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Anthocyanin Accumulation Mechanism in Highbush Blueberry Fruit by UV Radiation

UV 조사에 의한 블루베리의 안토시아닌 축적 기작에 대한 연구

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ABSTRACT

The aim of this work was to better understand the molecular mechanism of increasing anthocyanin contents in highbush blueberry fruit (*Vaccinium corymbosum* L.) after UV-B radiation at 6.0 kJ m^{-2} for 20 min. Blueberry fruits were applied by three kinds of UV lights (A, B and C) to find out UV-B and UV-C are the good radiations to maintain the quality and anthocyanin of blueberry fruit. Most of individual anthocyanins, namely Delphinidine-3-galactoside, Delphinidin-3-glucoside, Delphinidine-3-arabinoside, Petunidine-3-galactoside, Petunidine-3-glucoside, Petunidine-

3-arabinoside, Malvidine-3-galactoside, Malvidine-3-glucoside and Malvidine-3-arabinoside increased after 3 h by UV-B and UV-C radiation treatments. Then, the analysis on effects of UV-B radiation on the gene expressions of anthocyanin and ethylene related genes and transcription factors (TFs) of *VcBBX*, *VcMYB21*, *VcWD40*, *VcR2R3MYB*, *VcEIL4*, and *VcTDR4* in tissues of 'Duke' and 'Nelson' highbush blueberry fruit were investigated. Beside of that, the ethylene production and the main individual anthocyanin also were analyzed. These findings showed that the UV-B and UV-C radiation can improve the quality and anthocyanin in 'Duke' blueberry fruit. UV-B radiation increased transcript levels of anthocyanin biosynthesis genes such as *VcPAL*, *VcCHS*, *VcF3'H*, *VcDFR*, and *VcUFGT* and also up-regulated about transcript levels on TFs of *VcR2R3 MYB*, *VcMYB21*, *VcWD40*, *VcBBX*, and *VcEIL4* in the peels of 'Duke' blueberry fruit. This increasing was found in immediately after UV-B treatment (20 min) and became maximized within 3 h. However, UV-B radiation reduced the transcript levels of *VcTDR4*, *VcACO*, *VcETR1* in 'Duke' blueberry fruit. UV-B radiation also induces the reduction of ethylene production in 'Nelson' blueberry fruit. The contents of main individual anthocyanin (Delphinidin-3-Galactoside, Malvidin-3-Galactoside, and Malvidin-3-Arabinoside) were increased significantly after UV-B immediately or within 3 h depending on the analyzed tissues. The results indicated that UV-B

radiation stimulates the increase of anthocyanin biosynthesis which could be up-regulated by the anthocyanin related TFs and down-regulated by the ethylene related genes in ‘Duke’ blueberry fruit. This study supports that the pre-storage treatment of UV-B increases the phytochemical accumulation of the full-ripe highbush blueberry fruit after being picked.

Key words: Anthocyanin regulated genes, ethylene related genes, individual anthocyanin, postharvest quality, UV light, *Vaccinium corymbosum.*

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACO	Acyl-CoA Oxidase
ANS	Anthocyanidin synthase
BBX	B-box protein
bHLH	Basic helix loop helix
CHS	Chalcone synthase
CTAB	Cationic detergent cetyl-trimethylammonium bromide
DFR	Dihydroflavonol-4-reductase
EBG	Early stage biosynthesis gene
EIL3	Ethylene insensitive 3 like
ETR1	Ethylene receptor 1
F3H	Flavanone 3 ß-hydroxylase
HY5	Elongated hypocotyl 5
LBG	Late stage biosynthesis gene
MBW	MYB-bHLH-WD40 complex
PAL	Phenylalanine ammonia lyase
TDR4	FUL1 (Fruitfull1), a MADS-box transcription factor
TFs	Transcription factors
UFGT	UDP-glucose: flavonoid 3-O-glucosyltransferase
UV	Ultraviolet

GENERAL INTRODUCTION

Highbush blueberry fruit (*Vaccinium corymbosum* L.) belong to the family "Ericaceae," subfamily "Vacciniaceae," genus "*Vaccinium*," (Gough, 1993). Among berries of genus "*Vaccinium"* such as blueberries, bilberries, cranberries, huckleberries and lingonberries, highbush blueberry fruit are a kind of super power fruit consumed all around the world due to their high content of anthocyanin. The consumption of highbush blueberry fruit has been increasing considerably in South Korea. The fresh blueberry fruit are harvested from June to August, however they might rapidly deteriorate after harvest because of microbial development (Chun et al., 2013) and reduction of anthocyanin.

There are six main anthocyanidins in highbush blueberry fruit. These are cyanidin, delphinidin, malvidin, petunidin, and peonidin (Kader et al., 1996; Routray and Orsat, 2011). The anthocyanidins with the derivative of glucose, galactose and arabinose forms anthocyanin. Some major individual anthocyanins (Delphinidin-3-Galactoside, Delphinidin-3-Glucoside, Delphinidin-3-Arabioside, Petunidin-3-Galactoside, Petunidin-3-Glucoside, Petunidin-3-Arabioside, Malvinidin-3-Galactoside, Malvinidin-3-Glucoside, Malvinidin-3-Arabioside) were detected in 'Duke' blueberry

fruit which is cultivated in South Korea at full ripening stage (Nguyen et al., 2014). The accumulation of anthocyanin occurs in peel tissues of blueberry fruit (Riihinen et al., 2008) and follows the phenylpropanoid pathway which is a result of activation of anthocyanin biosynthetic genes (Routray and Orsat, 2011). Several transcription factors are involved in regulation of structural genes in the phenypropanoid pathway. They were MYBs family (MYB21 and R2R3 MYB), TTG1 (WD40) protein and a MADS box protein (TDR4) (Xu et al., 2015) or BBX proteins (Gangappa and Botto, 2014). Environmental factors affect the quality and the anthocyanin in blueberry fruit not only before harvest but also after being picked. Postharvest treatment technologies play an important role to maintain the quality as well as to improve anthocyanin content in harvested blueberry fruit.

Some studies investigated the effect of postharvest technologies on the quality, the anthocyanin and the expression of anthocyanin biosynthesis genes in harvested blueberry. In addition, some recent postharvest treatment studies applied to harvested blueberry fruit showed that methyl jasmonate induced the activation of PAL and CHS (Cocetta et al., 2015), and storage of blueberry under high CO_2 at 18 kPa CO_2 affected the expression of *VcCHS* (Harb et al., 2014)

UV radiation appears to be effective technique that can be applied for reduction of microbial growth rather than other immersing methods,

especially since it does not leave a residue after treatment (Ribeiro and Alvarenga, 2012). UV radiation at low doses from 0.25 to 8.0 kJ m^{-2} affects the DNA of microorganism, blocking their development (Terry and Joyce, 2004). It also induced stress response and secondary plant metabolites leading to enhanced antioxidant compounds and improved anthocyanin accumulation (Ribeiro and Alvarenga, 2012).

The accumulation of anthocyanins is also related to ripening and depends on the cultivar of blueberry fruit (Zifkin et al., 2012). Generally, ripening mechanism of non-climacteric and climacteric fruit is related to ethylene. Non-climacteric fruit cannot ripen after being picked. Climacteric fruit continue to ripen after harvesting and their ethylene production is higher than the one of non-climacteric fruits. In highbush blueberry fruit, ethylene production is the low range from 0.5 to 10 μL kg^{-1} h^{-1} (El-Agamy et al., 1982; Suzuki et al., 1997), although whether blueberry fruit belongs to non-climacteric fruits (Zifkin et al., 2012) or climacteric fruits (El-Agamy et al., 1982) is still under debate. In addition, the relationship between the accumulation of anthocyanin and ethylene during ripening is unclear. The mechanism of ethylene action in inducing the ripening of highbush blueberry fruit has not been resolved, especially after UV-B radiation treatment.

The main goal of this study was to develop a postharvest treatment

technology using UV radiation that maintains the quality as well as the anthocyanin of harvested blueberry fruit. The specific objective were (1) to determine which kind of UV light improves the quality and the anthocyanins of harvested blueberry fruit during cold storage, (2) to investigate the effect of UV-B radiation on the activation of anthocyanin biosynthesis genes and transcription factors that related to accumulation the anthocyanin, (3) to investigate the effect of UV-B on the expression levels of ethylene and anthocyanin regulated genes involve in natural ripening and by UV-B radiation treatment.

LITERATURE REVIEW

Anthocyanin biosynthesis and deterioration

Anthocyanins are one of the most common pigments in plants. They are responsible for blue dark pigments in blueberry fruit. Anthocyanins are glycosidic and acyloglycosidic forms of anthocyanidins (Tan et al., 2014). In blueberry fruit, 25 peaks of simple or acylated anthocyanins were analyzed in different species and anthocyanidin of delphinidin was found as a major compound (Kalt et al., 1999).

The accumulation of anthocyanin does not occur in the cells where they are synthesized but in flowers, fruit. They are also partly present in leaves and bark. There are different kinds of individual anthocyanin and in the different parts which have diverse functions. When plant meets stress, the defense mechanism can stimulate the accumulation of anthocyanins in different parts (Routray and Orsat, 2011). Accumulation of anthocyanins in the peel blueberry fruit and in the both of peel and flesh of bilberry fruit was reported earlier by Riihinen et al. (2008). Anthocyanin accumulation in red grapes was higher than this one in white grapes which is related to the expression of UFGT (Boss et al., 1996; Poudel et al., 2008). Unlike most of grapes, ‘Teinturier’ grapes accumulate anthocyanins both in skin and flesh. However, there are differences in the concentrations and compositions of

anthocyanins in both tissues (Guan et al., 2016). The synthesis of anthocyanin takes place by the phenylpropanoid pathway which affects phenylalanine conversion to coumaryl CoA (Stafford, 1990). Generally, anthocyanidins are weak and they are easy to be converted by glycosylation, a part of anthocyanin synthesis, and form glycosylated forms (Hendry and Houghton, 1996).

The anthocyanin composition and contents of blueberry fruit depends on cultivars, species, and varieties and analysis method (Lohachoompol et al., 2008). Normally, anthocyanin decline after harvesting and the reduction of anthocyanin depends on pH, enzymes, and temperature. These factors induced the differences in the structural properties of the anthocyanins (Routray and Orsat, 2011).

UV radiation and its application as postharvest technology

Extensive research on the effect of postharvest treatments such as such as CO_2-enrich atmosphere (Harb et al., 2014), sulfur dioxide (Cantin et al., 2012), calcium application before treatment (Angeletti et al., 2010), 1-methylcyclopropene (Chiabrando and Giacalone, 2011) and UV-C radiation (Perkins-Veazie et al., 2008) on the quality of highbush blueberry fruit has been done. Among postharvest treatment technologies, UV radiation appears to be effective for blocking microbial growth, and it does not leave a residue

after treatment.

There are three kinds of UV radiation (UV-A, -B, and -C) which subdivided by the range of wavelength. UV-A is in the range from 315 nm to 400 nm, UV-B is the range from 280 nm to 315 nm and UV-C is the range from 200 nm to 280 nm. Exposure to the proper dose of UV radiation with time scale induce changes of enzyme activities which delayed the development of microorganisms (Ribeiro and Alvarenga, 2012). In addition, UV radiation has been used on onions to increase health-enhancing phytonutrients (Rodov et al., 2010).

UV-C radiation was applied to many crops as a postharvest treatment technique. Handling of UV-C from 0.25 to 8.0 kJ m^{-2} can block microorganism development (Terry and Joyce, 2004). A recent report of Pinheiro et al. (2015) showed that UV-C radiation at 0.97 kJ m^{-2} and 4.83 0 kJ m^{-2} induces the lowest microbial load in tomato fruit. UV-C radiation also induced stress response and secondary plant metabolites which lead to enhanced antioxidant compounds and improved anthocyanin accumulations in several crops according to a review of Ribeiro and Alvarenga (2012).

Although UV-B radiation does not apply to as much as crops UV-C radiation does, but postharvest treatments with UV-B radiation have been investigated in blueberry fruit (Eichholz et al., 2011) and broccoli (Aiamla-or et al., 2009). UV-B light treatment increased the antioxidant contents in

highbush blueberry fruit and maintained the green color in broccoli. Moreover, it enhanced antioxidant capacity of carrot products (Avena-Bustillos et al., 2012), and increased soluble phenolic contents of some other crops (Du et al., 2014).

UV-A radiation is rarely applied for postharvest treatments. Aiamla-or et al. (2009) showed that UV-A radiation did not maintain the green color of broccoli like UV-B radiation did. However, a previous report showed that UV-A solar radiation inhibited the growth of lettuce (Krizek et al., 1998). Effect of UV-A radiation remains unclear in highbush blueberry fruit.

UV radiation induces the changes of anthocyanin and its biosynthesis pathway

Ultraviolet (UV)-B light was considered as a major environmental factor which could be applied to improve fruit quality and antioxidants of blueberry fruit (Perkins-Veazie et al., 2008). UV-B radiation in the range between 280 nm and 320 nm affected the plant defense system and led to an increased number of secondary metabolites (Teramura, 1983). UV-B radiation induced anthocyanin accumulation in hypocotyls of radish spout (Su et al., 2015) and also induced anthocyanin and flavonoid in apple skins (Ban et al., 2007a). Anthocyanin have higher capacity in the response to

UV-B light treatment for immature apple skin (Reay and Lancaster, 2001). The solar light is one of the vital factors adjusting the expression of anthocyanin biosynthesis genes (Guo et al., 2008). Genes which encode anthocyanin biosynthesis enzymes are PAL, CHS, F3H, DFR, ANS, and UFGT. The structural genes of the phenylpropanoid pathways catalyzed the conversion of phenylalanine and it lead to anthocyanin accumulation in blackberry fruit (Liu et al., 2013b). These biosynthesis enzymes are controlled by regulatory genes namely transcription factors (TFs) (Holton and Cornish, 1995; Zifkin et al., 2012). Liu et al. (2013a) reported that enzyme activity of F3H was related to the accumulation of the products in the flavonoid biosynthetic pathway because of UV-B radiation and drought stress.

UV-B radiation affected the resistance locus 8 as a signaling that mediates photomorphogenic which induced the expression of the gene encoding the elongated hypocotyl 5, a transcription factor controlling anthocyanin biosynthesis genes (Brown et al., 2009).

UV-B radiation, ethylene and fruit ripening

Ethylene is an important factor which is related to ripening of fruits. Fruits belonging to non-climacteric and climacteric have shown difference on respiration (Alexander and Grierson, 2002). While ethylene production

was low in non-climacteric fruits, it plays a significant role in the development of climacteric fruits such as grapes, citrus and strawberry (Bapat et al., 2010). Blueberry fruit produce very low amount of ethylene. According to Suzuki et al. (1997) and El-Agamy et al. (1982), ethylene production reached from 0.5 to 10.0 μL kg^{-1} h^{-1}. So, whether highbush blueberry fruit belong to non-climacteric (Zifkin et al., 2012) or climacteric fruits (El-Agamy et al., 1982) remains still under debate. In ethylene biosynthesis process, ACC synthase and ACC oxidase are two key enzymes involved in ethylene biosynthesis (Handa et al., 2011). Moreover, ethylene was encoded by receptors of ETRs family include ETR1, ETR2, ERS1, ERS2, and EIN4 in Arabidopsis (Moussatche and Klee, 2004).

The accumulations of anthocyanin in blueberry fruit may occur during ripening which might be affected by ethylene. Red light induces the reduction of ethylene production which leads to the stimulation of anthocyanin in cabbage seedlings (Kang and Burg, 1973). A recent study reported that anthocyanin accumulation is regulated negatively by ethylene and positively by light signaling (Jeong et al., 2010). However, the molecular mechanism of ethylene action in inducing the ripening of highbush blueberry fruit has not been clarified, especially after UV radiation treatment.

Transcription factors (BBX, R2R3MYB, MYB21, TDR4, and EIL4) are involve in anthocyanin accumulation, ripening regulation and their relationship to UV radiation

The dramatic physical and chemical changes associated with fruit ripening occur as a result of both catabolic and anabolic processes. In most instances, the question of whether the increase in activity was the result of enzyme synthesis, activation, disappearing of inhibitory substances or extraction artifacts arising from marked differences in the physical nature of unripe versus ripe tissue was not resolved. During ripen process of blueberry fruit, enzyme activity encode to anthocyanin biosynthesis and ethylene synthesis was increased (Handa et al., 2011).

Anthocyanin belongs to flavonoid compounds which the flavonoid biosynthesis genes were regulated by transcription factors of MYB families MYB families, TTG1 (WD40) family, TT8 (bHLH), and MADS box protein (Xu et al., 2015) and a B-box protein (BBX) (Gangappa and Botto, 2014). According to Bround (2005) and Zoratti et al. (2014) the MBW complex (MYB-bHLH-WDR) controlled anthocyanin biosynthesis. *MrMYB1* and *MrbHLH1* stimulated the activation of *MrCHI*, *MrF3H*, *MrDFR1*, *MrANS*, *MrUFGT* in bayberry fruits (Liu et al., 2013). Ambawat et al. (2013) reviewed that the function of MYB protein was transcription factors which they changed according to the number of the MYB DNA-binding domain.

Transcription factors in MYB families have been involved in the function of phenylpropanoid metabolism in plant when they had to response to biotic and abiotic stress (Liu et al., 2015). A previous study reported that MYB21 and R2R3 MYB regulated anthocyanin which related to jasmonate (Shan et al., 2009). Moreover, MYBs family and B-Box protein (BBX) family have the function as the light responsive regulatory factors. They were involved in controlling the transcription of apple anthocyanin genes (Ban et al., 2007b; Takos et al., 2006). The BBX has been also known as CO-like (COL) family that contained one or two B-box domains (Khanna et al., 2009). After UV-B treatment, the regulation of *MdCOL11* in *Arabidopsi*s were increased and lead to increase of anthocyanin in apples (Bai et al., 2014) which was induced by light and low temperature. MYBA was shown to control anthocyanin in grape and bilberry fruits (Jaakola et al., 2010; Kobayashi et al., 2002). The expression of MYBA was observed in response to light, tissue type, and maturity signals (Ambawat et al., 2013; Niu et al., 2010). Cutanda-Perez et al. (2009) showed that *VvMYBA* transcription factor controlled the expression of UGFT in berries.

