Effects of Exogenous Abscisic Acid on Soluble Sugars and Sugar Metabolizing Enzyme Activities in Highbush Blueberry (*Vaccinium corymbosum* cv. Jersey) Fruits

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ABSTRACT

Abscisic acid (ABA) is a non-climacteric fruit ripening regulator having a potential to increase soluble sugars and sugar metabolizing enzyme activities. This study was to examine whether an exogenous ABA treatment affects color, firmness, soluble sugar contents, and sugar metabolizing enzyme activities in highbush blueberry fruits (*Vaccinium corymbosum* cv. Jersey) during ripening. ABA dipping at 500 ppm at 53 days after full bloom enhanced fruit skin

coloration, and fruit softening, soluble sugar accumulation in blueberry fruits, suggesting a fruit ripening stimulatory effect. Analysis for the sugar contents revealed that the major soluble sugars in highbush blueberry fruits were fructose, glucose, and sucrose. With ABA treatment, the amounts of fructose, glucose, and sucrose were increased, and the increases of fructose and glucose were relatively higher than that of sucrose. Activities of the soluble sugar metabolizing enzymes, acid invertase, neutral invertase, sucrose synthase, and sucrose phosphate synthase were also affected by the ABA treatment. These results indicated that ABA stimulated ripening of highbush blueberry fruits by increasing sugar metabolizing enzyme activities.

Key words: abscisic acid, acid invertase, blueberry (*Vaccinium corymbosum*), fructose, glucose, neutral invertase, non-climacteric fruit, sucrose, sucrose phosphate synthase, sucrose synthase

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INTRODUCTION

Blueberry is known as a non-climacteric fruit whose ripening is regulated by abscisic acid (ABA) (Zhang et al., 2009a). The ripening mechanism of non-climacteric fruits, such as blueberries, whose respiration rates do not exhibit large peaks at around veraison and that evolve low levels of ethylene compared to climacteric fruits, is not well understood. Ethylene does not appear to be crucial to ripening in non-climacteric fruits, as it is in climacteric fruits. ABA is associated with accelerated ripening, because ABA increases rapidly at the beginning of fruit ripening and has a high correlation with the accumulation of sugar, reduction of acid, and production of fruit pigments (Sun et al., 2010). ABA has been reported to enhance carbohydrates unloading in fruits, to stimulate uptake of translocated carbohydrates by cells, and to regulate the sugar metabolism in cells (Loescher and Everand, 1996; Opaskornkul et al., 1999; Rock and Quatrano, 1995; Yamaki and Asakura, 1991).

Soluble sugars are generally low in immature fruits, but increase gradually with fruit ripening. In blueberry fruits, fructose and glucose are the major sugar followed by sucrose, but sucrose is present in smaller amounts (Darnell et al., 1994). Soluble sugar compositions and metabolisms are affected by soluble sugar metabolizing enzyme activities. Acid invertase (AI), neutral invertase (NI), sucrose synthase (SS), and sucrose phosphate synthase (SPS) are the major enzymes of soluble sugar metabolism. Fruit growth and carbohydrate

accumulation in blueberry fruits have been described, but the enzyme activity and exogenous ABA treatment for fruit ripening have received scarce attention.

In the present study, soluble sugars and sugar metabolizing enzyme activities in highbush blueberry fruits were assessed and correlated with exogenous ABA treatment. The objective was to determine how exogenous ABA treatment affects soluble sugar metabolism in highbush blueberry fruits and to understand non-climacteric fruit ripening.

LITERATURE REVIEW

Carbohydrates in fruits

Carbohydrates provide energy for fruit development and ripening. In most sink tissues, net sucrose degradation is occurring. Ultimately, reducing sugars or their derivatives are formed and metabolized for energy production or C-skeletons for growth or storage product accumulation (Quick and Schaffer, 1996). In fruits, carbohydrate are accumulated generally in the form of starch, non-reducing sugars, or reducing sugars during fruit development. During fruit ripening, there was a decrease in starch content and an increase in the reducing and non-reducing sugars. Sucrose is a major end product of photosynthesis and functions as a primary transport sugar (Winter and Huber, 2000). Although the main sugars in the fruits are fructose, glucose, and sucrose, the sugar composition varies among fruit species.

Sugar metabolizing enzyme activities in fruits

The amount and types of sugars in fresh fruit directly influence its quality and flavor. The sink strength and regulation of sugar metabolism in fruits have been investigated extensively based on the functions of AI, NI, SS, and SPS (Geiger et al., 1996; Ho, 1988; Miron and Schaffer, 1991; Table 1). The balance between AI, NI, SS, and SPS activities determines the soluble sugar concentrations in fruits

(Kubo et al., 2001). In fruits, two groups of enzymes with different properties catalyze the cleavage of sucrose; invertases and SS. These latter ones are

Table 1. Enzymes related to soluble sugar metabolism.

