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Softening in Highbush Blueberry Fruits: Cell Wall Modification and β-Aminobutyric Acid Effect

하이부시 블루베리 과실의 연화: 세포벽 변형과 β-Aminobutyric Acid의 효과

BY

SINATH CHEA

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MAJOR IN HORTICULTURAL SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

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ABSTRACT

Blueberry fruit quality deteriorates significantly after harvest due to its rapid softening characteristics. Fruit quality changes and cell wall-associated softening mechanism were investigated during ripening of 'Bluecrop' highbush blueberry fruits. The effects of preharvest β-aminobutyric acid (BABA) treatment on postharvest fruit quality and their softening were also evaluated during refrigerated storage. The fruit size greatly expanded along with a substantial accumulation of fruit weight between the reddish purple and dark purple stages. Fruit firmness declined significantly between the pale green and reddish purple stages, which corresponded to the increases in soluble solids content, pH, and total anthocyanin content. Respiration rate peaked at the reddish purple stage,

coinciding with the peak of ethylene production which accounted for approximately 2 $\mu L \cdot kg^{-1} \cdot h^{-1}$. The softening of 'Bluecrop' highbush blueberry fruits corresponded to extensively fractured microstructures of parenchyma tissues and alteration in cell wall ultrastructure, and significantly correlated with a reduction in fruit cell wall material contents. HCl-soluble pectins were converted to water-soluble pectins as ripening proceeded. Arabinose contents were substantially lost in HCl-soluble pectins, decreasing the ratio of arabinose plus galactose to rhamnose during ripening, and significantly correlated with fruit softening. Hemicellulose contents were significantly reduced with increasing ripening, showing a significant positive correlation with fruit firmness. Polygalacturonase, α-arabinofuranosidase, and β-galactosidase showed higher activities during early ripening, whereas the activities of α-mannosidase and endo-1,4-β-xylanase were higher during late ripening. Preharvest BABA treatment on fruit clusters at 20 mM significantly delayed color changes, enhanced individual soluble sugars and organic acids, and reduced decay incidence. BABA-treated fruits maintained higher skin firmness throughout the storage period. Cell wall material contents were significantly higher in BABA-treated fruits than in control. Moreover, preharvest BABA treatment significantly enhanced fruit pectin content at harvest and reduced activities of polygalacturonase and endo-1,4-β-xylanase, although hemicellulose content was

not changed. These results indicate that cell wall-related softening events in ‘Bluecrop’ highbush blueberry fruits extensively occur during early ripening and that preharvest BABA treatment improves the postharvest fruit quality including skin firmness during refrigerated storage. The results will be useful for managing softening-related fruit losses in highbush blueberries.

Key words: β-aminobutyric acid, ‘Bluecrop’ highbush blueberry, cell wall composition, cell wall modification, postharvest fruit quality, preharvest application

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LIST OF ABBREVIATIONS

BABA	β-Aminobutyric acid
CESA	Cellulose synthase A
CWME	Cell wall modifying enzyme
DMRT	Duncan's multiple range test
GalA	Galacturonic acid
GGM	Galactoglucomannan
HG	Homogalacturonan
LSD	Least significant difference
PBS	Phosphate buffered saline
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
XGA	Xylogalacturonan

GENERAL INTRODUCTION

Blueberry fruits are rich in anthocyanins (Vinson et al., 2001) that benefit human health via the prevention of a variety of diseases including certain types of cancers (Neto, 2007; Olsson et al., 2004; Stoner et al., 2008). The fruits ripen within 2 or 3 months after bloom (Gauthier and Kaiser, 2013). The fruit size gradually expands from fruitlet to large green stage and later the pink color initiates on the fruit surface. The fruit size and color continue to develop from pink stage toward blue stage and to a fully ripe stage.

In general, blueberry fruits are harvested at a fully ripe stage in order to obtain acceptable eating quality and to gain economic benefits as they do not properly ripen off the shrub despite being harvested at a nearly ripe stage. One of the most challenging issues in blueberry fruits is in that they are highly perishable and susceptible to rapid spoilage, thus increasing vulnerability to pathogen attacks. These intrinsic softening characteristics affect storability, transportability, and marketability, thus limiting their availability to consumers (Goulao and Oliveira, 2008; Vicente et al., 2007).

In blueberry fruits, firmness determines fruit quality, shelf life, and consumers' acceptance (Vicente et al., 2007) and cell wall strength has a huge impact on these characteristics (Goulao and Oliveira, 2008; Li et al., 2010). The

cell wall strength depends largely on cell wall compositions, linkages among cell wall polysaccharides, and dissolution of middle lamella (Brummell and Harpster, 2001; Goulao and Oliveira, 2008; Vicente et al., 2007).

Several cell wall modifying enzymes (CWMEs) including polygalacturonase, pectin methylesterase, pectate lyase, β-galactosidase, α-arabinofuranosidase, endo-1,4-β-glucanase, endo-1,4-β-xylanase, α-mannosidase, and β-xylosidase (Brummell, 2006) modify cell wall polysaccharides, causing modification and rearrangements which ultimately affect cell wall structures and lead to fruit softening (Brummell, 2006; Goulao and Oliveira, 2008; Vicente et al., 2007).

CWMEs have been studied in many fruits such as apple (Goulao et al., 2007; Ng et al., 2015), pawpaw (Koslanund et al., 2005), strawberry (Bustamante et al., 2009; D'Amour et al., 1993; Figueroa et al., 2010; Rosli et al., 2004, 2009; Villarreal et al., 2008), grape (Deng et al., 2005), pear (Tateishi et al., 2005), and peach (Manganaris et al., 2006). In blueberry fruits, however, cell wall modification by CWMEs is poorly understood and little has been done to search for fruit treatments to extend postharvest shelf life. Vicente et al. (2007) reported that pectin became more soluble and hemicellulose was depolymerized during softening, but the softening-related CWMEs are unknown.

The observation of cell microstructure and cell wall ultrastructure can

provide qualitative and comprehensive information useful for identifying the softening processes (Allan-Wojtas et al., 2001), which occur as a consequence of multiple cellular processes. Cell walls are structurally complex and might be synthesized during ripening (Brummell, 2006; Takizawa et al., 2014). Xylan is a major hemicellulose present in blueberry fruits (Vicente et al., 2007), but its distribution and role during the fruit softening remain largely unknown. Cell wall monoclonal antibody LM10 can be used to detect the epitope of (1,4)-β-xylan (Handford et al., 2003; Sutherland et al., 2009) with the support from either a confocal microscopy or a fluorescence microscopy. With this antibody, xylan could be visualized throughout fruit ripening and softening. Such information may complement the traditional fractionation of cell wall and analysis of cell wall monosaccharides.

Several synthetic compounds have been applied at either preharvest or postharvest to extend fruit shelf life. β-Aminobutyric acid (BABA), a non-protein amino acid, has been reported to enhance resistance of fruits and vegetables to biotic and abiotic stresses (Cohen et al., 2016; Jannatizadeh et al., 2018). Recently, BABA application has been expanded beyond stress tolerance and plant pathogen protection. Wang et al. (2015a) reported that postharvest BABA treatments at 30 mM enhanced cell wall contents and reduced CWME activities, leading to firmer sweet cherry fruits during storage.

To gain better insight into fruit softening mechanism and to search for appropriate treatments to extend shelf life of blueberry fruits, changes in fruit quality characteristics, cellular structures, xylan distribution, cell wall composition, and cell wall modifying enzyme activities during ripening were investigated. Moreover, the effect of preharvest BABA treatment on cell wall modification and changes in postharvest fruit quality were evaluated during refrigerated storage.

LITERATURE REVIEW

Blueberry fruit and changes in ripening-associated quality

Blueberry fruits belong to the Ericaceae family, genus *Vaccinium* (Pritts and Hancock, 1992) and rank second as the world's most important soft fruit (Chen et al., 2015; Giongo et al., 2013). The fruit is epigynous berry (Pritts and Hancock, 1992) and anatomically characterized as having a single-layered outer epidermis without stomata and covered with cuticle. Beneath the epidermis, hypodermis containing pigments is delineated by a ring of vascular bundles. Two more rings are established in the homogenous parenchyma mesocarp, in which five carpels with ten locules and a stony endocarp are present (Giongo et al., 2013; Gough, 1994). In general, fruits are made up of parenchyma tissues with polyhedral shape and thin cell wall (Gibson, 2012). Stone cells also exist in blueberry parenchyma tissues and are unevenly distributed within mesocarp, but generally occur just below the epidermis (Gough, 1994).

Blueberry fruits ripen within 2 or 3 months after bloom (Pritts and Hancock, 1992). Green and ripe fruits contain approximately 7 and 15% of soluble sugar content, respectively. Large amounts of pigments are accumulated and cell wall changes result in fruit softening. Best quality is obtained when fruits are harvested at fully ripe stage, but these fruits at this stage have a very short postharvest life. The fruits extensively soften, rapidly deteriorating quality and lowering resistance

to various fruit rots (Pritts and Hancock, 1992). Several factors such as varietal characteristics, harvest methods, postharvest handling, and storage temperature and duration affect blueberry fruit firmness (Li et al., 2011). The number and arrangement of stone cells and cell wall thickness in the parenchyma tissues may also contribute to blueberry fruit firmness (Allan-Wojtas et al., 2001). In a recent study, however, stone cells in 'Bluecrop' highbush blueberry fruits were not associated with the firmness, since cell wall thickness and the number of stone cells were not different at 35 and 70 days after anthesis (Konarska, 2015b), but they were involved in the grittiness (Blaker and Olmstead, 2014). Konarska (2015a) reported that more stone cells were present in 'Earliblue' mature blueberry fruits than in 'Patriot', but stone cell wall thickness in both cultivars was similar.

Despite variable reports on the contribution of stone cells to fruit firmness, the softening may be largely the result of changes in parenchyma cell walls that are predominant in blueberry fruits. Considering that cell walls are primarily constructed from pectins, hemicelluloses, and celluloses (Keegstra, 2010), modification of these polysaccharides during fruit development and ripening (Giongo et al., 2013) are definitely associated with blueberry fruit softening. Blueberry fruit firmness declines during ripening (Vicente et al., 2007) and postharvest storage (Chen et al., 2015), causing significant losses of quality during storage (Giongo et al., 2013). Excessive fruit softening affects its storability,

transportability, and marketability, and increases vulnerability to physical damage and susceptibility to decay and diseases, thus restricting its availability to consumers (Angeletti et al., 2010; Goulao and Oliveira, 2008; Li et al., 2011; Pritts and Hancock, 1992).

Several preharvest and postharvest technologies have been applied to reduce decay and extend fresh market life of blueberry fruits, including cold storage (Chen et al., 2015), UV irradiation (Nguyen et al., 2014; Perkins-Veazie et al., 2008), SO_2 fumigation and CO_2-enriched atmosphere (Cantín et al., 2012), and edible coating (Duan et al., 2011). However, harvested blueberry fruits responded differently to postharvest treatments. In the case of 1-methylcyclopropene-treated blueberry fruits, no significant effects on fruit firmness and other qualities were observed (Chiabrando and Giacalone, 2011). Since blueberry fruit softening occurs during on-shrub ripening and continues during storage (Chen et al., 2015; Vicente et al., 2007), preharvest treatment on fruits may promote the retention of postharvest firmness and other quality attributes during subsequent storage and market life. Angeletti et al. (2010) reported that preharvest calcium treatment reduced postharvest fruit softening and weight loss in blueberry. No literatures are currently available on the preharvest effects of BABA on postharvest fruit quality and cell wall modification of blueberry fruits during storage.

Cell wall polysaccharides in plants

Plant cell walls are constructed from pectins, hemicelluloses, celluloses, and a minor amount of glycoproteins (Bashline et al., 2014; Carpita and Gibeaut, 1993; Li et al., 2010; Verhertbruggen et al., 2009). They function to compartmentalize the cell and provide structural supports for the cells and organs (Keegstra, 2010; Yin et al., 2011).

Celluloses

Cellulose is a common constituent of the plant cell wall and occupies approximately 15-40% of dry weight. Cellulose consists of unbranched and long chains of 1,4-linked glucose (Ruiz-May and Rose, 2013; Somerville, 2006), which are assembled by hydrogen bonding into microfibrils (Burton et al., 2010; Cybulska et al., 2013). Most of celluloses in plant cells occur in the form of cellulose microfibrils that are largely crystalline and non-crystalline (Kumar and Turner, 2015). The general structure of cellulose is presented in Fig. 1.

Cellulose is synthesized at plasma membrane by cellulose synthase complex, containing as many as 36 cellulose synthase A (CESA) proteins (Somerville, 2006; Wightman and Turner, 2010), which are embedded in the plasma membrane (Cosgrove, 2005). The insoluble cellulose microfibrils are directly deposited into extracellular matrix (Keegstra, 2010). Cellulose biosynthesis at the primary cell wall is synthesized by CESA1, CESA2, and CESA6, whereas the secondary wall requires CESA4, CESA7, and CESA8 (Kumar and Turner, 2015; Wightman and

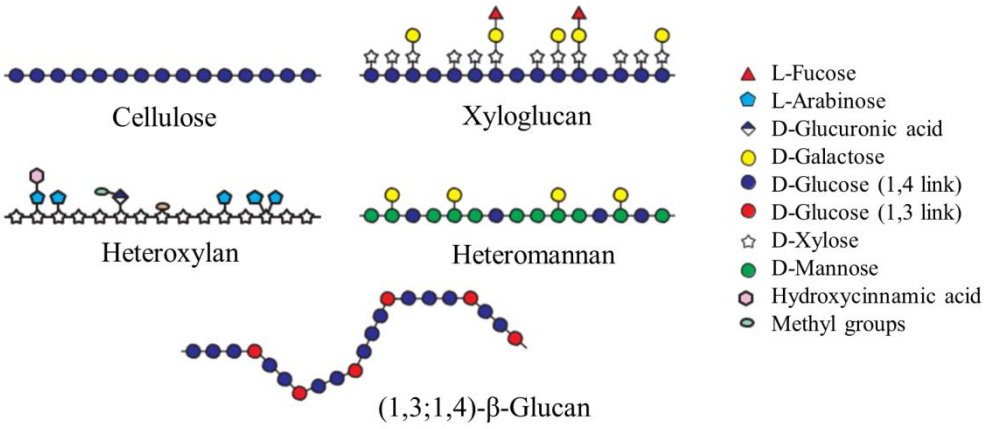

Fig. 1. Schematic structures of celluloses and structural heterogeneity of hemicelluloses (modified from Burton et al., 2010).

Turner, 2010).

Network between cellulose and hemicellulose establishes a strong complex polymer, strengthened by tensile strength of cellulose microfibers and cross-linking of multiple cellulose microfibers by hydrogen bonding of the hemicellulose (Bennett and Labavitch, 2008). New evidence of cross-link between cellulose and pectin was provided with an aid of a multidimensional solid-state nuclear magnetic resonance spectroscopy (Wang et al., 2015b).

Hemicelluloses

Primary cell walls are composed of 20-30% hemicellulose (Cosgrove and Jarvis, 2012; Ruiz-May and Rose, 2013). Major hemicelluloses include xyloglucans, xylans, mannans, glucomannans, and β-(1,3;1,4)-glucans, of which their backbones are built from β-(1,4)-link, and function to strengthen the cell wall by interacting with cellulose (Scheller and Ulvskov, 2010). The general structures of hemicelluloses are presented in Fig. 1. Hemicelluloses are synthesized in the Golgi by the action of glycosyltransferases (Scheller and Ulvskov, 2010). Xyloglucan is the most abundant hemicellulose and account for approximately 20-25% in the dicot cell wall component (Scheller and Ulvskov, 2010). However, this is not always the case. In blueberry fruits, xylan rather than xyloglucan is the principal hemicellulose (Vicente et al., 2007). Xyloglucan is highly branched and differs in structures. Xyloglucan is composed of (1,4)-linked β-D-glucopyranosyl

residues to which α-D-xylopyranosyl residues are linked at O-6 (Hoffman et al., 2005).

Xyloglucan tethers cellulose microfibers by non-covalent linkages (Tsuchiya et al., 2015). Xyloglucan was also depolymerized in apple fruits infected by *Penicillium expansum* (Miedes and Lorences, 2004) and in ripe tomato fruits (Maclachlan and Brady, 1994). Disassembly of xyloglucan and cellulose network resulted in fruit softening and xyloglucan depolymerization occurred in ripening tomato (Rose and Bennett, 1999). In the dicots, xyloglucan is the major cross-linking polysaccharide in the primary cell wall (Brummell and Schröder, 2009). However, xylans and glucomannans are also capable of binding to celluloses (Brummell and Schröder, 2009).

Xylans are the primary hemicellulose present in the secondary cell walls of the flowering plants (Peña et al., 2016) with a backbone of β-(1,4)-linked xylose residues (Scheller and Ulvskov, 2010). Xylans are variedly distributed within plant species and tissues (Brummell and Schröder, 2009). In guava, xylans were found in the parenchymatous cells, purified away from stone cells (Marcelin et al., 1993). In soft fruits like blueberry, xylans are the principal component of primary cell wall (Vicente et al., 2007). Xylan is commonly substituted with α-(1,2)-linked glucuronosyl and 4-O-methyl glucuronosyl residues, which are known as glucuronoxylans (Scheller and Ulvskov, 2010). Xylan also joins with pectin and xyloglucan making a complex as dicot growth stops (Brummell and Schröder,

2009). Depolymerization of xylan by endo-(1,4)-β-xylanase activity contributed to cell wall disassembly, leading to fruit softening (Brummell and Schröder, 2009; Iniestra-González et al., 2013; Manenoi and Paull, 2007). The degree of the depolymerization varied within species and depended on the linkage natures between xylan and microfibers or among xylan-pectin-xyloglucan (Brummell and Schröder, 2009). The linkage between xylans and cellulose microfibers were reported to affect cell mechanical properties (McCartney et al., 2005).

Mannans are another hemicellulose present in the plant cell wall, functioning as storage and structural polysaccharides (Schröder et al., 2009). The structures of mannans are more diverse than those of xyloglucan, but little has been known about the role of these hemicellulosic polymers (Schröder et al., 2009). Mannans are classified into pure mannan, galactomannan, glucomannan, and galactoglucomannan (GGM) with the backbones of unsubstituted (1,4)-β-D-mannose residues, (1,4)-β-D-mannose residues substituted with galactose, unsubstituted (1,4)-β-D-glucose and mannose residues, and (1,4)-β-D-glucose and mannose residues substituted with galactose, respectively. GGM plays roles in energy storage and structural components in the primary cell wall in dicots (Schröder et al., 2009). Among all types of mannans, only GGM is closely associated with cellulose microfibers (Schröder et al., 2009). In general, mannans are present in a small amount in fruit parenchyma, but they play an important role in cell wall structure, thus affecting tissue firmness (Prakash et al., 2012; Schröder

et al., 2009).

Pectins

Pectins constitute about 30-50% in the plant cell wall (Cosgrove and Jarvis, 2012). In blueberry fruits, pectins comprise 30–35% of the total cell wall (Vicente et al., 2007). Pectins contain as much as 70% of galacturonic acid (GalA) (Mohnen, 2008). The general structures of pectins are presented in Fig. 2. Pectins are synthesized by a large number of Golgi-localized glycosyltransferases, methyltransferases, and acetyltransferases, and transported to the cell wall by Golgi vesicles (Atmodjo et al., 2013; Mohnen, 2008). Homogalacturonan (HG), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), apiogalacturonan, and xylogalacturonan (XGA) are pectin polymers (Atmodjo et al., 2013). HG constitutes about 65% of pectin, whereas RG-I constitutes 20-35%. XGA and RG-II contain small amount, each less than 10% (Harholt et al., 2010; Mohnen, 2008).

HG is made up of a long chain of 1,4-α-D-GalA and secreted into the cell wall in a methylesterified form (Brummell, 2006). XGA is constructed from the same backbone as HG, but a haft of the GalA residues are substituted with α-D-xylose residues (Brummell, 2006). HG was demethylesterified prior to ripening in peach and pectin methylesterase is responsible for the removal of the methylester group (Brummell et al., 2004). HG is present in the cell wall, but more abundant in the middle lamella where they cement adjoining cells together (Jarvis et al.,

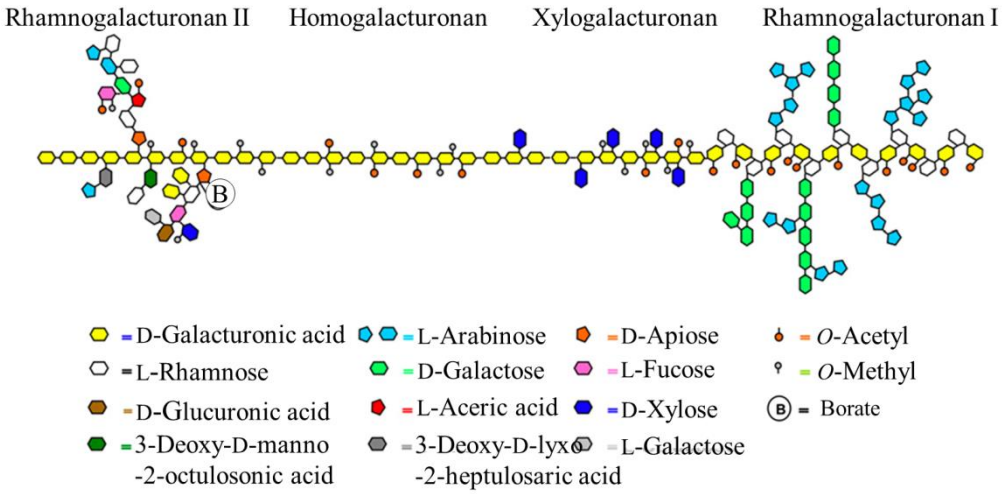

Fig. 2. Schematic structures of homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II, and xylogalacturonan (modified from Harholt et al., 2010).

2003). Therefore, HG degradation may contribute to alter the wall strength and integrity among the cells, causing fruit to soften.

Unesterified regions of HG was most abundantly found in the middle lamella and the methylesterified HG was evenly distributed within the cell wall (Knox et al., 1990). In apple fruits, methylestified HG were decreased as fruits become mature and ripen (Ng et al., 2013). The removal of methylester group from HG leaves a negatively charged group and may loosen the attachment to the cell wall in the absence of Ca^{2+}. In the cell wall with Ca^{2+}, unesterified HG was cross-linked Ca^{2+} to form Ca^{2+}-pectate complex, reinforcing the cell wall strength (Atmodjo et al., 2013; Brummell, 2006; Peaucelle et al., 2012; Wolf et al., 2009).

RG-I is highly heterogeneous and has alternating residues of GalA and rhamnose as a backbone. The large side chains in RG-I are attached to approximately half of the rhamnose residues. These side chains are linear galactans, branched arabinans, or brached arabinogalactans (Brummell, 2006; Mohnen, 2008).

Arabinan and galactan play structural and functional roles in the cell wall (Brummell, 2006) and are highly mobile (Harholt et al., 2010). Therefore, side chain degradation of RG-I alters cell wall property, thus contributes to fruit softening and textural changes (Brummell, 2006). Arabinan and galactan in the primary cell wall are present in the form of 1,4-β-D-galactan, 1,5-α-L-arabinan and branched arabinogalactan side chain of RG-I (Carpita and Gibeaut, 1993).