Ripening of fruit also related to TFs of TDR4 and EIL4. TDR4 belongs to MADS-box family which is involved in ripening regulation. It is known to be a likely *FUL* homolog, stimulating expression during ripening of fruit (Eriksson et al., 2004; Hileman et al., 2006). TDR is the

transcription factor which regulates the ripening of tomato (Bemer et al., 2012), and also relates to anthocyanin biosynthesis in bilberry fruits (Jaakola et al., 2010). TDR4 up regulate the anthocyanin biosynthesis genes (*VmCHS*) and relates to the accumulation of anthocyanins in bilberry fruits. Ethylene insentive 3 (EIN3) and other EIN3-like proteins (EILs) are transcription factors that adjust on ethylene regulated (Roman et al., 1995). EIN3 and EILs are involved in the expression of ethylene sensitive genes (Solano et al., 1998). Yokotani et al. (2009) reported that SlEIL1, SlEIL2, SlEIL3 and SlEIL4 regulated ethylene in tomato fruit. However, the expression of the TDR4 and EILs is not characterized in highbush blueberry fruit and so far no molecular information on main changes during ripening and after UV radiation of highbush blueberry fruit involved in accumulation of anthocyanin exists.

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CHAPTER 1

Effect of Pre-Storage UV-A, -B, and -C Radiation on Fruit Quality and Anthocyanin Content of 'Duke' Blueberries during Cold Storage

ABSTRACT

Ultraviolet (UV)-A, -B, and -C were radiated to full-ripe blueberry fruit (cv. 'Duke'), and their effects on fruit qualities and phytonutrients during subsequent cold storage were investigated. The blueberry fruit were exposed to each UV light at 6.0 kJ m^{-2} and then stored at 0 ℃ for 28 days. Weight loss and decay of the fruits after UV treatment were significantly reduced during the cold storage. The total phenolics and antioxidant activities of blueberry fruit after UV-B and -C treatments were higher than those of the control and UV-A treatment. Individual anthocyanins were markedly increased during the 3 h after the UV-B and -C treatments. The correlation matrix between total phenolics, anthocyanins, and antioxidant activity measured by the 2,2′- azinobis(3-ethylbenzthiazoline-6-sulfonic acid) assay indicated a significantly positively correlation with the

individual anthocyanin contents. It was confirmed that the pre-storage treatments of UV-B and -C increased the storability and phytochemical accumulation of the full-ripe ‘Duke’ blueberry fruit during cold storage.

Key words: Anthocyanins, antioxidant, berries, postharvest, quality, ultraviolet lights

INTRODUCTION

Highbush blueberry fruit are rich sources of anthocyanins, which have high radical scavenging activity, and thus are popularly known as healthy foods (Huang et al., 2012; Routray and Orsat, 2011). Anthocyanins are water-soluble pigments and commonly distributed in plants. Their basic structures, aglycones, are called anthocyanidins and can be obtained by acid hydrolysis of anthocyanins (Brouillard, 1983). However, aglycones are rarely found in living tissues (Wu et al., 2006). Although over 600 naturally occurring anthocyanidins have been reported, only 6 of them, cyanidin, malvidin, pelargonidin, delphinidin, peonidin, and petunidin, are generally observed in the flowers, pulp, and skin of berries (Francis and Markakis, 1989; Sarkis et al., 2013). Among these anthocyanins, cyanidin, delphinidin, or malvidin based anthocyanins have been reported to have an effect on the reduction of damage from free radical activity (Heinonen et al., 1998).

Postharvest treatment with ultraviolet (UV) radiation has been investigated in an attempt to inhibit storage rot and maintain the overall quality in blueberry fruit (Perkins-Veazie et al., 2008; Wang et al., 2009), strawberry fruit (Cote et al., 2013), peach fruit (El Ghaouth et al., 2003), tomato fruit (Liu et al., 2009) and mango fruit (González-Aguilar et al., 2001). UV radiation treatment increased the antioxidant contents in

blueberry fruit and tomatoes (Liu et al., 2009; Perkins-Veazie et al., 2008). In addition, UV light has been used on onions to increase health-enhancing phytonutrients (Yoo et al., 2013). These increases are primarily due to the increased phenylalanine ammonia-lyase (PAL) activity and/or rates (Pan et al., 2004).

Low doses of short UV-C radiation between 190 and 280 nm can reduce storage decay of horticultural crops (Terry and Joyce, 2004) by stimulating stress-induced phenylpropanoids. UV-C radiations between 0.25 and 8.0 kJ m^{-2} attack the DNA of microorganisms. Hence, UV-C treatment has been applied as a germicide. As compared with UV-A and -B, UV-C is more effective in sterilizing the surface of products (Bintsis et al., 2000). Radiation of UV-B in the range between 280 and 320 nm affected the plant defense system and led to an increased number of secondary metabolites (Teramura, 1983). The phenolic compound contents in black currants and the skin color of apples were increased or darkened by UV-B exposure (Hagen et al., 2007). Unlike UV-B or -C, UV-A has rarely been tested on harvested blueberry fruit.

The general beneficial effects of UV light in postharvest research have been demonstrated in various horticultural crops, but the persistence of UV-induced effects during subsequent cold storage of blueberry fruit are not available. The purposes of this study were to examine the effects of pre-

storage UV-A, -B, and -C treatments after harvest on overall fruit quality, total antioxidant levels, and total/individual anthocyanin contents of full-ripe, high-bush ‘Duke’ blueberry fruit during subsequent cold storage. To examine the relationships between the aforementioned variables, correlation analysis was also conducted for the UV-treated blueberry fruit.

MATERIALS AND METHODS

Plant materials

Fruits of high-bush blueberries (*Vaccinium corymbosum* L. cv 'Duke') were hand-picked from a farm at Gunpo, Korea, in June 20, 2013 at the full-ripe stage, as indicated by the dark-purple skin color. They are sorted into uniform fruit size and color before UV radiation treatment.

UV radiation

Within 2 h after the grading, blueberry fruit were treated with UV-A, -B, or -C inside the UV radiation device. The device was equipped with nine 40-W UV lamps and three analog timers (Kim et al., 2011). The lamp types of UV-A, -B, and -C were G40TBL (λmax = 352 nm), G40T10E (λmax = 306 nm), and G40T10 (λmax = 254 nm) (Sankyo Denki, Kanagawa, Japan), respectively. The distance of the UV lamp from the fruits was adjusted to obtain the required intensity (10 W m^{-2}) by using a KH-97503 photometer (Cole-Parmer, Vernon Hills, IL, USA). The fruits (about 2 kg) were placed in a mesh bag and radiated for 10 min each on the top and bottom side of the container until the fruits received a radiation dose of 6.0 kJ m^{-2}. For the control, fruits were not treated with any UV lights. We have selected the 6.0 kJ m^{-2} dose from our preliminary study with 1.6, 4.0, and

6.0 kJ m^{-2}, where a dose of 6.0 kJ m^{-2} remarkably reduced fruit decay of full-ripe blueberries without surface damage.

Storage condition

Upon UV treatments, fruits (200 g) were placed in polyethylene containers (15 × 10 × 10 cm) without covers and stored at 0 ℃ with 95% relative humidity for 28 days. Experimental fruits were collected at 3 h after UV treatment (AT) and at 7, 14, 21, and 28 days during cold storage. Blueberries from the initial harvest were used as 0 day samples. Three replicates (200 g per a container as a replicate) were employed for the experimental analysis.

Evaluation of fruit quality

Weight loss (%) was determined by the weight changes of samples between the control and each sampling date. The values were shown as the percent of weight loss/the initial fruit weight.

The decay (%) was calculated by the infected fruit number. In each replicate, the percentage of decay in 200 g of fruits were checked by counting the number of infected fruits in the total of fruit in each replicate after 7, 14, 21, and 28 days.

The fruit firmness (N) was tested using a texture tester (TA.XT2,

Stable Micro Systems, Scarsdale, NY, USA), and a 2 mm flat probe was used. Thirty fruits were randomly selected from each container, and individual fruits were penetrated at a speed of 0.5 mm s^{-1}.

Titratable acidity (TA) and soluble solids content (SSC) were determined using fruit juice that was extracted from a 50 g blueberry sample per each container. TA (%) was analyzed using a Fisher Titrimeter (model 35, Pittsburgh, PA, USA) and calculated as percent citric acid. The juice was titrated with sodium hydroxide (0.1 N) until the pH was 8.2. SSC (°Brix) was tested using a Atago PR-32 hand refractometer. The ratio of SSC/TA was expressed by dividing the SSC by the TA values.

Total phenolic, total anthocyanin contents and antioxidant activity

Blueberry fruit (about 50 g) in bags were blended with 0.5% hydrochloric acid (1:1, w/v) using a home blender. Slurry sample (5 g) was taken and mixed with 30 mL of 80% (v/v) acetone and then centrifuged at 15000g for 30 min at 4 °C. The collected supernatants were kept at −80 °C before experiment.

Total phenolics were measured at 765 nm according to the Folin-Ciocalteu method (Slinkard and Singleton, 1977) and calculated as milligrams of gallic acid equivalents (GAE) per 100 g of fresh weight.

Total anthocyanins were measured using the differences between

their absorbance at 515 nm (A_{515}) and 700 nm (A_{700}) in buffer solution at pH 1.0 and 4.5, where $A = [(A_{515} - A_{700})_{pH\ 1.0} - (A_{515} - A_{700})_{pH\ 4.5}]$ (Prior et al., 1998). The values were shown as milligrams of cyanidin-3-glucoside chloride equivalents per 100 g of fresh weight.

2,2-Di-(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) radical scavenging assay

The scavenging capacity of the DPPH radicals was determined by a spectrophotometric method (Brand-Williams et al., 1995) with slight modifications. Twenty-four milligrams of DPPH was dissolved in 100 mL of 80% ethanol (v/v), and the solution was kept at −20 °C until the day of the experiment. Ten milliliters of DPPH solution was mixed with 45 mL of 80% ethanol (v/v) to obtain 1.1 ± 0.02 value of absorbance at 515 nm. Blueberry fruit were uniformly blended using a home mixer, and fruit slurry was prepared for DPPH and ABTS assays. Five gram samples were homogenized with 25 mL of 80% acetone (v/v), and 150 μL of this homogenate was reacted with the DPPH solution (2.85 mL) for 40 min in total darkness. The absorbance was determined using a spectrophotometer (UV-2401, Shimadzu Co, Kyoto, Japan) at 515 nm. The standard graph was constructed from 0 to 1.0 μmol of Trolox. The data were calculated as the Trolox equivalent antioxidant capacity (TE) per gram of fresh weight.

2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical

scavenging assay

The ABTS assay is based on the deactivation of the antioxidant radical cation $ABTS^{\bullet+}$, which is measured by the decrease in absorbance at 734 nm. The essay was experimented according to the method of (Arnao et al., 2001) with some modifications. A stock of ABTS solution (7.0 mM) was mixed with potassium persulfate (2.45 mM) to make ABTS cation radicals ($ABTS^{\bullet+}$) and was enabled to react for 12 h at 24℃ in total darkness. The ABTS stock solution was diluted with 80% of ethanol (v/v) until the absorbance was 0.7 ± 0.01 at 734 nm. Slurry samples (5 g) were homogenized with 80% acetone (30 mL). Twenty microliters of fruit extract was combined with $ABTS^{\bullet+}$ solution (3 mL) and kept in total darkness. After 15 min, the absorbance was measured at 734 nm. The Trolox standard curve between 0 and 1.0 μmol was constructed to measure the antioxidant activity, and the activities were shown as TE per gram of fresh weight.

Extraction and analysis of individual anthocyanins

Three grams of blueberry fruit was macerated in 20 mL of 80% acetone using a Polytron P-10 tissue homogenizer (Brinkmann, NY, USA). The extracts were filtered and concentrated using a rotary evaporator NN series (EYELA, Tokyo Rikakikii Co. Ltd., Tokyo, Japan) in a water bath at 38 ℃ and then completely dissolved in 3% formic acid in water (v/v). All

samples were passed through a C18 Sepak cartridge (Waters, Milford, MA, USA). The anthocyanins were then recovered with absolute methanol (2 mL) consisting of 3% formic acid. Ten microliters of sample was separated by high-performance liquid chromatography (HPLC) after passing through a 0.42 μm membrane filter. Mixed or individual anthocyanin standards were analyzed for the confirmation of anthocyanins extracted from 'Duke' blueberry fruit. Three milligrams of cyanidin-3-glucoside chloride as an external standard was separately dissolved in 2% hydrogen chrolide in methanol (v/v). The individual anthocyanin content was calculated as milligrams of cyanidin-3-glucoside chloride equivalents per gram of fresh weight. Anthocyanins were analyzed using an YL 9100 HPLC system (Young Lin, Instrument Co. Ltd., Anyang, Korea) consisting of an YL9111 binary pump, an YL9160 diode array detector, and a YL9150 autosampler. An analytical column of Phenomenex Bondclon C18 (4.6 × 250 mm, 10 μm) was used. Mobile solvents were 5% formic acid in water (v/v) and acetonitrile (ACN), respectively. The solvent system was programmed from 5% ACN to 20% ACN for 50 min and flushed with 100% ACN for 5 min. The flow rate was 1 mL/min. The ten microliters of sample was injected. Anthocyanins were detected at 520 nm, and spectral data between 200 and 700 nm were collected at a time.

Identification of individual anthocyanin

Mass identification of anthocyanins was performed on a Surveyor HPLC system (Thermo Finnigan, San Jose, CA, USA). The system was fitted with an LCQ-DECA XP plus quadruple mass spectrometer. An analytical column ODS-N-120 (4.6 × 240 mm, 5 μm) (Innopia Technologies, Inc., Seongnam, Korea) was used. As mobile solutions, water and ACN, which both contained 5% formic acid, were eluted. The gradient started at 8% ACN and reached 40% ACN within 50 min. Positive electrospray ionization was performed by applying 5 kV. The temperature of the capillary was held at 275 ℃. The Xcalibur 2.0 software (Thermo Fisher Scientific Inc., Waltham, MA, USA package) was used to monitor the LC−mass system as well as to process the collected data. Identities were determined by comparison to authentic standards and previous studies (Gavrilova et al., 2011; Wu and Prior, 2005).

Anthocyanin standards

Cyanidin-3-galactoside, cyaniding-3-glucoside chloride, delphinidin-3-glucoside, malvidin-3-glucoside, and petunidin-3-glucoside were obtained from Polyphenols laboratories AS (Sandnes, Norway). Delphinidin-3-arabinoside, petunidin-3-arabinoside, malvidin-3-arabinoside, delphinidin-3-galactoside, and malvidin-3-galactoside were obtained from

Chromadex (Irvine, CA, USA).

Statistical analysis

The experiment was carried out using a completely randomized design. Measurements and analysis were repeated in triplicate. The means were expressed with standard errors. Descriptive statistics and correlation matrix between studied variables of data from UV-C treatment were analyzed using SAS 9.3 software, and statistically significant levels were considered at $P \leq 0.05$, 0.01, and 0.001, respectively.

RESULTS

Quality of blueberry fruit during cold storage

The weight loss (%) was comparable for all experimental treatments throughout the storage period, but the control showed ~1.3% higher rates compared to the UV treatments after 21 days (Fig. 1-1A). A significant reduction in fruit decay was found in the UV treatments, and these differences became greater toward the end of the storage time (Fig. 1-1B). At 28 days, the decay of the control was the highest (7.2%). The decay rate of the UV treatments ranged between 1.3 and 2.1% during storage of 28 days.

UV lights effectively inhibited the softening compared with the control; however, differences were not found in fruit firmness between the UV treatments (Fig. 1-2A). The control started to show softening after 7 days. The UV lights delayed further ripening of the fruits as seen by the SSC/TA ratio (Fig. 1-2B) compared with the control. During the storage period, fruits in the control showed lower acidity and higher sweetness compared with those in the UV treatments. However, there were no differences between UV treatments.

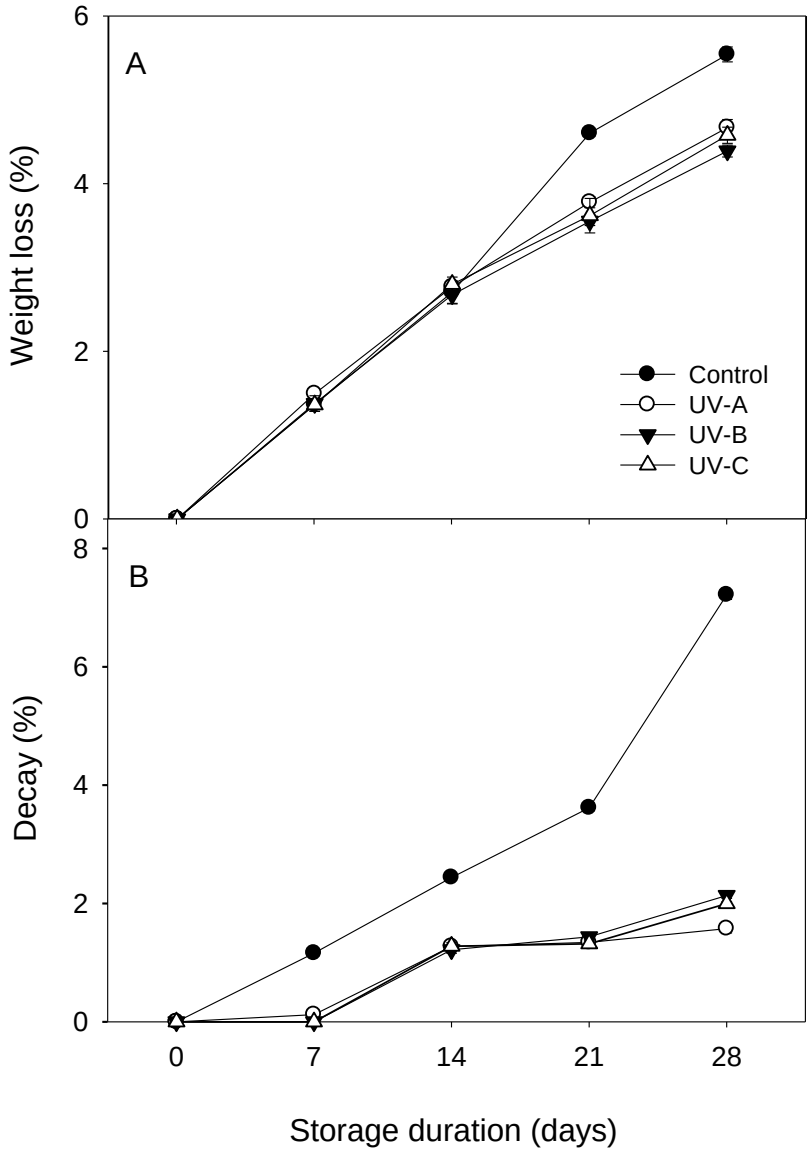


Fig. 1-1. Effects of pre-storage UV-A, B, and C treatment on fruit quality attributes of full-ripe 'Duke' blueberry fruits during subsequent cold storage at 0°C. Weight loss (%) (A); % decay (B). Data are the mean ± SE of three replications.

