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Abscisic acid stimulates anthocyanin accumulation in 'Jersey' highbush blueberry fruits during ripening

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ABSTRACT

Non-climacteric blueberry (*Vaccinium* spp.) fruits accumulate high levels of anthocyanins during ripening, which are a good source of dietary antioxidants. This study examined the effects of exogenous abscisic acid (ABA) application on fruit characteristics and anthocyanin accumulation in a northern highbush blueberry (*V. corymbosum* 'Jersey') during development. Fruits on shrubs were treated with 1 g L^{-1} ABA before the initiation of fruit colouration. Application of ABA temporarily increased the level of ABA in the fruits during development. Exogenous ABA had no obvious effect on fruit growth, but stimulated fruit colouration by accelerating the accumulation of individual anthocyanins, mainly malvidin, delphinidin and petunidin glycosides. This is the first report to show that ABA promotes the accumulation of anthocyanins in blueberry fruits. However, exogenous ABA also promoted fruit softening, which is undesirable during harvest and shelf life.

1. Introduction

Fruits are classified as climacteric or non-climacteric according to their ripening characteristics. Climacteric fruits, such as apple, peach and banana, generate a burst of ethylene followed by an increase in respiration rate at the onset of ripening (Giovannoni, 2001). These changes act as a signal for the initiation of ripening in all climacteric fruits. However, the hormonal control of ripening in non-climacteric fruits remains largely unknown. Non-climacteric fruits exhibit no dramatic changes in ethylene production or respiration (Frenkel, 1972), and ripening cannot be triggered by application of exogenous ethylene (Janes, Chin, & Frenkel, 1978).

Abscisic acid (ABA) may be important in the ripening of some non-climacteric fruits, such as grape (Berli, Fanzone, Piccoli, & Bottini, 2011; Jeong, Goto-Yamamoto, Kobayashi, & Esaka, 2004; Peppi, Walker, & Fidelibus, 2008; Sandhu, Gray, Lu, & Gu, 2011), strawberry (Jia et al., 2011; Li, Jia, Chai, & Shen, 2011) and sweet cherry (Shen et al., 2014). Acceleration of fruit softening by exogenous ABA has been observed in many grape (Peppi, Fidelibus, & Dokoozlian, 2006, 2007; Roberto et al., 2012) and strawberry (Li et al., 2014) cultivars. Fruit colouration is also promoted by exogenous ABA in grape (Jeong et al., 2004; Koyama, Sadamatsu, & Goto-Yamamoto, 2010; Koyama et al., 2014; Yamamoto et al., 2015), strawberry (Jia et al., 2011; Li et al., 2014) and sweet cherry (Shen et al., 2014). Shen et al. (2014) reported that silencing *NCED*, a gene encoding a key enzyme in the ABA biosynthesis pathway, resulted in a colourless phenotype in sweet cherry. These findings suggest that exogenous ABA up-regulates genes related to ripening, including those associated with cell wall modification and anthocyanin biosynthesis (Giribaldi, Gény, Delrot, & Schubert, 2010; Koyama et al., 2010). However, it is unclear whether these properties are shared by all non-climacteric fruits, since some reports suggest that exogenous ABA has no effect on anthocyanin accumulation in southern highbush blueberry (*Vaccinium darrowii*; Buran et al., 2012) or lowbush blueberry (*V. angustifolium*; Percival & MacKenzie, 2007).

Non-climacteric highbush blueberry (*V. corymbosum*) fruits accumulate high levels of anthocyanins during ripening, leading to a highly noticeable colouration process (Frenkel, 1972; Janes et al., 1978; Zifkin et al., 2012). This development-related colouration makes highbush blueberries suitable for studies of ripening. The present study was conducted to characterise the effects of exogenous ABA application on morphological and physiological characteristics, especially anthocyanin accumulation, during fruit development in 'Jersey' northern highbush blueberry.

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2. Materials and methods

2.1. Plant materials and ABA treatments

Ten-year-old northern highbush blueberry (*V. corymbosum* 'Jersey') shrubs were grown at the experimental orchard of Seoul National University, Suwon, Republic of Korea.