Pectic galactan and arabinan contents were extensively lost during the development of apple fruits (Ng et al., 2015). Higher contents of galactan and galactose residues in a slower softening ‘Scifresh’ apple fruits reduced cell wall porosity, thus restricting cell wall modifying enzymes and better retaining the cell wall integrity (Ng et al., 2015). Arabinose loss was high during ripening of pear (Brummell et al., 2004; Gross and Sams, 1984) and blueberry fruits (Gross and Sams, 1984). However, at least 25% loss of xylose was observed in blueberry fruits from green to 25% blue stage (Vicente et al., 2007), implying that xylan degradation is associated with cell wall alteration leading to fruit softening.

RG-II is built from HG backbone with four complex side chains consisting of at least 12 sugars and more than 20 different glycosyl linkages (Brummell, 2006; Ruiz-May and Rose, 2013). Borate diesters bridge the side chains of different RG-II molecules together.

HG, RG-I, and RG-II are all synthesized at Golgi and transported to the cell wall, where they are covalently linked via their backbones to create a complex pectin structure (Atmodjo et al., 2013). Pectins are also involved in cell wall strengthening via linkage with other cell wall polysaccharides (Atmodjo et al., 2013; Harholt et al., 2010; Mohnen, 2008). Wang et al. (2015b) found that cellulose contacted with pectin more than with xyloglucan. Low abundance linkage of pectin-xyloglucan was concluded by a recent review by Park and Cosgrove (2015).

Cell wall polysaccharide modification-associated fruit softening

Major cell wall alterations associated with fruit softening include pectin solubilization, depolymerization of polyuronide and hemicellulose, and loss of pectic galactan and arabinan. The sequence and degree at which these processes occur varied according to their cell wall constituents, structure as well as the nature of linkage among those polysaccharides in individual fruit species, developmental stages, and tissues (Brummell, 2006; Li et al., 2010).

Pectins became more soluble in most developing and ripening fruits. Water-soluble pectins were increased in apple (Ng et al., 2015), blueberry (Vicente et al., 2007), strawberry, and Chilean strawberry fruits (Figueroa et al., 2010). As water-soluble pectins were not attached to the cell wall, they did not contribute to the cell wall strength (Ng et al., 2015). The increases in water-soluble polyuronides in strawberry and Chilean strawberry fruits were correlated with fruit softening (Figueroa et al., 2010). Once pectins were solubilized, the cell wall became swollen, thus facilitating the mobility of CWMEs (Brummell, 2006; Redgwell et al., 1997).

The reductions in molecular size of both pectins and hemicelluloses were observed in various ripening fruits. Hemicelluloses were extensively depolymerized in blueberry fruits, whereas pectin depolymerization was not observed (Vicente et al., 2007). In Chilean strawberry fruits, the softening was involved in the early solubilization rather than depolymerization. The molecular

mass of the hemicelluloses and pectins were slightly reduced in Chilean strawberry fruits (Figueroa et al., 2010). Pectins and hemicelluloses were also depolymerized in peach fruits (Brummell et al., 2004). Ng et al. (2015) found that polyuronide in a faster softening 'Royal Gala' apple fruits became depolymerized to a lower molecular weight at mature stage, but this event did not occur until in ripening in a slower softening 'Scifresh' apple fruits. Similarly, continuous degradation of hemicelluloses enhanced softening in sweet cherry fruits during storage (Wang et al., 2015a).

Although cellulose is the primary constituent of the cell wall, cellulose contents remained constant throughout developmental stages in Chilean strawberry fruits and were not significantly correlated with fruit firmness (Figueroa et al., 2010). In blueberry fruits, however, cellulose content decreased gradually during cold storage at either 5 or 10^{o}C (Chen et al., 2015). Grapes stored in higher oxygen atmosphere had higher cellulose than those stored in air, but the content decreased over storage period (Deng et al., 2005). These results suggest that cellulose modification during postharvest storage contributes to fruit softening. Cellulose microfibrillar structure in ripening kiwifruit was not changed (Newman and Redgwell, 2002). However, a recent study on the nanostructures of six apple cultivars using an atomic force microscope found that fruits of the cultivar with thicker cellulose microfibers were firmer and crisper (Cybulska et al., 2013). From the literatures reviewed, the contribution of cellulose to cell wall

disassembly cannot be ignored, especially during postharvest storage.

Growing evidence reveals that cell wall modification significantly contributes to fruit softening and the processes initiate variedly among developing and ripening stages depending on fruit species and cultivars. The processes that commonly occur during early ripening include loss of arabinan and galactan side chains from RG-I, slow depolymerization of matrix glycans, HG demethylesterification, and polyuronide solubilization. Polyuronide depolymerization initiates during mid-ripening stages in avocado and tomato fruits, but in melon and peach fruits it occurs during late ripening stages (Brummell, 2006).

Cellular structure alteration and immunodetection of cell wall polysaccharides

Fruit ripening accompanies textural changes including softening (Li et al., 2010). Structural integrity of fruits is determined by cell size, shapes, cell wall integrity, and adjoining cell space (Allan-Wojtas et al., 2001). The observation of microstructure and ultrastructure can provide qualitative and comprehensive information, thus assisting in the identification of the softening causes (Allan-Wojtas et al., 2001). Cellular micro- and ultrastructure alteration in fruits can be evaluated with the advancement of microscopic technologies. The availability of antibodies has also made possible the visualization of cell wall polysaccharides

during fruit softening.

Several antibodies that have frequently been used to detect cell wall polysaccharides are listed in Table 1. The scanning electron micrographs of apple fruit cortical tissues revealed that the cells were densely packed at fruitlet, but they became ruptured and more separated as ripening proceeded (Ng et al., 2013). Cells in mature fruits of slower softening 'Scifresh' apple were larger and more angular than those in 'Royal Gala', reflecting less air space and greater cell-to-cell contact area (Ng et al., 2013). Cell junctions are where the cell meets and HG is rich in this region. Highly methylesterified HG immunolabeled with antibody LM20 were more intense in 'Scifresh' apple than in 'Royal Gala' throughout the fruit development (Ng et al., 2013). The scanning electron micrographs of sweet cherry fruit tissues revealed that BABA-treated fruits had smoother cuticle, more integrated sub-epidermal cell structure, and less ruptured than the control fruits (Wang et al., 2015a). The result suggested that BABA delayed fruit softening as did calcium (Alonso et al., 2005) by maintaining the cell microstructure.

Pectin modifying enzymes and gene expression

Fruit softening is associated with cell wall modification caused by combined actions of several CWMEs, which are encoded by multiple genes. The levels of activities and gene expression are variable among fruit species, cultivars, and developmental stages. Prominent enzymes involved in pectin degradation during

Table 1. Selected antibodies used to detect cell wall polysaccharides in fruit tissues.

Antibody	Specificity	Reference
LM5	Anti-(1,4)-β-galactan	Ng et al. (2015)
LM6	(1,5)-α-L-Arabinan	Ng et al. (2015)
LM8	Anti-xylogalacturonan	Willats et al. (2004)
LM10	Anti-(1,4)-β-xylan	McCartney et al. (2005)
LM15	Anti-xyloglucan	Marcus et al. (2008)
LM19	Non or low methylesterified homogalacturonan	Ng et al. (2013)
LM20	Highly methylesterified homogalacturoan	Ng et al. (2013)
LM21	Anti-(1,4)-β-mannan	Handford et al. (2003)
2F4	Calcium cross-linked homogalacturonan epitopes	Ng et al. (2013)

fruit ripening include polygalacturonase, pectin methylesterase, pectate lyase, β-galactosidase, and α-arabinofuranosidase.

Polygalacturonase is the most studied pectin modifying enzyme associated with fruit softening. The enzyme is divided into endo-polygalacturonase and exo-polygalacturonase (Brummell, 2006). Endo-polygalacturonase is considered in general involved in fruit softening (Li et al., 2010). Polygalacturonase hydrolyzes the cleavage of HG backbone at internal site (Brummell, 2006). Polygalacturonase was detected and its activity increased in various ripening fruits including apple (Goulao et al., 2007), strawberry (Figueroa et al., 2010), and peach (Brummell et al., 2004). In grape berries, *VvPG1* was highly expressed during color change and its transcript accumulation was correlated with berry softening (Deytieux-Belleau et al., 2008). Polygalacturonase activity in highbush blueberry fruits was highest at reddish purple stage, but not significantly correlated with fruit firmness (Proctor and Miesle, 1991).

In strawberry fruits, the expression of *FaPG1* was increased during ripening with decreasing firmness (Quesada et al., 2009). Transgenic fruit studies have confirmed the crucial role of polygalacturonase in polyuronide depolymerization and solubilization during ripening (Li et al., 2010). Once approximately 95% of *FaPG1* was suppressed in strawberry fruits, the softening was retarded, demonstrating that polygalacturonase played a central role in strawberry fruit softening (Quesada et al., 2009). In tomato fruits with 99% *PG* RNA suppression,

postharvest fruit firmness was much higher in red fruits (Carrington et al., 1993). The transient expression of *cpPG1* in papaya fruit brought about pulp softening, reflecting its critical role in ripening (Fabi et al., 2014). Critical role of polygalacturonase was also seen in apple fruits as apple lines with *PG* suppression inhibited softening (Atkinson et al., 2012). Posé et al. (2013) reported that antisense of *FaPG1* exhibited a 42% decrease in pectin solubilization, leading to firmer fruits.

Pectin is initially deposited into fruit cell wall in a highly esterified form (Brummell, 2006). Prior to fruit ripening, pectin methylesterase de-esterifies polyuronides by removing methylester groups from the C6 position of galacturonic acid (Brummell et al., 2004; Li et al., 2010). The process is necessary for the polyuronide depolymerization by polygalacturonase (Brummell and Harpster, 2001; Li et al., 2010). Pectin methylesterase activity also makes HG carboxylic group available for Ca^{2+} binding, decreasing access to cell wall hydrolases (Deytieux-Belleau et al., 2008; Micheli, 2001). Due to its activity to modify pectins in the fruit cell wall, pectin methylesterase has been studied in many ripening fruits. In strawberry and Chilean strawberry fruits, its activity was highest at turning stage and decreased at ripe stage (Figueroa et al., 2010). In grape berries, the activity of pectin methylesterase and the expression of *VvPME1* were detected prior to the onset of ripening. Its early activity induced grape ripening and the expression of *VvPME1* could be an early marker of veraison

(Deytieux-Belleau et al., 2008). As the pH in fruit apoplast decreases during ripening (Almeida and Huber, 1999), alkaline preferring enzymes such as pectin methylesterase and pectate lyase may be involved in an early stage of softening (Goulao et al., 2007). To further clarify the role of pectin methylesterase in ripening-associated softening, transgenic experiments have been carried out. The suppression of pectin methylesterase gene in tomato fruits enhanced pectin methylesterification, but did not alter fruit softening (Tieman et al., 1992).

Another pectin modifying enzyme is pectate lyase that acts on pectin in a different mode from polygalacturonase. Pectate lyase cleaves HG backbone by a β-elimination mechanism, generating 4,5-unsaturated oligogalacturonates (Li et al., 2010; Payasi et al., 2006). Transgenic strawberry with antisense pectate lyase gene exhibited a considerable reduction in fruit softening (Jiménez-Bermúdez et al., 2002). The result demonstrated an important role of pectate lyase in non-climacteric fruit softening. In Chilean strawberry fruits, pectate lyase gene was highly expressed as fruit ripening progresses which was proportional to the softening rate (Figueroa et al., 2008). Payasi and Sanwal (2003) found that pectate lyase activity peak coincided with climacteric peak in banana fruits, suggesting the significant role of pectate lyase in fruit ripening and softening. Pectate lyase in banana pulp was reported to be Ca^{2+}-dependent and the increased activity corresponded to an increase in soluble polyuronide (Marín-Rodríguez et al., 2003).

β-Galactosidase and α-arabinofuranosidase remove galactan and arabinan

side chains from RG-I, respectively (Brummell, 2006) and both enzymes contribute to the loss of neutral sugar residues (Tateishi, 2008). In a softer strawberry cultivar 'Toyonoka', the activity of α-arabinofuranosidase and the transcripts of *FaAra1*, *FaAra2*, and *FaAra3* were higher than those in a firmer cultivar at 50 and 70% red stages, suggesting the involvement in the softening process (Rosli et al., 2009). In Chillean strawberry fruits, however, α-arabinofuranosidase activity dramatically declined between large green and turning stages, which contradict to that in strawberry fruits (Figueroa et al., 2010). Differently from α-arabinofuranosidase, β-galactosidase activity increased throughout development stages in both Chilean strawberry and strawberry fruits (Figueroa et al., 2010).

Due to an increase in β-galactosidase activity during fruit ripening and its ability to hydrolyze native cell wall polysaccharides, β-galactosidase is considered as a fruit softening-related enzyme (Tateishi, 2008). In grape berries, β-galactosidase and the amount of transcripts were increased during ripening and consistent with a decrease in a type-I arabinogalactan of the cell wall (Nunan et al., 2001). The role of β-galactosidase in fruit softening was demonstrated with transgenic tomato plants. Antisense *TBG4* which encodes β-galactosidase II reduced exo-galactosidase activity and increased firmness (Smith et al., 2002).

Hemicellulose modifying proteins and gene expression

Hemicellulose is one of the main components of the fruit cell wall and disassembly of this polysaccharide is closely associated with fruit softening (Bennett and Labavitch, 2008; Brummell, 2006). Hemicellulose modifying proteins and enzymes include endo-1,4-β-glucanase, endo-1,4-β-xylanase, β-xylosidase, endo-1,4-β-mannanase, and xyloglucan endotransglycosylase. Endo-1,4-β-xylanase and β-xylosidase are the first two important hemicellulose degrading enzymes that play roles in fruit softening. Endo-1,4-β-xylanase degrades xylan by cleaving the β-1,4-glycosidic bonds between adjacent D-xylosyl residues to produce xylooligosaccharides. These oligosaccharides are then degraded by β-xylosidase, generating xyloxyl residues (Cleemput et al., 1997). An increase in endo-1,4-β-xylanase activity and gene expression were found to be correlated with papaya fruit softening (Manenoi and Paull, 2007). β-Xylosidase activity increased during ripening of Chilean strawberry and strawberry fruits (Figueroa et al., 2010). Hemicellulose contents decreased during strawberry fruit ripening. The accumulation of *FaXyl1* mRNA was ripening-related (Martínez et al., 2004). The softer strawberry cultivar ‘Toyonaka’ exhibited higher transcript of *FaXyl1* and corresponded to a higher activity of β-xylosidase throughout ripening stages (Bustamante et al., 2006).

Other hemicellulose modifying proteins include endo-1,4-β-glucanase, endo-1,4-β-mannanase, and xyloglucan endotransglycosylase (Bennett and

Labavitch, 2008; Brummell, 2006). Endo-1,4-β-glucanase hydrolyses β-1,4-linked glucans and is likely to target the glucan backbone of xyloglucan or non-crystalline cellulose chains at internal site (Bennett and Labavitch, 2008; Brummell, 2006). Although the increased transcriptional amounts of *faEG10* and *faEG30* (Trainotti et al., 1999) and endo-1,4-β-glucanase activity in both strawberry and Chilean strawberry fruits (Figueroa et al., 2010) were reported to contribute to softening, transgenic study with antisense downregulation of *Cel1* and *Cel2*, which encode endo-1,4-β-glucanase, showed no effect on fruit softening (Palomer et al., 2006). The results question the role of endo-1,4-β-glucanase in fruit softening, thus further studies are required.

Another hemicellulose degrading enzyme is xyloglucan endotransglycosylase. The enzyme cleaves glucan backbone of xyloglucan (Brummell, 2006) and an increased expression of *VXET*1 in grape was found to be most closely related to fruit softening (Ishimaru and Kobayashi, 2002). However, the role of xyloglucan endotransglycosylase in fruit softening is controversial. In apple, its activity was low in fruit set and increased till fully expanded stage, and then remained high throughout ripening (Goulao et al., 2007). However, xyloglucan was not depolymerized during apple fruit development and ripening (Percy et al., 1997). To clarify the role of xyloglucan endotransglycosylase in fruit softening, further evidence is required from a novel transgenic approach.

Endo-1,4-β-mannanase is mannan degrading enzyme. The enzyme

hydrolyzes the cleavage of mannan backbone of mannan, galactomannan, and glucomannan at internal site (Brummell, 2006). Its activity increased from pre-ripening, reaching a peak at mid-ripening, and coincided with respiratory and ethylene climacterics of pawpaw fruits, suggesting its involvement in the softening process (Koslanund et al., 2005). Endo-1,4-β-mannanase activity in mango fruits increased during ripening, reaching a peak at post-climacteric (yellowish green stage) (Yashoda et al., 2007). Although endo-1,4-β-mannanase was previously suggested to play a role in fruit softening, mannan in tomato fruits was not depolymerized during ripening despite the presence of this enzyme (Prakash et al., 2012). From these findings, further studies are required to redefine the role of endo-1,4-β-mannanase in fruit softening.

Other cell wall modifying proteins and genes

α-Mannosidase cleaves terminal mannosidic linkage from mannose rich complex and *N*-glycans (Liebminger et al., 2009). Its role in fruit softening was proved by a transgenic study in tomato fruits. Antisense a gene encoding for α-mannosidase increased tomato fruit firmness by 2-fold, enhancing 30 days of shelf life. Overexpression of the gene encoding for α-mannosidase caused an excessive fruit softening (Meli et al., 2010). In blueberry fruits, lower activities of α-mannosidase and other cell wall modifying enzymes contributed to maintaining fruit firmness during cold storage (Chen et al., 2015).

Expansins are non-enzymatic cell wall loosening proteins consisting of four subfamilies; α-expansin, β-expansin, expansin-like A, and expansin-like B (Cosgrove, 2015; Marowa et al., 2016; Sampedro and Cosgrove, 2005). Expansins are present in all plants and play various roles from germination to fruiting (Marowa et al., 2016). Among them, expansins are associated with loosening cell wall hemicellulose-cellulose linkage, thus facilitating other CWMEs to their substrates (Cosgrove, 2000; Marowa et al., 2016; Minoia et al., 2016; Sampedro and Cosgrove, 2005).

The role of expansin, *LeExp1*, in fruit softening was first reported in tomato by Rose et al. (1997). Since then several ripening-specific expansin genes and their involvement in fruit softening have been reported in tomato (*LeEXP1*) (Rose et al., 1997), strawberry (*FaEXP1*, *FaEXP2*, and *FaEXP5*) (Dotto et al., 2006; Figueroa et al., 2009), pear (*PcEXP2*, *PcEXP3*, *PcEXP5*, and *PcEXP6*) (Hiwasa et al., 2003), peach (*PpEXP3*) (Hayama et al., 2003; Zhang, 2012), kiwifruit (*AdEXP1* and *AdEXP2*) (Yang et al., 2007), durian (*DzEXP1* and *DzEXP2*) (Palapol et al., 2015), and grape (*VlEXP1*) fruits (Ishimaru et al., 2007). The role of expansin genes associated with fruit softening was proved in tomato fruits (Minoia et al., 2016), demonstrating that mutations in α-expansin *SlEXP1* altered cell wall metabolism and delayed the softening.

Cooperative actions of cell wall modifying proteins

Considering fruit cell walls are constructed from multiple polysaccharides and proteins, their disassembly during ripening and softening is definitely a complex process. Cell wall modifying proteins have been held responsible for cell wall disassembly, but a single enzyme is unlikely to control the process. Prevention of fruit softening by an individual gene modification has been failed in many cases (Goulao and Oliveira, 2008). In order to elucidate the combined actions of these enzymes in fruit softening, transgenic approach by the suppression of more than one gene was carried out in ripening tomato fruits. The results exhibited the importance of cooperative action of CWMEs. Tomato fruits suppressed for both *LeEXP1* and polygalacturonase gene were significantly firmer than control fruits and fruits with either suppression of *LeEPX1* or polygalacturonase gene (Cantu et al., 2008). In fruit softening study, therefore, actions of several CWMEs should be collectively investigated (Goulao et al., 2007). Furthermore, both pre- and postharvest treatments that lower the activities of those enzymes and gene expression would contribute to extend fruit postharvest shelf life.

BABA and its effect on defense mechanism and cell wall modification

BABA, a non-protein amino acid naturally found in plants (Thevenet et al., 2017), enhanced resistance of fruits and vegetables to biotic and abiotic stresses (Cohen et al., 2016; Jannatizadeh et al., 2018). In postharvest BABA-treated apple

fruits, BABA effectively controlled the postharvest blue mold by damaging plasma membrane of *Penicillium expansum* and activated defense-related enzymes (Zhang et al., 2011). Recently, BABA application has been expanded beyond stress tolerance and plant pathogen protection. Wang et al. (2015a) reported that postharvest BABA treatment at 30 mM enhanced cell wall contents and reduced activities of CWMEs, resulting in firmer sweet cherry fruits during storage. However, the effects of preharvest BABA treatment on postharvest quality and cell wall modification of highbush blueberry fruits during storage have not been reported. Since BABA is considered safe enough to use in fruits and vegetables (Cohen et al., 2016; Jannatizadeh et al., 2018), searching for the effect of preharvest BABA on postharvest quality and cell wall modification of highbush blueberry fruits during storage could contribute to blueberry postharvest quality improvement.

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Chapter I

Changes of Firmness and Quality Attributes during Ripening in 'Bluecrop' Highbush Blueberry Fruits

ABSTRACT

Understanding physiological and biochemical changes during fruit ripening is crucial for achieving optimal eating qualities with longer shelf life and higher economic value. This study was carried out to investigate major changes of quality attributes in 'Bluecrop' highbush blueberry fruits during ripening. The fruit size greatly expanded along with increasing fruit weight between the reddish purple and dark purple stages. Fruit firmness declined significantly between the pale green and reddish purple stages, which corresponded to the increases in soluble solids content, pH, and total anthocyanin content. Respiration rate peaked at the reddish purple stage, coinciding with the peak of ethylene production which accounted for approximately 2 $\mu L \cdot kg^{-1} \cdot h^{-1}$. These results suggest that highbush blueberry fruits soften extensively during on-shrub ripening and the optimal qualities including fruit weight were obtained when they are harvested at a fully ripe stage, although 'Bluecrop' highbush blueberry fruits exhibit a climacteric rise

in respiration during ripening.

Key words: ‘Bluecrop’ highbush blueberry, ethylene production, fruit firmness, respiration rate

INTRODUCTION

Blueberry fruits belong to the Ericaceae family, genus *Vaccinium* (Pritts and Hancock, 1992) and are ranked second as the most important soft fruit in the world (Chen et al., 2015; Giongo et al., 2013). Blueberry fruit is developed from an inferior ovary (Pritts and Hancock, 1992) and anatomically characterized as having a single-layered outer epidermis without stomata and covered with a cuticle and a waxy bloom. Beneath the epidermis, the hypodermis containing pigments is present.

