 exhibited relatively low total phenolics content and no anthocyanin accumulation. Foliar spray of $CaCl_2$ boosted fruit total phenolics content, especially anthocyanins, by more than 20% in both Seascape and RF7-4. Expression levels of almost all the flavonoid pathway genes were comparable in Ruegen F7-4 and YW5AF7 green-stage fruit. However, at the turning and ripe stages, key anthocyanin structural genes, including flavanone 3-hydroxylase (*F3H1*), dihydroflavonol 4-reductase (*DFR2*), anthocyanidin synthase (*ANS1*), and UDP-glucosyltransferase (*UGT1*), were highly expressed in Ruegen F7-4 compared with YW5AF7 fruit. Calcium treatment further stimulated the expression of those genes in Ruegen F7-4 fruit. Anthocyanins isolated from petioles of YW5AF7 and Ruegen F-7 had the same HPLC–DAD profile, which differed from that of Ruegen F-7 fruit anthocyanins. All the anthocyanin structural genes except *FvUGT1* were detected in petioles of YW5AF7 and Ruegen F-7. Taken together, these results indicate that the "yellow" gene in YW5AF7 is a fruit specific regulatory gene(s) for anthocyanin biosynthesis. Calcium can enhance accumulation of anthocyanins and total phenolics in fruit possibly via upregulation of anthocyanin structural genes. Our results also suggest that the anthocyanin biosynthesis machinery in petioles is different from that in fruit.

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1. Introduction

Anthocyanins are phenolic pigments in fruits and vegetables that impart from red to blue coloration. Anthocyanins play a role in fruit and vegetable tolerance to environmental stresses and disease resistance, and promote postharvest quality and shelf-life (Jaakola, 2013; Petroni and Tonelli, 2011). Furthermore, anthocyanins are recognized as compounds with potential human health-benefits against oxidative stress mediated diseases (He and Giusti, 2010). Strawberry, a fruit rich in anthocyanins and other phenolic compounds, is an economically important rosaceous crop. In 2011, worldwide production of strawberries was nearly 4.6 million tons and strawberry production in the U.S. was valued at over $2 billion (www.faostats.fao.org) (Floegel et al., 2010). Commercial strawberry (*Fragaria* × *ananassa* Duch.) is an octoploid hybrid with a complex genetic background (Sun and Shi, 2008). The diploid woodland strawberry (*Fragaria vesca* L. ssp. *vesca*) has become an attractive model system for rosaceous functional genomics studies because of its small stature, small genome size, and available genome sequence (Kim et al., 2003; Shulaev et al., 2011; Slovin et al., 2009). Like octoploid strawberry, most woodland strawberry varieties produce anthocyanins and yield red fruit after ripening. However, some accessions such as Yellow Wonder bear

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yellow fruit (Slovin et al., 2009), which are resulted from spontaneous mutation of a "yellow" gene and provides an excellent model to study the mechanisms of anthocyanin biosynthesis in fruit.

Anthocyanins are synthesized from phenylalanine via the phenylpropanoid and flavonoid pathways (Jaakola, 2013; Petroni and Tonelli, 2011). Biosynthesis starts with metabolism of phenylalanine to phenylpropanoids that then enter the flavonoid pathway through the action of chalcone synthase (CHS) (Supplement Fig. 1). Further sequential reactions involve chalcone isomerase (CHI), flavanone 3β-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS), which yield anthocyanidin pigments, the aglycones of anthocyanins. In this pathway, there are three other branches: flavonol synthase (FLS) produces flavonols, and leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), respectively, synthesize *trans*- and *cis*-flavan-3-ols, the precursors of proanthocyanidin polymers. An array of UDP-glycosyltransferases (UGTs) modifies anthocyanins and flavonoids by glycosylation, thereby increasing their polarity, water solubility, and stability.

Anthocyanin and phenolic compound contents in fruit are affected by genetic, developmental and environmental factors. The expression of anthocyanin biosynthesis structural genes is regulated by internal and external factors including sugar concentration, light, hormones, chilling stress, and nutrient status (Carbone et al., 2009). Calcium is a universal second messenger in the signal transduction pathways of hormones and environmental stimuli (touch, wind, chilling, light, and elicitors) (DeFalco et al., 2010; Kudla et al., 2010; Poovaiah and Reddy, 1987, 1993). The effect of calcium on fruit anthocyanin and phenolic content has received little attention. Increasing evidence indicates that calcium plays an import role in fruit anthocyanin biosynthesis. The calcium signaling pathway was implicated in sucrose-induced anthocyanin accumulation in grapes by activating flavonoid pathway genes, such as *DFR* (Gollop et al., 2002; Vitrac et al., 2000). In *Arabidopsis*, changes in endogenous calcium levels can modulate sucrose-induced sugar uptake and regulate anthocyanin accumulation (Shin et al., 2013). Here we compared the expression profiles of flavonoid pathway genes, and anthocyanin content and composition, in two *F. vesca* accessions, Ruegen F7-4 (RF7-4, a red-fruited inbred line from Ruegen) and Yellow Wonder 5AF7 (YW5AF7, a yellow-fruited inbred line from Yellow Wonder) (Slovin et al., 2009). In addition we evaluated the effects of calcium treatment on the contents of total anthocyanins and phenolic compounds in fruit, and its impact on the expression of flavonoid pathway genes.