Enzyme	Catalyzed reaction
Acid (AI) or neutral invertase (NI)	Sucrose → Fructose + Glucose
Sucrose synthase (SS)	Sucrose + UDP ↔ UDP-Glucose + Fructose
Sucrose phosphate synthase (SPS)	UDP-Glucose + Fructose-6-phosphate → Sucrose-6-phosphate

glycosyltransferases, which, in the presence of uridine 5'-diphosphate (UDP), convert sucrose into UDP-glucose and fructose in a reversible manner. The main distinctive feature of invertases is the production of glucose instead of UDP-glucose (Nonis et al., 2007). AI is mostly located in the vacuole, but also located in apoplast. SPS is a sucrose biosynthetic enzyme. The changes in sugar metabolizing enzyme activities vary among plant materials.

Effect of ABA on fruit ripening

Fruits can commonly be divided into the climacteric and non-climacteric fruits. Climacteric fruits exhibit an increase in ethylene evolution and respiration at the commencement of ripening (Tucker, 1993). In contrast, respiration rates of non-climacteric fruits do not exhibit large peaks at around veraison. Ethylene does not appear to be crucial to ripening in non-climacteric fruits, as it is in climacteric fruits. Exogenous ABA application has shown to affect growth and ripening in several fruits including avocado (*Persea americana*; Chernys and Zeevaart, 2007), orange (*Citrus sinesis*; Rodrigo et al., 2006), grape (*Vitis vinifera*; Wheeler et al., 2009), peach (*Prunus persica*; Zhang et al., 2009b), strawberry (*Fragaria ananassa*; Jia et al., 2011), and tomato (*Solanum lycopersicum*; Zhang et al., 2009a), which have widely been used as a model for studying climacteric and non-climacteric fruits. Free ABA levels have been shown to increase at about the time of veraison. The increase in ABA levels at around the time of veraison is

consistent with it having a role in the control of fruit ripening. ABA application has been reported to enhance both sugar and anthocyanin accumulation (Düring et al., 1978; Hale and Coombe, 1974; Matsushima et al., 1989). ABA levels were increased by sugar treatment of *Arabidopsis* seedlings, and treatment with ABA increases sugar sensitivity, suggesting that regulation of ABA synthesis plays an important role in plant sugar responses. Although specific role of ABA during fruit ripening is not clear yet, several studied have pinpointed ABA as the signal triggering berry ripening. Controversially, ABA did not induce blueberry fruit ripening (Buran et al., 2012). Thus, the direct effects of ABA on blueberry fruits are still unclear. In addition, the sugar metabolizing enzyme activities of ABA-treated blueberry fruits has not been studied. This study was designed to examine the effect of exogenous ABA on fruit ripening by evaluating soluble sugars and sugar metabolizing enzyme activities in highbush blueberry.

MATERIALS AND METHODS

Plant materials and ABA treatment

Fifteen-year-old highbush blueberry shrubs (*Vaccinium corymbosum* cv. Jersey) were used for ABA treatment. Uniformly sized fruits were harvested from 53 days after full bloom (DAFB) before veraison stage, because blueberry fruits in any given cluster do not all ripen at the same time (Young, 1952). Harvested fruits were directly soaked in an ABA solution consisting of 500 ppm (+)-ABA, 0.1 N KOH, and 0.1% Tween 80 as a wetting agent for 10 min. The control solution contained 0.1 N KOH with 0.1% Tween 80. Fruits were frozen in liquid N_2 and stored at −80°C until the sugar content determination and enzyme assays.

Physical characteristics determination

Fruit firmness was measured using a texture analyzer (Brookfield CT3, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a 2-mm probe and calculated in Newton (N). Fruit color was measured at the surface using a colorimeter (CR-400, Minolta Co., Tokyo, Japan). The color of fruit was recorded using the CIELAB L*, a*, and b* color spacecoordinates, in which the L* scale represents the lightness of color and ranged from (L* = 0; black) to (L* = 100; white). The a* scale is negative for green and positive for red. The b* scale is negative for blue and positive for yellow.

Carbohydrate analysis

Fruits were immediately frozen in liquid N_2 to stop metabolism, then ground in a chilled mortar and stored as powder at −80°C until use. Soluble sugars were analyzed using the method of González-Rossia et al. (2008) with slight modifications. One hundred milligrams of the powder were put in a 2-mL test tube containing 1 mL of 80% ethanol. After incubating at 85°C for 15 min and centrifuging at 15,000 g for 5 min, the supernatant was collected in a 15-mL test tube and the pellet was re-extracted twice as above. The collected supernatant was evaporated using a N_2 evaporator (N-EVAP™, Organomation Associates, Inc., West Berlin, MA, USA) at 60°C; pellets were stored in a dry oven for starch analysis. The solution of sugar extracts was dissolved in 3 mL of distilled water, passed through 0.45 μm nylon filter (Acrodisc® 13 mm syringe filter, Pall Co., Washington, NY, USA) and C18 Sep-Pak cartridge (Water Associates, Milford, MA, USA). Sugars were analyzed using an HPLC (UltiMate 3000, Dionex, Sunnyvale, CA, USA) connected to a Shodex RI-101 detector (Showa Denko K.K., Kawasaki, Japan). The filtered extracts of 10 μL were injected into a Sugar-Pak column which temperature was kept at 75°C and distilled water was as a solvent at a flow rate of 0.5 $mL \cdot min^{-1}$.