Blueberry fruits ripen within 3 months after bloom (Pritts and Hancock, 1992). Major changes in fruit quality attributes related to ripening include soluble sugar, acidity, color, nutritional value, and softening. The decline in fruit firmness during ripening has an effect on other fruit quality attributes and consumers' acceptance. These physiological and biochemical events occur in a close relation with fruit respiration and ripening hormone. Two major hormones that play an important role during fruit ripening are ethylene and abscisic acid. Ethylene is vital in climacteric fruits, while abscisic acid in non-climacteric fruits.

In blueberry fruits, however, the detection of ethylene and its production rate during ripening have been variably reported and are cultivar-dependent (Suzuki et al., 1997). In general, blueberry fruits are required to be harvested at fully ripe stage to obtain acceptable quality, because the fruit qualities do not

improve after harvest. However, the harvested blueberry fruits at this stage are very soft, which may have a great adverse effect on postharvest shelf life.

Understanding physiological and biochemical changes during ripening is crucial for managing blueberry fruits to achieve the optimal eating qualities with longer shelf life and higher economic value. In the present study, changes of fruit quality attributes, respiration rate, and ethylene production were examined during maturation and ripening of ‘Bluecrop’ highbush blueberry fruits.

MATERIALS AND METHODS

Plant materials

Twelve-year-old ‘Bluecrop’ highbush blueberry shrubs were grown in the field at the experimental orchard of Seoul National University, Suwon, Republic of Korea (37° 15’ N, 126° 98’ E). One hundred and forty fruits each at pale green, reddish purple, dark purple, and dark blue stages were harvested for analysis (Fig. I-1). Reddish purple, dark purple, and dark blue stages indicated fruits at turning point, fully ripe, and over-ripe stages, respectively. Over-ripe fruits were obtained by storing the harvested ripe fruits at room temperature for 3 days. Thirty fresh fruits at each stage were used to measure fruit length, diameter, weight, and firmness. The rest were frozen in liquid nitrogen and stored at –80ºC until they were used to analyze soluble solids content, pH, and total anthocyanin content.

Determination of fruit size, weight, soluble solids content, pH, and total anthocyanin content

Fruit length and diameter were measured using a caliper (Mitutoyo, Kawasaki, Japan). Fruits were weighed using a digital balance (GT4100, Ohaus corporation, Florham Park, NJ, USA).

Soluble solids content and pH were determined according to the methods of

Fig. I-1. Ripening stages of ‘Bluecrop’ highbush blueberry fruits used for monitoring changes of quality attributes.

Figueroa et al. (2012). Soluble solids content in the juice was determined using a digital refractometer (GMK-701R, G-Won Hitech, Seoul, Korea) and expressed as ºBrix. pH was measured using a pH meter (Orion Star A215, Thermo Scientific, Waltham, MA, USA).

Total anthocyanin content was determined as described by Angeletti et al. (2010) with slight modifications. Briefly, 0.2 g of frozen fruit powder was added to 1.5 mL of 1% (v/v) HCl in methanol. The suspension was vigorously vortexed and centrifuged at 12,000 *g* at 4°C for 10 min. The absorbance of the supernatant was recorded at 515 nm using a spectrophotometer (UV-Vis 2550, Shimadzu, Kyoto, Japan). The results were expressed as mg cyanidin-3-glucoside per 100 g fresh weight.

Measurements were duplicated at each stage for each of three biological replicates.

Determination of fruit firmness

Fruit firmness at each stage was determined using a texture analyzer (CT3-4500, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) fitted with a 2-mm flat probe. The equatorial side of each fruit was compressed by 4 mm at a speed of 2 $mm \cdot s^{-1}$. The maximum force was recorded and expressed as Newtons (N).

Measurements of carbon dioxide and ethylene production

Fruits were harvested and grouped into five stages (small green, pale green, reddish purple, reddish with 50% blue, and dark purple). Twenty grams of fruits at each stage were placed in a 50-mL tube sealed with a septum in the lid and incubated at room temperature for 4 h. Carbon dioxide and ethylene production were measured in 1 mL of gas from each tube by using a gas chromatography system (YL6500, YoungLin, Anyang, Korea). The measurements were triplicated. The rate of ethylene production was calculated using the following formula: ethylene production = [ethylene (ppm) × free volume (L)]/[fruit weight (kg) × (h)].

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 24.0 (IBM Corp., Armong, NY, USA). The data were analyzed by one-way analysis of variance. The means were compared using Duncan's multiple range test (DMRT) at $P < 0.05$. A correlation matrix among parameters was generated.

RESULTS AND DISCUSSION

Fruit size, weight, soluble solids content, pH, and total anthocyanin content during ripening

The size of ‘Bluecrop’ highbush blueberry fruits significantly increased during ripening via expansion of both fruit length and diameter, particularly between the reddish purple and dark purple stages (Table I-1). The highest fruit weight was at the dark purple stage, which was comparable to that reported by Castrejón et al. (2008). Soluble solids content and pH significantly increased during ripening (Table I-2), indicating the conversion of starch and organic acids to sugars during ripening (Castrejón et al., 2008; Sun et al., 2013). Total anthocyanins significantly accumulated during ripening, reaching the highest level at the dark blue stage (Table I-2). The contents obtained at the dark purple stage were comparable to those reported for ‘Bluecrop’ highbush blueberry fruits by Chung et al. (2016). Although anthocyanin content was highest at the dark blue stage, the fruits at this stage were too soft to be marketed and consumed. These results indicate that maximal fruit weight and acceptable quality of ‘Bluecrop’ highbush blueberry fruits are achieved by harvesting at the fully ripe stage.

Fruit firmness during ripening

Fruit firmness declined nearly 6-fold between the pale green and reddish

Table I-1. Growth of 'Bluecrop' highbush blueberry fruits during ripening.

Ripening stage[z]	Length (cm)	Diameter (cm)	Weight (g)
PG	1.01 b[y]	1.37 b	1.17 c
RP	1.03 b	1.38 b	1.29 c
DP	1.19 a	1.58 a	2.09 a
DB	–	–	1.94 b

[z]PG, pale green; RP, reddish purple; DP, dark purple; DB, dark blue.

[y]Means with different letters within columns indicate significant difference among ripening stages by DMRT at $P < 0.05$.

Table I-2. Quality attributes of 'Bluecrop' highbush blueberry fruits during ripening.

Ripening stage[z]	SSC[y] (%)	pH	Total anthocyanin content (mg·100 g^{-1} FW[y])	Firmness (N)
PG	9.0 d[x]	2.82 d	4.9 c	9.2 a
RP	12.5 c	2.99 c	7.3 c	1.6 b
DP	15.0 b	3.38 b	70.0 b	1.4 b
DB	16.3 a	3.83 a	148.6 a	0.7 c

[z]PG, pale green; RP, reddish purple; DP, dark purple; DB, dark blue.

[y]SSC, soluble solids content; FW, fresh weight.

[x]Means with different letters within columns indicate significant difference among ripening stages by DMRT at $P < 0.05$.

purple stages and then remained unchanged until it reached the lowest level at the dark blue stage (Table I-2). During strawberry ripening, substantial softening also occurred once fruits transited from the large green to turning point stages (Figueroa et al., 2010).

Respiration rate and ethylene production during ripening

Respiration rate in ‘Bluecrop’ highbush blueberry fruits increased as fruits developed and ripening progressed, reaching the highest levels at the reddish purple and reddish with 50% blue stages and declined thereafter (Fig. I-2). The ethylene production was detected only at the reddish purple and reddish with 50% blue stages with the production rates of approximately 2 and 1 $\mu L \cdot kg^{-1} \cdot h^{-1}$, respectively. The burst of ethylene production in the present study was comparable to that in ‘Collins’ highbush blueberry fruits reported by Suzuki et al. (1997), suggesting that ‘Bluecrop’ highbush blueberry fruits are grouped into the climacteric fruits.

In tomato fruits, a model climacteric fruit, ethylene production increased rapidly from the breaker to turning stages (Kim et al., 1991). The change of ethylene regulates key genes that control the changes of color, flavor, and fruit softening (Tucker et al., 2017). In the present study, however, it is less appropriate to correlate the ethylene evolution with other physiological changes during ripening in ‘Bluecrop’ highbush blueberry fruits due to its transient and lower

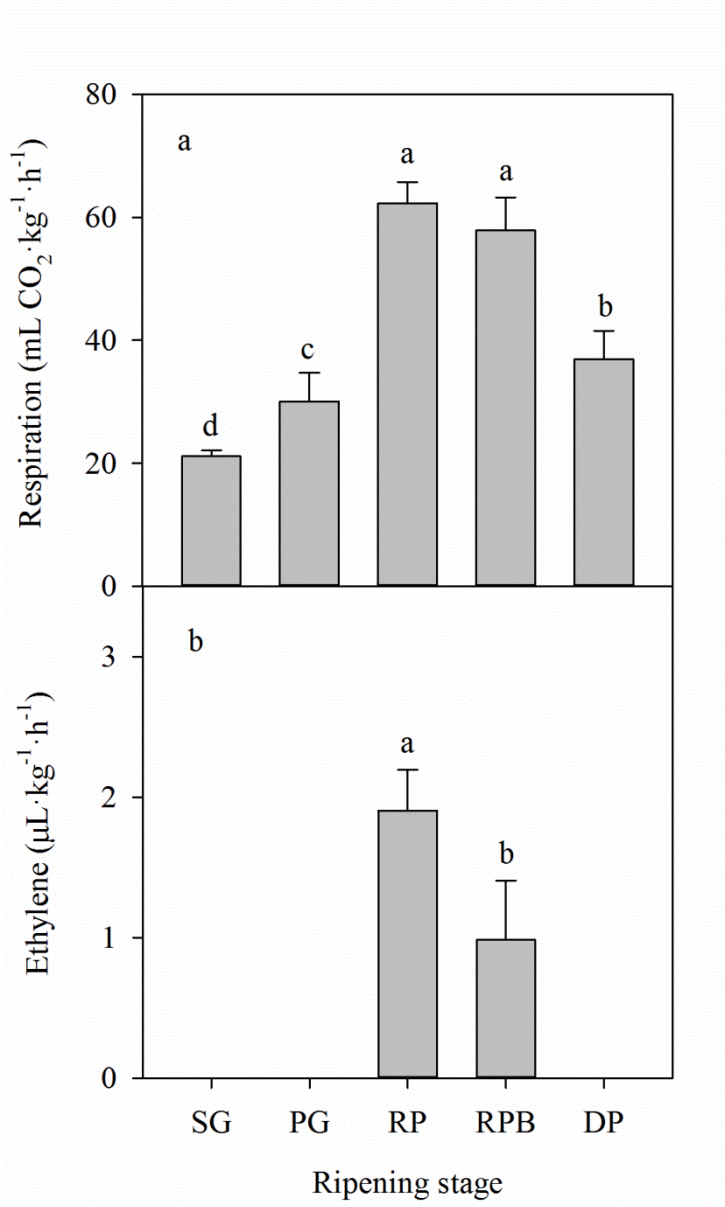

Fig. I-2. Respiration rate (a) and ethylene production (b) in 'Bluecrop' highbush blueberry fruits during ripening. Vertical bars represent standard errors of the means (n = 3). Different letters indicate significant differences among ripening stages by DMRT at $P < 0.05$. SG, small green; PG, pale green; RP, reddish purple; RPB, reddish with 50% blue; DP, dark purple.

production rate compared to other climacteric fruits such as banana, apple, and pear (Paul et al., 2012; Suzuki et al., 1997; Yan et al., 2011). Moreover, previous research demonstrated that abscisic acid was involved in the ripening and softening of 'Jersey' highbush blueberry fruits (Oh et al., 2018). Further studies are required to conclude the relationship between hormonal changes and other physiological changes during fruit ripening in various highbush blueberry cultivars.

Relationships of fruit firmness with other physicochemical properties during ripening in 'Bluecrop' highbush blueberry

Reductions in fruit firmness significantly correlated with increases in soluble solids content, whereas pH and total anthocyanin content significantly positively correlated with soluble solids content (Table I-3). These correlations imply that fruit firmness predicts other quality attributes during ripening-related softening.

Table I-3. Correlation among physicochemical properties of 'Bluecrop' highbush blueberry fruits during ripening.

Variable	Firmness	Soluble solids content	pH
Soluble solids content	-0.96^{**}		
pH	-0.70^{NS}	0.91^{**}	
Total anthocyanin content	-0.59^{NS}	0.84^{**}	0.95^{**}

$^{NS, **}$ Not significant or significant at $P < 0.01$.

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Chapter II

Changes of Cellular Structure and Xylan Distribution during Ripening in 'Bluecrop' Highbush Blueberry Fruits

ABSTRACT

The integrity of fruit tissues is largely determined by the cell shape, cell wall strength, cell to cell adhesion, and tissue anatomy. This study was carried out to investigate the alteration in cellular structures and xylan distribution during ripening and softening of 'Bluecrop' highbush blueberry fruits. Fruits at pale green, reddish purple, and dark purple stages, which represented large green, turning point, and ripe stages, respectively, were used in this study. Microstructures of parenchyma tissues were intact at the pale green stage, but became extensively altered at the reddish purple and dark purple stages. At ultrastructural level, cell walls and middle lamella were degraded, while plasma membranes were separated from primary cell walls as ripening proceeded. The immunofluorescence labelling of xylan was abundant at the pale green stage once the fruits were firm. However, the labelling was weak at the reddish purple and dark purple stages and it was not well confined to cell wall areas, implying cell

wall breakdown at late ripening stages. These results demonstrated that cell walls, middle lamella, and xylans were extensively degraded during the ripening and softening of ‘Bluecrop’ highbush blueberry fruits.

Key words: ‘Bluecrop’ highbush blueberry, cell microstructure, immunofluorescence labelling, ultrastructure

INTRODUCTION

Firmness is one of the most important fruit textures that are mainly determined by cell size and shape, cell wall thickness and strength, cell to cell adhesion, and tissue property and arrangement (Allan-Wojtas et al., 2001; Cornuault et al., 2018). During fruit ripening, the softening occurs as consequences of multiple cellular processes that may have undesirable effects on fruit quality and storage. Thus, the observation of cell microstructure and ultrastructure using modern microscopies can provide qualitative and comprehensive information required to understand anatomy-related softening processes (Allan-Wojtas et al., 2001).

Fruit cell walls are structurally complex and may be synthesized during ripening (Brummell, 2006; Takizawa et al., 2014). Specific monoclonal antibodies could be used to detect the distribution of major epitopes of pectins and hemicelluloses in the cell wall during ripening. Cell wall monoclonal antibody LM10 can be used to detect epitope of (1-4)-β-xylan (Handford et al., 2003; Sutherland et al., 2009) with the support from either a confocal microscopy or a fluorescence microscopy. Such information is of great importance to understand cell wall remodeling-related softening processes and to complement the interpretation of the results obtained from the traditional fractionation of cell wall

and analysis of monosaccharides.

In blueberry fruits, little is known about the alteration in cell wall ultrastructure. Xylan is a major hemicellulose present in blueberry fruits (Vicente et al., 2007), but its distribution and role during fruit softening remain unknown. Moreover, no information is available regarding the xylan distribution using cell wall monoclonal antibodies throughout fruit development and ripening in blueberries. In the present study, cellular microstructure was investigated and xylan was visualized to obtain information regarding cellular structural changes and the role of xylan during ripening and softening in highbush blueberry fruits.

MATERIALS AND METHODS

Plant materials

Twelve-year-old ‘Bluecrop’ highbush blueberry shrubs were grown in the field at the experimental orchard of Seoul National University, Suwon, Republic of Korea (37° 15’ N, 126° 98’ E). Fruits at pale green, reddish purple, and dark purple stages, indicating fruits at large green, turning point, and ripe stages, respectively (Fig. II-1). Ten fresh fruits at each ripening stage were used for the microscopic observation and immunofluorescence labelling.

Cell microstructure observation by scanning electron microscopy (SEM)

The fruit flesh tissues at the pale green, reddish purple, and dark purple stages were excised, fixed, and dehydrated as described by Wang et al. (2015a). Critical point drying was performed using an automated critical point dryer (Leica EM CPD300, Leica Microsystems, Wetzla, Germany) with liquid CO_2 as a transitional fluid. The samples were mounted on a copper film and sputter coated with platinum in a SEM coating unit (Leica EM ACE200, Leica Microsystems). Cell microstructures were observed using a SEM (Supra 55 VP, Carl Zeiss AG, Oberkochen, Germany).

Ultrastructural observation by transmission electron microscopy (TEM)

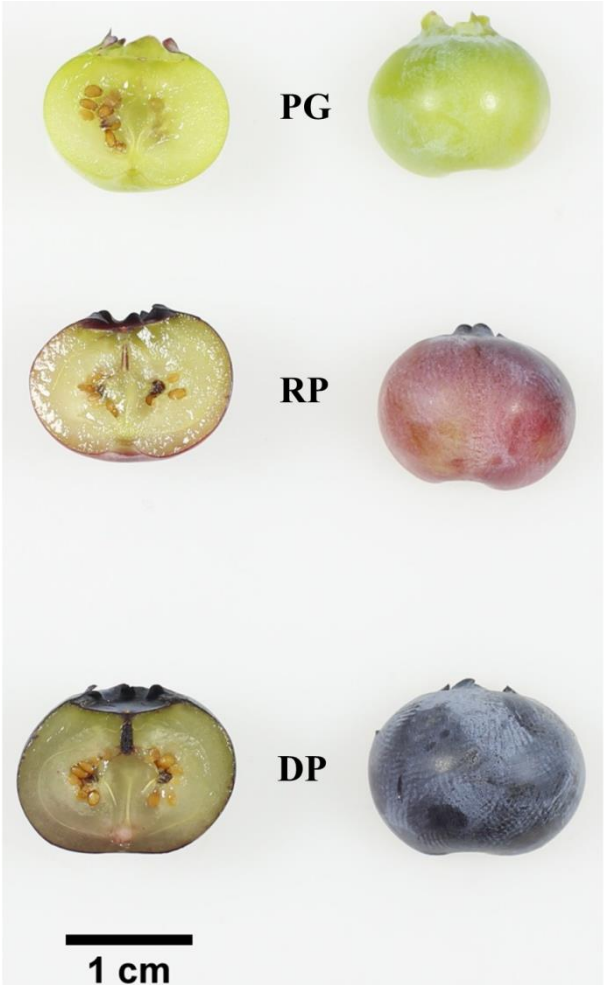

Fig. II-1. Ripening stages of ‘Bluecrop’ highbush blueberry fruits used in the study. PG, pale green; RP, reddish purple; DP, dark purple.

The pieces about 3 × 4 mm diameter of fruit flesh tissues at each stage were excised and fixed in 2% *p*-formaldehyde and 0.25% glutaraldehyde in phosphate buffered saline (PBS, pH 7.4). The fixed samples were washed three times in the distilled water for 10 min each, post-fixed with 2% OsO_4 for 2 h, and washed again in distilled water. After dehydration in a graded series of ethanol solutions (30, 50, 70, 80, 90, 100, 100, and 100%), the samples were transited twice in 100% xylene and infiltrated in LR white resin for 5 h by exchanging the solution every hour for the first 2 h. The infiltrated tissues were then embedded in LR white resin and polymerized at 55^{o}C for 48 h. Ultrathin sections approximately 85 nm were cut using an ultramicrotome (EM UC7, Leica Microsystems), mounted onto Formvar-coated copper grids, and stained with uranyl acetate and lead citrate for 30 and 10 min, respectively. Cell wall ultrastructures in the tissues were observed using a TEM (JEM-1010, Jeol, Tokyo, Japan) at 80 kV.

Immunofluorescence labelling of xylan epitope

Polysaccharide visualization was utilized to observe the changes in major cell wall polymers during fruit ripening. In this study, antibody LM10 was employed to detect the epitope of (1,4)-β-xylan. Fruit segments as described above were excised, fixed, and immunolabelled as described by Ng et al. (2013) and Sutherland et al. (2009). Samples without embedding in LR white resin were visualized using a confocal microscopy (SP8 X, Leica Microsystems). Samples

without primary antibody (200 mL of goat anti-rat IgG AlexaFluor 488 diluted 1:600 in PBS) were also examined as a control.

RESULTS AND DISCUSSION

Cell microstructural alteration during ripening

Parenchyma tissues of 'Bluecrop' highbush blueberry fruits were extensively altered during ripening (Fig. II-2). The parenchyma tissues were intact at the pale green stage, but they became largely deformed as ripening progressed, particularly at the reddish purple stage. Considering that the progressive changes of cell microstructure may be caused by cell breakage and separation (Gwanpua et al., 2016), blueberry fruit tissues were extensively deformed during on-shrub ripening prior to the harvest time.

Alteration of cell wall ultrastructure during ripening

Fruit parenchyma cell walls exhibited well-integrated structures at the pale green stage. As ripening progressed, however, the cell wall and middle lamella were greatly degraded (Fig. II-3). During late ripening, cell walls were wavy, thinner, and partially degraded probably due to localized swelling as previously observed in grape cell wall (Huang et al., 2005). Plasma membrane stayed closely to the primary cell wall, but became separated at late ripening stage. Cell separation became clear at the reddish purple and dark purple stages. In apple fruits, greater intercellular air space and less cell to cell contacts were related to a soft perception (Allan-Wojtas et al., 2003; Ng et al., 2013).

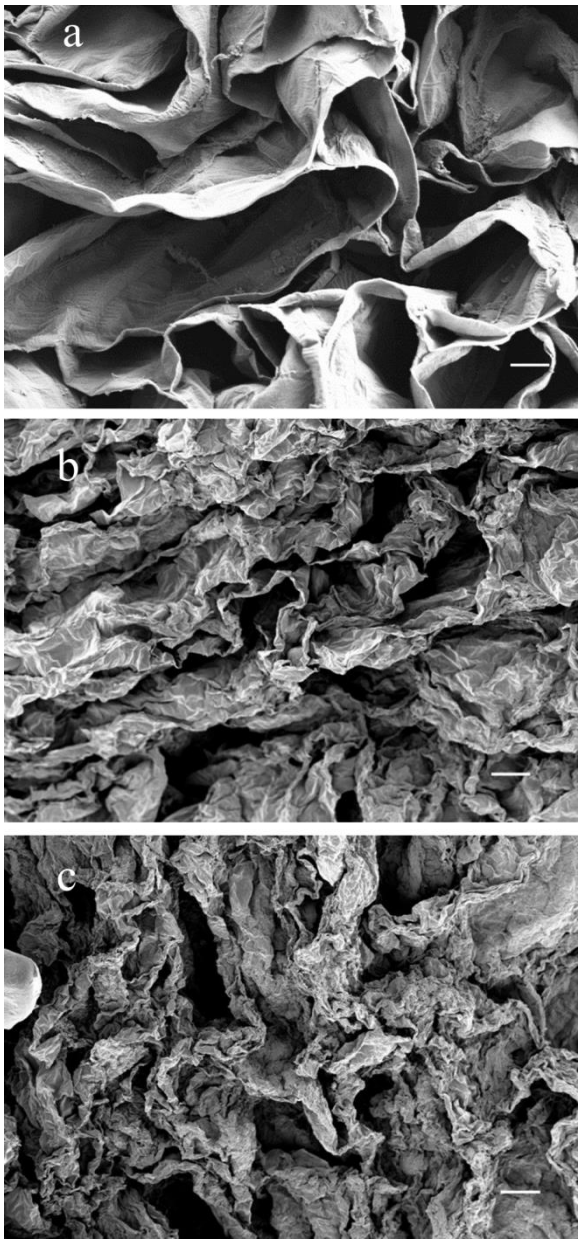

Fig. II-2. Scanning electron micrographs of parenchyma tissues of ‘Bluecrop’ highbush blueberry fruits at pale green (a), reddish purple (b), and dark purple (c) stages. Scale bars indicate 10 μm.