2. Materials and methods

2.1. Plant materials and calcium treatments

Diploid woodland strawberry (*F. vesca* ssp. *vesca*) 7th generation inbred lines Yellow Wonder 5AF7 (YW5AF7) and Ruegen F7-4 (RF7-4), as well as octoploid strawberry (*F.* × *ananassa*) cv. Seascape were grown in a greenhouse at 26 °C with a diurnal cycle of 16 h light and 8 h darkness following normal cultivation practices. Fruit samples were collected from 4 to 5 individual plants at three different stages based on size and on the color of achenes and receptacles: Green, small size fruit with green achenes and receptacle; Turning, white receptacles starting to change color and achenes showing tan (YW5AF7 and Seascape) or red (RF7-4); Ripe, full size fruit with fully yellow (YW5AF7) or red (RF7-4 and Seascape) receptacles (Fig. 1A). After harvest, all the fruit from different stages were rinsed with distilled water, cut into quarters, immediately frozen in liquid nitrogen, and kept at −80 °C for future use.

For calcium treatment, different concentrations (10, 20 and 50 mM) of $CaCl_2$ solution were used to spray whole plants every four days after pollination. Seascape fruit were harvested at commercial maturity for analysis of total phenolics and anthocyanins, and diploid strawberry fruit were harvested at similar maturity. Fruit at the green, turning, and ripe stages were harvested for gene expression studies and phenolics measurements.

2.2. Determination of total phenolics and anthocyanins

The content of total phenolics was determined using a method described by Lester et al. (2012) with modifications as follows. Briefly, 2.5 g of frozen strawberry fruit or non-fruit tissue powder was extracted with 10 mL of 80% methanol. The samples were then centrifuged at 6650 *g* for 7 min. Then 100 μL of 0.1% Fast Blue BB solution were added to 1.0 mL aliquots of the supernatants, followed by 100 μL of 5% NaOH. The absorbance of the mixtures was read at 420 nm. Results were expressed as micrograms of gallic acid equivalent per gram of fresh fruit. Total anthocyanins were estimated by the method of Sanchez-Moreno et al. (2003). Briefly, 2.5 g of frozen tissue powder was extracted with 10 mL of methanol acidified with 0.1% HCl. The samples were then centrifuged at 6650 *g* for 15 min. The supernatants were then passed through a 0.45 μm filter. Total anthocyanins were estimated by a pH differential method. Absorbance was measured at 510 nm and at 700 nm in buffer at pH 1.00 and pH 4.5, using $A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$ with a molar extinction coefficient for cyanidin-3-glucoside of 29,600. Results were expressed as micrograms of cyanidin-3-glucoside equivalent per gram of fresh weight using a calibration curve for cyanidin-3-glucoside. The calibration curve ($y = 0.01805x + 0.0041$, where y is absorbance and x is sample concentration) ranged from 10 to 1000 μg/mL ($R^2 = 0.9967$). Data were analyzed by Student's t test using SAS (version 8.5). A probability of $P > 0.05$ was considered not significant. Regression analyses were done by Origin (Microcal Software Inc., Northampton, MA, USA).

2.3. Extraction and HPLC–DAD analysis of anthocyanins

Leaf petiole and ripe fruit pericarp tissues were excised from plant organs of inbred *F. vesca* lines RF7-4 and YW5AF7. The tissues were flash frozen in liquid N_2, ground to a powder with a chilled mortar and pestle, and extracted twice with 70% aqueous acetone. Acetone was removed from the extracts by N_2 evaporation at 35 °C in dim light and the aqueous concentrates were acidified by addition of HCl to 0.1%. The acidified extracts were loaded onto 6 mL × 200 mg Strata-X SPE cartridges (Phenomenex, Torrance, CA) after pre-equilibration with methanol followed by deionized water and 0.05% aqueous HCl. The cartridges were washed with 4 mL of 20% aqueous methanol plus 0.05% HCl and then total anthocyanins were eluted with 4 mL of 50% aqueous methanol plus 0.05% HCl. Strata-X anthocyanin eluates were reduced to 0.5 mL by N_2 evaporation at 40 °C and transferred to 2 mL HPLC vials, which were flushed with N_2, capped, and stored at −20 °C until analyzed.

HPLC–DAD analysis of leaf petiole and ripe fruit anthocyanins was performed using an Agilent 1100 Series system including a quaternary pump, a solvent degasser, an autosampler, and a photodiode array detector. Anthocyanins were separated on a 4.6 mm i.d. × 250 mm, 5 μm particle size Luna C18(2) column (Phenomenex) using a gradient of A, aqueous 0.3% H_3PO_4, and B, methanolic 0.3% H_3PO_4, as follows: 0–6 min, linear increase from 25 to 30% B at 1.0 mL/min; 6–16 min, linear increase from 30 to 60% B at 1.0 mL/min; 16–20 min, linear increases from 60 to 80% B and from 1.0 to 1.2 mL/min; 20–26 min, linear decrease from 80 to 25% B at 1.2 mL/min; 26–28 min, hold at 25% B with a linear decrease from 1.2 to 1.0 mL/min. Identification of major constituents was