Starch was analyzed using the remaining pellet according to the method conducted by Smith and Zeeman (2006). The pellet was dissolved in 1 mL of distilled water, and then boiled to gelatinize starch granules. After cooling on ice for 1 min, 0.5 mL of 0.2 M Na-acetate (pH 5.5) buffer, 15 units of

amyloglucosidase (A7095, Sigma-Aldrich Korea Ltd., Yongin, Korea), and 5 units of α-amylase (A3404, Sigma-Aldrich Korea Ltd.) were added and incubated at 55°C for 2 h. After centrifugation at 15,000 g for 15 min, the supernatants were collected, evaporated, and dissolved in 3 mL of distilled water. The extracts were filtered through 0.45 μm nylon filter and C18 Sep-Pak cartridge, and then the released glucose was determined by HPLC as above.

Enzyme extraction

Enzyme extraction was performed using the method of Hubbard et al. (1989) with slight modifications. All procedures were carried out at 4°C or lower. Frozen fruit tissues were ground in a chilled mortar using a tissues to buffer ratio of 1:5. The extraction buffer contained 50 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM dithiothreitol (DTT), 0.05% (v/v) Triton X-100, 0.5 $mg \cdot mL^{-1}$ bovine serum albumin (BSA), 10% (v/v) glycerol, and 10% (w/v) polyvinylpyrrolidone (PVP). The homogenate was agitated for 15 min on ice and centrifuged at 15,000 g at 4°C for 10 min. Supernatants were desalted immediately by passing Sephadex G-25 columns (PD-10 columns, GE Healthcare, Uppsala, Sweden) equilibrated with 50 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 2.5 mM DTT, and 0.5 $mg \cdot mL^{-1}$ BSA.

Protein determination

Protein content was estimated in crude extracts by the method of Bradford

(1976) using BSA as a standard.

Enzyme assay

Acid invertase

AI was assayed in cleavage direction by determining the amount of glucose produced from sucrose using the method of Dreier et al. (1998) with slight modifications. AI activity was measured by adding 20 μL of desalted extracts to 300 μL with 0.2 M acetate buffer (pH 4.5). The enzyme reaction was proceeded by adding 400 μL of 0.225 M sucrose and incubated at 30°C for 20 min. The reaction was stopped by adding 1 mL of 1% 3,5-dinitrosalicylic acid (DNSA) and incubated in a water bath at 100°C for 10 min. Control was performed by adding 1 mL of 1 % DNSA-reagent prior to the desalted extract. After boiling, the reaction mixtures were immediately cooled on ice for 5 min and at room temperature for 10 min, and then color development was measured at 560 nm.

Neutral invertase

NI was assayed in cleavage direction by determining the amount of glucose produced from sucrose. NI assay was identical to that of AI except that the 0.2 M acetate buffer (pH 4.5) was used instead of 50 mM Hepes-NaOH (pH 7.5).

Sucrose synthase

SS for synthetic way was assayed by determining the amount of sucrose

produced from uridine 5'-diphosphoglucose (UDPG) and fructose. The reaction mixture at a volume of 70 μL contained 50 mM Hepes-NaOH (pH 7.5), 15 mM $MgCl_2$, 10 mM fructose, 10 mM UDPG, and 45 μL desalted extract. Reaction was proceeded at 30°C for 20 min and terminated at 0 and 20 min with 30% KOH and by heating at 100°C for 10 min. After cooling for 1 min on ice, 1 mL of 0.14% anthrone in 13.8 M H_2SO_4 was added and incubated in a water bath at 100°C for 10 min. After boiling, the reaction mixtures were immediately cooled on ice for 3 min and at room temperature for 3 min, and then color development was measured at 620 nm.

Sucrose phosphate synthase

SPS was assayed in synthesis direction by determining the amount of sucrose produced from UDPG, fructose-6-phosphate, and glucose-6-phosphate. SPS assay was identical to that of SS except that 10 mM fructose was used instead of 5 mM fructose-6-phosphate and 15 mM glucose-6-phosphate.

Statistical analysis

Statistical differences were analyzed by using the SigmaPlot 10.0 package (SAS Inst. Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Fruit characteristics in response to ABA treatment

The skin color of blueberry fruit gradually changed from pale green to dark purple after ABA treatment. Compared to the control, fruits treated with ABA showed denser blue/purple color during experiment days after ABA treatment (Figs. 1, 2). Fruit color parameters were significantly different with days after ABA treatment (Fig. 2). The L* and b* scales of fruits treated ABA decreased with days after ABA treatment, but the a* scales of fruits treated ABA increased with days after ABA treatment (Fig. 2). These were consistent with enhancing effects of ABA on fruit skin coloration (Guan et al., 2012) and ripening (Kuhn et al., 2014). The firmness of fruits treated ABA decreased than the control during days after ABA treatment (Fig. 3). This result indicates that softening of fruits treated with ABA result from fruit ripening.