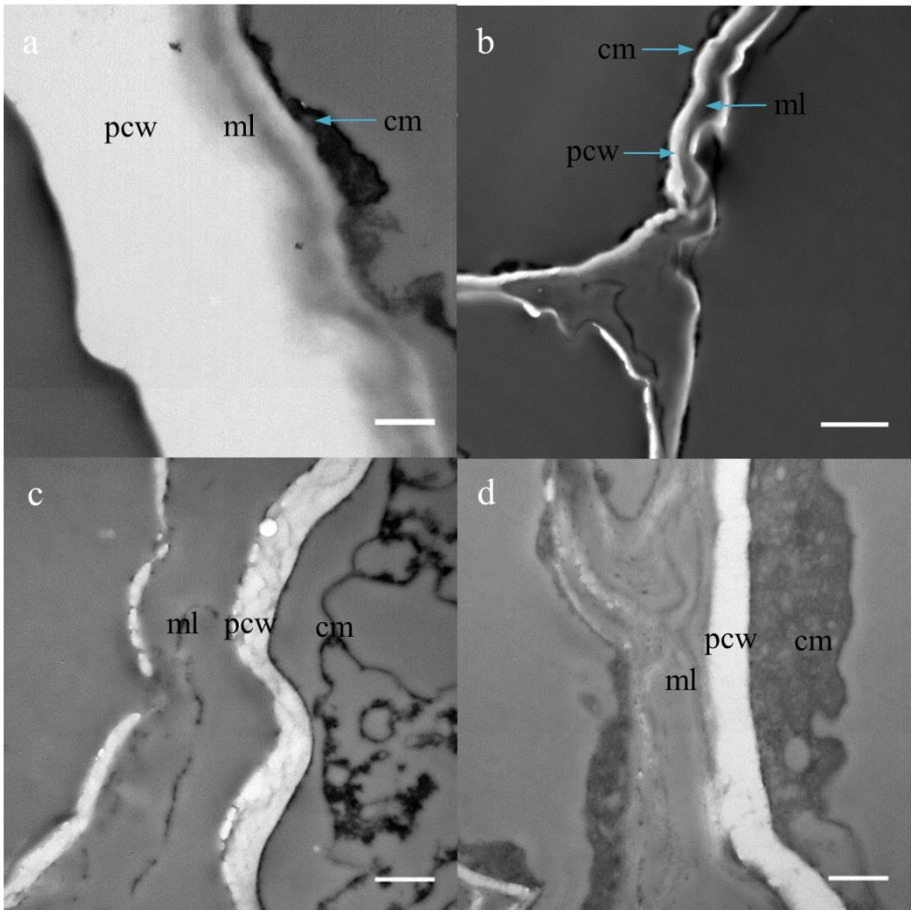

Fig. II-3. Transmission electron micrographs of parenchyma cell wall of 'Bluecrop' highbush blueberry fruits at pale green (a, b), reddish purple (c), and dark purple (d) stages. cm, cell membrane; pcw, primary cell wall; ml, middle lamella. Scale bars indicate 500 nm for a, c, and d, and 2 μm for b.

Since middle lamella connects cell together and the region is rich in pectins, the present results suggested that cell separation extensively occurred during early ripening, resulting in fruit softening in 'Bluecrop' highbush blueberries. These transmission electron micrographs confirmed the results observed in parenchyma tissues using SEM and corresponding fruit firmness.

Immunolocalization of xylan during ripening

There are various structures of xylans, non-substituted or substituted structures (Brummell and Schröder, 2009), with a backbone of β-(1,4)-linked xylose residues. These xylans are variedly distributed within plant species, cultivars, and tissues (Brummell and Schröder, 2009), and have also been reported to be present in various fruit parenchyma cells including guava and blueberry (Marcelin et al., 1993; Vicente et al., 2007). In the present study, immunolabelling of xylan in the fruit cell walls was more intense at the pale green stage, but the labelling became weaker as ripening progressed (Fig. II-4). The labelling at the reddish purple and dark purple stages were not well confined to the cell wall areas presumably due to broken cell walls at these stages that release cell wall components to other parts of cells. Since LM10 is specific to xylans with short side chain of either α-L-arabinose or α-D-glucuronic acids (Brummell and Schröder, 2009; McCartney et al., 2005), the present results suggest that xylans in

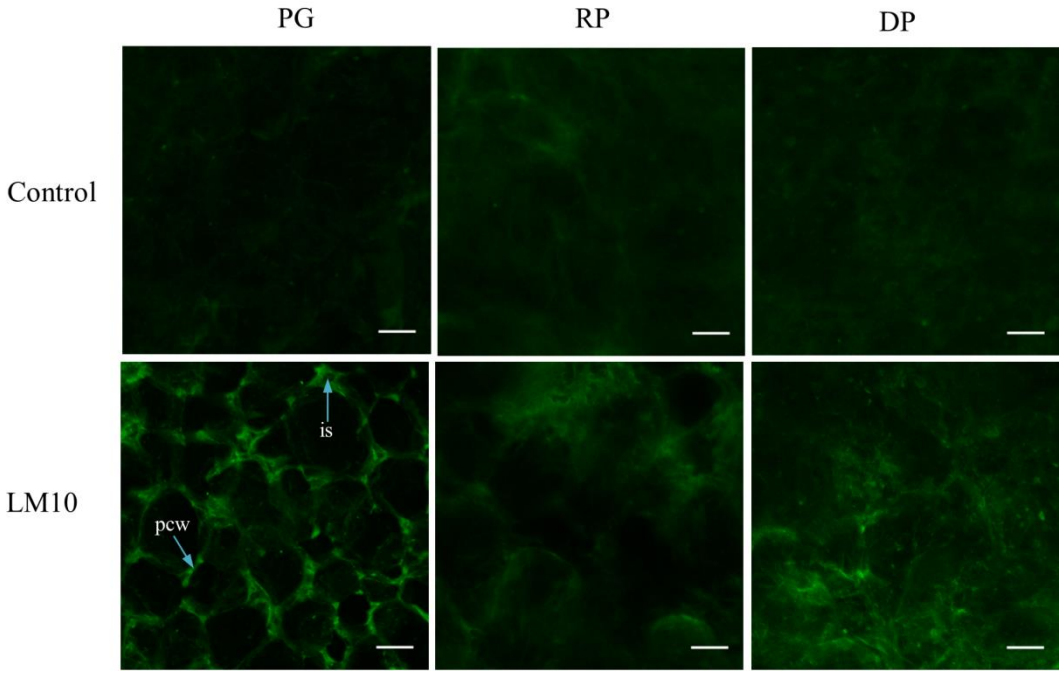

Fig. II-4. LM10 immunolabelling of xylan in parenchyma tissues of ‘Bluecrop’ highbush blueberry fruits during ripening. PG, pale green; RP, reddish purple; DP, dark purple; is, intercellular space; pcw, primary cell wall. Scale bars indicate 50 μm.

‘Bluecrop’ highbush blueberry fruits are present as low substituted types.

The cell wall strength is determined by the cross-linkages among hemicelluloses with celluloses (Bennett and Labavitch, 2008), pectins with celluloses (Wang et al., 2015b), and xylan with pectins (Cornuault et al., 2018). Since xylan is the major hemicellulose in the cell wall of blueberry fruits (Vicente et al., 2007), its degradation contributes to the weakening of the cross-linkage, leading to softening as observed in some fruits including papaya (Brummell and Schröder, 2009; Iniestra-González et al., 2013; Manenoi and Paull, 2007). Therefore, the present results suggest that the early softening of ‘Bluecrop’ highbush blueberry fruits is associated with xylan degradation.

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Chapter III

Changes of Cell Wall Composition and Cell Wall Modifying Enzyme Activities during Ripening in 'Bluecrop' Highbush Blueberry Fruits

ABSTRACT

Blueberry fruit quality deteriorates significantly after harvest due to its rapid softening characteristics. However, the mechanism of softening is not well understood. Here, cell wall-associated fruit softening in 'Bluecrop' highbush blueberry was examined by investigating cell wall composition, fruit cell wall neutral sugars, and cell wall modifying enzyme activities at different fruit ripening stages. The amount of fruit cell wall materials declined significantly during ripening and softening. The increased amount of water-soluble pectins correlated with a decline in HCl-soluble pectins during ripening. A substantial loss of arabinose and a decreased ratio of arabinose plus galactose to rhamnose in HCl-soluble pectins significantly correlated with fruit softening. Hemicellulose contents were significantly reduced, mainly between the pale green and reddish purple stages, showing a significant positive correlation with fruit firmness. Polygalacturonase, α-arabinofuranosidase, and β-galactosidase showed higher

activities during early ripening, whereas the activities of α-mannosidase and endo-1,4-β-xylanase were higher during late ripening. These results indicate that cell wall-related processes are a key feature of early ripening in ‘Bluecrop’ highbush blueberry fruits. These results set the stage for efforts to improve fruit shelf life by managing softening-related losses in highbush blueberries.

Key words: ‘Bluecrop’ highbush blueberry, cell wall composition, cell wall modifying enzyme, fruit firmness, softening

INTRODUCTION

Softening of blueberry fruits occurs during on-shrub ripening and postharvest storage (Chen et al., 2015; Vicente et al., 2007). The characteristics of fruit softening differ among species and cultivars and are largely determined by cell wall modifications (Angeletti et al., 2010; Chen et al., 2015; Vicente et al., 2007). Cell walls are constructed from pectin, hemicellulose, cellulose, and a minor amount of glycoproteins (Bashline et al., 2014; Carpita and Gibeaut, 1993; Li et al., 2010; Verhertbruggen et al., 2009). These cell wall components are synthesized at different locations and are assembled into a functional cell wall matrix that structurally supports cells and organs (Keegstra, 2010). Solubilizing and depolymerizing these polysaccharides alters cell wall structure and cell-to-cell adhesion, leading to softening and other effects on fruit quality (Goulao and Oliveira, 2008; Li et al., 2010). In blueberry fruits, however, little is known about the changes in the cell wall composition during ripening and over-ripening.

Various cell wall modifying enzymes are responsible for cell wall modifications during the ripening and softening of fruits (Brummell, 2006; Goulao and Oliveira, 2008; Li et al., 2010; Matas et al., 2009), including polygalacturonase, α-arabinofuranosidase, β-galactosidase, α-mannosidase, β-xylosidase, and endo-1,4-β-xylanase (Brummell, 2006; Rosli et al., 2004). These

enzymes have extensively been studied in many fruits, including apple (Goulao et al., 2007; Ng et al., 2015), pawpaw (Koslanund et al., 2005), strawberry (Bustamante et al., 2009; D'Amour et al., 1993; Figueroa et al., 2010; Martínez and Civello, 2008; Martínez et al., 2004; Rosli et al., 2004, 2009; Villarreal et al., 2008), grape (Deng et al., 2005), pear (Tateishi et al., 2005), and peach (Manganaris et al., 2006). Vicente et al. (2007) reported that pectin became more soluble and hemicellulose was depolymerized during the ripening and softening of blueberry fruits, but the enzymatic mechanisms of blueberry fruit softening remain unknown.

Poor understanding of fruit softening mechanisms has remained a main constraint on improving the postharvest shelf life of blueberry fruits. The present study was conducted to investigate cell wall composition, neutral sugars in the fruit cell wall, and cell wall modifying enzyme activities in 'Bluecrop' highbush blueberry (*Vaccinium corymbosum*) fruits during ripening. This information will be useful for managing softening related fruit losses in highbush blueberries.

MATERIALS AND METHODS

Plant materials

Twelve-year-old ‘Bluecrop’ highbush blueberry shrubs were grown in the field at the experimental orchard of Seoul National University, Suwon, Republic of Korea (37° 15’ N, 126° 98’ E). Based on size and skin coloration, fruit ripening was categorized into four different ripening stages: pale green, reddish purple, dark purple, and dark blue. Reddish purple, dark purple, and dark blue stages indicated fruits at turning point, fully ripe, and over-ripe stages, respectively. Over-ripe fruits were obtained by storing the harvested ripe fruits at room temperature for 3 days. One hundred fruits at each stage were harvested and frozen in liquid nitrogen and stored at –80ºC until they were used to analyze cell wall composition, fruit cell wall neutral sugars, and cell wall modifying enzyme activities.

Isolation, fractionation, and quantification of cell wall materials

Cell wall materials were isolated in the form of alcohol-insoluble residue as described by Figueroa et al. (2012). Two independent extractions were performed at each stage. Isolated cell wall materials were fractionated into water-soluble pectins, EDTA-soluble pectins, HCl-soluble pectins, NaOH-soluble polymers (hemicelluloses), and H_2SO_4-soluble polymers (celluloses) as described by

Figueroa et al. (2010).

Pectin contents were estimated by measuring uronic acid contents in each pectin fraction using the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) with galacturonic acid as a standard and expressed as μg galacturonic acid per mg alcohol-insoluble residue. Hemicellulose and cellulose contents were estimated using the anthrone method (D'Amour et al., 1993) with glucose as a standard and expressed as μg glucose per mg alcohol-insoluble residue. The measurements were duplicated in each fraction.

Analyses of fruit neutral sugar composition in pectin fractions

Fruit neutral sugar composition was analyzed only in water- and HCl-soluble pectins, since EDTA-soluble pectin contents were relatively low. Fruit neutral sugars were extracted as described by Gwanpua et al. (2014) and quantified by using an ion chromatograph (ICS 5000+, Thermo Dionex, Dreieich, Germany). Commercial neutral sugars (L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose) were used as external standards at 0.1-10 $mg \cdot L^{-1}$.

Assays of cell wall modifying enzymes

To extract cell wall modifying enzymes, 0.4 g of frozen fruit powder was

mixed with 1.2 mL of the respective extraction buffer. Four independent extractions were performed at each stage. Polygalacturonase, α-arabinofuranosidase, β-galactosidase, and β-xylosidase were extracted and assayed as described by Figueroa et al. (2010), whereas α-mannosidase and endo-1,4-β-xylanase were assayed according to the methods of Chen et al. (2015) and Manenoi and Paull (2007), respectively, with slight modifications.

Polygalacturonase was assayed with 0.3% (w/v) polygalacturonic acid (Sigma-Aldrich, St. Louis, MO, USA) by incubating at 37ºC for 3 h. The amount of galacturonic acid released was monitored with 2-cyanoacetamide (Gross, 1982) and determined using a standard curve constructed with D-(+)-galacturonic acid. The activity was expressed as nmol galacturonic acid·min^{-1}·mg^{-1} protein.

α-Arabinofuranosidase was assayed with 4-nitrophenyl-α-L-arabinofuranoside (Sigma-Aldrich) as a substrate by incubating at 37°C for 30 min. β-Galactosidase was assayed with *p*-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich) as a substrate by incubating at 37°C for 15 min. α-Mannosidase was assayed with *p*-nitrophenyl-α-mannopyranoside (Sigma-Aldrich) as a substrate by incubating at 37°C for 60 min. After incubation, the reactions were stopped by adding 0.4 M Na_2CO_3. β-Xylosidase was assayed with 4-nitrophenyl-β-D-xylopyranoside (Sigma-Aldrich) as a substrate by incubation at 55°C for 30 min, followed by the addition of 1% (w/v) Trizma base solution. The amount of *p*-

nitrophenol released by each enzyme was calculated using a standard curve constructed with *p*-nitrophenol. Their activities were expressed as nmol *p*-nitrophenol·min^{-1}·mg^{-1}protein.

Endo-1,4-β-xylanase was assayed with remazol brilliant blue-xylan (Sigma-Aldrich) as a substrate by incubating at 37°C for 4 h. The reaction was stopped by adding 3,5-dinitrosalicylic acid reagent. The reducing sugar released was calculated using a standard curve constructed with xylose (Sigma-Aldrich) as a standard. The activity was expressed as μmol xylose·h^{-1}·mg^{-1} protein.

Protein content of each extract was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 24.0 (IBM Corp., Armong, NY, USA). The data were analyzed by one-way analysis of variance. The means were compared using Duncan's multiple range test (DMRT) at $P < 0.05$. A correlation matrix among parameters was generated.

RESULTS AND DISCUSSION

Cell wall materials and cell wall composition during ripening

Fruit cell wall material contents significantly declined nearly to half from the pale green to dark purple stages, but no further decreases were observed between the dark purple and dark blue stages (Table III-1). Reductions in cell wall material content have been reported for various fruits (Figueroa et al., 2010; Rosli et al., 2004; Sun et al., 2013) and could be ascribed to cell wall turnover, fruit growth, and starch degradation during ripening (Raffo et al., 2011).

During fruit ripening and softening, water-insoluble pectins are converted to water-soluble pectins (Li et al., 2010; Sun et al., 2013). In the present study, water- and EDTA-soluble pectins, representing loosely and ionically bound pectins, respectively, significantly increased during ripening, whereas HCl-soluble pectins, representing covalently bound pectins, significantly declined. This result implies that the water-soluble pectins are derived from the HCl-soluble pectin fraction, and that this process occurs extensively at the early ripening stage. Similar results were found in strawberry and Chilean strawberry fruits (Figueroa et al., 2010). The displacement of pectins from one fraction to another indicates that pectin structural modifications occur during fruit ripening and softening (Sun et al., 2013).

Fruit hemicelluloses cross-link multiple cellulose microfibrils, thus

Table III-1. Cell wall materials and cell wall composition in 'Bluecrop' highbush blueberry fruits during ripening.

Ripening stage[z]	CWM[y]	WSP	ESP	HSP	Hemicellulose	Cellulose
	(mg AIR·g^{-1} FW)	(μg GalUA·mg^{-1} AIR)			(μg Glu·mg^{-1} AIR)	
PG	56.4 a[x]	20.6 c	5.7 b	60.0 a	25.2 a	21.2 a
RP	47.2 b	89.6 a	7.9 b	18.8 b	17.1 b	18.2 a
DP	30.5 c	77.6 b	7.7 b	21.6 b	20.6 b	21.4 a
DB	31.9 c	73.8 b	23.1 a	21.2 b	20.2 b	22.7 a

[z]PG, pale green; RP, reddish purple; DP, dark purple; DB, dark blue.

[y]CWM, cell wall materials; WSP, water-soluble pectins; ESP, EDTA-soluble pectins; HSP, HCl-soluble pectins; AIR, alcohol-insoluble residue; FW, fresh weight; GalUA, galacturonic acid; Glu, glucose.

[x]Means with different letters within columns indicate significant difference among ripening stages by DMRT at $P < 0.05$.

determining the integrity of the cell wall (Bennett and Labavitch, 2008). The decline in hemicellulose contents during ripening varies with species and cultivars and is closely related to softening (Brummell, 2006; Figueroa et al., 2010; Rosli et al., 2004; Sun et al., 2013; Vicente et al., 2007). Here, hemicellulose content was greatly reduced between the pale green and reddish purple stages and remained unchanged thereafter. However, no significant changes were observed for cellulose content throughout ripening, as in the previous studies (Bennett and Labavitch, 2008; Figueroa et al., 2010). These results suggest that changes in hemicellulose content rather than cellulose content are important in the blueberry fruit softening.

Changes in neutral sugar composition in pectin fractions

Glucose represented the most abundant neutral sugar in water-soluble pectins, followed by arabinose, galactose, and xylose (Table III-2). Glucose level significantly declined at the reddish purple stage, but markedly increased thereafter, reaching a level similar to that at the pale green stage. The abundance of glucose in this fraction distinguishes blueberry fruits from other fruits, in which arabinose and galactose are typically the most abundant neutral sugars (Basanta et al., 2014; Ponce et al., 2010; Raffo et al., 2012).

No clear pattern of glucose solubility during ripening were observed in this fraction. By contrast, the amount of arabinose in water-soluble pectins

Table III-2. Cell wall neutral sugar composition of water-soluble and HCl-soluble pectins in 'Bluecrop' highbush blueberry fruits during ripening.

Fraction[z]	Ripening stage[y]	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
					(mol %)			
WSP	PG	nd[x]	nd	10.3 d[w]	11.6 b	1.3 a	8.8 b	68.0 b
	RP	nd	nd	25.4 a	12.3 a	0.8 c	8.2 d	53.3 d
	DP	nd	nd	19.5 b	4.3 c	0.9 c	8.5 c	66.8 c
	DB	nd	nd	10.8 c	4.6 c	1.1 b	10.6 a	72.9 a
HSP	PG	2.5 b	0.8 d	51.3 a	14.6 d	1.9 d	15.5 d	13.4 b
	RP	4.6 a	1.0 c	40.8 b	19.8 c	2.6 c	22.5 c	8.7 d
	DP	4.5 a	1.2 a	31.8 c	24.0 b	4.3 a	24.5 b	9.7 c
	DB	4.6 a	1.1 b	24.4 d	25.0 a	3.9 b	25.8 a	15.2 a

[z]WSP, water-soluble pectins; HSP, HCl-soluble pectins.

[y]PG, pale green; RP, reddish purple; DP, dark purple; DB, dark blue.

[x]nd, not detected.

[w]Means with different letters within columns indicate significant difference among ripening stages by DMRT at $P < 0.05$.

accumulated by 2.5- and 1.9-fold at the reddish purple and dark purple stages, respectively (Table III-2). As fruit ripening progressed, arabinose became highly soluble as observed in the previous studies (Brummell, 2006; Goulao and Oliveira, 2008; Raffo et al., 2012; Vicente et al., 2007).

HCl-soluble pectins enriched covalently bound pectins (Figueroa et al., 2010). The fraction was rich in arabinose, followed by galactose and xylose (Table III-2). Over the course of ripening, arabinose levels significantly decreased whereas galactose levels increased (Table III-2). The increase in galactose in this fraction has previously been reported for various sweet cherry cultivars (Basanta et al., 2014). The larger amounts of arabinose and galactose and the detectable amount of rhamnose in HCl-soluble pectins indicated that rhamnogalacturonan I was present in the fraction. Since arabinose and galactose are the neutral sugar side chains of rhamnogalacturonan I, which can be covalently linked to hemicellulose and cellulose, the removal and/or rearrangement of the two sugars greatly determines cell wall strength and porosity (Brummell, 2006; Guillon et al., 2017; Ng et al., 2015). The high proportion of xylose in HCl-soluble pectins in the present study suggests that this fraction contains either xylogalacturonan or xylose from the hemicellulose fraction.