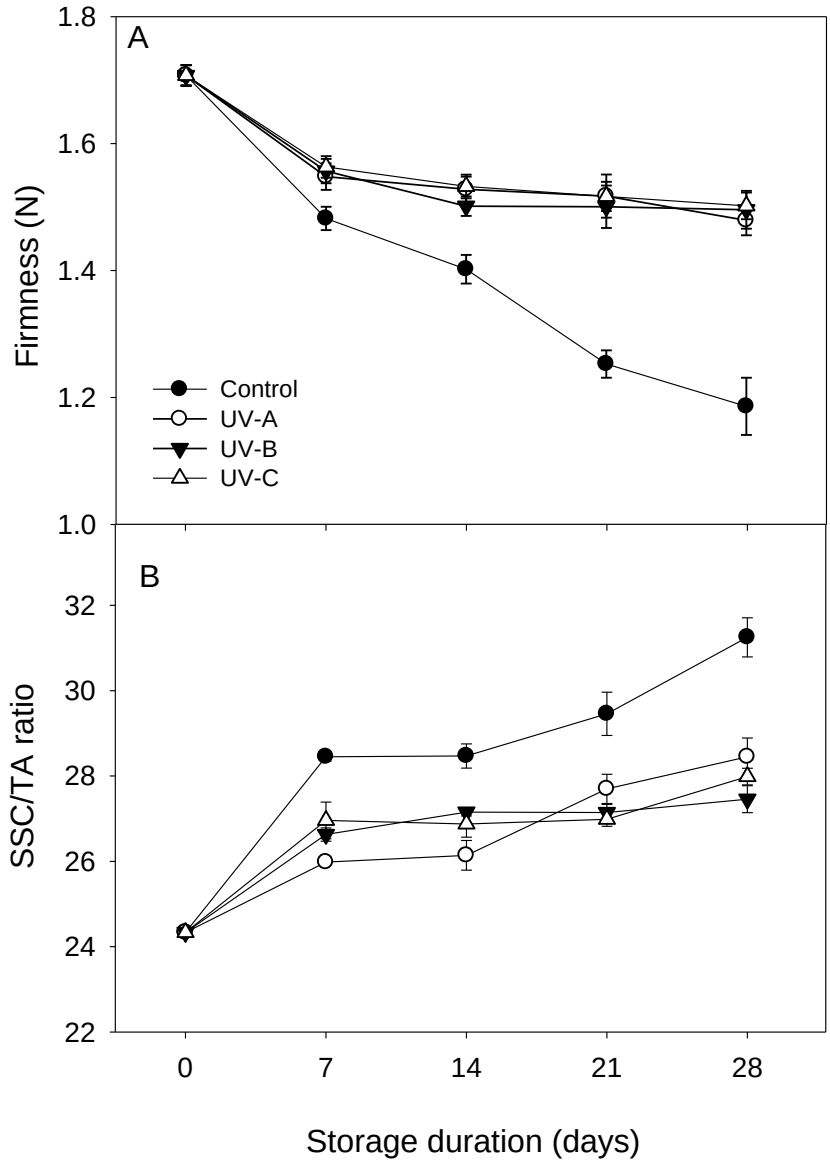


Fig. 1-2. Effects of pre-storage UV-A, B, and C treatment on fruit quality attributes of full-ripe 'Duke' blueberry fruit during subsequent cold storage at 0°C. Firmness (A). SSC (Soluble solids content)/TA (Titratable acidity) ratio (B). Data are the mean ± SE of three replications.

Changes of total phenolic, total anthocyanin and antioxidant activities of blueberry fruits during cold storage.

During the storage period, effects of prestorage UV-A, -B, and -C treatments on total phenolics, total anthocyanin, and antioxidant activities of full-ripe 'Duke' blueberry fruit during subsequent cold storage at 0 ℃ with 95% RH showed in Figs. 1-3 and 1-4.

The total phenolics of blueberry fruit during cold storage were greatly affected by the prior UV treatments. There was a ~17 mg GAE 100 g^{-1} fresh weight increase in the total phenolics due to the UV-B and –C treatments after 3 h, whereas UV-A showed a slight reduction (Fig. 1-3A).

The total anthocyanin changes during storage are shown in Fig. 1-3B. The total anthocyanins remarkably increased by UV-B and -C treatments in 3 h, but sharply decreased at 7 days. After the sharp decrease, the contents of total anthocyanins steadily stabilized. The total anthocyanins in UV-B and -C treatments were still ~88 mg 100 g^{-1} higher than the control and UV-A treatment at 28 days.

The antioxidant activity measured by the DPPH assay in UV-B and -C treated blueberry fruit immediately increased after UV treatments. Contrary to an increased antioxidant activity in the UV-B and -C treatments, there was a slight reduction in the UV-A treatment at 3 h (Fig. 1-4A). The

activities determined by the ABTS assay in the UV-B and -C treatments were also much higher than in both the control and UV-A treatment (Fig. 1-4B). This trend did not change throughout the duration of the cold storage. The control showed the lowest antioxidant levels at 28 days.

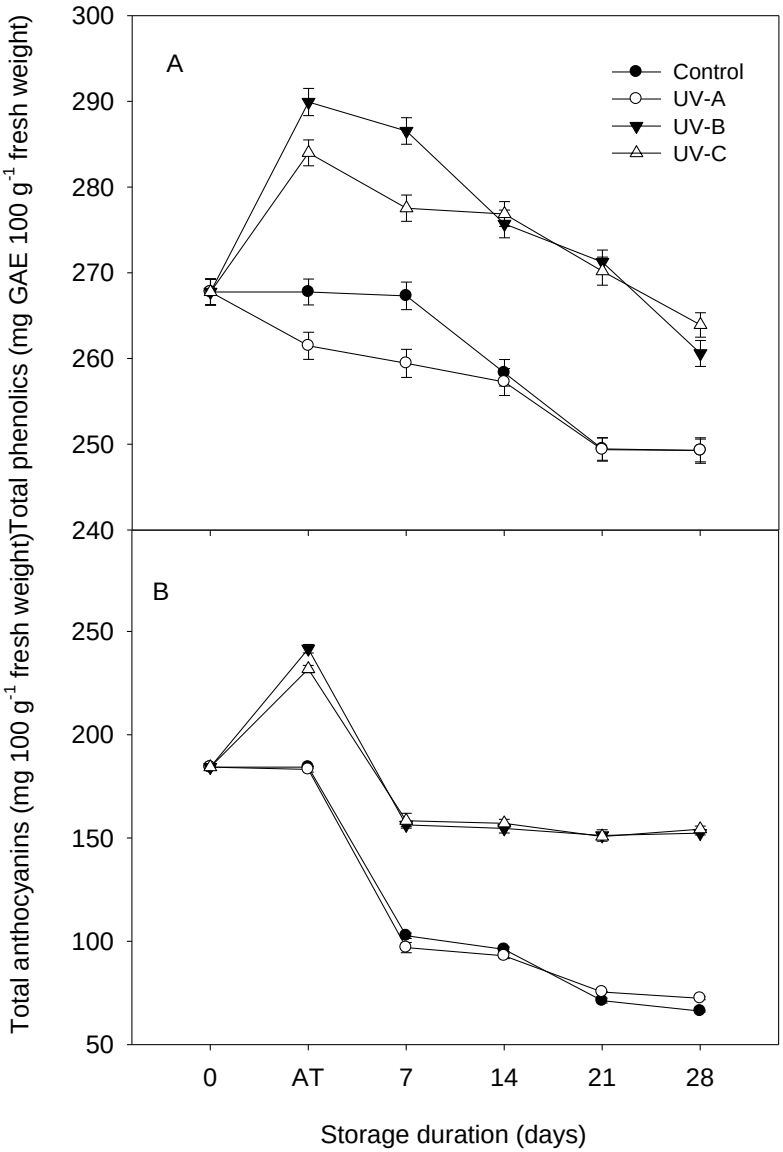


Fig. 1-3. Effects of pre-storage UV-A, B, and C treatment on total phenolics (A) and total anthocyanins (B) of full-ripen 'Duke' blueberry fruit during subsequent cold storage at 0°C. AT, measured at 3 h after UV treatments. Data are the mean ± SE of three replications.

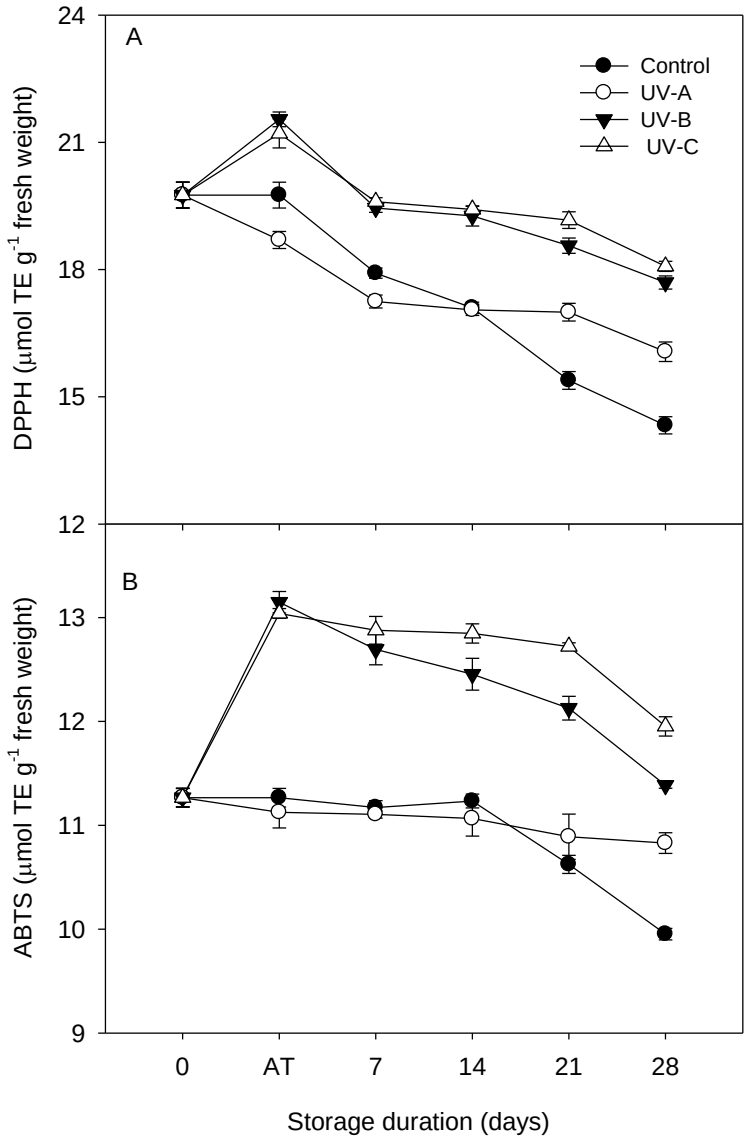
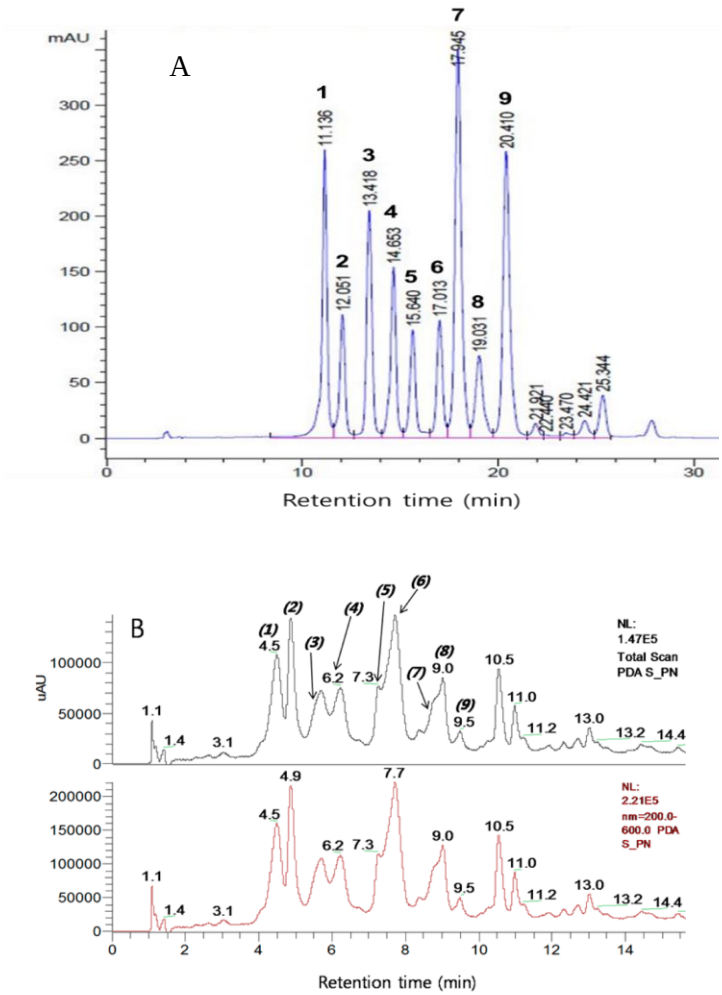


Fig. 1-4. Effects of pre-storage UV-A, B, and C treatment on antioxidant activities according to the DPPH (A) and ABTS (B) assays of full-ripe 'Duke' blueberry fruit during subsequent cold storage at 0°C. AT, measured at 3 h after UV treatments. Data are the mean ± SE of three replications.

Analysis of individual anthocyanins in ‘Duke’ blueberry fruit

The typical HPLC and LC-MS profiles of anthocyanins in blueberry extracts are presented in Fig. 1-5 and the peak number, retention time, and exact mass of each anthocyanin are listed in Table 1-1. This study has obtained similar anthocyanin profiles by HPLC and LC-MS analysis and detected nine major anthocyanins in full ripe ‘Duke’ blueberry fruit. Three major anthocyanins of full-ripe blueberry fruit were malvidin-3-galactoside, delphinidin-3-galactoside, and petunidin-3-galactoside (Figs. 1-6, 1-7, and 1-8).



	Delphinidin	Malvidin	Petunidin
R_1	OH	OCH_3	OCH_3
R_2	OH	OH	OH
R_3	OH	OCH_3	OH
MWs	303	331	317

Fig. 1-5. Typical HPLC (A) and LC-MS (B) profiles of anthocyanin in full-ripen ‘Duke’ blueberry fruit extracts.

Table. 1-1. Peak retention time (RT), identification, and molecular mass of anthocyanins in full-ripe ‘Duke’ blueberry fruit after harvest.

Peak	RT (min)	Identification	M^+	Fragment
1	11.1	Delphinidine-3-galactoside	465	303
2	12	Delphinidine-3-glucoside	465	303
3	13.4	Delphinidine-3-arabinoside	435	303
4	14.6	Petunidine-3-galactoside	479	317
5	15.6	Petunidine-3-glucoside	479	317
6	17	Petunidine-3-arabinoside	449	317
7	17.9	Malvidine-3-galactoside	493	331
8	19	Malvidine-3-glucoside	493	331
9	20.4	Malvidine-3-arabinoside	463	331

Changes in individual anthocyanin contents

Changes in the individual anthocyanins during cold storage are shown in Figs. 1-6, 1-7, and 1-8. All of the individual anthocyanins increased significantly within 3 h when treated with UV-B and UV-C. This increase was especially distinct in the UV-B treatment. These effects diminished as the storage time increased, although there were apparent UV-induced effects in the UV-B and -C treatments. The contents of all individual anthocyanins in the UV-B and -C treatments were higher than those in the control and UV-A during cold storage but not for delphinidin- and malvidin-3-arabinoside at 28 days.

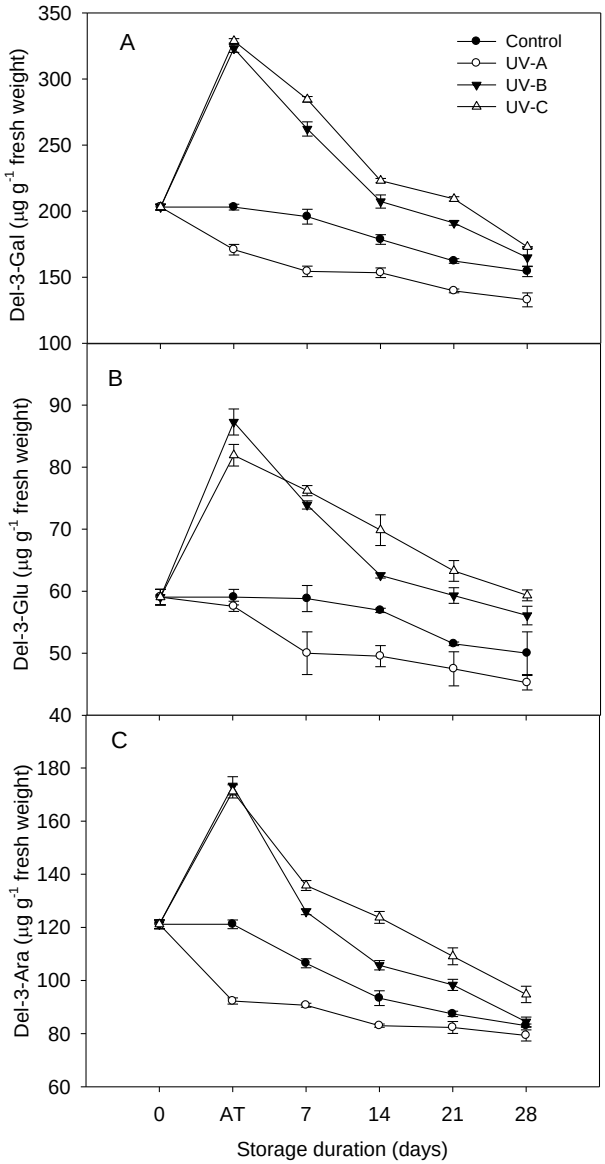


Fig. 1-6. Effect of pre-storage UV-A, B, and C treatments on delphinidin glycoside changes of full-ripe 'Duke' blueberry fruit during cold storage at 0ºC. AT, measured at 3 h after UV treatments. The data are expressed as cyanidin-3-glucoside chloride equivalents. Data are the mean ±SD of three replications.