Starch degradation in blueberry fruits was not associated with fruit ripening and total soluble sugar contents (Fig. 4). Similarly, Eck (1988) reported that after coloration, starch and other complex carbohydrates did not change appreciably as the berry matured. The total soluble sugar content in fruit increased with days after ABA treatment (Fig. 5). Small fruits, such as blueberries and strawberries, often store primarily glucose and fructose (Shaw, 1988; Woodruff et al., 1960). Fructose, glucose, and sucrose were found in 'Jersey' fruits (Fig. 6). In blueberries, fructose and glucose accumulate predominantly during fruit ripening (Kader et al.,

1993).

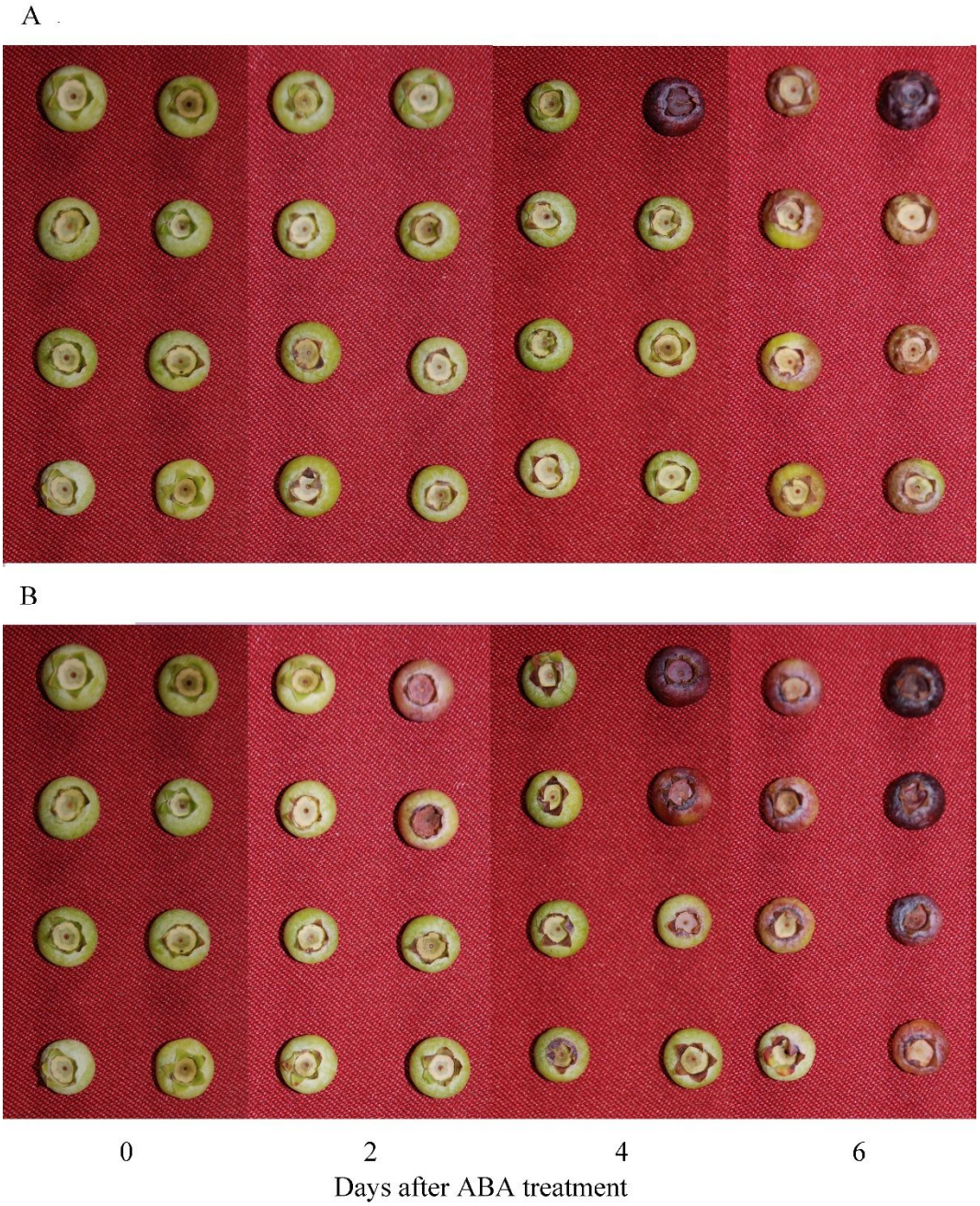

Fig. 1. Changes of color in 'Jersey' highbush blueberry fruits with ABA dipping at

500 ppm. A, control; B, ABA-treated.