The ratio of galactose plus arabinose to rhamnose is typically used to indicate the average length of neutral sugar side chains attached to the backbone

of rhamnogalacturonan I (Gwanpua et al., 2016; Ng et al., 2015). In the present study, the ratios of arabinose to rhamnose and arabinose plus galactose to rhamnose markedly declined as ripening progressed (Fig. III-1), indicating a substantial loss of arabinose in 'Bluecrop' highbush blueberry fruits during ripening. The decreased ratio of arabinose plus galactose to rhamnose in the covalently bound pectin fraction have been reported for various fruits during ripening (Gwanpua et al., 2016; Ponce et al., 2010). Therefore, the present result suggests that rhamnogalacturonan I is highly degraded during ripening and softening of 'Bluecrop' highbush blueberry fruits.

Cell wall modifying enzyme activities during ripening

Polygalacturonase degrades pectins and promotes their solubilization by hydrolyzing the homogalacturonan backbone (Brummell, 2006; Goulao et al., 2007). Polygalacturonase activity was high at the pale green and reddish purple stages and significantly decreased thereafter (Table III-3), which is consistent with the previous results (Proctor and Miesle, 1991). The higher activity of polygalacturonase during early ripening stages corresponded to the increase of loosely bound pectins and to the decrease of covalently bound pectins (Tables III-1, III-3), indicating the conversion of pectin substrates by polygalacturonase. After the reddish purple stage, however, the polygalacturonase activities

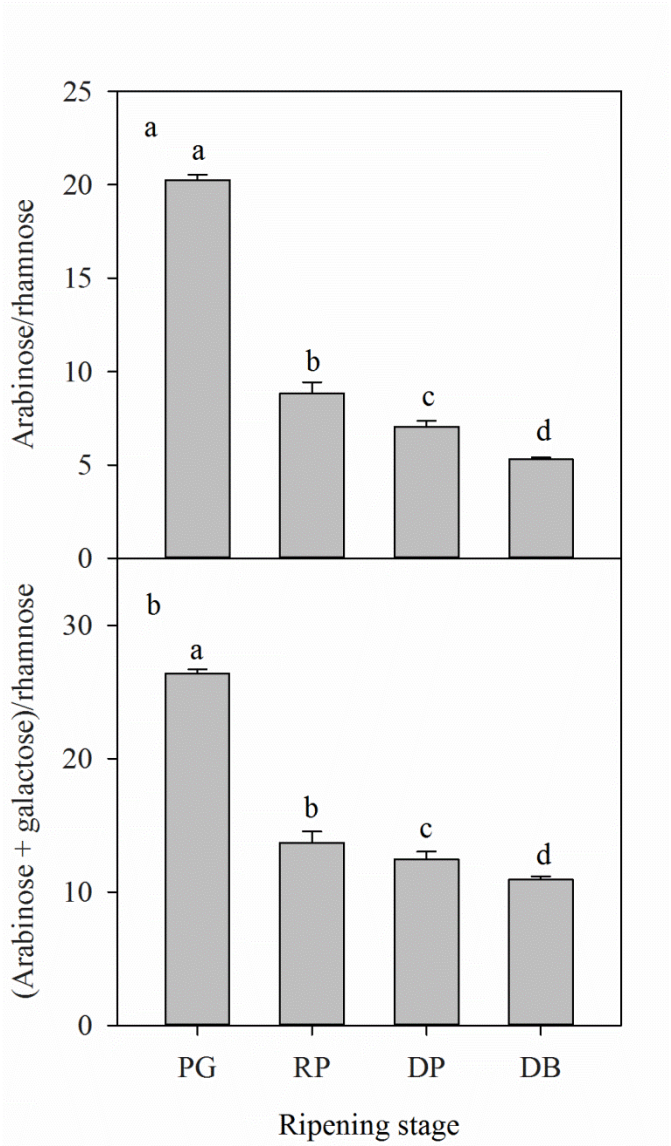

Fig. III-1. Ratios of arabinose to rhamnose (a) and arabinose plus galactose to rhamnose (b) in HCl-soluble pectins of 'Bluecrop' highbush blueberry fruits during ripening. Vertical bars represent standard errors of the means (n = 3). Different letters indicate significant differences among ripening stages by DMRT at $P < 0.05$. PG, pale green; RP, reddish purple; DP, dark purple; DB, dark blue.

Table III-3. Cell wall modifying enzyme activities in 'Bluecrop' highbush blueberry fruits during ripening.

Ripening stage[z]	Polygalacturonase (nmol GalUA[y]·min^{-1} ·mg^{-1} protein)	α-Arabinofuranosidase (nmol *p*NP·min^{-1}·mg^{-1} protein)	β-Galactosidase (nmol *p*NP·min^{-1}·mg^{-1} protein)	α-Mannosidase (nmol *p*NP·min^{-1}·mg^{-1} protein)	β-Xylosidase (nmol *p*NP·min^{-1}·mg^{-1} protein)	Endo-1,4-β-xylanase (μmol Xyl·h^{-1} mg^{-1}· protein)
PG	70.8 a[x]	27.1 a	312.0 a	9.4 b	247.7 a	12.6 a
RP	80.7 a	14.1 b	357.3 a	12.7 b	246.3 a	10.0 a
DP	51.1 b	11.8 b	289.6 a	54.8 a	213.2 b	15.3 a
DB	44.2 b	13.5 b	186.6 b	54.0 a	114.5 c	15.8 a

[z]PG, pale green; RP, reddish purple; DP, dark purple; DB, dark blue.

[y]GalUA, galacturonic acid; *p*NP, *p*-nitrophenol; Xyl, xylose.

[x]Means with different letters within columns indicate significant difference among ripening stages by DMRT at $P < 0.05$.

were lowered and the amounts of the two pectin fractions were not significantly changed (Tables III-1, III-3), presumably due to a limited pectin substrates and/or varying amounts of proteins.

α-Arabinofuranosidase and β-galactosidase affect cell wall structures and promote pectin solubilization by removing arabinose and galactose from pectin side chains, thus increasing the access of other cell wall modifying enzymes, including polygalacturonase, to their substrates (Goulao et al., 2007; Ng et al., 2015; Paniagua et al., 2016). In the present study, the activity of α-arabinofuranosidase significantly decreased during ripening (Table III-3). Similar decreases have been found in Chilean strawberry (Figueroa et al., 2010) and tomato fruits (Takizawa et al., 2014). However, the increases of α-arabinofuranosidase activity have also been reported in apple (Gwanpua et al., 2016) and strawberry fruits (Rosli et al., 2009).

These contradictory results may stem from the presence of different α-arabinofuranosidase isoforms in different species (Bustamante et al., 2009; Tateishi et al., 2005). Similarly to the observation for α-arabinofuranosidase, β-galactosidase activity was high at the reddish purple stage and then significantly decreased between the dark purple and dark blue stages (Table III-3). The high activity of this enzyme during early ripening was accompanied by a large accumulation of water-soluble pectins (Tables III-1). In ‘Golden Delicious’ apple

fruits, β-galactosidase activity was high before the ripe stage but became lower at the over-ripe stage (Gwanpua et al., 2016). Furthermore, this enzyme produced more soluble pectins in strawberry fruits (Paniagua et al., 2016). The present results indicate the important role of this enzyme in pectin solubilization during early ripening and softening of blueberry fruits.

However, α-mannosidase activity markedly increased during ripening, reaching the highest activity at the dark purple and dark blue stages (Table III-3). α-Mannosidase cleaves the terminal mannosidic linkage from the mannose rich complex and *N*-glycans (Liebminger et al., 2009). Silencing the gene encoding α-mannosidase delayed softening of non-climacteric fruits of capsicum (Ghosh et al., 2011), demonstrating that the enzyme played a significant role in the softening. The activity pattern of α-mannosidase in the present study implies that the enzyme is involved in firmness loss during late ripening and softening of blueberry fruits.

β-Xylosidase and endo-1,4-β-xylanase are hemicellulose modifying enzymes that degrade xylan (Bustamante et al., 2009; Cleemput et al., 1997). In the present study, β-xylosidase activity significantly decreased from the pale green to dark blue stages (Table III-3), similarly to the results found in tomato fruits (Takizawa et al., 2014). In strawberry and Chilean strawberry fruits, however, β-xylosidase activity increased during similar stages of ripening (Figueroa et al., 2010). These differences may stem from species-specific

contributions of other proteins to β-xylosidase activity, and/or from variation in cell wall composition among species (Bustamante et al., 2009; Tateishi et al., 2005). The activity of endo-1,4-β-xylanase was approximately 21% higher at the dark purple stage than at the pale green stage (Table III-3). This enzyme has been suggested to play a crucial role during fruit softening due to its enzymatic activity and the high expression of its corresponding genes (Manenoi and Paull, 2007). Since xylan is a main hemicellulose in blueberry fruits (Vicente et al., 2007), an increase in endo-1,4-β-xylanase activity may interrupt the cellulose-hemicellulose network, resulting in the softening of blueberry fruits. However, the greatly reduced hemicellulose content in the present study suggests that other hemicellulose modifying enzymes are also involved. The enzymatic activity and immunolocalization of other major hemicelluloses should be investigated to clarify the mechanism of hemicellulose modification during the ripening and softening of blueberry fruits.

Relationships among fruit firmness, cell wall composition, and cell wall modifying enzyme activities

The softening of various fruits during ripening depends on enzymatic modifications of the architecture and chemical composition of the cell wall (Brummell, 2006; Figueroa et al., 2010; Goulao and Oliveira, 2008; Vicente et al.,

2007). In the present study, fruit softening was closely related to a decline in cell wall material content, suggesting that the cell wall materials can be used to predict the softening of ‘Bluecrop’ highbush blueberry fruit during ripening.

Fluctuations in the amounts of fruit cell wall materials are determined by compositional changes of pectins, hemicelluloses, and celluloses. The amounts of water- and HCl-soluble pectins negatively and positively correlated with fruit firmness, respectively (Table III-4). The reduction in hemicellulose content significantly correlated with a decrease in fruit firmness. Such correlations have previously been found in Chilean strawberry fruits (Figueroa et al., 2010). These results indicate that the fruit softening of ‘Bluecrop’ highbush blueberry is caused by a reduction of tightly bound pectins and hemicelluloses. Furthermore, fruit firmness showed a significant positive correlation with arabinose content and the ratio of arabinose plus galactose to rhamnose in HCl-soluble pectin, suggesting that softening of blueberry fruits is strongly related to the degradation of rhamnogalacturonan I.

The relationship between fruit softening and cell wall modifying enzyme activities varies among species and cultivars (Brummell, 2006; Goulao and Oliveira, 2008; Goulao et al., 2007). For example, α-arabinofuranosidase activity negatively correlated with firmness in strawberry fruits, but positively correlated with firmness in Chilean strawberry fruits (Figueroa et al., 2010). The activities of all of the studied pectin modifying enzymes positively correlated with

Table III-4. Correlations among firmness, cell wall compositions, and cell wall modifying enzyme activities.

Variable	Firmness	AIR	WSP	ESP	HSP	NSP	SSP	AraWSP	AraHSP	(Ara + gal)/rha	PG	AFase	BGal	AMan	EXase
AIR	0.83*														
WSP	–0.94**	–0.64													
ESP	–0.41	–0.55	0.14												
HSP	0.99**	0.75*	–0.98**	–0.27											
NSP	0.86**	0.95**	–0.70	–0.45	0.79*										
SSP	0.46	0.12	–0.61	0.49	0.58	0.33									
AraWSP	–0.48	–0.07	0.71*	–0.55	–0.60	–0.22	–0.87**								
AraHSP	0.87**	0.94**	–0.67	–0.73*	0.78*	0.89**	0.07	0.00							
(Ara + gal)/rha	0.99**	0.85**	–0.93**	–0.43	0.97**	0.86**	0.44	–0.43	0.89**						
PG	0.39	0.74*	–0.11	–0.64	0.29	0.63	–0.20	0.46	0.75*	0.45					
AFase	0.90**	0.79*	–0.87**	–0.24	0.90**	0.80*	0.50	–0.51	0.77*	0.90**	0.33				
BGal	0.30	0.57	0.01	–0.85**	0.18	0.49	–0.35	0.61	0.68	0.34	0.82*	0.28			
AMan	–0.64	–0.92**	0.42	0.57	–0.55	–0.77*	0.15	–0.16	–0.86**	–0.67	–0.82*	–0.61	–0.62		
EXase	–0.31	–0.71*	0.02	0.70	–0.19	–0.64	0.38	–0.51	–0.67	–0.34	–0.84**	–0.14	–0.72*	0.77*	
BXyl	0.54	0.70	–0.29	–0.97**	0.41	0.59	–0.41	0.46	0.84**	0.57	0.73*	0.39	0.83*	–0.70	–0.73*

*, ** Significant at $P < 0.05$ and 0.01, respectively.

AIR, alcohol-insoluble residue; WSP, water-soluble pectins; ESP, EDTA-soluble pectins; HSP, HCl-soluble pectins; NSP, hemicelluloses; SSP, celluloses; AraWSP, arabinose in water-soluble pectins; AraHSP, arabinose in HCl-soluble pectins; (Ara + gal)/rha, ratio of arabinose plus galactose to rhamnose; PG, polygalacturonase; AFase, α-arabinofuranosidase; BGal, β-galactosidase; AMan, α-mannosidase; EXase, endo-1,4-β-xylanase; BXyl, β-xylosidase.

fruit firmness (Table III-4), accounting for their relatively high activities during early ripening. By contrast, the activities of α-mannosidase and endo-1,4-β-xylanase negatively correlated with fruit firmness, hemicellulose levels, and the amounts of other cell wall materials. The findings suggest that early softening in 'Bluecrop' highbush blueberry fruits is caused, at least in part, by higher activities of polygalacturonase, α-arabinofuranosidase, and β-galactosidase, while late softening stems from the increased activities of α-mannosidase and endo-1,4-β-xylanase.

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Chapter IV

Effect of Preharvest β-Aminobutyric Acid Treatment on Postharvest Quality and Cell Wall Modification of 'Bluecrop' Highbush Blueberry Fruits during Refrigerated Storage

ABSTRACT

This study was conducted to examine the effect of preharvest β-aminobutyric acid (BABA) treatment on postharvest quality and the softening of 'Bluecrop' highbush blueberry fruits during refrigerated storage. Reddish purple-colored fruits on shrubs were treated with 20 mM BABA and harvested 7 days after treatment when they turned dark purple. The harvested fruits were stored in clear polyethylene terephthalate clamshells at 2°C for up to 20 days. Preharvest BABA treatment significantly delayed color changes, enhanced individual soluble sugars and organic acids, and reduced decay incidence. BABA-treated fruits also retained higher skin firmness throughout the storage period. Cell wall materials, closely associated with fruit softening, were significantly higher in BABA-treated fruit than in control. Moreover, preharvest BABA treatment significantly enhanced fruit pectin content at harvest and reduced activities of

polygalacturonase and endo-1,4-β-xylanase, although hemicellulose content was not changed. These results suggest that preharvest BABA treatment improves key postharvest fruit quality attributes of ‘Bluecrop’ highbush blueberry including skin firmness during refrigerated storage.

Key words: β-aminobutyric acid, ‘Bluecrop’ highbush blueberry, cell wall modification, postharvest fruit quality, preharvest application, refrigerated storage

INTRODUCTION

Blueberry fruit quality deteriorates significantly after harvest due to its rapid softening characteristics. Excessive fruit softening affects its storability, transportability, and marketability, thus limiting its availability to consumers (Angeletti et al., 2010; Goulao and Oliveira, 2008). Major causes of blueberry fruit softening include water loss (Paniagua et al., 2013) and cell wall modification (Chen et al., 2015; Vicente et al., 2007). Cell wall-associated fruit softening is generally due to the structural and compositional modifications of primarily pectins and hemicelluloses by several cell wall modifying enzymes, including polygalacturonase, pectin methylesterase, α-arabinofuranosidase, α-mannosidase, β-xylosidase, and endo-1,4-β-xylanase (Goulao and Oliveira, 2008).

Many approaches to maintain blueberry postharvest fruit quality have been explored, including combined treatments of aqueous chloride dioxide and UV-C (Xu et al., 2016), nitric oxide and 1-methylcyclopropene (Grozeff et al., 2017), chitosan and *Aloe vera* coating (Vieira et al., 2016), biofumigation (Mehra et al., 2013), and refrigerated storage (Chen et al., 2015). However, such treatments are potentially costly and require additional fruit handling that increases the risk of fruit damage (Mirdehghan and Rahimi, 2016), in particular of soft fruit like blueberry. Since blueberry fruits soften extensively during on-shrub ripening and

are typically harvested at a fully ripe stage, preharvest treatment may help the retention of postharvest fruit quality during subsequent storage.

β-Aminobutyric acid (BABA) is a non-protein amino acid that has been reported to be safe enough in enhancing resistance of fruit and vegetables to biotic and abiotic stresses (Cohen et al., 2016; Jannatizadeh et al., 2018; Wu et al., 2010; Yan et al., 2015). Moreover, recent studies have described a further role for BABA in enhanced health-related compounds including anthocyanin contents (Wu et al., 2010), total phenols, and antioxidant capacity (Wang et al., 2016b). In sweet cherry, postharvest application of BABA improved fruit storage quality attributes and reduced fruit cell wall degradation (Wang et al., 2015, 2016b). However, little or no information is available regarding the effect of preharvest BABA treatment on postharvest blueberry fruit quality, especially softening and cell wall changes. Preharvest BABA application was hypothesized to improve blueberry postharvest fruit quality. In the present study, the effects of preharvest BABA treatment on fruit quality characteristics and cell wall modification were evaluated in ‘Bluecrop’ highbush blueberry (*Vaccinium corymbosum*) fruits during refrigerated storage.

MATERIALS AND METHODS

Plant materials and BABA application

Twelve-year-old ‘Bluecrop’ highbush blueberry shrubs were grown in the fields at the experimental orchard of Seoul National University, Suwon, Republic of Korea (37° 15’ N, 126° 98’ E). Once fruit skins turned reddish purple as an initial sign of ripening (Chung et al., 2016), fruit clusters on three shrubs were sprayed with 20 mM BABA (Sigma-Aldrich, St. Louis, MO, USA) containing 0.05% Tween 80 until running off, while those of three control shrubs were sprayed with distilled water containing 0.05% Tween 80. The BABA treatment concentration was selected based on the preliminary experiments. One hundred twenty uniformly ripe fruit from each shrub were harvested 7 days following BABA treatment and used to determine fruit quality, cell wall composition, and cell wall modifying enzyme activities.

A further two hundred ripe fruits were harvested from each shrub to determine weight loss and decay incidence. Harvested fruits were transported to the laboratory within 2 h and stored in clear polyethylene terephthalate clamshells in a completely randomized design with three replications at 2°C for 20 days. Samples were taken following 0, 10, and 20 storage days. Thirty fruits from each treatment and storage day were used to measure skin color and firmness. Immediately following these measurements, these thirty fruits were combined

with sixty further fruits taken from each treatment and storage day, frozen in liquid nitrogen and stored at –80°C until they were used to analyze fruit quality attributes, cell wall composition, and cell wall modifying enzyme activities.

Determination of fruit skin color and total anthocyanin content

Skin color was determined at two equatorial points of each fruit using a spectrophotometer (CM-2500d, Minolta Co., Osaka, Japan) and expressed as CIELAB scale (L^*, a^*, and b^*). Hue angle ($h°$) and chroma (C^*), indicating color saturation or intensity, were calculated according to the following formulas: $h° = \text{arctangent}\,[b^*/a^*]$ and $C^* = [a^{*2} + b^{*2}]^{1/2}$ (McGuire, 1992). Hue angle represents visual color appearance; 0°, red-purple; 90°, yellow; 180°, blueish green; 270°, blue (McGuire, 1992).

Total anthocyanin content was determined as described by Angeletti et al. (2010) with slight modifications. Briefly, 0.2 g of frozen fruit powder was added to 1.5 mL of 1% (v/v) HCl in methanol. The suspension was vigorously vortexed and centrifuged at 12,000 × *g* at 4°C for 10 min. After supernatant dilution (1:5, v/v) in the same solution, the absorbance was recorded at 515 nm using a spectrophotometer (UV-Vis 2550, Shimadzu, Kyoto, Japan). The measurements were duplicated in each treatment. The results were expressed as mg cyanidin-3-glucoside equivalent per 100 g fresh weight by using molar extinction coefficient

(ε) = 29,000 $M^{-1} \cdot cm^{-1}$.

Determination of soluble solids content, pH, and titratable acidity

Soluble solids content, pH, and titratable acidity were determined according to the methods of Figueroa et al. (2012). Briefly, 2 g of frozen fruit powder were added to 25 mL of distilled water and the mixture was vortexed. After filtering the mixture through one layer of Miracloth (EMD Millipore Corp., Billerica, MA, USA), soluble solids content in the juice was determined using a digital refractometer (GMK-701R, G-Won Hitech, Seoul, Korea) and expressed as percentage (%). pH was measured using a pH meter (Orion Star A215, Thermo Scientific, Waltham, MA, USA). Titratable acidity was determined using a fruit acidity meter (GMK 708, G-Won Hitech) and expressed as percentage of citric acid.

Analyses of individual soluble sugars and organic acids

Soluble sugars and organic acids were extracted as described by Selcuk and Erkan (2015) with slight modifications. Briefly, 2 g of frozen fruit powder were added to 25 mL of deionized water, vigorously vortexed, filtered with one layer of Miracloth (EMD Millipore Corp.) and centrifuged at 20,000 × *g* at 4°C for 20 min. The supernatant was filtered with a 0.45-µm membrane filter (Acrodisc, Pall Co., Washington, NY, USA). Ten microliters of the samples were injected into a high

performance liquid chromatography (Ultimate 3000, Thermo Dionex, Sunnyvale, CA, USA). Soluble sugars were separated with a Sugar-Pak column (10 μm, 300 mm × 6.5 mm, Waters, Milford, MA, USA) and detected with an RI-101 detector (Shodex, Showa Denko K.K., Kawasaki, Japan). Deionized water was used as a mobile phase at a flow rate of 0.5 $mL \cdot min^{-1}$. Organic acids were separated with an Aminex 87H column (9 μm, 300 mm × 10 mm, Bio-Rad, Hercules, CA, USA) and detected with a UV detector (RefractoMAX520, ERC Inc., Kawaguchi, Japan) at 210 nm. Sulfuric acid at 0.01 N was used as a mobile phase at a flow rate of 0.5 $mL \cdot min^{-1}$. The contents of individual soluble sugars and organic acids were expressed as $g \cdot kg^{-1}$ fresh weight.

Determination of fruit firmness, weight loss, and decay incidence

Fruit skin firmness was determined using a texture analyzer (CT3-4500, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) fitted with a 2-mm flat probe. The equatorial side of each fruit was compressed by 4 mm at a speed of 2 $mm \cdot s^{-1}$. The maximum force was recorded and expressed as Newtons (N).

Weight loss was calculated using two hundred fruits in each treatment and replication by subtracting the initial fresh weight prior to being stored from the weight determined following 10 and 20 storage days. Fruits showing decay and

fungal infection were counted following 10 and 20 storage days from each treatment, and decay incidence (%) was calculated.