Six weeks after full bloom, fruit clusters were dipped into 1 g L^{-1} (±)-ABA (Sigma–Aldrich, St. Louis, MO, USA) solution for 1 min, according to the methods of Jeong et al. (2004) and Zhang, Yuan, and Leng (2009). The ABA concentration was chosen from our preliminary experiment. This study was in a randomised complete block design with three replications consisting of five shrubs each. ABA solution was prepared with 5% ethanol containing 0.1% (v/v) Tween 80. All treatments were conducted after sunset to avoid photodegradation of ABA (Zaharia, Walker-Simmon, Rodríguez, & Abrams, 2005). Control fruits were treated with 5% ethanol solution containing 0.1% Tween 80 without ABA. Fifty fruits were randomly sampled each from the replication block at 0, 3, 6, 9 and 12 days after treatment (DAT). Ten fruits sampled were used to determine fruit characteristics such as length, diameter, weight, colour and firmness. The rest were immediately frozen in liquid nitrogen and stored at −80 °C for later determination of ABA and anthocyanin concentrations.

2.2. Determination of ABA concentration

ABA was extracted using the method described by Forcat, Bennett, Mansfield, and Grant (2008), with minor modifications. Freeze-dried fruit tissues were ground with liquid nitrogen. Approximately 200 mg of each sample was suspended in a 2-mL microfuge tube with acetone:water:acetic acid (80:19:1, v/v/v), and powdered using a TissueLyser II (Qiagen, Venlo, Netherlands) with two 3-mm beads at 25 Hz for 3 min. Samples were stirred for 30 min and then centrifuged at 13,000 rpm for 2 min. The supernatant was lyophilised at room temperature, dissolved in 100 μL of methanol:acetic acid (99:1, v/v), combined with 900 μL of water:acetic acid (99:1, v/v) and centrifuged at 13,000 rpm for 1 min. The supernatant was then drawn through a C_{18} Sep-Pak cartridge (Waters Corp., Milford, MA, USA). ABA was eluted from the cartridge using 1 mL 80% methanol containing 1% acetic acid, and then lyophilised at room temperature. The dried sample was resuspended in 200 μL 10% methanol, sonicated for 5 min and then centrifuged at 13,000 rpm for 10 min.

ABA was identified in a Triple TOF 5600 Q-TOF liquid chromatography–tandem mass spectrometry (LC/MS/MS) system (AB Sciex, Foster City, CA, USA) using an Ultimate 3000 rapid separation (RS) high performance liquid chromatography (HPLC) system (Thermo Dionex, Waltham, MA, USA) equipped with a diode array detector (DAD). LC separation was performed on a Hypersil GOLD column (2.1 × 50 mm, 1.9 μm; Thermo Fisher Scientific, Waltham, MA, USA). ABA was eluted with a gradient of mobile phase A (aqueous 0.1% (v/v) formic acid) and mobile phase B (0.1% (v/v) formic acid in acetonitrile) in the following sequence: 0–3 min, 5% B; 3–11 min, 5–30% B; 11–14 min, 30–100% B; 14–16 min, 100% B. The flow rate was 0.25 mL min^{-1} and the injection volume was 5 μL. Mass spectra were acquired under negative electrospray ionisation with an ion spray voltage of −4500 V. The source temperature was 500 °C. The curtain gas, ion source gas 1 and ion source gas 2 were set to 25, 50 and 50 psi, respectively. Multiple reaction monitoring was used to quantify ABA (*m/z* 263.1–153.1).

2.3. Determination of fruit colour and firmness

Fruit skin colours were measured using a spectrophotometer (CM-2500d, Minolta Co., Osaka, Japan) and described using the International Commission on Illumination a^* and b^* colour space coordinates (Hunter & Harold, 1987). The a^* and b^* values range between –100 and 100. The a^* value is negative for green and positive for red. The b^* value is negative for blue and positive for yellow. Values were measured at two different points on the equator of each fruit.