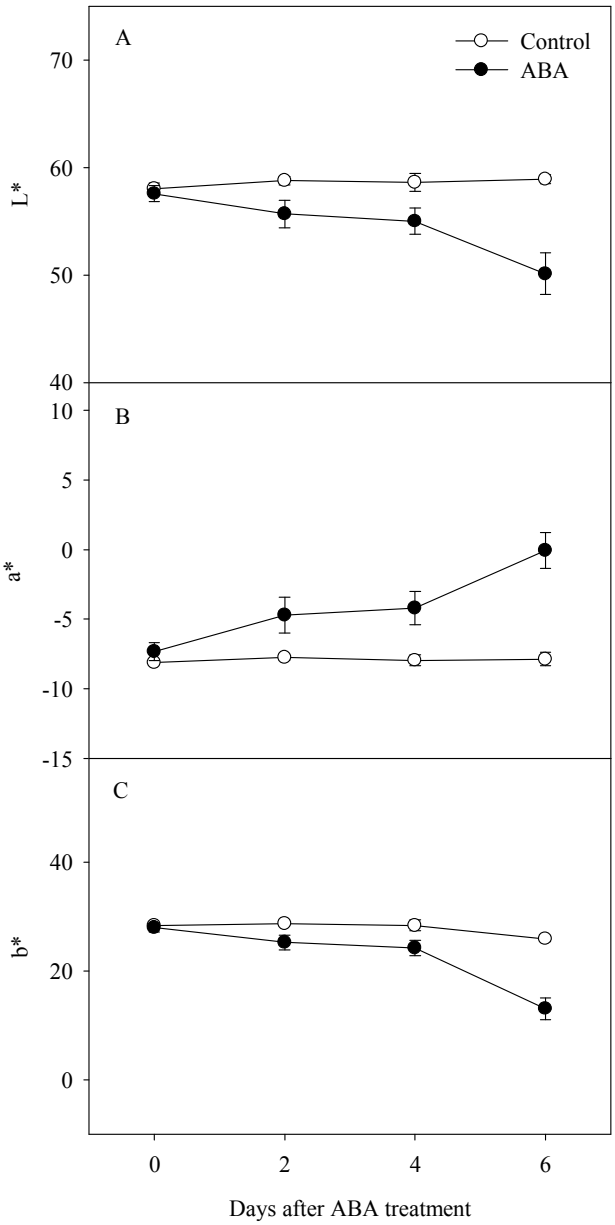

Fig. 2. Changes of L* (A), a* (B), and b* (C) color scales in 'Jersey' highbush

blueberry fruits with ABA dipping at 500 ppm. Vertical bars are the standard deviations of the means.

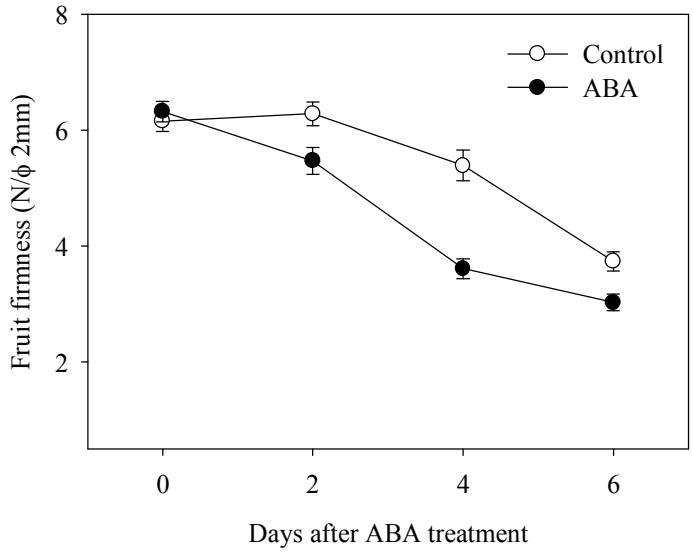

Fig. 3. Changes of firmness in 'Jersey' highbush blueberry fruits with ABA dipping at 500 ppm. Vertical bars are the standard deviations of the means.

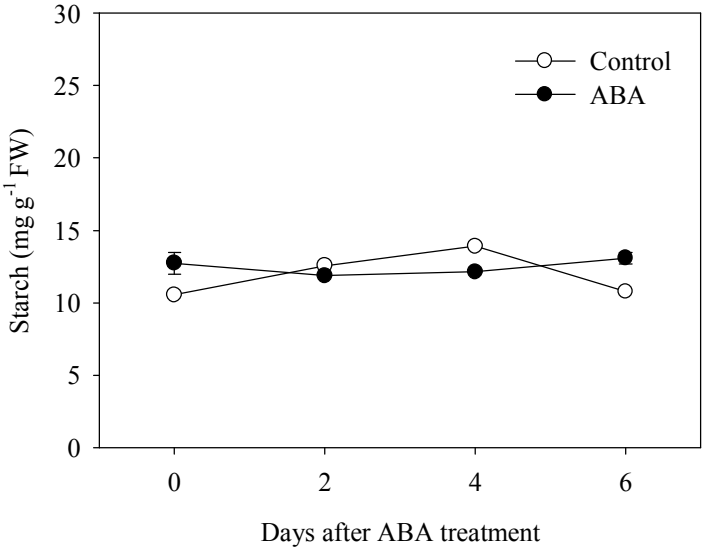

Fig. 4. Changes of starch content in 'Jersey' highbush blueberry fruits with ABA dipping at 500 ppm. Vertical bars are the standard deviations of the means.