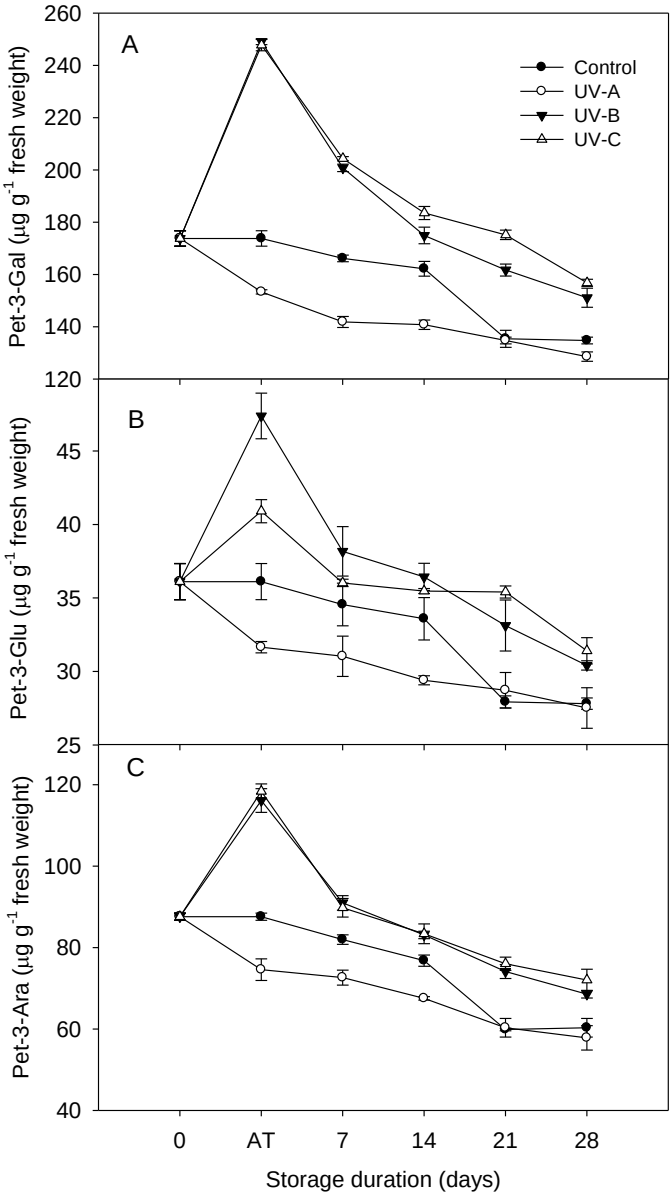


Fig. 1-7. Effect of pre-storage UV-A, B, and C treatments on petunidin glycoside of 'Duke' blueberry fruit during cold storage at 0°C. AT, measured at 3 h after UV treatments. The data are expressed as cyanidin-3-glucoside chloride equivalents. Data are the mean ±SD of three replications.

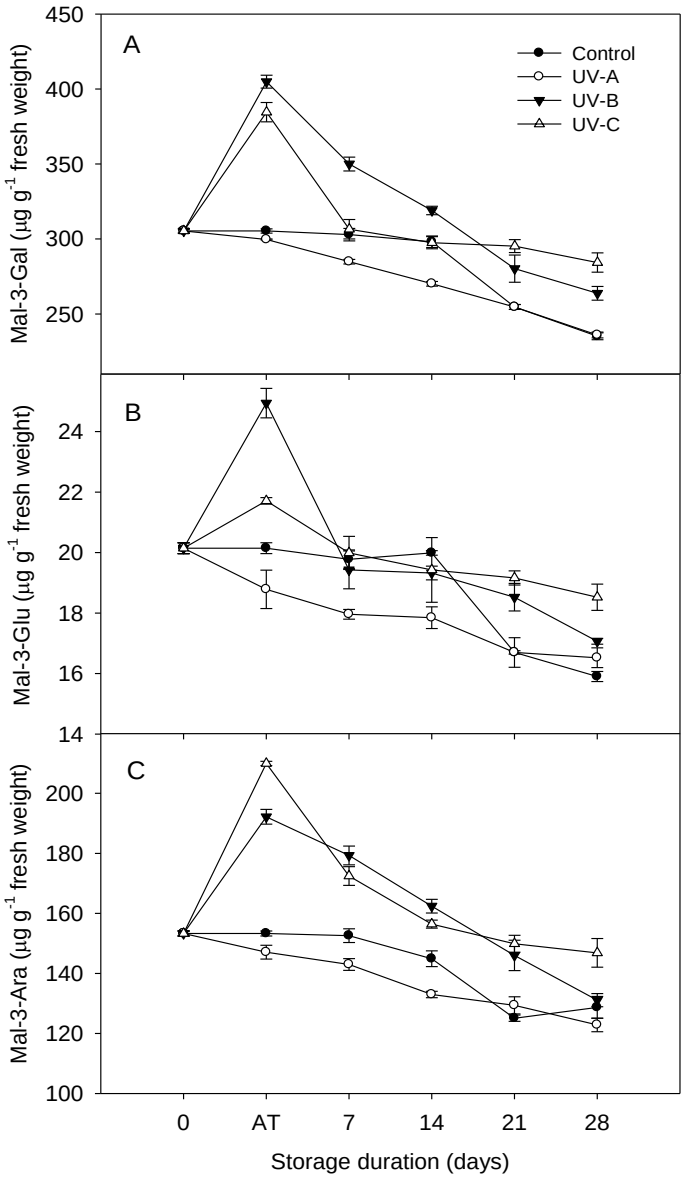


Fig. 1-8. Effect of pre-storage UV-A, B, and C treatments on malvidin glycoside of 'Duke' blueberry fruit during cold storage at 0°C. AT, measured at 3 h after UV treatments. The data are expressed as cyanidin-3-glucoside chloride equivalents. Data are the mean ±SD of three replications.

Table 1.2. Descriptive statistics and correlation matrix between studied variables

	Del-3-Gal	Del-3-Glu	Del-3-Ara	Pet-3-Gal	Pet-3-Glu	Pet-3-Ara	Mal-3-Gal	Mal-3-Glu	Mal-3-Ara	TPs[b]	DPPH	ABTS	TAs[c]
Del-3-Gal	1.00												
Del-3-Glu	0.96**	1.00											
Del-3-Ara	0.96**	0.89*	1.00										
Pet-3-Gal	0.98***	0.93**	0.98***	1.00									
Pet-3-Glu	0.87*	0.77NS	0.95**	0.92**	1.00								
Pet-3-Ara	0.90*	0.81*	0.98***	0.96**	0.93**	1.00							
Mal-3-Gal	0.86*	0.77NS	0.93**	0.94**	0.91*	0.98***	1.00						
Mal-3-Glu	0.87*	0.75NS	0.96**	0.92**	0.96**	0.98***	0.94**	1.00					
Mal-3-Ara	0.95**	0.90*	0.96**	0.98***	0.87*	0.97**	0.96**	0.91*	1.00				
TPs[b]	0.93**	0.97**	0.92**	0.93**	0.85*	0.83*	0.78NS	0.79NS	0.87*	1.00			
DPPH	0.66NS	0.80NS	0.51NS	0.62NS	0.45NS	0.39NS	0.41NS	0.29NS	0.54NS	0.78NS	1.00		
ABTS	0.86*	0.76NS	0.96**	0.91*	0.99***	0.95**	0.91*	0.98***	0.88*	0.84*	0.38NS	1.00	
TAs[c]	0.68NS	0.51NS	0.83*	0.76NS	0.85*	0.91*	0.87*	0.94**	0.79NS	0.56NS	-0.02NS	0.90*	1.00

*** Correlation is significant at the 0.001 level, ** at the 0.01 level, * at the 0.05 level, or is NS non-significant. [a] Abbreviations are presented in Table 1-2; [b] Total phenolic contents, [c] Total anthocyanin contents

Correlations between studied variables

The correlation analysis between studied variables is shown in Table 1-2. Most anthocyanins had a high positive correlation coefficient with each other, except delphinidin-3-glucoside with petunidin-3-glucoside (r = 0.77), malvidin-3-galactoside (r = 0.77), and malvidin- 3-glucoside (r = 0.75). Some pairs had a significant correlation (r = 0.98) at the 0.001 level, such as delphinidin- and petunidin-based anthocyanins, as well as petunidin- and malvidin-based anthocyanins. Total phenolic content also had a significant correlation with most individual anthocyanins, except malvidin-3-galactoside (r = 0.78) and malvidin-3- glucoside (r = 0.79). Although all of the anthocyanins had no significant correlation with the antioxidant activity by the DPPH assay, they had a close correlation in the ABTS assay, except delphinidin-3-glucoside (r = 0.76). The total anthocyanins had a significant correlation with delphinidin-3-galactoside (r = 0.98), petunidin-3-glactoside (r = 0.96), and malvidin-3- galactoside (r = 0.97), which were highly abundant anthocyanins in blueberry fruit. In addition, the antioxidant activity determined by the ABTS analysis had significant correlations with the total phenolics (r = 0.84) and total anthocyanins (r = 0.9) at the 0.05 level, respectively. The antioxidant capacity, total phenolics, and total anthocyanins in blueberry fruit were positively correlated, from 0.87 to 0.99.

DISCUSSION

The benefits of low-dose UV lights on fresh fruits have been found in other studies, particularly with UV-C from 0.25 to 8.0 kJ m^{-2}. Low-dose UV-C promoted the synthesis of phytoalexins (Shama and Alderson, 2005) or delayed the weight loss and softening of fruits during storage (Barka et al., 2000; Erkan et al., 2008). With UV-C treatment between 1.0 and 4.0 kJ m^{-2}, the decay in blueberry fruit decreased by 10% (Perkins-Veazie et al., 2008). However, a high dose of UV-B treatment from 15 to 30.0 kJ m^{-2} induced damage to living organisms, because cellular compounds such as proteins and nucleic acid absorbed the energy-rich radiation (Smirnoff, 1998). In this experiment, all UV lights of 6.0 kJ m^{-2} effectively maintained fruit quality by reducing the weight loss, decay, and ripening of blueberries during subsequent cold storage for 28 days (Fig. 1-1). Therefore, it is concluded that the UV treatments regardless of UV light type were beneficial to maintaining fruit qualities of blueberries during cold storage.

Although the total phenolics gradually declined with storage time, the content in blueberries in the UV-B and -C treatments always remained higher than the control and UV-A treatment. Similarly, the UV-C treatment significantly increased all major individual phenolics as well as the

flavonoid compounds in blueberries, but there were no compositional changes of individual anthocyanins (Wang et al., 2009). These beneficial effects of UV-C were linked to the PAL activation (Pan et al., 2004). In this study, UV-B also showed an effect similar to that of UV-C; however, the total phenolics of blueberry fruit with UV-B and -C decreased quickly with storage time. The data observed that the anthocyanin contents were rapidly increased by the UV-B and -C treatments, then rapidly decreased in 7 days, and a steady decrease thereafter. The initial differences in the increases were maintained during cold storage. The total anthocyanin contents increased with the treatments of 2.0 or 4.0 kJ m^{-2} UV-C in 'Bluecrop' blueberry fruit, particularly immediately after treatment (Wang et al., 2009). UV-B also showed a rapid increase after treatment, and the contents were nearly identical to those for UV-C treatment during storage. These trends were similar to those of the total phenolics and antioxidant

In this chapter, delphinidin-, malvidin-, and petunidin-based anthocyanins were detected as major compounds and those based on cyanidin, pelargonidin, and peonidin were not detected or were in very little amounts. In another study, cyanidin-3-galactoside and malvidin-3-galactoside were the main compounds in the same 'Duke' cultivar (Wang et al., 2009) but the cyanidin-based anthocyanins were not detected or the minor compounds in this study. In highbush 'Bergitta' cultivar, dephinidin-,

malvidin-, and petunidin-based anthocyanins were detected as major compounds and cyanidin-based anthocyanins were minor (Wang et al., 2000). The anthocyanin composition in berries could be different according to cultivar (Prior et al., 1998) species (Määttä-Riihinen et al., 2004; Wu and Prior, 2005) or fruit development stage (Jaakola et al., 2002). Therefore, the difference from or similarity to other studies seemed to be due to wide variation in the composition of individual anthocyanins or their contents among the cultivars and locations of production.

UV-light induced anthocyanin accumulation in plant tissues via the activation of anthocyanin biosynthetic genes (Jaakola et al., 2002; Mol et al., 1996). In addition to PAL induction, UV radiation also increased the activity of other flavonoid synthesis enzymes, such as chalcone synthase/isomerase or dihydroflavonol-4-reductase (Tomas-Barberan and Espin, 2001). In apples, anthocyanin synthesis was regulated by UV-B (Arakawa, 1988). Anthocyanins are one of the most common pigments in plants. However, the effect of UV-A, -B, and -C radiation on anthocyanin biosynthetic genes in harvested blueberry fruit is very little understood. However, this study could assume that the nature of UV-induced anthocyanin accumulation in blueberry fruit would be very similar to that of previous reports in other crops (Jaakola et al., 2002; Tomas-Barberan and Espin, 2001). This data found the most individual anthocyanins in blueberry

fruit were induced by UV-B and -C light and subsequently decreased in the same manner during cold storage. It also showed that doses of UV-B and -C at 6.0 kJ m^{-2} maintained the postharvest quality of blueberry fruit better than the control, as indicated in Figs. 1-1 and 1-2 and greatly enhanced the total phenolics and antioxidant activities by accumulating phytonutrients in the blueberry fruit (Figs. 1-3 and 1-4). These positive effects of UV-light appeared immediately (in 3 h) after the treatments and gradually diminished during the subsequent cold storage time. The degradation of these compounds in full ripe blueberry fruit was possibly slowed by the cold temperature, but could not be stopped.

This study also demonstrated that the total phenolic contents were positively correlated with all individual anthocyanins except malvidin-based anthocyanins. Malvidin-based anthocyanins showed higher antioxidant activities, as measured by the oxygen radical absorbance capacity assay, compared to petunidin- or cyanidin-based anthocyanins (Wang et al., 2012). The results from Table 1-2 showed that all individual anthocyanins except delphinidin-3-glucoside were positively correlated significantly with total phenolics and antioxidant activity in ‘Duke’ blueberry fruit, as measured by the ABTS assay. The correlations between delphinidin-based anthocyanins and ABTS assay were relatively low compared to the other anthocyanin compounds. The antioxidant activity in blueberry fruit according to the

DPPH assay did not show significant correlations with any of the studied variables. It means that the ABTS assay represents the antioxidant activity better than the DPPH assay in individual anthocyanins in blueberry extracts. The antioxidant activities of anthocyanins are influenced by hydroxylation and glycosylation patterns of their chemical structures (Bors et al., 1998; Wang et al., 1997). The OH group on the positions of both 3′ and 4′ of the B ring contributed to the high antioxidant activity except for delphinidin-based anthocyanins. On the basis of these criteria, anthocyanin compounds with 3′,4′-di-OH substitution in the B ring such as cyanidin- or petunidinbased anthocyanins had high antioxidant capacities compared with the malvidin-based anthocyanins, which show only one OH group in the B ring. In this chapter, delphinidin-based anthocyanins showed low correlations with ABTS assay compared to the other anthocyanin compounds, but those based on petunidin were correlated significantly with ABTS assay. There were differences in the response of blueberry fruit to the three types of UV light (A, B, and C). The synthesis of phytonutrient compounds and associated antioxidant activities were stimulated or maintained by a prestorage UV-B or C treatment during subsequent cold storage. UV-A had little ability to improve or maintain fruit quality during cold storage. UV-B and –C at 6.0 kJ m^{-2} were effective in inhibiting the loss of fruit weight, the development of fruit decay, and the decreases of anthocyanins and antioxidant activities.

The UV-B and -C treatments were also effective in the stimulation of individual anthocyanins in ‘Duke’ blueberry fruit, and the persistence of UV induced effects was maintained during the cold storage. These results also showed a strong correlation between these increased individual anthocyanins in blueberry fruit and total phenolics, total anthocyanins, and the antioxidant ability tested by the ABTS assay.

Further studies on the dynamics of UV mediated structural or regulatory gene expression levels involved in anthocyanin biosynthesis in blueberry fruit would elucidate more detailed biosynthesis processes.

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CHAPTER 2

UV-B Radiation Accumulates Anthocyanin in the Peels of 'Duke' Blueberry Fruit by up-Regulation of Anthocyanin Biosynthesis-Related Genes

ABSTRACT

The general beneficial effects of ultraviolet (UV) radiation have been demonstrated in various horticultural crops. UV-B light after harvest can increase the anthocyanin content in blueberry fruit and the freshness of the treated fruits can be retained longer during cold storage. However, little is known about the molecular mechanism underlying UV-induced anthocyanin biosynthesis in harvested blueberry fruit. The aim of this work was to better understand the mechanism responsible for increasing the anthocyanin content in the high-bush blueberry (*Vaccinium corymbosum* 'Duke') after 6.0 kJ m^{-2} of UV-B radiation. The effects of UV-B radiation on the gene expression of anthocyanin biosynthesis genes and the three transcription factors (TFs) *VcBBX*, *VcMYB21,* and *VcR2R3 MYB* in the peel

of blueberry fruit were investigated. The findings showed that UV-B radiation induced over-expression of genes involved in anthocyanin biosynthesis in blueberry fruit compared to a non-treated control. Phenylalanine ammonia lyase, chalcone synthase, and flavanone 3β-hydroxylase are enzymes functioning upstream of anthocyanin biosynthesis were significantly expressed in response to UV-B. Expression levels of *VcBBX*, *VcMYB21*, and *VcR2R3 MYB* were up-regulated by UV-B in the same manner as the anthocyanin biosynthesis genes. The significant increase in the expression of TFs to UV-B occurred immediately after UV-B treatment, was maximized within 3 h, and then sharply declined. In accordance with these changes, the contents of individual anthocyanins in the fruits treated with UV-B significantly increased within 6 h and were 2-3 fold higher than the control. The results indicated that UV-B radiation stimulates an increase in anthocyanin biosynthesis which could be up-regulated by TFs. This study supports the practice of pre-storage UV-B treatment to increase phytochemical accumulation in full-ripe blueberry fruit after harvest.

Key words: Anthocyanin biosynthesis genes, anthocyanin content, BBX, blueberry fruit, MYB21, R2R3MYB, WD40.

INTRODUCTION

Highbush blueberry fruit (*Vaccinium corymbosum*) contain large amounts of anthocyanins, which are blue-colored pigments in the skin of fruits found in a wide assortment of healthy foods. Anthocyanins have a positive effect on human health and a recent study showed that taking individual anthocyanins such as dietary flavonoids can reduce the risk of myocardial infarction in young women (Cassidy et al., 2013). Five major anthocyanins identified as cyanidin, delphinidin, malvidin, petunidin, and peonidin are commonly observed in blueberry fruit (Routray and Orsat, 2011). In the previous study, three of the major anthocyanins malvidin-3-galactoside, delphinidin-3-galactoside, and petunidin-3-galactoside were only found in full-ripe blueberry fruit (Nguyen et al., 2014). The anthocyanin composition in blueberry fruit may be different based on the cultivar, species, location or fruit development stage (Zifkin et al., 2012). Anthocyanin biosynthesis has been found to involve genes in the phenylpropanoid pathway (Routray and Orsat, 2011). Genes which encode anthocyanin biosynthesis enzymes include phenylalanine ammonium lyase (PAL), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT). These

biosynthesis enzymes are controlled by regulatory genes, namely, transcription factors (TFs) (Holton and Cornish, 1995; Zifkin et al., 2012).