Extraction, fractionation, and quantification of cell wall materials

Cell wall materials were isolated in the form of alcohol-insoluble residue as described by Figueroa et al. (2012). The results were expressed as mg alcohol-insoluble residue per g fresh weight. The cell wall materials isolated were fractionated into water-, EDTA-, and HCl-soluble pectins as described by Figueroa et al. (2010) with slight modifications. The pectin fractions were sequentially extracted with distilled water, 40 mM EDTA in 50 mM sodium acetate (pH 4.5), and 50 mM HCl, respectively. NaOH-soluble polymers representing hemicelluloses were also extracted with 4 M NaOH from residues of the HCl-soluble pectin fraction.

Uronic acid contents in the pectin fractions were determined using the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) with galacturonic acid as a standard and expressed as μg galacturonic acid per mg alcohol-insoluble residue. Neutral sugars in the pectin and hemicellulose fractions were estimated using the anthrone method (D'Amour et al., 1993) with glucose as a standard and expressed as μg glucose·mg^{-1} alcohol-insoluble residue. The measurements were duplicated in each fraction.

Analysis of fruit neutral sugar composition in pectin fractions

Fruit neutral sugar composition was analyzed only in water- and HCl-soluble pectins, since EDTA-soluble pectin contents were relatively low. Fruit neutral sugars were extracted as previously described by Gwanpua et al. (2014) and quantified by an ion chromatography (ICS 5000+, Thermo Dionex, Dreieich, Germany). Commercial neutral sugar standards (L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose) at 0.1-10 $mg \cdot L^{-1}$ were used as external standards.

Assays of cell wall modifying enzymes

For extracting cell wall modifying enzymes, 0.4 g of frozen fruit powder was mixed with 1.2 mL of the respective extraction buffer. Polygalacturonase, α-arabinofuranosidase, β-galactosidase, and β-xylosidase were extracted and assayed as described by Figueroa et al. (2010), whereas α-mannosidase and endo-1,4-β-xylanase were assayed according to the methods of Chen et al. (2015) and Manenoi and Paull (2007), respectively, with slight modifications.

Polygalacturonase was assayed with 0.3% (w/v) polygalacturonic acid (Sigma-Aldrich) as a substrate by incubating at 37°C for 3 h. The released amount of galacturonic acid was determined with 2-cyanoacetamide (Gross, 1982) and calculated using a standard curve constructed with D-(+)-galacturonic acid. The

activity was expressed as nmol galacturonic acid·min^{-1}·mg^{-1} protein.

α-Arabinofuranosidase was assayed using 4-nitrophenyl-α-L-arabinofuranoside (Sigma-Aldrich) as a substrate by incubating at 37°C for 30 min. β-Galactosidase was assayed with *p*-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich) as a substrate by incubating at 37°C for 15 min. α-Mannosidase was assayed with *p*-nitrophenyl-α-mannopyranoside (Sigma-Aldrich) as a substrate by incubating at 37°C for 60 min. β-Xylosidase was assayed with 4-nitrophenyl-β-D-xylopyranoside (Sigma-Aldrich) as a substrate by incubating at 55°C for 30 min. The amount of *p*-nitrophenol released was calculated using a standard curve constructed with *p*-nitrophenol. The activity was expressed as nmol *p*-nitrophenol·min^{-1}·mg^{-1} protein.

Endo-1,4-β-xylanase was assayed with remazol brilliant blue-xylan (Sigma-Aldrich) as a substrate by incubating at 37°C for 4 h. The reducing sugar released was calculated using a standard curve constructed with xylose (Sigma-Aldrich) as a standard. The activity was expressed as μmol xylose·h^{-1}·mg^{-1} protein.

Protein content of each enzyme extract was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 24.0 (IBM

Corp., Armong, NY, USA). The data were analyzed by two-way analysis of variance with treatment and storage days as factors. The means were compared using least significant difference (LSD) test at $P < 0.05$. Graphs were plotted using a SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA). Correlation heatmap among parameters was generated using the R 3.2.2 software package (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS AND DISCUSSION

Effects of BABA on fruit skin color and total anthocyanin content

Fruit skin color gradually developed during refrigerated storage (Table IV-1). Values of a^* and $h°$ increased during refrigerated storage and were significantly lower in BABA-treated fruits than in control fruits following 0 and 10 storage days, indicating that BABA treatment delayed color changes. A maximum $h°$ value was recorded in control and BABA-treated fruits following 10 and 20 storage days, respectively. The $h°$ value may reflect accumulated total anthocyanin content. Despite no significant differences, BABA-treated fruits displayed higher anthocyanin accumulation at all storage days and these contents were significantly correlated with $h°$ (Table IV-1, Fig. IV-1), which is comparable to the results of Chung et al. (2016). BABA was previously reported to stimulate anthocyanin accumulation by enhancing the expression of chalcone synthase and dihydroflavonol-4-reductase genes in *Arabidopsis* (Wu et al., 2010).

Effects of BABA on soluble solids content, pH, and titratable acidity

Soluble solids content and pH were not significantly changed during refrigerated storage, but were significantly lower in BABA-treated fruits than in control fruits following 20 storage days (Table IV-2). The pH measured in the

Table IV-1. Skin color and total anthocyanin content of BABA-treated 'Bluecrop' highbush blueberry fruits during refrigerated storage.

BABA (mM)	Storage days at 2°C	Color parameter					TAC[z] (mg c3g·100 g^{-1} FW)
		L^*	a^*	b^*	C^*	$h°$	
0	0	39.4 aA[y]	–0.49 bB	–7.6 bA	7.6 aA	266.5 bA	60.3 bA
	10	37.2 bA	–0.23 aB	–6.9 bA	6.9 bA	268.6 aA	114.2 aA
	20	33.4 cA	–0.37 aA	–6.3 aA	6.3 cA	266.8 abA	91.7 aA
20	0	40.0 aA	–0.83 bA	–7.8 bA	7.8 aA	263.8 bB	98.9 bA
	10	38.8 bA	–0.55 aA	–7.6 bA	7.6 bA	265.8 aB	117.0 aA
	20	32.7 cA	–0.44 aA	–6.3 aA	6.3 cA	266.3 abA	125.7 aA

[z]TAC, total anthocyanin content; c3g, cyanidin-3-glucoside; FW, fresh weight.

[y]Significant differences are marked with lowercase letters within storage day and with uppercase letters within treatment by LSD test at $P < 0.05$.

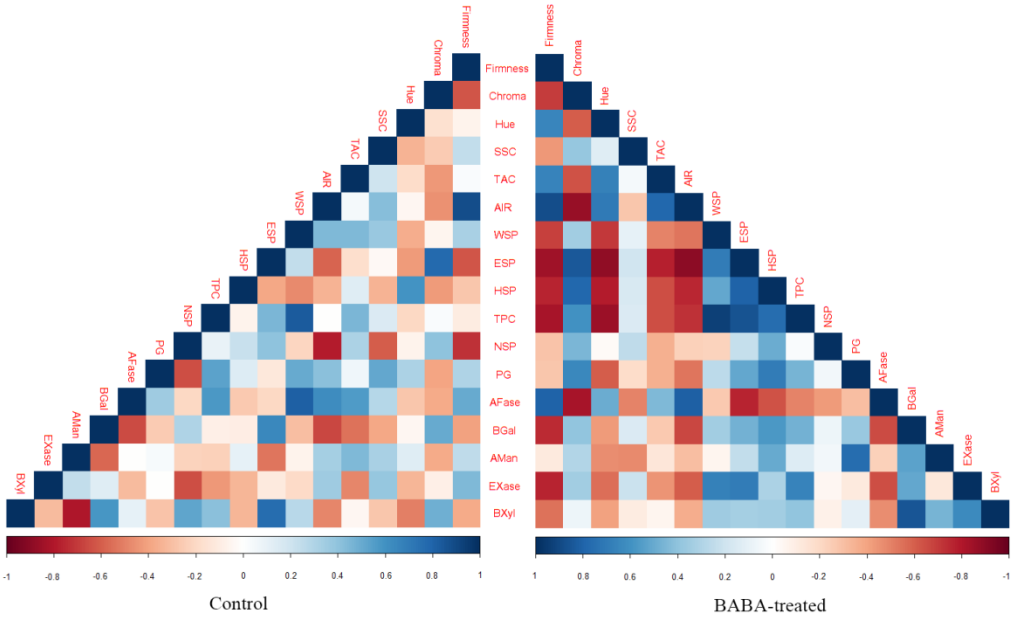

Fig. IV-1. Correlation heatmap of fruit quality attributes with cell wall materials and cell wall modifying enzyme activities in BABA-treated 'Bluecrop' highbush blueberry fruits during refrigerated storage. SSC, soluble solids content; TAC, total anthocyanin content; AIR, alcohol-insoluble residue; WSP, water-soluble pectins; ESP, EDTA-soluble pectins; HSP, HCl-soluble pectins; TPC, total pectin contents; NSP, hemicellulose; PG, polygalacturonase; AFase, α-arabinofuranosidase; BGal, β-galactosidase; AMan, α-mannosidase; EXase, endo-1,4-β–xylanase; BXyl, β-xylosidase.

Table IV-2. Soluble solids content, pH, and titratable acidity of BABA-treated 'Bluecrop' highbush blueberry fruits during refrigerated storage.

BABA (mM)	Storage days at 2°C	SSC[z] (%)	pH	Titratable acidity (%)
0	0	15.6 aA[y]	3.27 aA	0.73 bB
	10	15.2 aA	3.30 aA	0.81 aB
	20	16.7 aA	3.36 aA	1.08 aB
20	0	15.0 aA	3.20 aA	1.17 bA
	10	15.0 aA	3.26 aA	1.85 aA
	20	14.7 aB	3.20 aB	1.85 aA

[z]SSC, soluble solids content.

[y]Significant differences are marked with lowercase letters within storage day and with uppercase letters within treatment by LSD test at $P < 0.05$.

present study was similar to that reported for ‘Bluecrop’ highbush blueberry fruits by Angeletti et al. (2010). Titratable acidity significantly increased during storage and was significantly higher in BABA-treated fruit than in control fruits following each storage day. The increases in titratable acidity were mainly due to fruit dehydration, which has been reported in cold-stored blueberry fruits (Chiabrando and Giacalone, 2011). Higher titratable acidity in BABA-treated fruits may relate to less conversion of organic acids to sugars during refrigerated storage. Thus, the higher titratable acidity in BABA-treated fruits suggests that preharvest BABA treatment delays quality deterioration in highbush blueberry fruits during refrigerated storage. Higher titratable acidity was also reported in postharvest BABA-treated sweet cherry fruits (Wang et al., 2016b).

Effects of BABA on individual soluble sugars and organic acids

Glucose and fructose were the main soluble sugars in ‘Bluecrop’ highbush blueberry fruits (Table IV-3). Both sugars significantly accumulated during refrigerated storage and were significantly higher in BABA-treated fruits than in control fruits following 10 storage days. Sucrose was not detected in the present study probably due to its low concentration, similarly to the report of Wang et al. (2008).

Citric acid was the most predominant organic acid in ‘Bluecrop’ highbush blueberry fruits, followed by malic acid. All detected organic acids significantly

Table IV-3. Individual soluble sugars and organic acids of BABA-treated 'Bluecrop' highbush blueberry fruits during refrigerated storage.

BABA (mM)	Storage days at 2°C	Glucose	Fructose	Citric acid ($g \cdot kg^{-1}$ fresh weight)	Malic acid	Shikimic acid
0	0	53.0 bA[z]	57.5 bA	8.8 bB	2.4 cA	0.019 bA
	10	56.9 abB	61.5 bB	9.2 bB	2.6 bB	0.021 abB
	20	64.9 aA	73.1 aA	13.9 aA	3.1 aB	0.031 aA
20	0	58.1 bA	61.9 cA	11.8 bA	2.5 cA	0.025 aA
	10	63.0 abA	74.6 bA	14.5 aA	3.2 bA	0.030 aA
	20	74.9 aA	83.1 aA	11.1 bB	3.7 aA	0.028 aA

[z]Significant differences are marked with lowercase letters within storage day and with uppercase letters within treatment by LSD test at $P < 0.05$.

increased during refrigerated storage. Preharvest BABA treatment significantly enhanced citric acid following 0 and 10 storage days, malic acid following 10 and 20 storage days, and shikimic acid following 10 storage days. Citric and malic acids were also enhanced in sweet cherry fruits by postharvest BABA treatment during storage (Wang et al., 2016b).

Effects of BABA on fruit weight, skin firmness, and decay incidence

Fruit weight significantly decreased during refrigerated storage, but no statistically significant differences regarding weight loss were observed between control and BABA-treated fruits (Table IV-4). This result is contradictory to that reported by Wang et al. (2016a) in sweet cherry fruits, presumably due to different fruit characteristics, timing of BABA treatment, and storage temperature and duration.

Fruit skin firmness significantly increased during refrigerated storage. The firmness was similar in control and BABA-treated fruits at 0 storage day, but became significantly higher in BABA-treated fruits following 10 and 20 storage days (Table IV-4, Fig. IV-2). Although fruit firmness generally decreases during storage, the increase in fruit firmness observed here might be due to cold temperature effects of tightening and dehydration, similarly to that reported for cold-stored blueberry (Chiabrando and Giacalone, 2011), strawberry (Chen et al., 2011; Lara et al., 2004), and ‘Celeste’ sweet cherry fruits (Belge et al., 2017).

Table IV-4. Weight loss, skin firmness, and decay incidence of BABA-treated 'Bluecrop' highbush blueberry fruits during refrigerated storage.

BABA (mM)	Storage days at 2°C	Weight loss (%)	Skin firmness (N)	Decay incidence (%)
0	0	0.00 cA[z]	1.48 cA	0.00 bA
	10	2.71 bA	1.80 bB	0.00 bA
	20	5.63 aA	1.98 aB	2.33 aA
20	0	0.00 cA	1.52 cA	0.00 bA
	10	3.31 bA	2.02 bA	0.00 bA
	20	6.72 aA	2.13 aA	0.33 aB

[z]Significant differences are marked with lowercase letters within storage day and with uppercase letters within treatment by LSD test at $P < 0.05$.

Fig. IV-2. Appearance of ‘Bluecrop’ highbush blueberry fruits as affected by BABA treatment following 20 storage days at 2°C.

Preharvest BABA treatment significantly reduced decay in blueberry fruits during refrigerated storage (Table IV-4). Similarly, Wang et al. (2016a) reported that BABA applied at 10-100 mmol·L^{-1} inhibited *Botrytis cinerea* infection in strawberry fruits by suppressing fungal growth and triggering disease resistance.

Effects of BABA on cell wall material contents

Cell wall material contents in 'Bluecrop' highbush blueberry fruits increased during refrigerated storage and were significantly higher in BABA-treated fruits than in control fruits following 10 and 20 storage days (Fig. IV-3). This result suggests that refrigerated storage and preharvest BABA treatment enhances the amount of cell wall materials in blueberry fruits, thus promoting the retention of fruit skin firmness. Similar increases in cell wall material contents have previously been observed in cold-stored strawberry (Lara et al., 2004), calcium- and auxin-treated Chilean strawberry (Figueroa et al., 2012), and sweet cherry fruits (Belge et al., 2017). However, BABA effects on fruit cell wall materials have not been reported. Here, cell wall materials, which determine fruit softening, were significantly correlated with fruit skin firmness (Fig. IV-1).

Effects of BABA on cell wall composition

Galacturonic acid represents uronic acid present in the cell wall (D'Amour

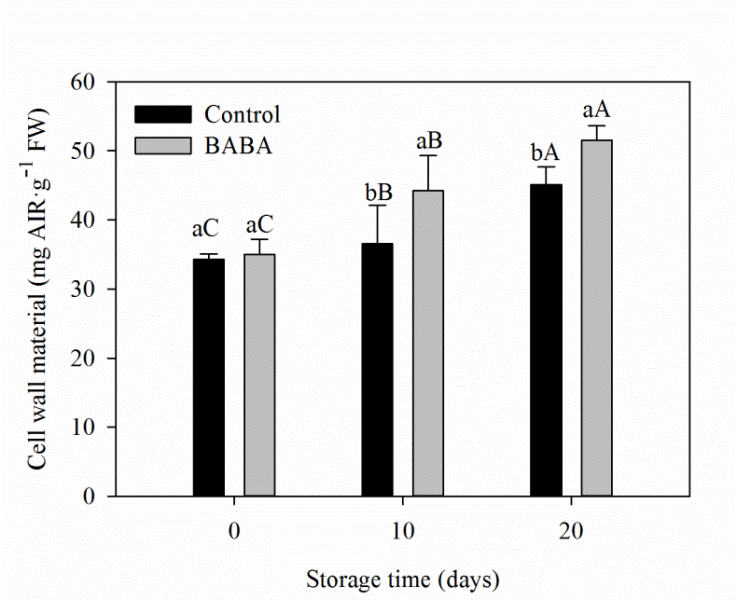

Fig. IV-3. Cell wall material (alcohol-insoluble residue, AIR) in BABA-treated 'Bluecrop' highbush blueberry fruits during refrigerated storage. Vertical bars represent standard errors of the means (n = 3). Significant differences are marked with lowercase letters within treatment and with uppercase letters within storage day by LSD test at $P < 0.05$. FW, fresh weight.

et al., 1993) and some pectins contain neutral sugars as side chains (Mohnen, 2008). In water-soluble pectins, uronic acid decreased during refrigerated storage, but neutral sugars increased (Fig. IV-4a, d). Preharvest BABA-treated fruits had higher uronic acid contents at harvest, but neutral sugar contents in this fraction were not different between control and BABA-treated fruits. Since water-soluble pectins are not attached to the cell wall, they do not contribute to cell wall strength (Ng et al., 2015). Uronic acid and neutral sugars in EDTA-soluble pectins declined significantly following 20 storage days, but difference between control and BABA-treated fruits was not significant (Fig. IV-4b, e). Similarly, a decrease in EDTA-soluble pectins was reported for highbush blueberry fruits during refrigerated storage (Wang et al., 2017). Since a loss in blueberry fruit firmness is associated with increases in water- and EDTA-soluble pectins (Vicente et al., 2007), a rapid decline in EDTA-soluble pectins following refrigerated storage suggests that low temperatures retard pectin solubilization. Unlike other pectin fractions, EDTA-soluble pectins were present in a very low amount, which have been reported in other berries (Figueroa et al., 2010; Rosli et al., 2004), suggesting that no association with fruit firmness loss.

Higher uronic acid content in HCl-soluble pectins was measured at the beginning of storage (0 storage day), but the contents of tightly bound neutral sugars in HCl-soluble pectins remained unchanged during refrigerated storage (Fig. IV-4f). Chen et al. (2015) reported that higher contents of tightly bound

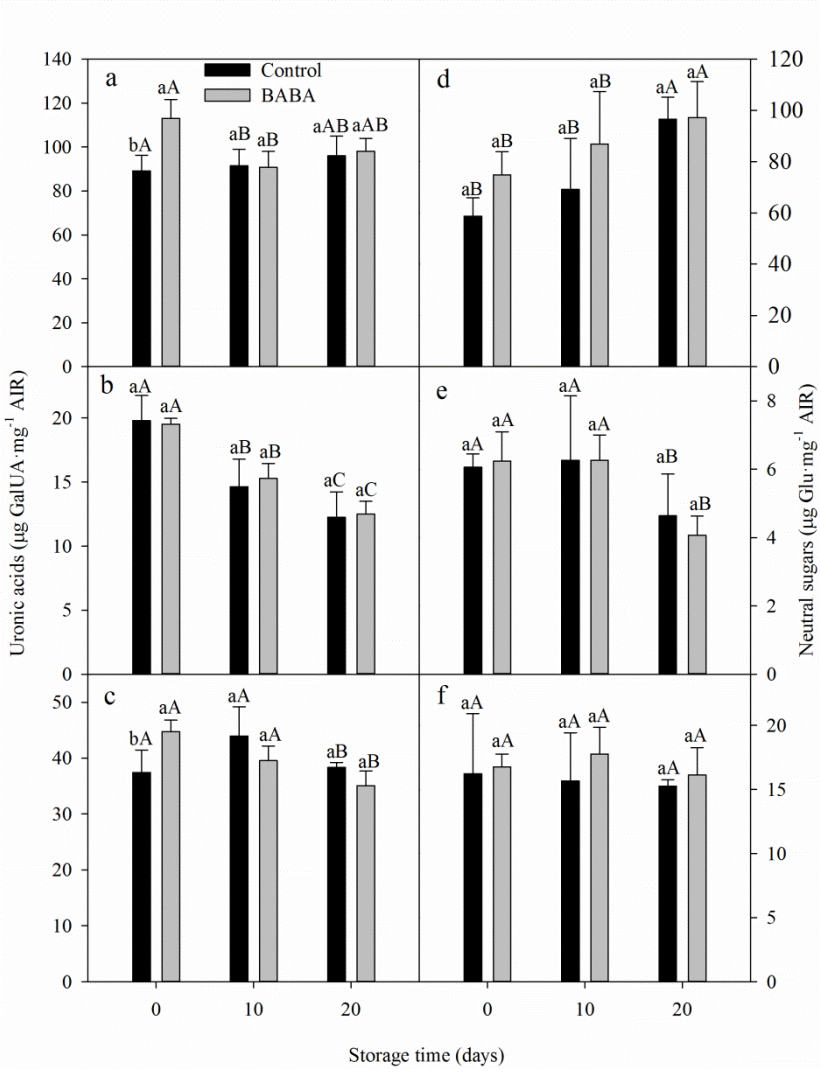

Fig. IV-4. Contents of uronic acids and neutral sugars in water-soluble (a, d), EDTA-soluble (b, e), and HCl-soluble pectins (c, f) in BABA-treated 'Bluecrop' highbush blueberry fruits during refrigerated storage. Vertical bars represent standard errors of the means (n = 3). Significant differences are marked with lowercase letters within treatment and with uppercase letters within storage day by LSD test at $P < 0.05$. GalUA, galacturonic acid; AIR, alcohol-insoluble residue; Glu, glucose.

pectins, as found in HCl-soluble pectins in the present study, contributed significantly to blueberry fruit skin firmness during storage. Postharvest BABA treatment was found to delay softening in sweet cherry fruits by enhancing pectin contents (Wang et al., 2015). The results presented here suggest that, at least during initial refrigerated storage, preharvest BABA treatment partly contributes to a delay in pectin degradation in blueberry fruits.

Hemicellulose content remained unchanged following 10 storage days, but decreased significantly thereafter (Fig. IV-5). Hemicellulose is extensively depolymerized during ripening and softening of blueberry fruits (Vicente et al., 2007). In the present study, hemicellulose contents decreased by 25 and 19% in control and BABA-treated fruits, respectively, following 20 storage days. Wang et al. (2015) reported that postharvest BABA treatment enhanced hemicellulose content in sweet cherry fruits once stored at 20°C.

The changes of each cell wall fraction did not correspond to a significant increase in cell wall materials in BABA-treated fruits following 10 and 20 storage days, presumably due to the variation effects of BABA on other cell wall compositions such as celluloses and structural proteins, which were also present in the cell wall. Another reason could be accounted for a high fruit weight loss, which could generate more alcohol-insoluble residues (Figueroa et al., 2012) in BABA-treated fruits following these storage days.