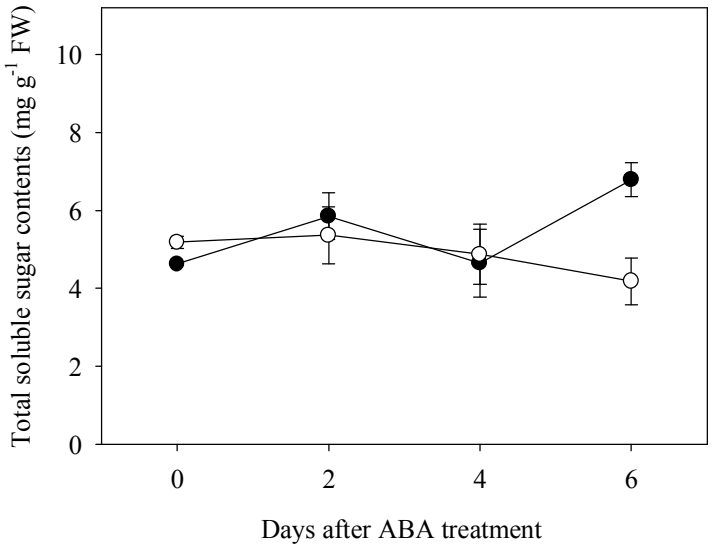

Fig. 5. Changes of total soluble sugar contents in 'Jersey' highbush fruits with ABA dipping at 500 ppm. Vertical bars are the standard deviations of the means.

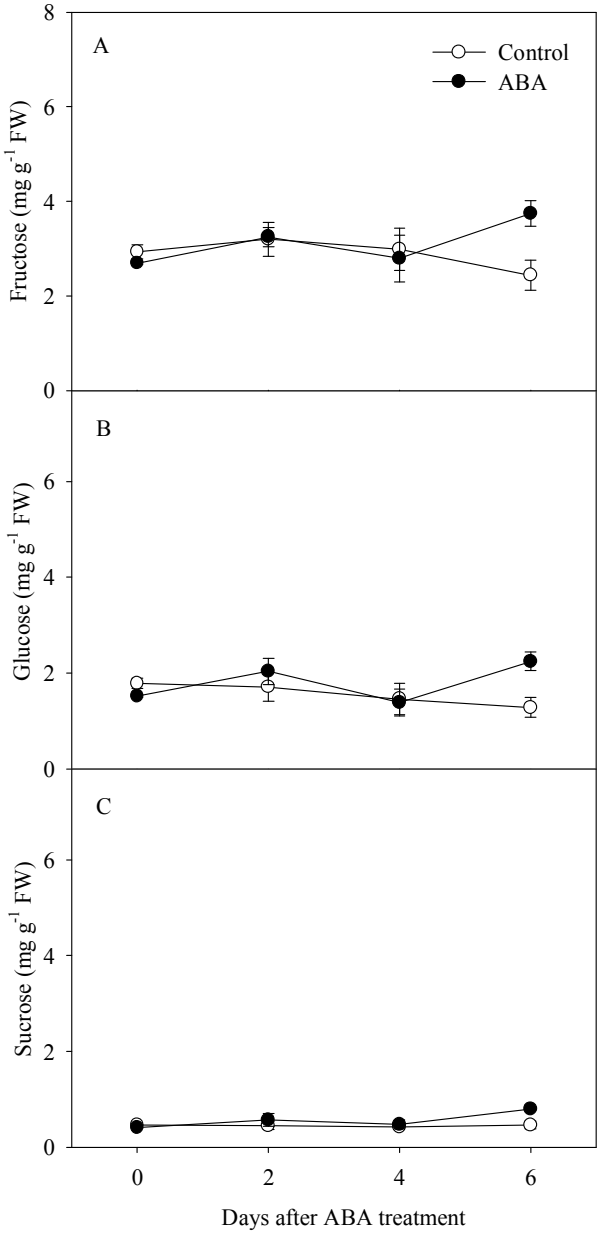
A
Control
ABA
Fructose (mg g-1 FW)
B
Glucose (mg g-1 FW)
C
Sucrose (mg g-1 FW)
0
2
4
6
8
Days after ABA treatment

Fig. 6. Changes of fructose (A), glucose (B), and sucrose (C) contents in 'Jersey' highbush blueberry fruits with ABA dipping at 500 ppm. Vertical bars are the standard deviations of the means.