Light is one of the most important factors for regulating gene expression (Guo et al., 2008). Ultraviolet (UV)-C light has been considered as a major environmental factor which could be applied to improve fruit quality and antioxidant content in blueberry fruit (Perkins-Veazie et al., 2008), to enhance antioxidant capacity of carrot products (Avena-Bustillos et al., 2012), and to enhance soluble phenolic contents of some other crops (Du et al., 2014). UV-B radiation in the range between 280 nm and 320 nm affected the plant defense system and led to an increase in the number of secondary metabolites (Teramura, 1983). UV-B radiation induced the accumulation of anthocyanins in hypocotyls of radish sprouts (Su et al., 2015) and anthocyanins and flavonoids in apple skins (Ban et al., 2007a).

In research on TFs, low-dosage UV-B radiation accumulated more hydroxycinnamic acid derivatives and anthocyanins by up-regulation of MYB TFs (Ban et al., 2007b; Crifo et al., 2012), COL family, B-box protein (Gangappa and Botto, 2014), and MYB-bHLH-WDR (MBW) complex (Zoratti et al., 2014). MYB TFs play important roles in many secondary metabolic pathways including synthesis of anthocyanins and flavonols in plants. Several studies have shown that MYB family (including MYB1 and R2R3MYB) are involved in the regulation of anthocyanin synthesis in

apples (Takos et al., 2006), berries (Primetta et al., 2015), and grapes (Czemmel et al., 2012). R2R3MYB and MYBA are key players that determine apple peel color (Ban et al., 2007b; Takos et al., 2006). *MrMYB1* and a R2R3 MYB homolog were identified as activators of anthocyanin biosynthesis in Chinese bayberries (Niu et al., 2010). In Arabidopsis, MYB21 and R2R3 MYB involved in the increase of anthocyanin which induced by jasmonate (Shan et al., 2009). Additionally, UV-B induced anthocyanin accumulation related to *MdCOLL11* in the peels of apple fruits was found to be due to light induced signal transduction and photomorphogenesis through elongated hypocotyl 5 (HY5). HY5 regulated *MdCOL11* which further induces the expression of *MdMYBA* (Bai et al., 2014).

However, the expression levels of anthocyanin biosynthesis genes in blueberry fruit after UV-B radiation are still unknown; in particular, the role of TFs is still unclear. Thus, this study hypothesized that application of UV-B radiation on harvested blueberry fruit stimulate the accumulation of anthocyanins at a certain time, and this could occur through the activation of the major genes and related TFs in the anthocyanin biosynthesis pathway. Therefore, this study aims to determine the mechanism mediating the effects of UV-B on expression levels of anthocyanin biosynthesis genes as well as TFs such as *VcB-box zinc finger protein* (*VcBBX*, accession number

KX300037, Table 2.1), *VcMYB21*, and *VcR2R3 MYB* that are present in the peel tissues of blueberry fruit which is picked at full-ripe stage. Moreover, *VcWD40* (TTG) (KX447762.1), a domain of COP1 protein also was investigated. To examine the relationships between these genes, correlation analysis was also conducted. Individual anthocyanins were also analyzed to confirm the accumulation of anthocyanins after UV-B treatment.

MATERIALS AND METHODS

Blueberry fruit and UV-B radiation

High-bush blueberry fruit (*Vaccinium corymbosum* L. cv 'Duke') was hand-picked from a farm in Korea, in June 22, 2015 at the full-ripe stage, as indicated by the dark-purple skin color. Approximately 10 kg of blueberry fruit were harvested and sorted by uniform fruit size and color. Within 1 h after grading, blueberry fruit were treated with UV-B inside the UV radiation device described in (Nguyen et al., 2014). Approximately 2 kg of the fruits were placed in a mesh bag and radiated for 10 min each on the top and bottom side of the container until the fruits received a radiation dose of 6.0 kJ m^{-2}. Control fruits were not treated with UV-B radiation. The dose of 6.0 kJ m^{-2} was selected based on the preliminary study using dosages of 1.6, 4.0, and 6.0 kJ m^{-2}, where a dose of 6.0 kJ m^{-2} remarkably reduced fruit decay of full-ripe blueberry fruit without causing surface damage (Nguyen et al., 2014). After UV-B treatment, blueberry fruit were stored in polyethylene containers (20 cm × 10 cm) and kept at room temperature for 24 h. The peel tissues were removed and collected from the blueberry fruit (about 50 fruits per container) immediately after harvest without UV-B treatment, and immediately, 20 min, 3 h, 6 h, and 24 h after UV-B treatment. Samples were immediately frozen in liquid nitrogen and kept at –80ºC until

analysis.

Isolation of total RNA

Total RNA was extracted from peel tissues (about 3-5 g) according to the protocol described by (Harb et al., 2014; Jaakola et al., 2001) with some modifications. Fruit peels were ground in a mortar under liquid nitrogen and 3 g of the frozen powder samples were added to 15 mL of cationic detergent cetyl-trimethylammonium bromide (CTAB) based extraction buffer which was then preheated at 65ºC and incubated for 10 min at 65ºC. Next, 15 mL of chloroform/isoamyl alcohol (v/v, 24:1) was added and the mixture was vortexed and centrifuged at 11,000 *g* for 10 min a 4ºC. The supernatant was collected and mixed with 6.43 mL of lithium chloride before precipitation overnight at 4ºC. After 12 h, the residues were added to 0.5 mL of pre-warmed (65ºC) sodium chloride, sodium dedocyl sulfate, tris and EDTA (SSTE) buffer after centrifugation at 21,000 *g* for 20 min at 4ºC. The suspended RNA was washed once with 0.5 mL of chloroform/isoamyl alcohol (24:1, v/v) and twice with 0.5 mL of phenol/chloroform/isoamyl alcohol (PCI, 20:20:1, v/v/v). The samples were then centrifuged at 11,000 *g* for 10 min at 4ºC, and washed one more time with 0.7 mL of cold isopropanol. The pellet was collected after centrifugation at 21,000 *g* for 15 min at 4ºC. After washing with 75%

ethanol, the RNA pellet was suspended in diethylpyrocarbonate (DEPC) treated water and stored at –20^{o}C until analysis. The concentration of RNA samples was assessed by the spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, MA, USA) and the integrity of RNA samples was evaluated by electrophoresis using a 2% agarose gel (Fig. 2-1). Following the manufacturer's protocol, the first strand cDNA was synthesized from 2000 ng of treated total RNA using the amfiRivert cDNA Synthesis Platium Master Mix (GenDEPOT, Katy, TX, USA). The cDNA was diluted 20-fold with DEPC-treated water and stored at -20^{o}C until analysis by Real-time PCR.

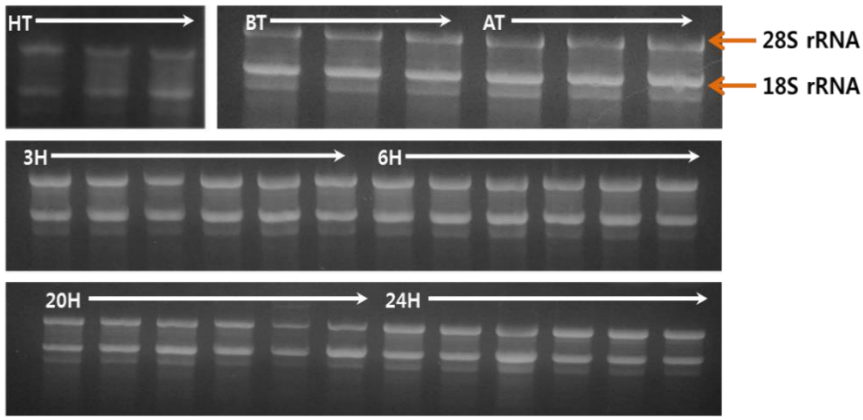

Fig. 2-1. RNA quality by gel electrophoresis of peel samples.
HT (harvest time), BT: before treatment; AT: after treatment; 3H, 6H, 20H and 24H (after 3 h, after 6 h, 20 h and after 24 h, respectively).

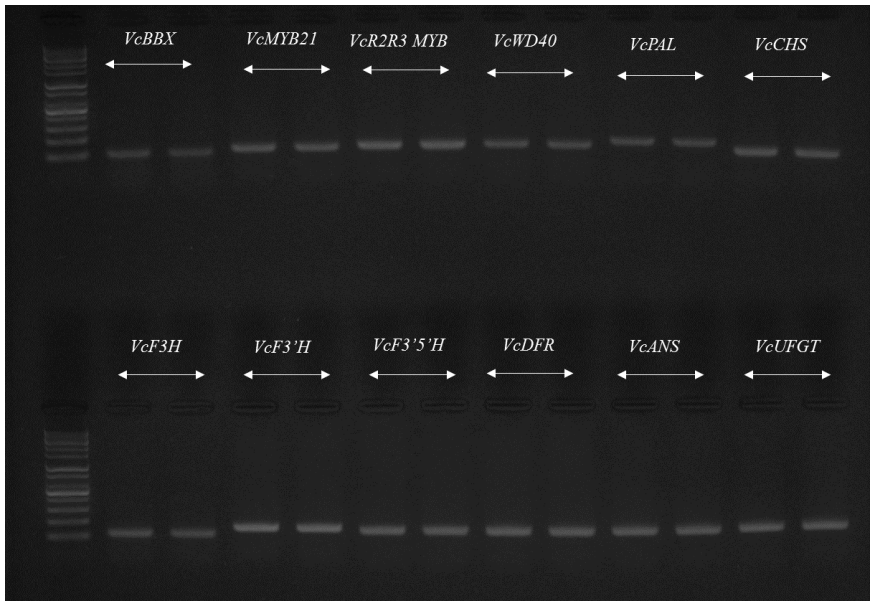

Fig. 2-2. Gel electrophoresis analysis for qRT-PCR products.

Analysis of gene expression

Gene expression was analyzed using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Masek et al. 2005). Six genes involved in the anthocyanin biosynthesis pathway *VcPAL*, *VcCHS*, *VcF3'H*, *VcDFR*, *VcANS*, and *VcUFGT* were selected for amplification from the NCBI database based on references (Cocetta et al., 2015; Harb et al., 2014; Naik et al., 2007; Zifkin et al., 2012). Specific primers for *VcBBX*, *VcMYB21,* and *VcR2R3 MYB* were designed using the NCBI primer blast tool and were based on the sequences reported by (Bai et al., 2014; Jaakola et al., 2010). *VcMYB21* and *VcR2R3 MYB* primers were designed based on the nucleotide sequences for *Vaccinium corymbosum* and *Vaccinium myrtillus,* respectively, in the NCBI database. The *VcBBX* primer was designed from *MdCOL11*, an apple B-box zinc finger protein which is involved in UV-B and temperature induced anthocyanin biosynthesis in apple skin (Bai et al. 2014). Information on all primers used in our study is shown in Supplementary Table 2-1.

PCR products of target genes were amplified by RT-PCR using a Bio-Rad T100TM Thermal Cycler (Bio-Rad, Foster, USA) and cleaned up for sequencing. All confirmation results are presented in Fig. 2-2, Supplementary Fig. 2-1. The qRT-PCR was performed on the CFX Connect^{TM} using Labopass SYBR Green Q Master Mix (2X) by the CFX

Connect real-time system (Bio-Rad, Hercules, USA). A total reaction volume of 10 µL was used. Each reaction contained 2 µL of cDNA, 0.4 µL of forward primer, 0.4 µL of reverse primer, and 5 µL of SYBR Green Q Master (Cosmogenetech, Seoul, Korea). The thermal cycling parameters were 95°C for 30 s; 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s; 95°C for 10 s and a melting curve of 65°C to 95°C, increment of 0.5°C for 10 s. The $2^{-\Delta\Delta Ct}$ method was used to normalize and calibrate transcript values relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously (Tran et al., 2015). *VcGAPDH* was used as an internal control (Harb et al., 2014).

Analysis of individual anthocyanins

High performance liquid chromatography (HPLC) was used to separate and identify individual anthocyanins in blueberry peel tissues according to protocols described by (Nguyen et al., 2014). Three grams of peel tissues were macerated in 50 mL of methanol using a Polytron P-10 tissue homogenizer (Brinkmann, NY, USA). The extracts were filtered and concentrated using a rotary evaporator series (EYELA, Tokyo Rikakikii Co. Ltd., Tokyo, Japan) in a water bath at 38°C and then completely dissolved in 3% formic acid in water (v/v). All samples were passed through a C18 Sepak cartridge (Waters, Milford, MA, USA). The anthocyanins were then

recovered with 2 mL absolute methanol consisting of 3% formic acid (v/v). Ten microliters of sample were separated by HPLC after passing through a 0.42 μm membrane filter.

Mixed or individual anthocyanin standards were analyzed to confirm the anthocyanins extracted from blueberry fruit. Delphinidin-3-galactoside, malvidin-3-arabinoside, and malvidine-3-galactoside were purchased from Chromadex (Irvine, CA, USA). They were dissolved in 10 mL of 3% formic acid in methanol (v/v) and used as an external standard stock solution for generating calibration curves.

Anthocyanins were analyzed using a YL 9100 HPLC system (Young Lin, Instrument Co. Ltd., Anyang, Korea) consisting of a YL9111 binary pump, a YL9160 diode array detector, and a YL9150 autosampler. A Phenomenex Bondclon C18 analytical column (4.6 × 250 mm, 10 μm) was used. Mobile solvents were 5% formic acid in water (v/v) and acetonitrile (ACN). The solvent system was programmed from 5% ACN to 20% ACN for 50 min and flushed with 100% ACN for 5 min. The flow rate was 1 mL min^{-1}. Ten microliters of sample were injected. Spectral data between 200 and 700 nm were collected and anthocyanins were detected at 520 nm.

Statistical analysis

The data were statistically evaluated using SAS 9.3 (TS1M2)

statistical software (SAS Institute Inc., Cary, NC, USA). The experiment was performed in triplicate. The means and standard deviations were calculated using least square means. Means were differentiated using Duncan's multiple range test at $P < 0.05$ (*), $P < 0.01$ (**), and at $P < 0.001$ (***). Correlation analysis between two sets of variables was conducted using SPSS software.

RESULTS

Expression of anthocyanin biosynthesis genes

The qRT-PCR results for the anthocyanin biosynthesis genes *VcPAL*, *VcCHS*, *VcF3'H*, *VcDFR*, *VcANS*, and *VcUFGT* are shown in Fig. 2-3 and Fig. 2-4. This study analyzed gene expression in peel tissues of blueberry fruit at harvest time, and immediately, 20 min, 3 h, 6 h, and 24 h after UV-B treatment. The results showed that gene expression was affected by UV-B treatment. UV-B treated blueberry fruit showed higher gene expression levels compared to those without UV-B. The induction of *VcPAL*, *VcCHS*, and *VcUFGT* expression in harvested blueberry fruit gradually increased according to the experiment time regardless of UV-B treatment, although this phenomenon was more clearly observed in UV-B treated fruits (Figs. 2-3A, 2-3B, and 2-4C).

In the UV-treated fruits, a statistically significant induction of gene expression was observed for all genes within 3 h of the treatment, except for *VcANS* which had a slight increase in transcript level with treatment that was not statistically significant. The significant increase in gene expression for *VcPAL* ($P < 0.01$), *VcCHS* ($P < 0.05$), *VcF3'H* ($P < 0.01$), *VcDFR* ($P < 0.05$), and *VcUFGT* ($P < 0.05$) appeared at 3 h after treatment in the UV-treated fruits (Figs. 2-3 and 2-4). The induction of gene expression in

blueberry fruit was not significantly different between the control and treated samples after 6 h. The UV-B treatment appeared to be effective in stimulating the expression of anthocyanin biosynthesis genes. *VcPAL*, *VcCHS*, and *VcF3'H* which are key enzymes involved upstream of anthocyanin biosynthesis significantly responded to UV-B within a short time of 3 h.

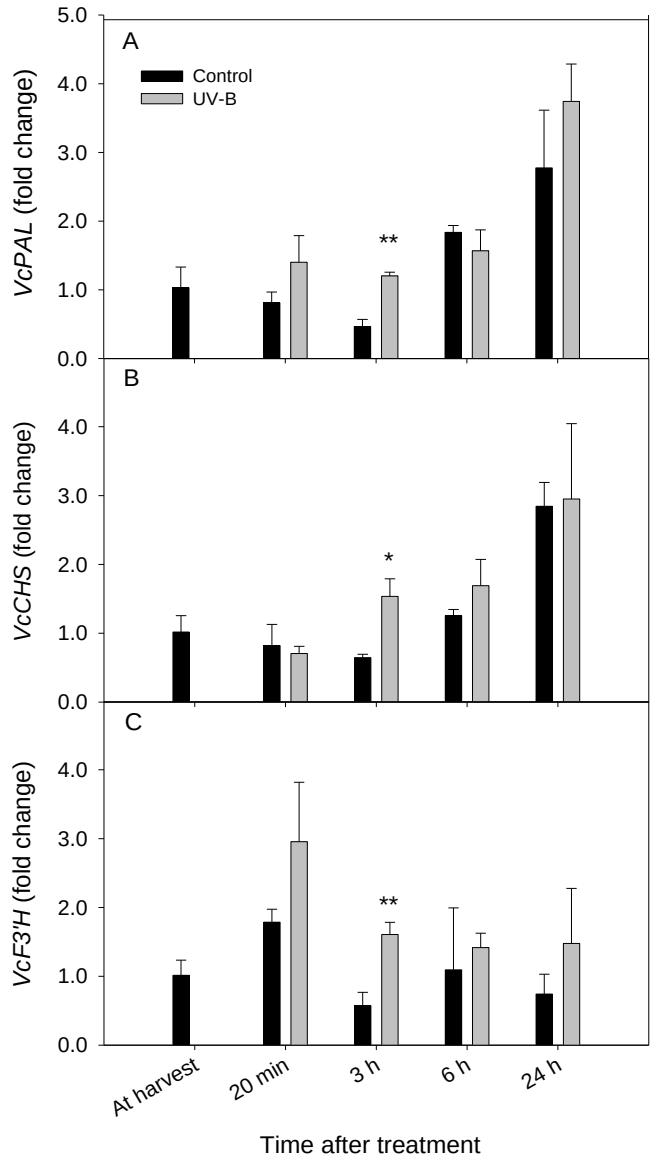

Fig. 2-3. Real-time quantitative PCR analysis of changes in expression of anthocyanin biosynthesis genes (PAL, CHS, and F3'H) in the peels of harvested full-ripe 'Duke' blueberry fruit without (control) or with UV-B treatment at 6.0 kJ m^{-2} for 20 min (UV-B). Data were obtained from samples at harvest, at 20 min, and at 3, 6, and 24 h at room temperature. Data were expressed as the mean ± SD of three replicates. *, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$.