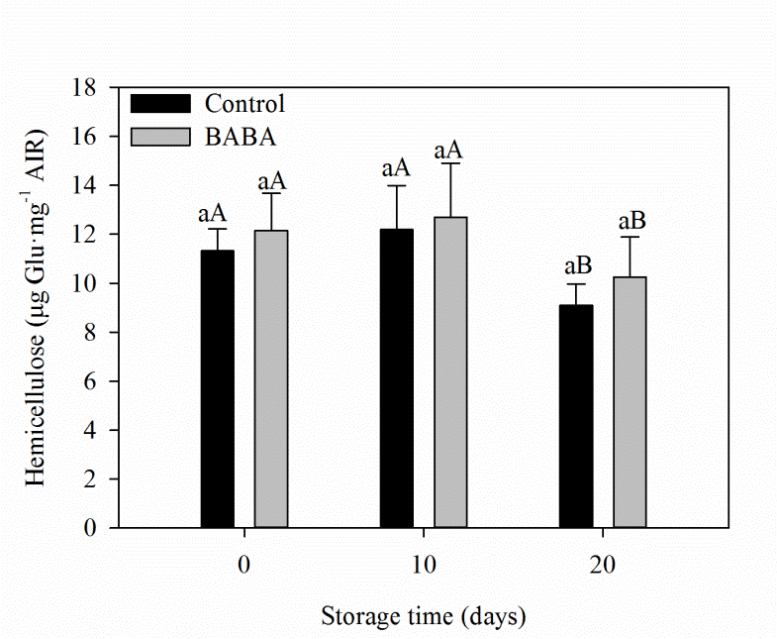

Fig. IV-5. Hemicellulose contents in BABA-treated 'Bluecrop' highbush blueberry fruits during refrigerated storage. Vertical bars represent standard errors of the means (n = 3). Significant differences are marked with lowercase letters within treatment and with uppercase letters within storage day by LSD test at $P < 0.05$. Glu, glucose; AIR, alcohol-insoluble residue.

Effects of BABA on fruit neutral sugar composition in pectin fractions

Glucose was the most predominant neutral sugar in water-soluble pectins and its content in both control and BABA-treated fruits increased during refrigerated storage (Table IV-5). These results suggest that glucose largely contributes to the increases of total neutral sugars in water-soluble pectins as demonstrated in Fig. IV-4d. Higher glucose contents in this fraction was not common, but has also been reported for Chinese bayberry fruits (Sun et al., 2013). Arabinose, galactose, and xylose decreased during refrigerated storage, suggesting that refrigerated storage suppresses the solubility of these neutral sugars. Mannose present in the smallest amount in this fraction and preharvest BABA treatment significantly suppressed mannose solubility following 20 storage days.

Galactose and arabinose were the most abundant neutral sugars in HCl-soluble pectins (Table IV-5), indicating that the fraction contains abundant rhamnogalacturonan I. Galactose contents were not affected by either preharvest BABA treatment or cold temperature. Since substantial amounts of arabinose were lost from rhamnogalacturonan I during the ripening of blueberry fruits (Gross and Sams, 1984), a significant higher content of arabinose in BABA-treated fruits following 20 storage days indicated that preharvest BABA treatment could reduce loss of arabinose, thus protect pectin side chain degradation. Xylose was also present in a similar amount to arabinose and its content increased with increasing storage period, suggesting that HCl-soluble pectins contain neutral

Table IV-5. Cell wall neutral sugar composition of water-soluble and HCl-soluble pectins in BABA-treated 'Bluecrop' highbush blueberry fruits during refrigerated storage.

Fraction[z]	BABA (mM)	Storage days at 2°C	Rhamnose	Fucose	Arabinose	Xylose (mol %)	Mannose	Galactose	Glucose
WSP	0	0	nd	nd	8.8 aA[y]	9.6 aA	2.2 bA	7.4 aA	72.0 bA
		10	nd	nd	5.3 bA	2.1 bA	1.5 bA	3.4 bA	87.7 aA
		20	nd	nd	5.3 bA	3.0 abA	4.2 aA	3.5 bA	84.0 aA
	20	0	nd	nd	9.4 aA	3.6 aA	1.8 aA	6.1 aA	79.1 aA
		10	nd	nd	5.2 bA	2.8 aA	1.9 aA	3.2 bA	86.9 aA
		20	nd	nd	4.5 bA	2.8 aA	1.9 aB	2.8 bA	88.0 aA
HSP	0	0	4.2 aA	1.1 abA	27.9 aA	18.1 bA	4.6 bB	28.9 aA	15.2 aA
		10	3.9 abA	1.2 aA	25.7 abA	20.6 abA	5.1 abA	29.2 aA	14.3 aA
		20	3.4 bA	1.0 bB	23.4 bB	24.8 aA	5.4 aA	28.8 aA	13.0 aA
	20	0	3.9 aA	1.2 aA	26.2 abA	18.9 bA	5.3 aA	30.2 aA	14.2 aA
		10	3.6 aA	1.1 aA	23.7 bA	24.6 aA	5.6 aA	28.2 aA	13.3 aA
		20	3.8 aA	1.2 aA	26.8 aA	21.4 abA	5.6 aA	27.9 aA	13.3 aA

[z]WSP, water-soluble pectins; HSP, HCl-soluble pectins.

[y]Significant differences are marked with lowercase letters within storage day and with uppercase letters within treatment by LSD test at $P < 0.05$. nd, not detected.

sugars from hemicellulose fraction. Mannose increased during refrigerated storage and was significantly higher in BABA-treated fruits than in control fruits following 0 storage day. The reorganization of individual neutral sugars in the two pectin fractions provided further evidence that preharvest BABA treatment and cold temperature could suppress solubility of some neutral sugars and that BABA could enhance arabinose content in 'Bluecrop' highbush blueberry fruits.

Effects of BABA on cell wall modifying enzyme activities

Polygalacturonase activity was not changed during refrigerated storage, but preharvest BABA treatment more significantly suppressed the activity compared to that in control fruits following 20 storage days (Fig. IV-6a). Similar suppressive effects on polygalacturonase activity were observed in sweet cherry fruits (Wang et al., 2015). α-Arabinofuranosidase activity increased significantly, whereas β-galactosidase activity declined during storage (Fig. IV-6b, c). Preharvest BABA treatment had no effect on the activities of these two cell wall modifying enzymes. The increase in α-arabinofuranosidase activity (Fig. IV-6b) and the decrease in β-galactosidase activity (Fig. IV-6c) during refrigerated storage and their association with fruit skin firmness (Fig. IV-1) indicated that low temperatures, but not preharvest BABA treatment, caused these effects on enzyme activities and fruit firmness.

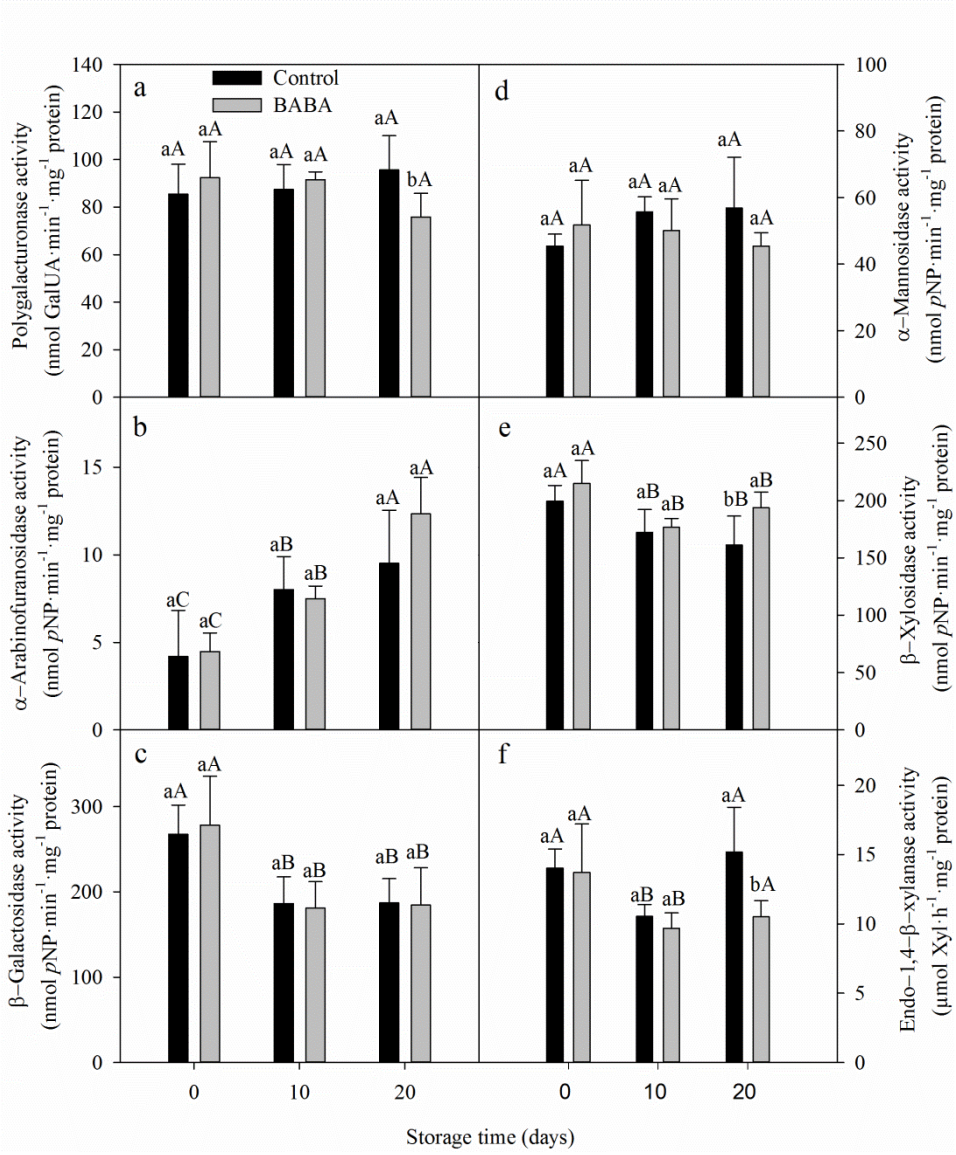

Fig. IV-6. Cell wall modifying enzyme activities in BABA-treated 'Bluecrop' highbush blueberry fruits during refrigerated storage. Vertical bars represent standard errors of the means (n = 3). Significant differences are marked with lowercase letters within treatment and with uppercase letters within storage day by LSD test at $P < 0.05$. GalUA, galacturonic acid; *p*NP, *p*-nitrophenol; Xyl, xylose.

α-Arabinofuranosidase and β-galactosidase can hydrolyze α-L-arabinofuranosyl and β-D-galactosyl residues, respectively (Rosli et al., 2009). The removal of neutral sugars by the action of these two enzymes may affect cell wall structure and increase the accessibility of cell wall modifying enzymes, including polygalacturonase, to their respective substrates (Paniagua et al., 2016). In apple fruits during refrigerated storage, for example, increased activity of α-arabinofuranosidase was associated with a loss in fruit firmness (Gwanpua et al., 2014; Timm et al., 2015). In Chilean strawberry (Figueroa et al., 2010) and tomato fruits (Takizawa et al., 2014), α-arabinofuranosidase activity decreased along with a decline in fruit skin firmness. In the present study, however, α-arabinofuranosidase activity was positively correlated with fruit skin firmness (Fig. IV-1). These contradictory results may be due to the presence of different α-arabinofuranosidase isoforms (Bustamante et al., 2009; Timm et al., 2015).

The α-mannosidase activities were 10 and 20% lower in BABA-treated fruits following 10 and 20 storage days, respectively, than in control fruits but no statistical differences were observed (Fig. IV-6d). Activity of α-mannosidase, which cleaves terminal α-mannosidic linkage from mannose rich complex and *N*-glycans (Liebminger et al., 2009), was found to increase during the ripening of non-climacteric fruit of capsicum (Ghosh et al., 2011). When α-mannosidase was assayed at different ripening stages of ‘Bluecrop’ highbush blueberry fruits, its

activity was closely related to fruit softening (data not shown). During refrigerated storage, however, α-mannosidase activity remained unchanged and did not display close association with postharvest blueberry fruit softening (Fig. IV-1).

β-Xylosidase and endo-1,4-β-xylanase modify xylan (Bustamante et al., 2009), which is a major component of hemicellulose in blueberry fruits (Vicente et al., 2007). β-Xylosidase activity was suppressed during refrigerated storage, but was not significantly different between control and BABA-treated fruits following 0 and 10 storage days (Fig. IV-6e). Endo-1,4-β-xylanase activity was significantly reduced by both low temperatures and preharvest BABA treatment (Fig. IV-6f) and was significantly correlated with fruit skin firmness in BABA-treated fruits (Fig. IV-1), similarly to that observed for papaya fruits (Manenoi and Paull, 2007). These results suggest that lower endo-1,4-β-xylanase activity caused by preharvest BABA treatment partly contributes to higher skin firmness of 'Bluecrop' highbush blueberry fruits as shown in Table IV-1.

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CONCLUSIONS

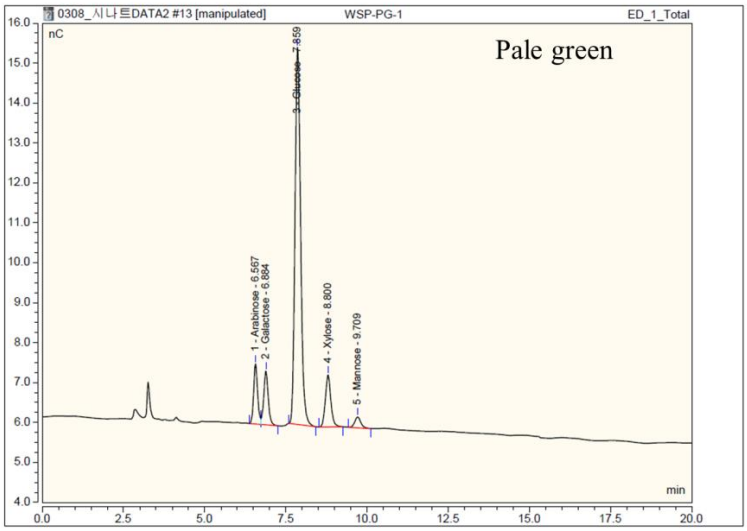
During the course of blueberry fruit softening, cell microstructures were extensively fractured and ultrastructures of fruit cell walls were also extensively altered. The softening of 'Bluecrop' highbush blueberry fruits were the results of a reduction in cell wall material contents, an increase in water-soluble pectins and a significant reduction in HCl-soluble pectins. An accumulated amount of arabinose and a substantial loss of arabinose in water- and HCl-soluble pectins, respectively, during early ripening coincided with fruit softening. Polygalacturonase, α-arabinofuranosidase, and β-galactosidase were found to play significant roles during early ripening stages of blueberry fruits. Hemicellulose contents were significantly reduced during early ripening, showing a significantly positive correlation with fruit firmness. The activities of α-mannosidase and endo-1,4-β-xylanase negatively correlated with fruit firmness during ripening. These results together with extensively deformed surfaces of parenchyma tissues at reddish purple stage indicate that 'Bluecrop' highbush blueberry fruits soften extensively during on-shrub ripening prior to the harvest time.

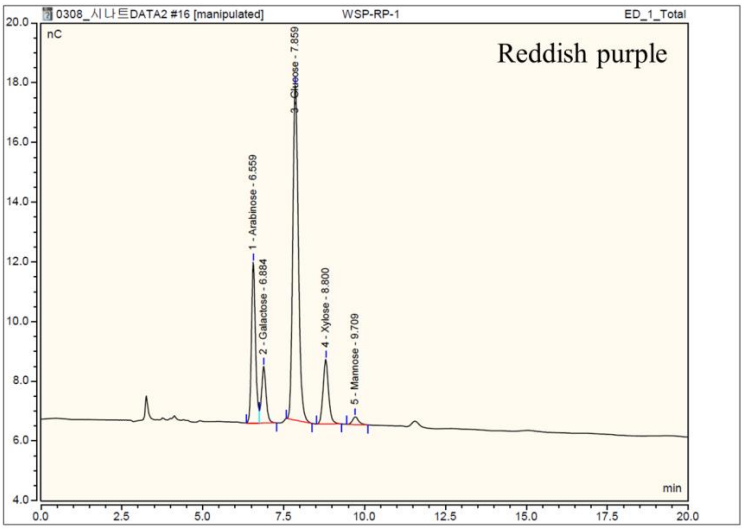
Preharvest BABA treatment delayed changes in skin colors, lowered pH, enhanced acidity and individual soluble sugars, and maintained higher skin firmness in 'Bluecrop' highbush blueberry fruits. Reduced softening caused by

preharvest BABA treatment was partly the result of increased cell wall materials, enhanced pectin contents at least during initial refrigerated storage, and reduced the activities of polygalacturonase and endo-1,4-β-xylanase. These data also showed that cold temperature exerted effects on fruit quality attributes, including skin firmness. These results will be useful for managing softening-related fruit losses in highbush blueberries.

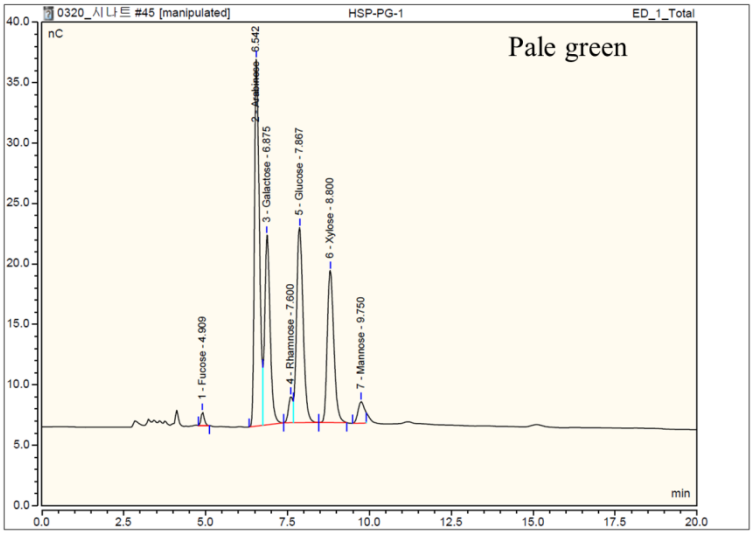
APPENDICES

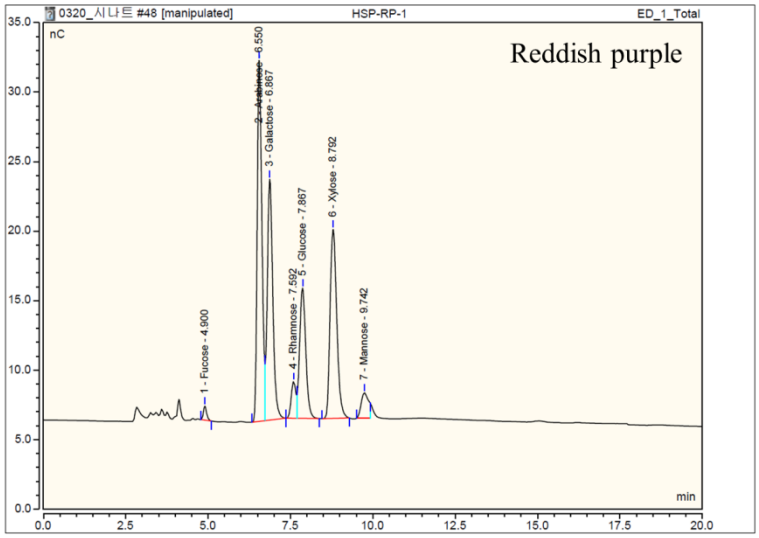
Appendix 1. Typical Bio-LC chromatograms of neutral sugar composition in water-soluble pectins during ripening in 'Bluecrop' highbush blueberry fruits.



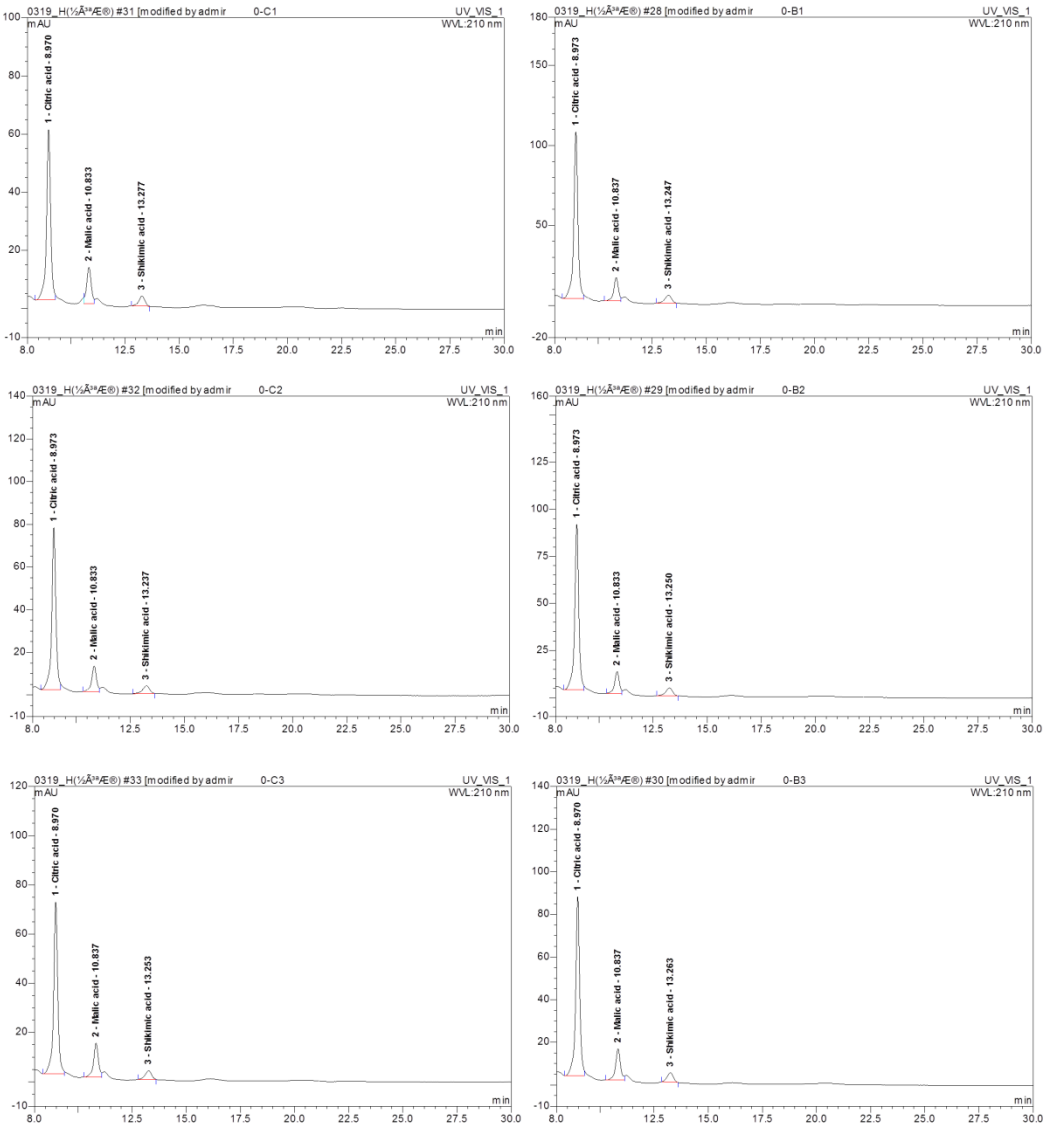


Appendix 2. Typical Bio-LC chromatograms of neutral sugar composition in HCl-soluble pectins during ripening in 'Bluecrop' highbush blueberry fruits.