Total soluble sugars negligibly changed until 4 days after ABA treatment and then significantly increased (Fig. 5). Of all the soluble sugars, fructose concentration was highest in mature fruit, which was about 1.7 times of glucose, and 4.7 times of sucrose concentration. The concentrations of sucrose, glucose, and fructose in blueberry fruits increased following ABA treatment (Fig. 6). On the other hand, control fruits showed no significant changes of soluble sugar concentrations with days. These results indicate that ABA is involved in sugar accumulation in highbush blueberry fruits. Changes in total soluble sugar contents coincided with changes of L*, a*, and b* color scales (Figs. 2, 5). The positive relationship between sugar concentration in the tissue and pigmentation has been suggested in several plants (Weiss and Halevy, 1991; Kawabata et al., 1999) including grape (Pirie and Mullins, 1977). These results suggest that ABA increases rapidly at the beginning of fruit ripening and has a high correlation with the accumulation of sugar and synthesis of fruit pigments (Sun et al., 2010).

Sugar metabolizing enzyme activities in response to ABA treatment

ABA treatment promoted soluble sugar metabolizing enzyme activities. The

activities of the enzymes showed similar trends. The activities of soluble sugar metabolizing enzymes generally increased, but subsequently declined. These results suggested that sugar metabolizing enzyme activities were inactivated. The activities of AI and NI increased from 0 to 4 days after ABA treatment. The increases in AI and NI activities correlated with increases in fructose and glucose concentrations (Figs. 6A-B, 7, 8). Because sucrose is cleaved into fructose and glucose by invertases (Hawker, 1985). This agrees with the report that invertases were the dominant enzymes in hexose-accumulating fruits, such as blueberries (Darnell et al., 1994). Thus, the activation of AI and NI by ABA treatment accounted for the most portion of increased fructose and glucose concentrations in blueberry fruits. Both AI and NI activities of control also changed during days after ABA treatment but not as much as ABA treated (Figs. 7, 8). Sucrose accumulation appeared to be that was attributed by SS activities (Figs. 6C, 9). According to this study, SS was assayed sucrose synthesis direction. The activities of SS increased from 0 to 4 days after ABA treatment (Fig. 9). However, both invertases and SS activities were increased. SPS also sucrose synthetic enzyme (Winter and Huber, 2000). In contrast, the activity of SPS decreased from 0 to 2 days after ABA treatment, and then increased (Fig. 10). Similarly, SPS did not affect total soluble sugars in blueberry fruit during ripening (Cano-Medrano and Darnell, 1997; Darnell et al., 1994). After AI, NI, and SS activities increased from 0 to 4 during days after ABA treatment, total soluble sugars increased from 4 to 6 during days after ABA treatment (Figs. 7, 8, 9). Low level of total soluble sugar

contents in early stages of fruits does not match with enzyme activities, but these results indicated that these enzyme activities were associated with generating total soluble sugars.

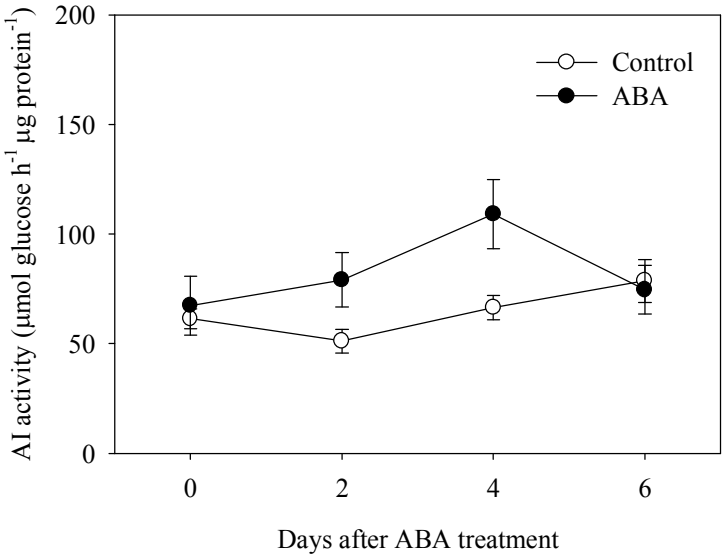

Fig. 7. Changes of acid invertase activities in 'Jersey' highbush blueberry fruits with ABA dipping at 500 ppm. Vertical bars are the standard deviations of the means.

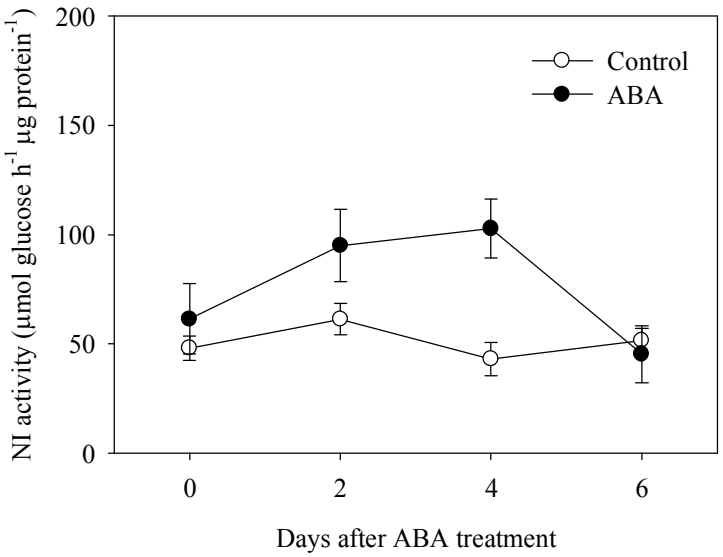

Fig.8. Changes of neutral invertase activities in 'Jersey' highbush blueberry fruits with ABA dipping at 500 ppm. Vertical bars are the standard deviations of the means.