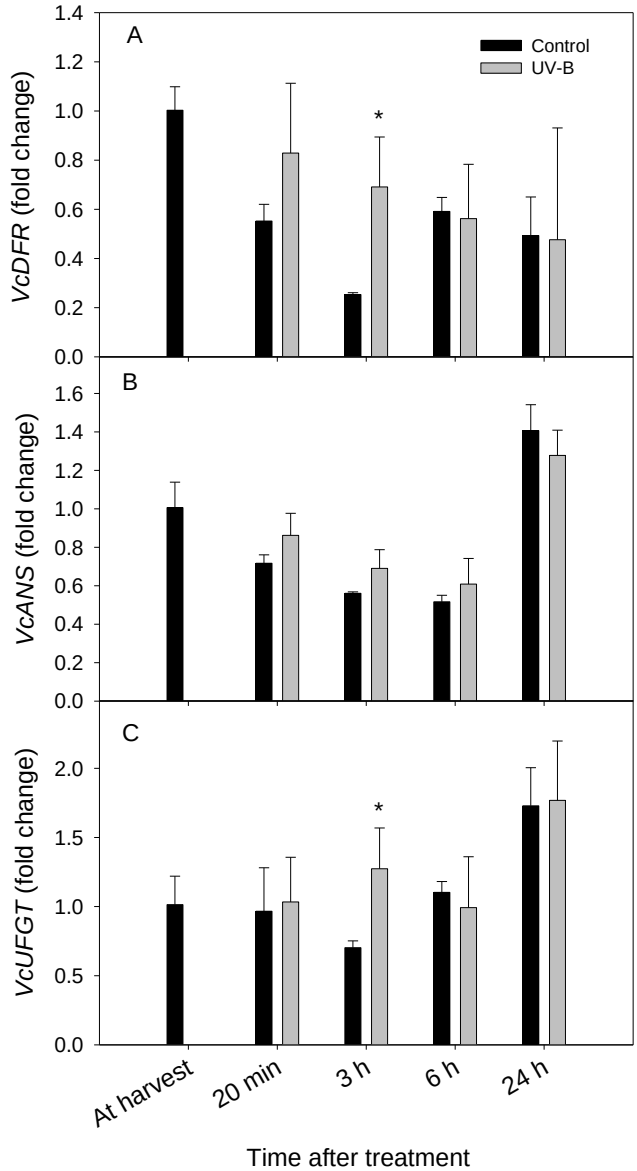

Fig. 2-4. Real-time quantitative PCR analysis of changes in expression of anthocyanin biosynthesis genes (DFR, ANS, and UFGT) in the peels of harvested full-ripe 'Duke' blueberry fruit without (control) or with UV-B treatment at 6.0 kJ m^{-2} for 20 min (UV-B). Data were obtained from samples at harvest, at 20 min, and at 3, 6, and 24 h at room temperature. Data were expressed as the mean ± SD of three replicates. *, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$.

Expressions of transcription factors

Figure 2-5 and Fig. 2-6 showed the expression levels of 4 TFs: *VcBBX*, *VcMYB21*, *VcWD40* and *VcR2R3 MYB*. Both *VcBBX* and *VcMYB21* were significantly up-regulated at 20 min by UV-B and their expression levels reached a 2.66-fold change for *VcBBX* and 3.04-fold change for *VcMYB21* (Fig. 2-5). The significant increase of *VcDW40* and *VcR2R3 MYB* occurred 3 h after UV-B treatment and reached to 2.32 fold and 3.87-fold respectively (Fig. 2-6). After 6 and 24 h, the expression levels of all TFs decreased and stably maintained at around a 1-fold change without a statistically significant difference. The highest level of expression of *VcBBX* and *VcMYB21* were recorded at 20 min after UV-B treatment (Fig. 2-5), whereas the expression of *VcWD40* and *VcR2R3 MYB* increased after 3 h (Fig. 2-6). These findings suggested that *VcBBX* is a major TF for inducing an immediate response to UV-B for the accumulation of anthocyanins in the peel of blueberry fruit. The expression of *VcBBX* after UV-B treatment was clearly distinguished from the control compared to the other TFs analyzed.

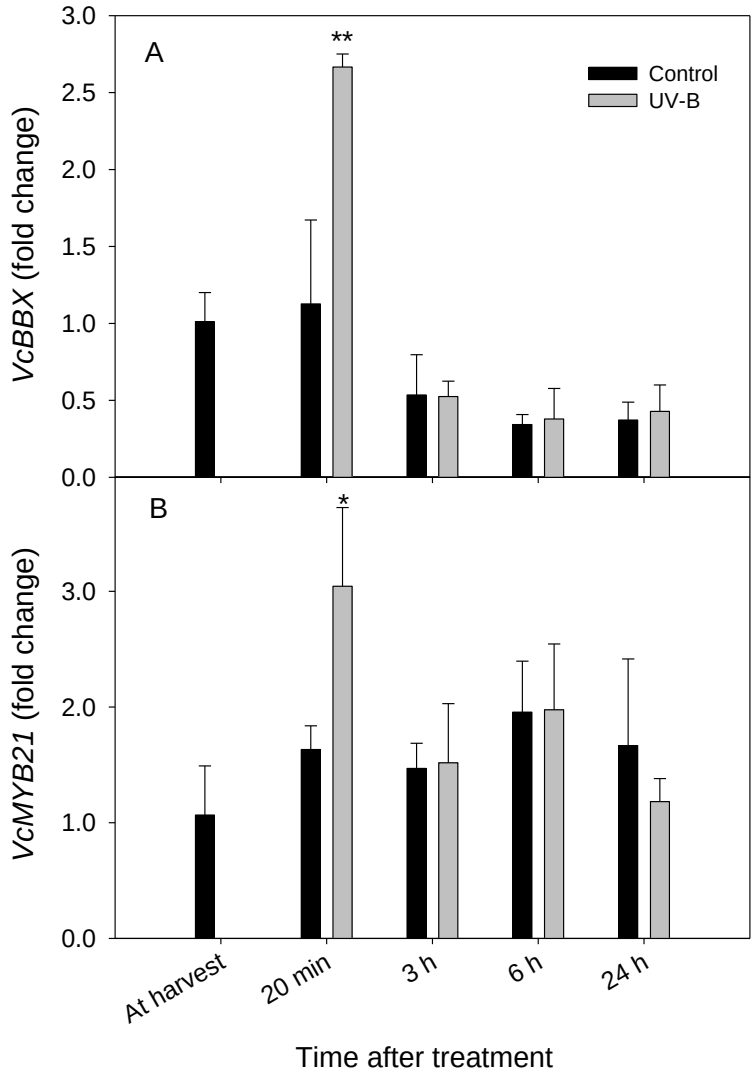

Fig. 2-5. Real-time quantitative PCR analysis of changes in gene expression of transcription factors (*VcBBX* and *VcMYB21*) in the peel of harvested full-ripe 'Duke' blueberry fruit without (control) or with UV-B treatment at 6 kJ m^{-2} for 20 min (UV-B). Data were obtained from samples at harvest, at 20 min, and at 3, 6, and 24 h at room temperature. Data are the mean ± SD of three replicates. *, significant at $P < 0.05$; **, significant at $P < 0.01$. ***, significant at $P < 0.001$.

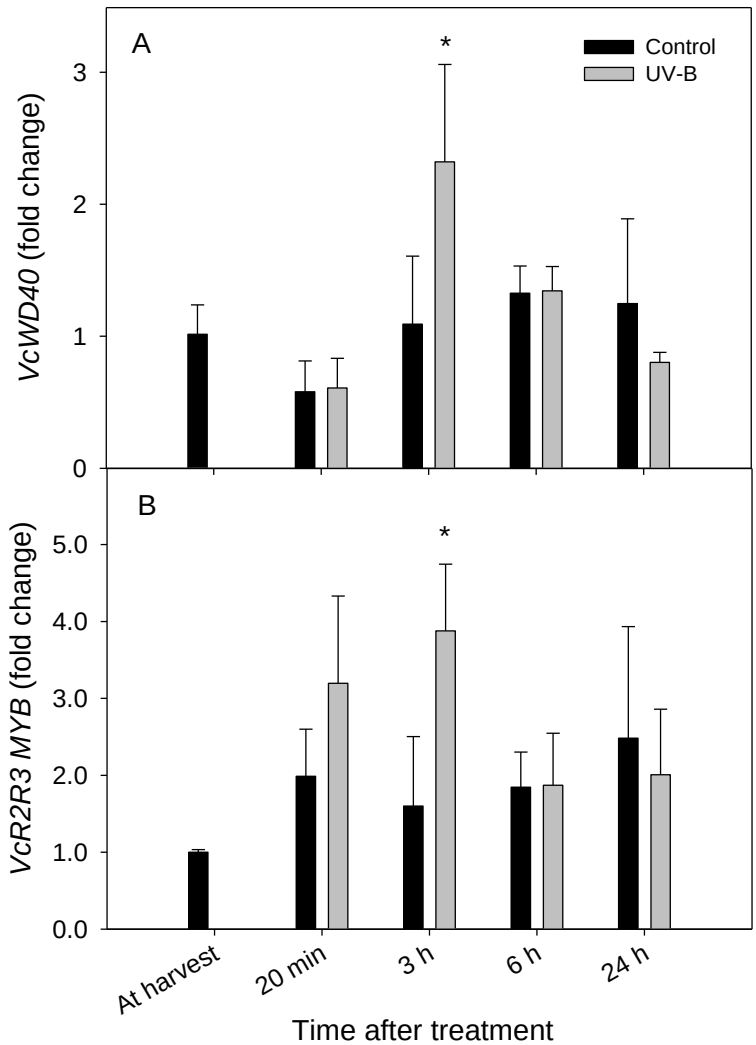

Fig. 2-6. Real-time quantitative PCR analysis of changes in gene expression of transcription factors (*VcWD40* and *VcR2R3 MYB*) in the peel of harvested full-ripe 'Duke' blueberry fruit without (control) or with UV-B treatment at 6.0 kJ m^{-2} for 20 min (UV-B). Data were obtained from samples at harvest, at 20 min, and at 3, 6, and 24 h at room temperature. Data are the mean ± SD of three replicates. *, significant at $P < 0.05$; **, significant at $P < 0.01$. ***, significant at $P < 0.001$.

Correlation analysis

The correlation analysis between all studied genes is shown in Table 2-1. *VcBBX* had a high significant correlation with *VcMYB21* and *VcF3'H* (r = 0.62 and r = 0.66 at $P < 0.001$). *VcMYB21* had a significant correlation with *VcR2R3 MYB* and *VcF3'H* (r = 0.46 and r = 0.50 at $P < 0.05$ and $P < 0.01$, respectively). *VcR2R3 MYB* had not only a high correlation with *VcF3'H* (r = 0.48 at $P < 0.05$) but also had a significant correlation with *VcUFGT* (r = 0.46 at $P < 0.05$). The genes involved in the anthocyanin biosynthesis pathway had positive correlations with each other.

Table 2-1. Descriptive statistics and correlation matrix between studied genes

	VcBBX	*VcMYB21*	*VcR2R3 MYB*	*VcPAL*	*VcCHS*	*VcF3'H*	*VcDFR*	*VcANS*	*VcUFGT*
VcBBX	1								
VcMYB21	0.62^{***}	1							
VcR2R3 MYB	0.35^{ns}	0.46^{*}	1						
VcPAL	0.34^{ns}	0.11^{ns}	0.06^{ns}	1					
VcCHS	-0.07^{ns}	-0.27^{ns}	0.12^{ns}	0.56^{**}	1				
VcF3'H	0.66^{***}	0.50^{**}	0.48^{*}	0.29^{ns}	0.05^{ns}	1			
VcDFR	0.19^{ns}	0.30^{ns}	0.34^{ns}	0.43^{*}	0.43^{*}	0.27^{ns}	1		
VcANS	0.23^{ns}	-0.22^{ns}	-0.04^{ns}	0.59^{***}	0.64^{***}	0.02^{ns}	0.29^{ns}	1	
VcUFGT	0.22^{ns}	0.09^{ns}	0.46^{*}	0.66^{**}	0.65^{**}	0.13^{ns}	0.64^{***}	0.58^{**}	1

*** Correlation is significant at the 0.001 level, ** at the 0.01 level, * at the 0.05 level, or is ^{NS} non-significant.
Red and green colors represent positive and negative correlations, respectively.

Analysis of individual anthocyanin compounds

Changes in the three individual anthocyanins in the peel of blueberry fruit are shown in Fig. 2-7: delphinidin-3-galactoside (Del-3-Gal) (Fig. 2-7A), malvidin-3-galactoside (Mal-3-Gal) (Fig. 2-7B), and malvidin-3-arabinoside (Mal-3-Ara) (Fig. 2-7C). All of the individual anthocyanins increased significantly within 3 h after UV-B treatment. At 3 h, the concentrations of Del-3-Gal and Mal-3-Gal reached 197.38 µg g^{-1} and 228.05 µg g^{-1} fresh weigh in the UV-treated fruits, respectively. After 6 h, the concentrations of the three anthocyanins (Del-3-Gal, Mal-3-Gal, and Mal-3-Ara) reached maximum levels of 248.56 µg g^{-1}, 409.75 µg g^{-1}, and 376.63 µg g^{-1} fresh weights, respectively, in the UV-B treated fruits. The total amount of anthocyanins after UV-B treatment was ~103 mg 100 g^{-1} higher than the control (data not shown).

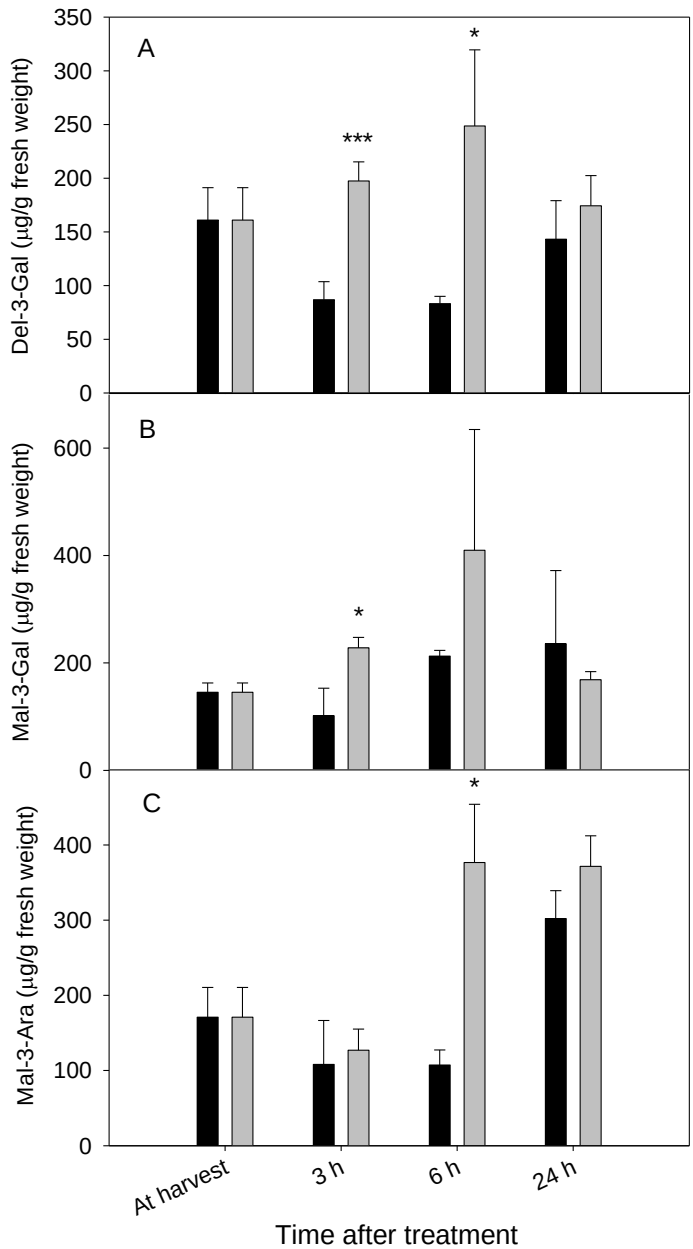

Fig. 2-7. Individual anthocyanin contents in the peels of harvested full-ripe 'Duke' blueberry fruit without (control) or with UV-B treatment (UV-B) at 6.0 kJ m^{-2} for 20 min. Data are the mean ± SD of three replicates. **, significant at $P < 0.01$; ***, significant at $P < 0.001$.

DISCUSSION

Among postharvest technologies, UV radiation is a technique that may be more effective in reducing microbial growth than other methods, especially since it does not leave a residue after treatment (Ribeiro and Alvarenga, 2012). UV radiation at low doses from 0.25 to 8.0 kJ m^{-2} affects the DNA of microorganisms by blocking microorganism development (Terry and Joyce, 2004). UV radiation also induces stress responses and secondary plant metabolites which lead to enhanced antioxidant compounds and improved anthocyanin accumulation (Ribeiro and Alvarenga, 2012). In the previous work (Nguyen et al., 2014), three types of UV-light (A, B, and C) at 6.0 kJ m^{-2} were applied to harvested blueberry fruit in order to investigate the effect of UV on fruit quality and anthocyanins during 4 weeks of cold storage at 0^{o}C. The data found a reduced decay in UV-treated fruits and a remarkable increase in individual anthocyanins after 3 h of UV-B and -C treatment. In this work, UV-B radiation was selected because compare with UV-C radiation, UV-B radiation showed more effectively to enhance anthocyanin accumulation after 3 h. The results confirmed that UV-B light at 6.0 kJ m^{-2} was effective in increasing the levels of anthocyanin compounds (Fig. 2-7) by increasing the expression levels of the anthocyanin biosynthetic genes *VcPAL*, *VcCHS*, *VcF3'H*, *VcDFR*, and *VcUFGT* (Figs. 2-

3, 2-4) and all of the studied TFs of *VcBBX*, *VcMYB21*, *VcWD40* and *VcR2R3 MYB* (Fig. 2.5, 2-6).