Fruit firmness was analysed using a CT3-4500 texture analyser (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) equipped with a flat probe of 2 mm diameter. The maximum peak force was recorded in N/ϕ 2 mm.

2.4. Identification and quantification of individual anthocyanins

Anthocyanins were extracted according to the method described by Gavrilova, Kajdžanoska, Gjamovski, and Stefova (2011), with some modifications. Approximately 5 g of ground fruit tissues were homogenised with 10 mL of acetone:acetic acid (99:1, v/v) solution. Homogenates were sonicated for 15 min and then centrifuged for 15 min at 3000 rpm. Supernatants were evaporated until dry using a rotary evaporator (EYELA N-1000S-W, Tokyo Rikakikai Co., Tokyo, Japan) at 37 °C, completely redissolved in 10 mL 20% methanol and then filtered through a 0.45-μm pore-size polytetrafluoroethylene filter (Whatman Inc, Florham Park, NJ, USA).

Individual anthocyanins were identified using a Triple TOF 5600 Q-TOF LC/MS/MS system (AB Sciex) with an Ultimate 3000 RS HPLC (Thermo Dionex) equipped with a DAD. LC separation was performed on a Kinetex F5 100-Å column (2.1 × 100 mm, 2.6 μm; Phenomenex, Torrance, CA, USA). Anthocyanins were eluted with a gradient of mobile phase A (aqueous 0.1% (v/v) formic acid) and mobile phase B (0.1% (v/v) formic acid in acetonitrile) in the following sequence: 0–1 min, 5% B; 1–25 min, 5–100% B; 25–27 min, 100% B; 27–27.1 min, 100–5% B; 27.1–33 min, 5% B. The flow rate was 0.25 mL min^{-1} and the injection volume was 1 μL. Mass spectra were acquired under positive electrospray ionisation with an ion spray voltage of 4500 V. The source temperature was 450 °C. The curtain gas, ion source gas 1 and ion source gas 2 were set at 35, 65 and 55 psi, respectively. Two full-scan mass spectra were acquired over an *m/z* range of 50–2000 on the MS mode. Data were collected using Analyst TF 1.7 (AB Sciex) software and analysed using PeakView 2.2 (AB Sciex) and MarkerView 1.2.1.1 (AB Sciex). Anthocyanins were identified by comparing UV–Vis and mass spectral data to previous work (Gavrilova et al., 2011).

Identified anthocyanins were quantified using an HPLC-DAD system (Ultimate 3000, Thermo Dionex) equipped with VDSpher PUR C-18 column (4.6 × 150 mm, 3.5 μm; VDS Optilab, Berlin, Germany). Anthocyanins were eluted using a gradient of mobile phase A (aqueous 5% (v/v) formic acid) and mobile phase B (5% (v/v) formic acid in acetonitrile) in the following sequence: 0–30 min, 5–45% B; 30–35 min, 45% B; 35–36 min, 45–5% B; 36–40 min, 5% B. The flow rate was 0.8 mL min^{-1} and detections were made at 520 nm. Amounts of anthocyanins were expressed as the external standard equivalent (malvidin 3-*O*-glucoside) from the calibration curve.

2.5. Statistical analysis

Statistical differences were analysed using SAS Enterprise 4.3 (SAS Inst. Inc., Cary, NC, USA). Figures were plotted using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA).

3. Results and discussion

3.1. Effects of exogenous ABA on fruit ABA concentration

Before exogenous ABA application, ABA concentrations in 'Jersey' highbush blueberry fruits were maintained below 2 μg g^{-1} dry weight (DW) (data not shown). In untreated fruits, ABA concentration steadily increased and reached a maximum concentration of 9.6 μg g^{-1} DW during the 12-day observation period (Fig. 1). Similar patterns were observed in 'Rubel' highbush blueberry (Zifkin et al., 2012), grape (Owen et al., 2009; Sun et al., 2010) and strawberry (Jia et al., 2011).