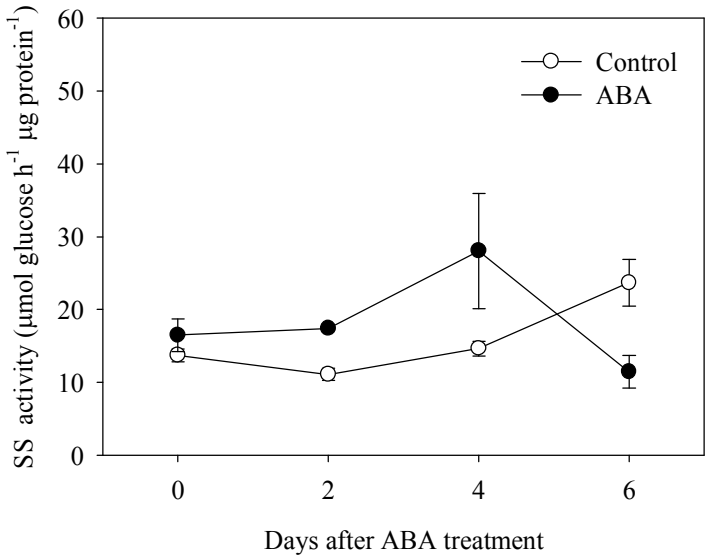

Fig. 9. Changes of sucrose synthase activities in 'Jersey' highbush blueberry fruits with ABA dipping at 500 ppm. Vertical bars are the standard deviations of the means.

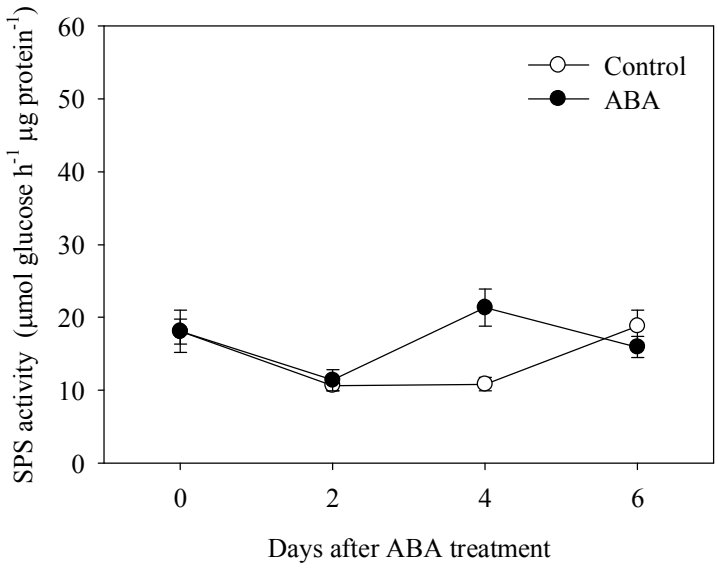

Fig. 10. Changes of sucrose phosphate synthase activities in 'Jersey' highbush blueberry fruits with ABA dipping at 500 ppm. Vertical bars are the standard deviations of the means.

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ABSTRACT IN KOREAN

앱시스산(ABA)은 호흡 비급등형 과실의 성숙 조절 인자로 관여하고 있다고 알려져 있다. 이러한 앱시스산이 과실 성숙에 끼치는 영향 중, 과실의 색, 경도, 가용성 당 함량과 당 관련 효소 활성을 하이부시 블루베리 'Jersey' 품종 과실을 대상으로 연구하였다. 만개 53일 후에 'Jersey' 품종 과실에 앱시스산 500ppm을 처리한 결과 과피의 색이 짙어지고 경도가 감소하며 과실 내 당 축적이 이루어짐을 관찰하였다. 당 분석 결과, 'Jersey' 품종 과실의 가용성 당의 대부분은 과당, 포도당, 자당이었다. 이때 앱시스산을 처리한 경우, 과당, 포도당, 자당의 양이 모두 증가하였고, 이 중 상대적으로 과당과 포도당이 자당에 비해 많이 증가하였다. 이러한 결과와 함께 가용성 당 관련 효소인 acid invertase, neutral invertase, sucrose synthase, sucrose phosphate synthase의 활성 또한 앱시스산 처리에 의해 증가하는 경향을 보였다. 이러한 결과들을 통해 앱시스산이 하이부시 블루베리 과실들의 당 관련 효소들의 활성을 증가시키고 이를 통해 가용성 당들의 함량이 증가시키는 데 관여하고 있음을 알 수 있었다.