There have been several studies investigating the effect of UV radiation on the expression of anthocyanin biosynthesis-related genes in *Arabidopsis*, apple, grape, and bilberry fruits (Bai et al., 2014; Boss et al., 1996; Jaakola, 2013; Liu et al., 2015; Ribeiro and Alvarenga, 2012; Scattino et al., 2014; Zhang et al., 2013; Zoratti et al., 2014a). Many studies have focused on the expression of main genes in the phenylpropanoid biosynthetic pathway and well demonstrated the effect of UV on gene expression. However, the effects of UV-B on the genes involved in the anthocyanin biosynthesis pathway in the peel of harvested blueberry fruit have not yet been characterized, so this work examined this hypothesis in the current study. The increases in expression levels of the early-stage biosynthetic genes CHS and F3'H induced the expression of the late-stage biosynthetic genes DFR, ANS, and UFGT, which is similar to some reports (Martin et al., 1991; Tanaka et al., 2008). In this study, up-regulation of *VcF3'H*, *VcDFR*, and *VcUFGT* was detected after 3 h by UV-B treatment (Fig. 2-3). Similar results were found in the up-regulation of *VcPAL*, *VcCHS*, and *VcDFR* in skin tissues of peach fruits (Scattino et al., 2014) and apple fruits (Ban et al., 2007a; Ubi et al., 2006; Zoratti et al., 2014a), as well

as the over expression of UFGT in Asian pear (*Pyrus pyrifolia* ('Nakai') (Qian et al., 2014; Zhang et al., 2013) after UV-B treatment.

In addition, the expression of *VcPAL* was significantly positively correlated with *VcCHS*, *VcDFR*, *VcANS*, and *VcUFGT* (Table 2-1). This result indicated that although *VcPAL* was not involved in the early-biosynthetic gene pathway, it may affect the expression of *VcCHS*, *VcDFR*, *VcANS*, and *VcUFGT*. It has been shown that stress responses can affect the plant defense system and lead to an increase in secondary metabolites (Cantarello et al., 2005; Teramura, 1983). CHS plays an important role in providing a common chalcone precursor for the production of all intermediate and final products in the flavonoid biosynthesis pathway (Czemmel et al., 2012). In this work, the expression level of *VcCHS* had positive correlations with *VcDFR*, *VcANS*, and *VcUFGT* (Table. 2-1) which clearly confirmed that the expression of the early-biosynthetic genes of CHS and F3H related to the expression of the late-biosynthetic genes.

The MYB and BBX families have been identified as light responsive regulatory factors and are involved in controlling the transcription of anthocyanin genes in apples (Ban et al., 2007b; Takos et al., 2006). The BBX family is a class of zinc finger TFs that contain one or two B-box domains with specialized tertiary structures that are stabilized though the binding of Zn ions (Khanna et al., 2009). BBX has also been known as a

CO-like (COL) family protein. After UV-B treatment, the over-expression of *MdCOL11* in *Arabidopsi*s was observed (Bai et al., 2014). Similar to the increase in *MdCOL11* in apple skin under UV-B light (Bai et al., 2014), the data found an over-expression of *VcBBX* in the peel of blueberry fruit for 20 min after treatment with UV-B radiation (Fig. 2-5). It was proposed a hypothetical working mechanism that *MdHY5* directly binds to the *MdMYBA* promoter to induce its expression. *MdHY5* also regulates the expression of *MdCOL11* which induces the expression of *MdMYBA* (Bai et al., 2014). MYBA and R2R3 MYB are known TFs that are related to fruit flavonoid synthesis. MYBA has been shown to control anthocyanin biosynthesis in grape and bilberry fruits (Jaakola et al., 2010; Kobayashi et al., 2002). The expression of MYBA responded to light, tissue type, and maturity of fruits (Ambawat et al., 2013; Niu et al., 2010).

The role of the MYB TFs, WD40 and BBX families in harvested blueberry fruit after UV-B light treatment has not been well characterized. In this study, analysis of *VcMYB21* was designed based on nucleotide sequences of MYBA in studies on *Vaccinium myrtillus* (Jaakola et al., 2010) and *VcR2R3 MYB* analysis was based on nucleotide sequences of *Vaccinium corymbosum* (Zifkin et al., 2012) (Table 2-1). *VcMYB21* and *VcR2R3 MYB* had a high significant correlation with each other (Table 2-1) which suggested that they have a close relationship. The R2R3 MYB protein has a

long C-terminal sequence (Kui et al., 2010) and it specifically controls the anthocyanin biosynthetic pathway genes as well as anthocyanin conjugation and transport into the vacuole (Tohge et al., 2005). R2R3 MYB is also a key player that determines the effect of anthocyanins on the color of apple fruits (Ban et al., 2007b; Takos et al., 2006). During the ripening stage in bilberry fruits, *VmR2R3 MYB* was involved in the process of anthocyanin accumulation (Jaakola et al., 2010). In this work, the over-expression of *VcR2R3 MYB* was induced at 3 h after the over-expression of *VcBBX* and *VcMYB21* that occurred immediately after UV-B treatment (Figs. 2-5 and 2-6). Moreover, the close correlation between TFs and *VcF3'H*, especially between *VcR2R3 MYB* and *VcF3'H* or *VcUFGT* (Table 2-1), confirmed that the over-expression of these TFs activated the expression of *VcUFGT*, a key enzyme in the last consecutive step in the anthocyanin biosynthesis pathway. On the other hand, UV-B radiation induced stress in the peel of blueberry fruit immediately, which activated an increase in the late-biosynthetic genes leading to anthocyanin accumulation after 3 h.

Anthocyanins are one of the most common pigments in plants. UV-B induces anthocyanin accumulation in plant tissues via the activation of anthocyanin biosynthetic genes (Mol et al., 1996). Anthocyanins are extremely unstable and rapidly converted either to anthocyanins or epicatechin in a competitive manner by the action of UFGT and ANS (Boss

et al., 1996; Poudel et al., 2008). In this work, levels of Del-3-Gal, Mal-3-Gal, and Mal-3-Ara were quickly increased by UV-B treatment in peels of harvested blueberry fruit which confirmed the previous results that UV-B and -C at 6.0 kJ m^{-2} induced an increase in nine individual anthocyanins of blueberry fruit after 3 h (Nguyen et al., 2014). This anthocyanin accumulation might be induced by significant increases in the expression levels of the phenylpropanoid pathway related genes (Figs. 2-3 and 2-4), and by UV-B acting on stresses that induce the plant defense system and lead to an increased number of secondary metabolites (Cantarello et al., 2005; Teramura, 1983). A recent report on the red Chinese sand pear found that anthocyanins did not accumulate in the dark (Qian et al., 2014). Under light, individual anthocyanins accumulated in bilberry fruits (Zoratti et al., 2014b). Anthocyanins accumulated in both the pulp and peel of bilberry fruits while the accumulation occurred in the peel of blueberry fruit (Riihinen et al., 2008).

In conclusion, anthocyanins accumulated in the peels of harvested 'Duke' blueberry fruit due to treatment with UV-B at 6.0 kJ m^{-2}. UV-B induced increased expression of the TFs *VcBBX*, *VcMYB21*, and *VcR2R3 MYB*, and the anthocyanin biosynthesis genes *VcPA*L, *VcCHS*, *VcF3'H*, *VcDFR*, and *VcUFGT*, thereby promoting the accumulation of individual anthocyanins that appeared quickly after UV-radiation. Nucleotide

sequences of *VcBBX* were determined using an online alignment search (BLAST) program to obtain the accession number KX300037, and the study confirmed that this TF was regulated by UV-B light. Similar to the anthocyanin accumulation in the skins of apple fruits by UV-light (Bai et al., 2014), anthocyanin accumulation in the peel of blueberry fruit might also be due to the activation of MYB21 and R2R3 MYB. The findings could aid in revealing the molecular mechanism of anthocyanin biosynthesis by UV-light and contribute to the development of postharvest technologies allowing healthier fruit consumption.

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CHAPTER 3

UV-B Radiation Accelerates the Transcript Levels of *VcTDR4* and Decreases Ethylene Production in Highbush Blueberry Fruit

ABSTRACT

In the previous study, the increasing of transcript levels of anthocyanin related genes by UV-B radiation induces anthocyanin accumulation in 'Duke' blueberry fruit. *VcTDR4* transcription factor is belongs to MADS-box protein, an important factor in regulation of anthocyanin biosynthesis in bilberries. However, the change of *VcTDR4* transcription factor is still unclear during natural ripening, especially after UV-B radiation treatment in highbush blueberry fruit. The purpose of this study was to examine the changes of *VcTDR4* in 'Nelson' highbush blueberry fruit during natural ripening as well as by 6.0 kJ m^{-2} of UV-B radiation. Moreover, *VcTDR4* is the transcription factor which is regulated the ripening of fruits. So, the ethylene production and some ethylene regulated genes was also analyzed in samples during natural ripen stage and

after UV-B radiation treatment. The study showed that the transcript levels of *VcTDR4* not only significantly reduced during ripening stages, but also negative accelerated by UV-B radiation at full-ripe 'Nelson' blueberry fruit. Moreover, the expression levels of anthocyanin biosynthesis genes, *VcMYB21*, *R2R3 MYB* as well as ethylene related genes increased during ripening stages. The ethylene production reached the peak at stage 3 before reducing at stage 5. After 3 h by UV-B radiation treatment, ethylene production significantly decreased while individual anthocyanin contents significantly increased in full ripe 'Nelson' blueberry fruit. This study suggests that UV-B radiation might improve anthocyanin by reducing of ethylene and negative accelerating of *VcTDR4* transcription factor.

Key words: Anthocyanin regulated genes, ethylene production, ethylene related genes, ripening stages, UV-B radiation, *VcTDR4*.

INTRODUCTION

Highbush blueberry fruit (*Vaccinium corymbosum*) are an excellent source of health promoting bioactive components because they contain a high content of anthocyanin. Anthocyanin has a positive effect on human health and a recent review showed that anthocyanin-rich extracts from blueberry fruit decreased weight gain and inflammatory response (Lila et al., 2016). The major anthocyanin found in blueberry fruit are anthocyanidins such as cyanidin, delphinidin, malvidin, petunidin and peonidine with their derivatives (Routray and Orsat, 2011). The content of anthocyanin reached from 25 mg 100 g^{-1} of fresh weight to 495 mg 100 g^{-1} of fresh weight in blueberry fruit (Michalska and Łysiak, 2015). However, the anthocyanin content in highbush blueberry fruit depends on the cultivars (Prior et al., 1998). Riihinen et al. (2008) reported that anthocyanin was accumulated on the peel tissues of blueberry fruit (Riihinen et al., 2008). The anthocyanin biosynthesis had been happened in the cell and it was involved by structural genes in the phenylpropanoid pathway (Routray and Orsat, 2011).

Light plays an important role in control of plant up-growth and gene expression (Guo et al., 2008; Zoratti et al., 2014a). The regulation of gene expression depends on the quality and quantity of light (Cominelli et al., 2008). The artificial ultraviolet radiation induced stress responses and

secondary metabolite (Ribeiro and Alvarenga, 2012). Perkins-Veazie et al. (2008) announced that application of UV-C radiation (2.0-4.0 kJ m^{-2}) can improve fruit quality and antioxidant activity in blueberry fruit. UV-B radiation affect the plant's secondary metabolism which is result of accumulation of anthocyanin (Schreiner et al., 2012). UV-B radiation enhanced anthocyanin in hypocotyls of radish sprouts (Su et al., 2015) and flavonoids in apple skins (Ban et al., 2007a). In the previous research on red cabbage seedling, red light inhibited ethylene production which lead to the increase of anthocyanin content (Kang and Burg, 1973).

Ethylene is consider as a key hormone which is adjusting the ripening of fruits and the main different of ethylene in non-climacteric and climacteric fruits depends on the presence of autocatalytic ethylene production (Bapat et al., 2010). Blueberry fruit are one kind of fruit which produce ethylene very low. Ethylene production reached from 0.5 to 2 μL kg^{-1} h^{-1} in highbush blueberry fruit (Suzuki et al., 1997), reached 10 μL kg^{-1} h^{-1} in rabbiteye blueberry fruit (El-Agamy et al., 1982). So, there are still having argument that blueberry belongs to non-climacteric fruits (Zifkin et al., 2012) or climacteric fruits (El-Agamy et al., 1982). The ACC oxidase (ACO) plays a vital role in adjusting ethylene synthesis which induces the differences on the autocatalytic ethylene production during ripening (Handa et al., 2011). Moreover, ethylene is recognized by encoded ETR receptors.

ETRs family are identified by ETR1, ETR2, ERS1, ERS2, and EIN4 genes were in Arabidopsis (Moussatche and Klee, 2004). A previous study on the pigmentation of Arabidopsis showed that the accumulation of anthocyanin is related to the increase of light and the decrease of ethylene (Jeong et al., 2010).

TDR4 is belongs to MADS-box family which these genes of MADS-box family was reported that all their functions are as positive regulators of ripening (Bemer et al., 2012). TDR4 is a likely *FUL* homolog, stimulated expression levels during ripening of tomato fruits (Eriksson et al., 2004; Hileman et al., 2006). *VmTDR4* transcription factor also related to anthocyanin biosynthesis in bilberries which was up regulated the anthocyanin biosynthesis genes (*VmCHS*) (Jaakola et al., 2010). Moreover, TDR4 is involved in the activation of anthocyanin biosynthesis genes during developmental plant process and under the light (Zoratti et al., 2014a). However, this transcription factor was not characterized in highbush blueberry fruit during natural ripen as well as by UV-B light.

Thus, this study hypothesized that UV-B radiation induced the reduction about transcript levels of *VcTDR4* and ethylene production which maybe indirectly related to accumulation of anthocyanin in highbush blueberry fruit. In order to justify the hypothesis, this study aimed to investigate the changes of ethylene production as well as the transcript

levels of genes related to anthocyanin and ethylene at natural ripening process in ‘Nelson’ blueberry fruit in order to supplement for the hypothesis.

MATERIALS AND METHODS

Fruit materials and UV-B treatment

High-bush blueberry fruit (*Vaccinium corymbosum* L. 'Nelson') were hand-picked from a farm in Korea, in July 22, 2016.

For the first experiment about ripening stages, 5 different developmental stages were sorted (Fig. 3-1). Stage 1 (ST1) and stage 2 (ST2) were sorted by increasing size (ST1 around 8 mm in diameter, ST2 around 11 mm). Stage 3 (ST3), stage 4 (ST4) and stage 5 (ST5) were sorted by fruit color (ST3, 25%-50% red skin; ST4, predominantly purple skin with some red or blue; ST5, entirely dark blue and soft texture).

For the second experiment with UV-B and ethylene treatments, fruits were harvested at the full-ripe stage (ST5), as indicated by the dark-purple skin color. Approximately 10 kg of blueberry fruits were harvested and sorted by uniform fruit size and color. Within 1 h after grading, the blueberry fruits were treated with UV-B inside the UV radiation device described in (Nguyen et al., 2014) or treatment by ethylene (1000 ppm). Approximately 2 kg of the fruits were placed in a mesh bag and radiated for 10 min each on the top and bottom side of the container until the fruits received a radiation dose of 6.0 kJ m^{-2}. Control fruits were not treated with

UV-B light. This study selected the 6.0 kJ m^{-2} dose based on the preliminary study using dosages of 1.6, 4.0, and 6.0 kJ m^{-2}, where a dose of 6.0 kJ m^{-2} remarkably reduced fruit decay of full-ripe blueberry fruit without causing surface damage (Nguyen et al., 2014). After UV-B treatment, blueberry fruit were stored in polyethylene containers (20 cm × 10 cm) and kept at room temperature for 24 h. The peel tissues, flesh tissues and whole fruits were separated and collected from the blueberry fruit (about 50 fruits per container) immediately after harvest without UV-B treatment, and immediately, 20 min, 3 h, 6 h, and 24 h after UV-B treatment. Samples were immediately frozen in liquid nitrogen and kept at –80^{o}C until analysis.

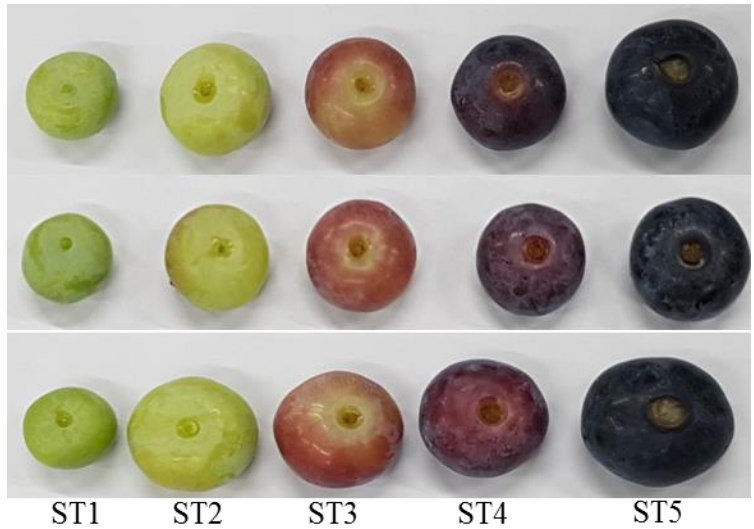

Fig. 3-1. Ripening stages of ‘Nelson’ blueberry fruit. Stage 1 (ST1), Stage 2 (ST2), Stage 3 (ST3), Stage 4 (ST4), and Stage 5 (ST5).

Isolation of total RNA and cDNA synthesis

The Ribospin ™ Seed/Fruit RNA mini kit of GENEALL BIOTECHNOLOGY COMPANY (Made in Korea) was used. Place up 100 mg of ground blueberry samples into 1.5 ml microcentrifuge tube. Then add 500 µL buffer SL, 500 µl buffer ML and 10 µl of ß-mercaptoethanol to the samples. After vortex and incubate at room temperature, samples were centrifuged at 13 000 rpm for 1 min then moved to the filter column in order to centrifuge 1 min more at 13 000 rpm. Transfer 500 µL of samples to a new tube and add 250 µL of absolute ethanol and mix well. The mixture was transferred into a mini spin column and centrifuge at 13 000 rpm for 1 min. After adding 500 µL of buffer RBW for washing by centrifuge at 13 000 rpm for 30 sec, apply 70 µL of DNaseI and incubate for 10 min. 500 µL of buffer RBW one more time and centrifuge at 13 000 rpm for 30 sec. Before drying the samples, add 500 µL of buffer RNW into the column and centrifuge at 13 000 rpm for 30 sec. Finally add 50 µL of nuclease-free water to the center of the membrane in the column and centrifuge at 13 000 rpm for 1 min. Collected RNA samples were stored at –80^{o}C until analysis. The concentration of RNA samples was assessed by the spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, MA, USA) and the integrity of RNA samples was evaluated by electrophoresis using a 2% agarose gel (Fig. 3-2). Following the manufacturer's protocol, the first

strand cDNA was synthesized from 2000 ng of treated total RNA using the amfiRivert cDNA Synthesis Platium Master Mix (GenDEPOT, Katy, TX, USA). The cDNA was diluted 20-fold with DEPC-treated water and stored at -20^{o}C until analysis by Real-time PCR (Masek et al., 2005).