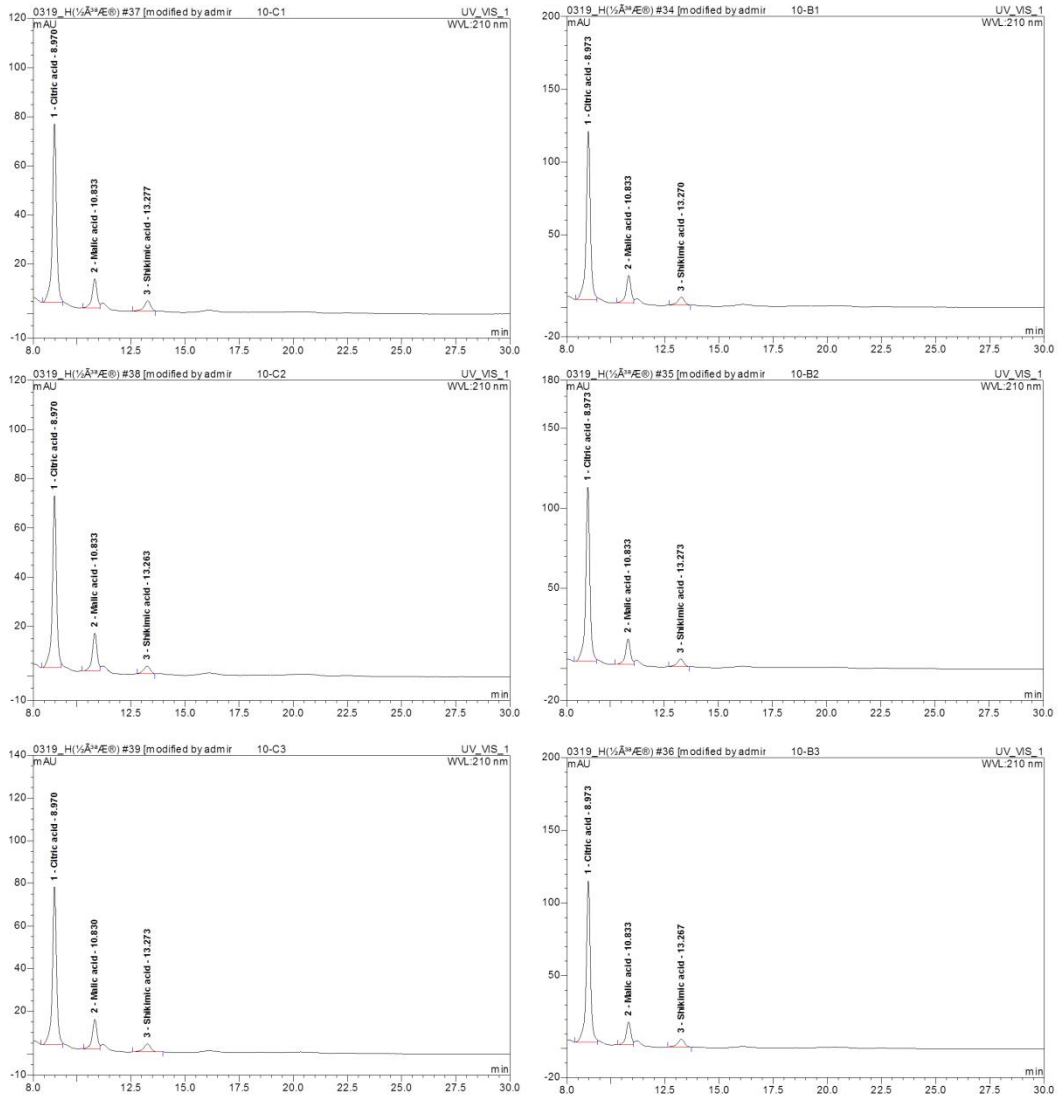


Appexdix 3. HPLC chromatograms of organic acids in control and BABA-treated ‘Bluecrop’ highbush blueberry fruits following 0 storage day.



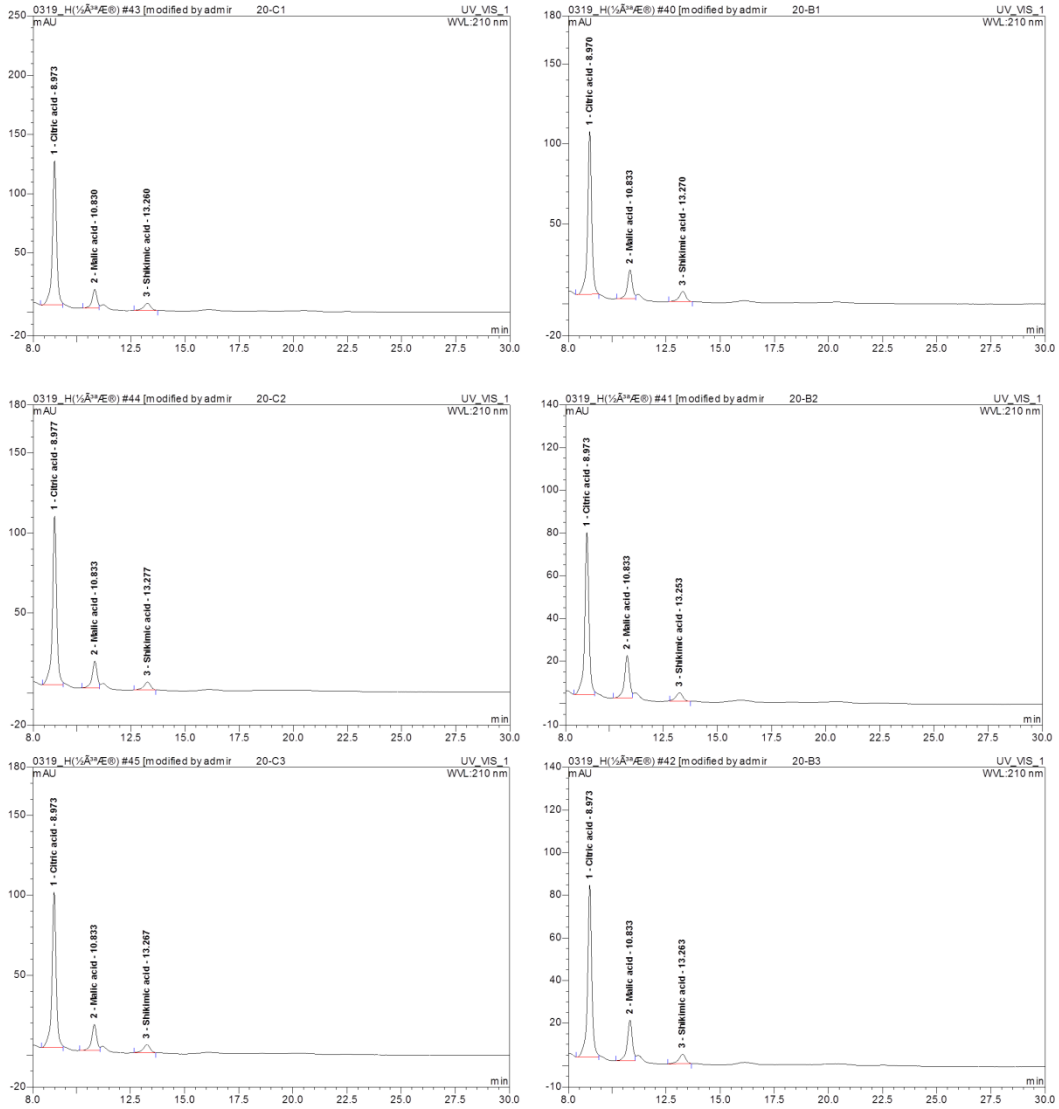
Control BABA

Appendix 4. HPLC chromatograms of organic acids in control and BABA-treated 'Bluecrop' highbush blueberry fruits following 10 storage days.



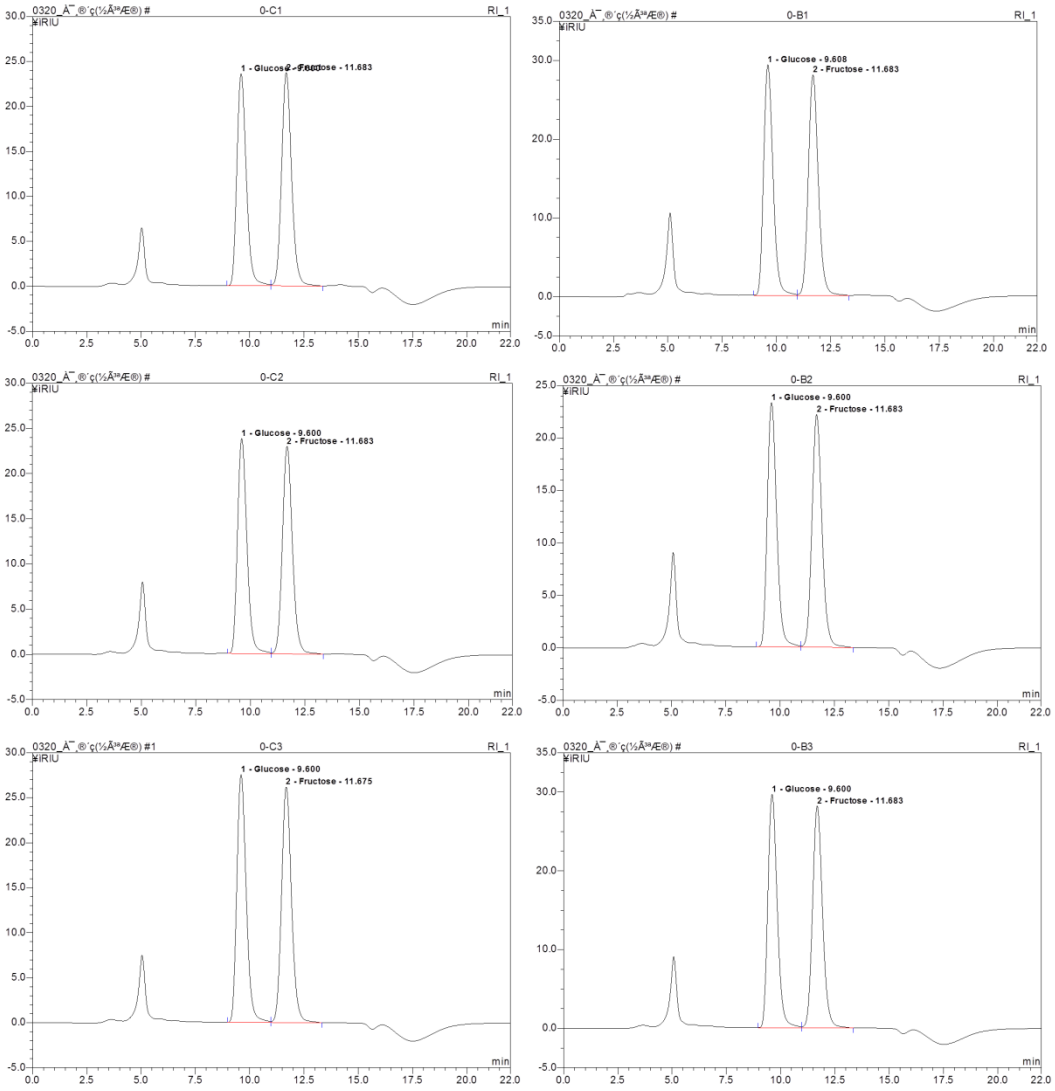
Control BABA

Appendix 5. HPLC chromatograms of organic acids in control and BABA-treated 'Bluecrop' highbush blueberry fruits following 20 storage days.



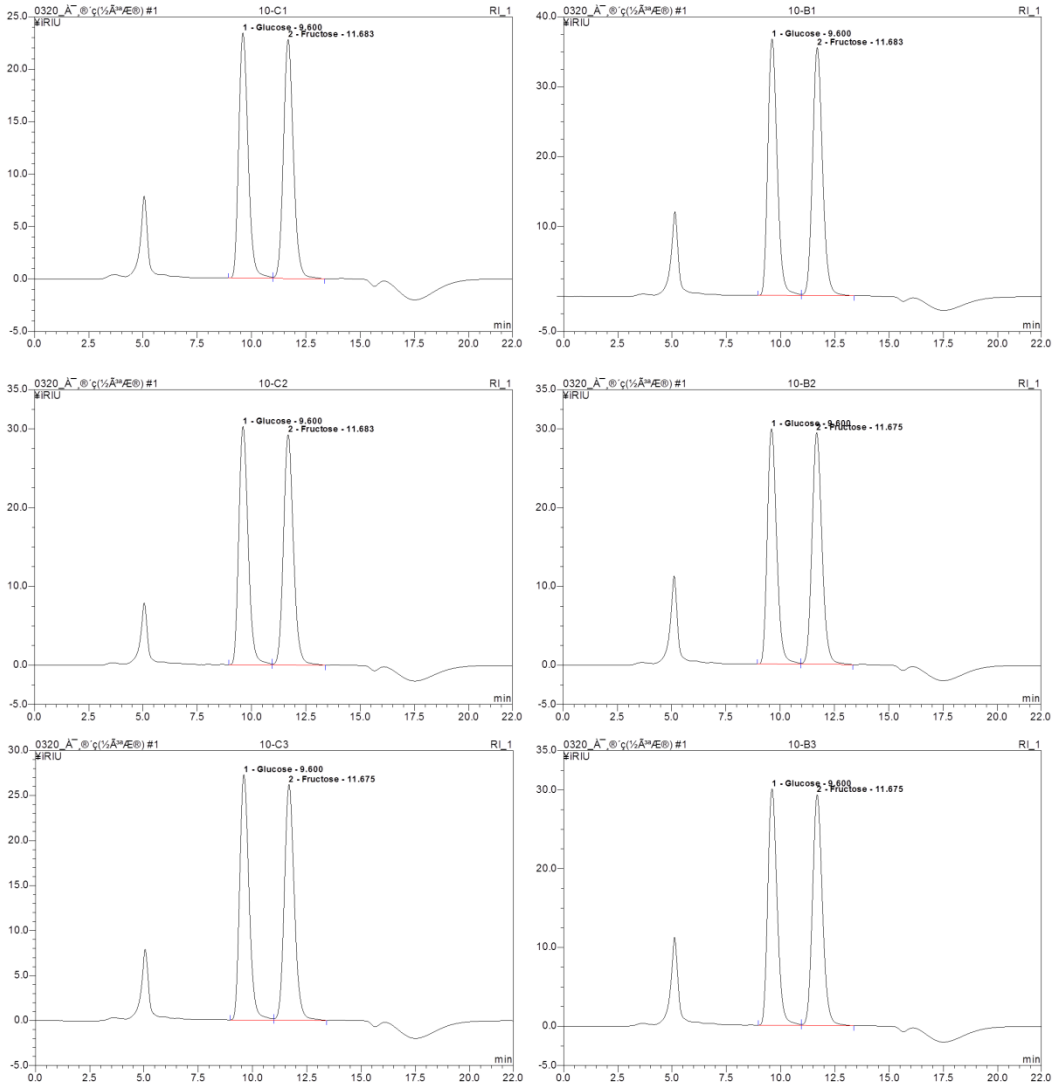
Control BABA

Appendix 6. HPLC chromatograms of soluble sugars in control and BABA-treated ‘Bluecrop’ highbush blueberry fruits following 0 storage day.



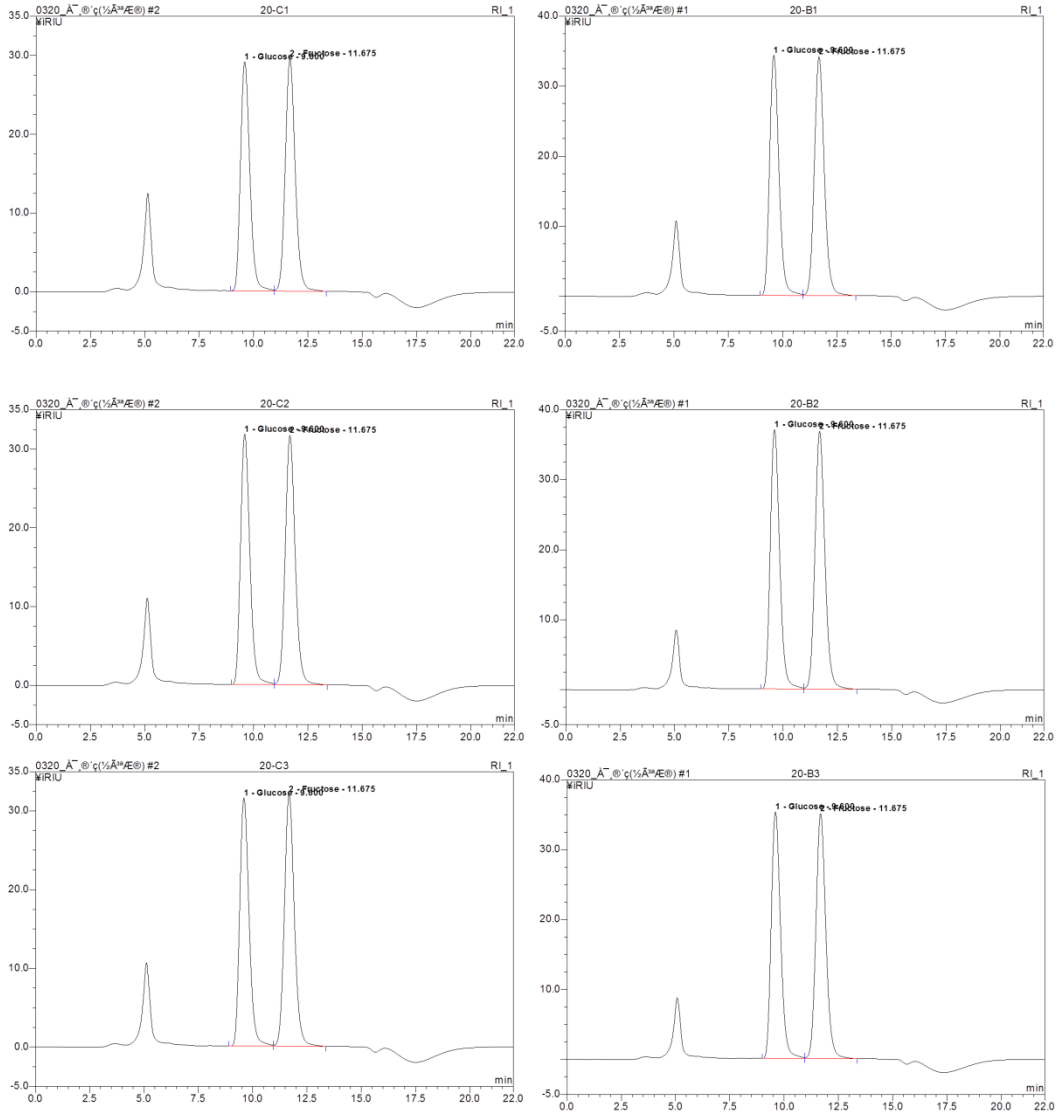
Control BABA

Appendix 7. HPLC chromatograms of soluble sugars in control and BABA-treated ‘Bluecrop’ highbush blueberry fruits following 10 storage days.



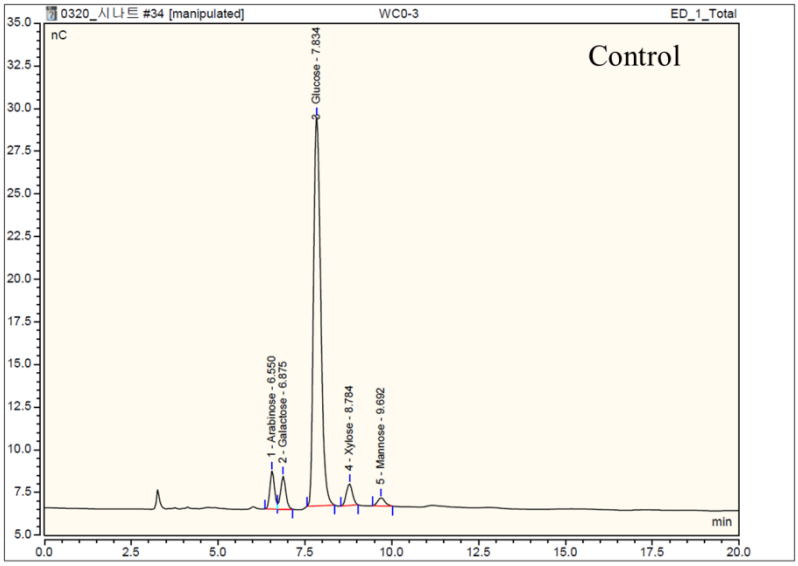
Control BABA

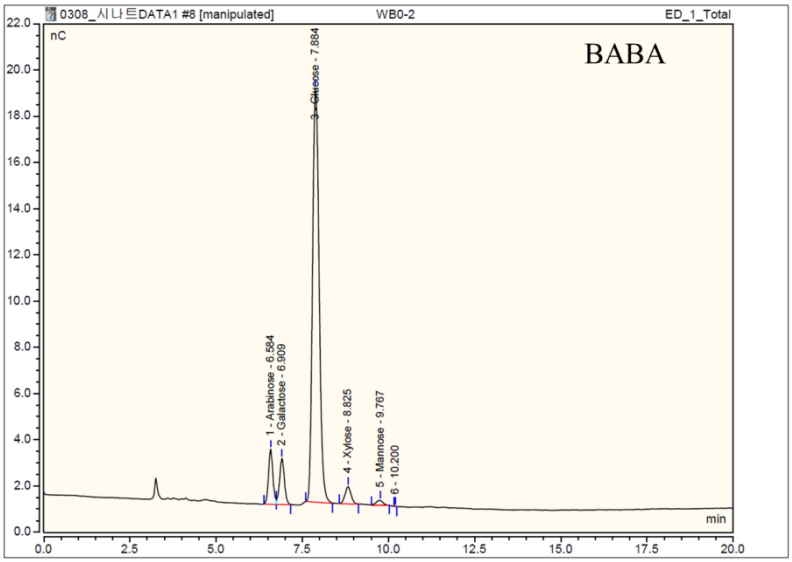
Appendix 8. HPLC Chromatograms of soluble sugars in control and BABA-treated ‘Bluecrop’ highbush blueberry fruits following 20 storage days.



Control BABA

Appendix 9. Typical Bio-LC chromatograms of neutral sugar composition in water-soluble pectins of control and BABA-treated ‘Bluecrop’ highbush blueberry fruits.





Appendix 10. Typical Bio-LC chromatograms of neutral sugar composition in HCl-soluble pectins of control and BABA-treated ‘Bluecrop’ highbush blueberry fruits.