Analysis of gene expression

Gene expression was analyzed using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Masek et al. 2005). Six genes involved in the anthocyanin biosynthesis pathway *VcPAL*, *VcCHS*, *VcF3H*, *VcDFR*, *VcANS*, and *VcUFGT* were selected for amplification from the NCBI database based on references (Cocetta et al., 2015; Harb et al., 2014; Naik et al., 2007; Zifkin et al., 2012). Specific primers for *VcMYB21*, *VcR2R3 MYB*, *VcTDR4*, *VcACO*, *VcETR1*, *VcEIL* were designed using the NCBI primer blast tool and were based on the sequences reported by (Bai et al., 2014; Jaakola et al., 2010). *VcMYB21* and *VcR2R3 MYB* primers were designed based on the nucleotide sequences for *Vaccinium corymbosum* and *Vaccinium myrtillus*, respectively, in the NCBI database. The *VcBBX* primer was Information on all primers used in this study is shown in Supplementary Table. 3-1 and were confirmed by PCR (Fig. 3-3).

PCR products of target genes were amplified by RT-PCR using a Bio-Rad T100TM Thermal Cycler (Bio-Rad, Foster, USA) and cleaned up for

sequencing. All confirmation results are presented in Supplementary Fig 3-1. The qRT-PCR was performed on the CFX Connect^{TM} using Labopass SYBR Green Q Master Mix (2X) by the CFX Connect real-time system (Bio-Rad, Hercules, USA). A total reaction volume of 10 µL was used. Each reaction included 2 µL of template, 0.4 µL of forward primer, 0.4 µL of reverse primer, and 5 µL SYBR Green along with an additional 2.2 µL of RNA-free water. The qRT-PCR was performed using the following amplification program: 95^{o}C for 15 min followed by 40 cycles at 95^{o}C for 30 sec and 60^{o}C for 30 sec. The $2^{-\Delta\Delta Ct}$ method was used to normalize and calibrate transcript values relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously (Tran et al., 2015). *VcGAPDH* was used as an internal control (Harb et al., 2014).

Ethylene production measurement

About 20 g of harvested blueberry fruit from each replicate were placed in 50 mL sealed tubes. After closing the lids for 4 h, 1 mL sample of headspace gas was removed from each tube using a gas tight syringe. Ethylene production was measured with the gas chromatography system (YL6500, Young Lin, Korea) equipped with a flame ionization detector and Porapak column (1.5 m x 6 mm) for ethylene. Oven, injector, and detector temperatures were set at 50^{o}C, 50^{o}C and 250^{o}C respectively.

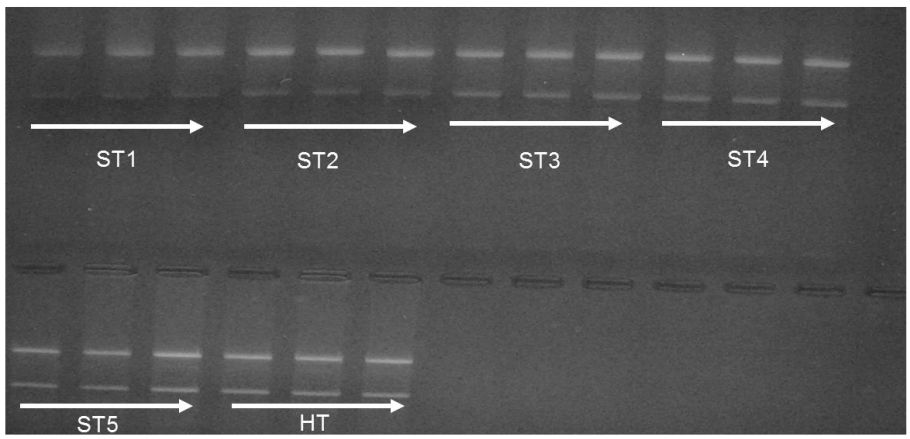

Fig. 3-2. RNA quality by gel electrophoresis in ‘Nelson’ blueberry fruit.
ST: stage, HT: harvest time.

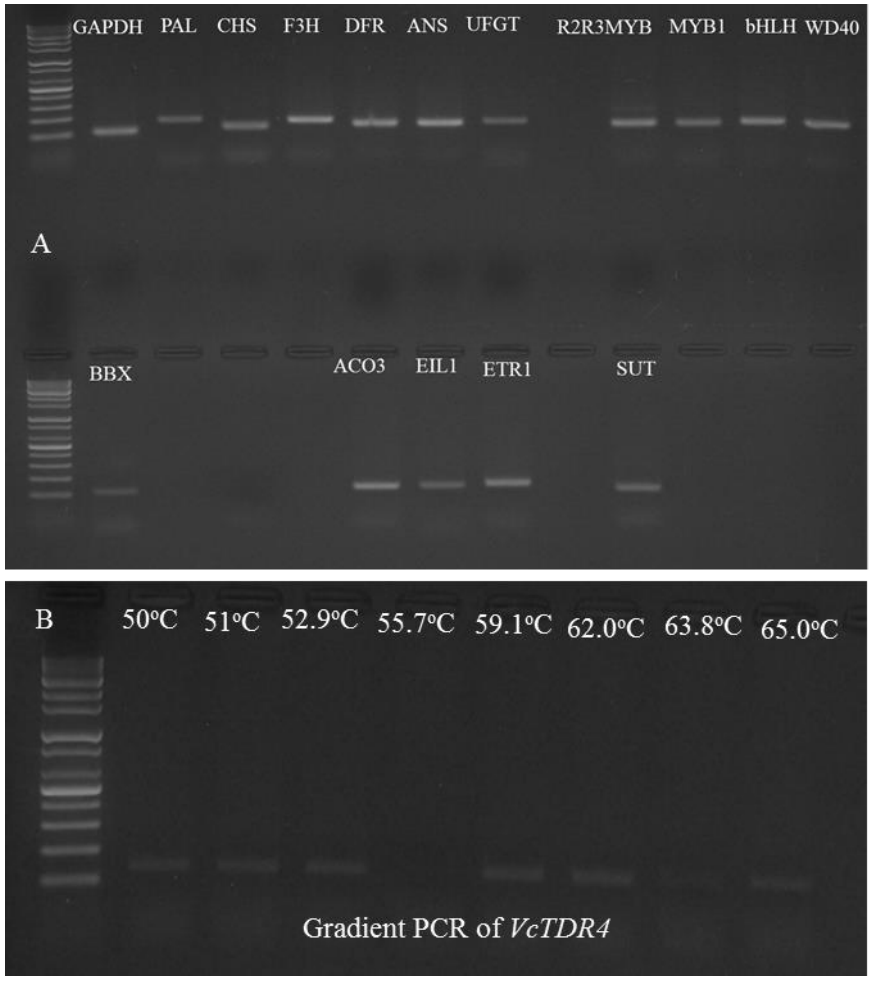

Fig. 3-3. PCR products of all studied genes in 'Nelson' blueberry fruit. A, PCR products at 55°C; B, PCR product of TDR4 at different temperatures.

Analysis of individual anthocyanins

High performance liquid chromatography (HPLC) was used to separate and identify individual anthocyanins in blueberry peel tissues according to protocols described by (Nguyen et al., 2014). Three grams of peel tissues were macerated in 50 mL of methanol using a Polytron P-10 tissue homogenizer (Brinkmann, NY, USA). The extracts were filtered and concentrated using a rotary evaporator series (EYELA, Tokyo Rikakikii Co. Ltd., Tokyo, Japan) in a water bath at 38℃ and then completely dissolved in 3% formic acid in water (v/v). All samples were passed through a C18 Sepak cartridge (Waters, Milford, MA, USA). The anthocyanins were then recovered with 2 mL absolute methanol consisting of 3% formic acid (v/v). Ten microliters of sample were separated by HPLC after passing through a 0.42 μm membrane filter.

Mixed or individual anthocyanin standards were analyzed to confirm the anthocyanins extracted from blueberry fruit. Delphinidin-3-galactoside, malvidin-3-arabinoside, and malvidine-3-galactoside were purchased from Chromadex (Irvine, CA, USA). They were dissolved in 10 mL of 3% formic acid in methanol (v/v) and used as an external standard stock solution for generating calibration curves.

Anthocyanins were analyzed using a YL 9100 HPLC system (Young Lin, Instrument Co. Ltd., Anyang, Korea) consisting of a YL9111 binary pump, a YL9160 diode array detector, and a YL9150 autosampler. A Phenomenex Bondclon C18 analytical column (4.6 × 250 mm, 10 μm) was used. Mobile solvents were 5% formic acid in water (v/v) and acetonitrile (ACN). The solvent system was programmed from 5% ACN to 20% ACN for 50 min and flushed with 100% ACN for 5 min. The flow rate was 1 mL min^{-1}. Ten microliters of sample were injected. Spectral data between 200 and 700 nm were collected and anthocyanins were detected at 520 nm.

Statistical analysis

The data were statistically evaluated using SAS 9.3 (TS1M2) statistical software (SAS Institute Inc., Cary, NC, USA) statistical software. The experiment was performed in triplicate and using standard deviations. Means were differentiated using Duncan's multiple range test at $P < 0.05$ (*), $P < 0.01$ (**), and at $P < 0.001$ (***).

RESULTS

Expression of anthocyanin regulated genes

The qRT-PCR results for the expression levels of anthocyanin biosynthesis genes *VcPAL*, *VcCHS*, *VcF3H*, *VcDFR*, *VcANS*, *VcUFGT* and anthocyanin regulated transcription factors are shown in Figs. 3-4 and 3-5. This study analyzed gene expression levels at different development stages and the data showed that most of genes in anthocyanin biosynthesis pathway increased during ripening and reach the peak at stage 4 (Fig. 3-4). There was a significant declined at stage 5 in the transcript levels of *VcF3H* and *VcUFGT*. The transcript levels reduced from 9.52 to 5.49 fold (Fig. 3-4C) and from 231.13 to 106.3 fold (Fig. 3-4F) at *VcF3H* and *VcUFGT* respectively.

The expression levels of both transcription factors *VcMYB21* and *VcR2R3 MYB* increased during ripening (Figs. 3-5B, 3-5C), while the expression levels of *VcTDR4* decreased during developmental stages (Fig. 3-5A). The expression of *VcR2R3 MYB (*Fig. 3-5C) was stronger than *VcMYB21* (Fig. 3-5B). The significant increase started from stage 3 and reached to the peak at stage 5 (2.58 fold) in *VcMYB21*, at ST4 (29.75 fold) in *VcR2R3 MYB*.

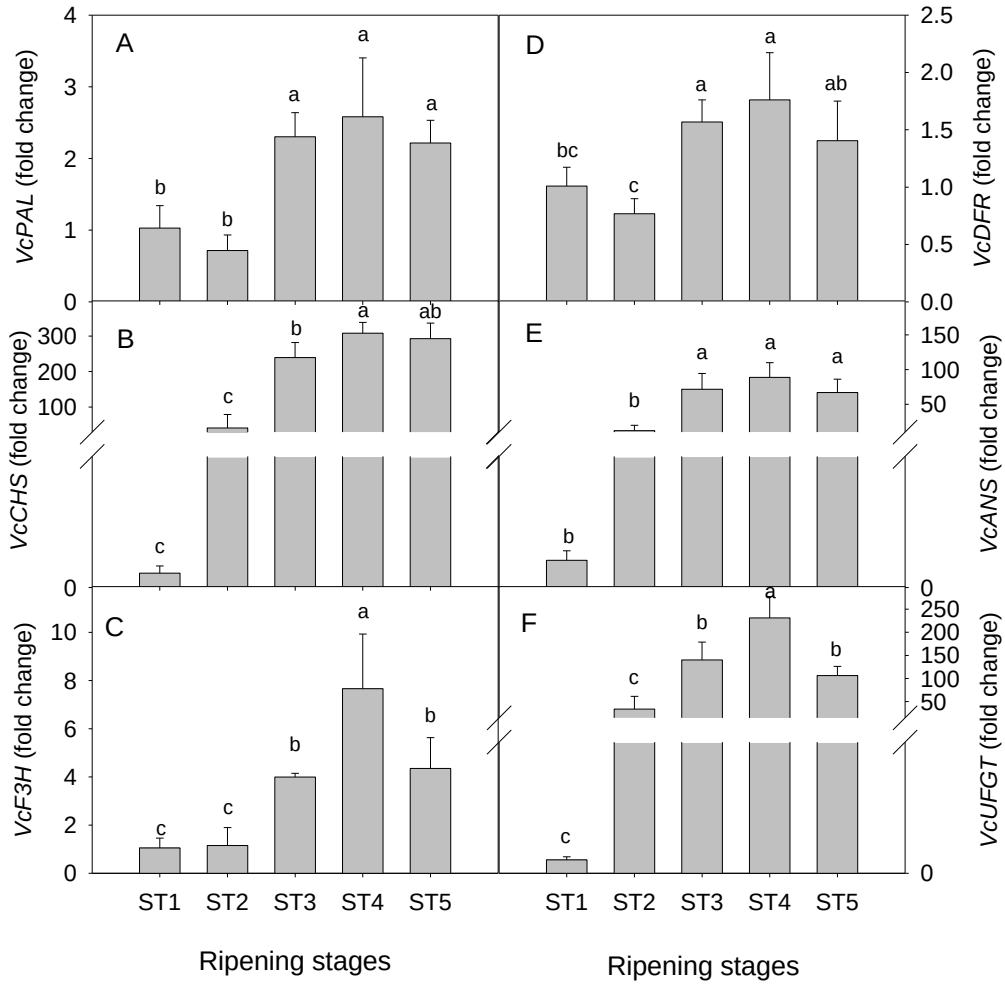

Fig. 3-4. Changes in the transcript levels of anthocyanin biosynthesis genes in 'Nelson' blueberry fruit during ripening. *VcPAL* (A), *VcCHS* (B), *VcF3H* (C), *VcDFR* (D), *VcANS* (E), and *VcUFGT* (F). The values are expressed as means ±SD of triplicate samples. Data in columns with different letters at the same stage are significantly different according to Duncan's multiple range test at $p < 0.05$.

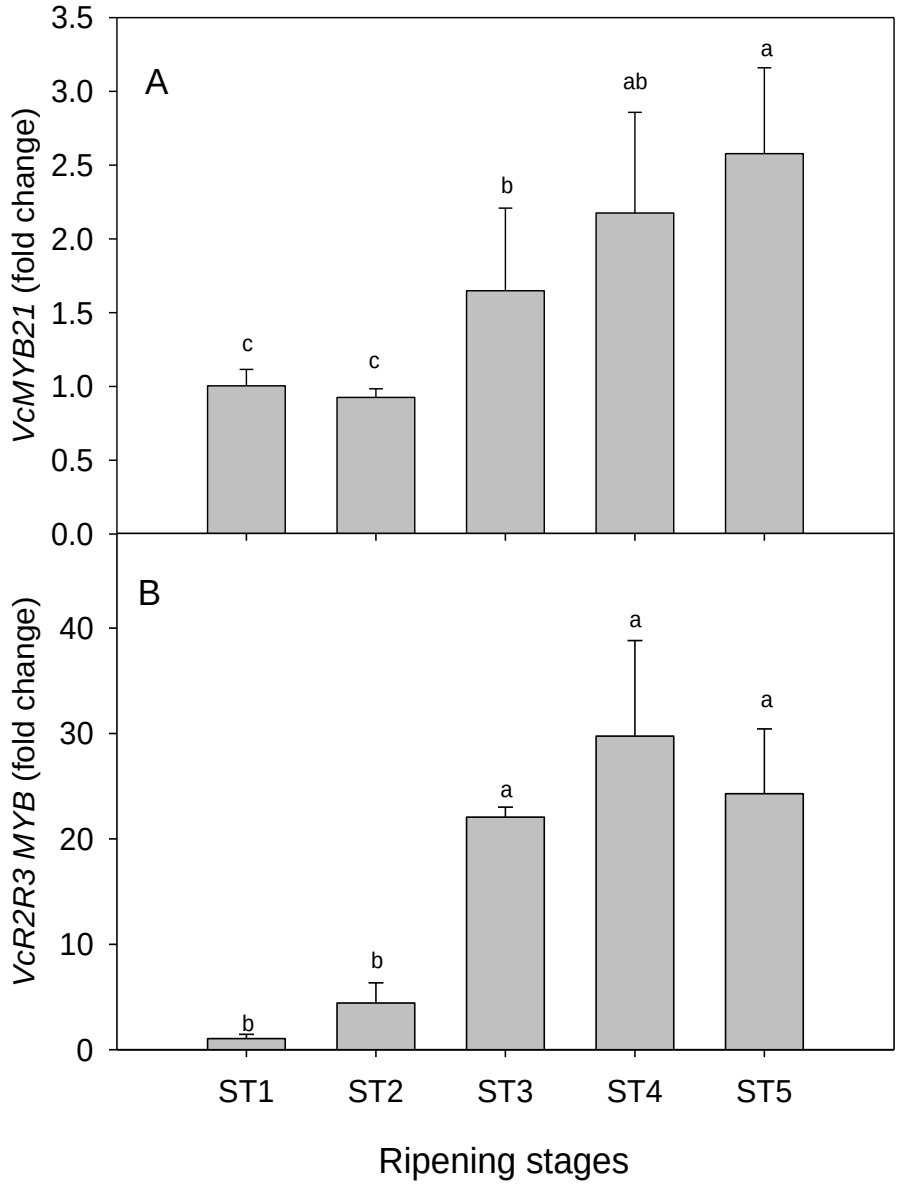

Fig. 3-5. Changes in the transcript levels of anthocyanin regulated transcription factors in 'Nelson' blueberry fruit during ripening. *VcMYB21* (A) and *VcR2R3 MYB* (B). The values are expressed as means ±SD of triplicate samples. Data in columns with different letters at the same stage are significantly different according to Duncan's multiple range test at $p < 0.05$.

Expression of ethylene related genes

+ During ripening at 'Nelson' blueberry fruit

The expression levels of *VcACO, VcETR1* and TF *VcEIL4* were analyzed at different ripe stages as well as after UV-B treatment in order to understand the relationship between ethylene regulation genes and UV-B on skin tissue of blueberry fruit. Fig. 3-6 showed the expression levels of *VcACO* and transcription factor *VcEIL4* increased during the ripening and reached to the top at ST5 (Figs. 3-6A and 3-6C). The fold change of the receptor gene *VcETR1* reached the peak at ST4 (1.41-fold) then decreased to 1.01-fold at ST5 (Fig. 3-6B).

+ After UV-B radiation at 'Duke' blueberry fruit

However, after 3 h treatment by UV-B on 'Duke' blueberry fruit, the expression levels of *VcACO* were down-regulated (Figs. 3-7A) while the expression of *VcEIL4* transcription factor was up-regulated (Fig. 3-7C). Figure 3-7A showed the fold of *VcACO* reduced from 8.37 to 2.38 after 3 hours with UV-B treatment, fall from 14.01 to 4.63 fold after 6 h by UV-B light.

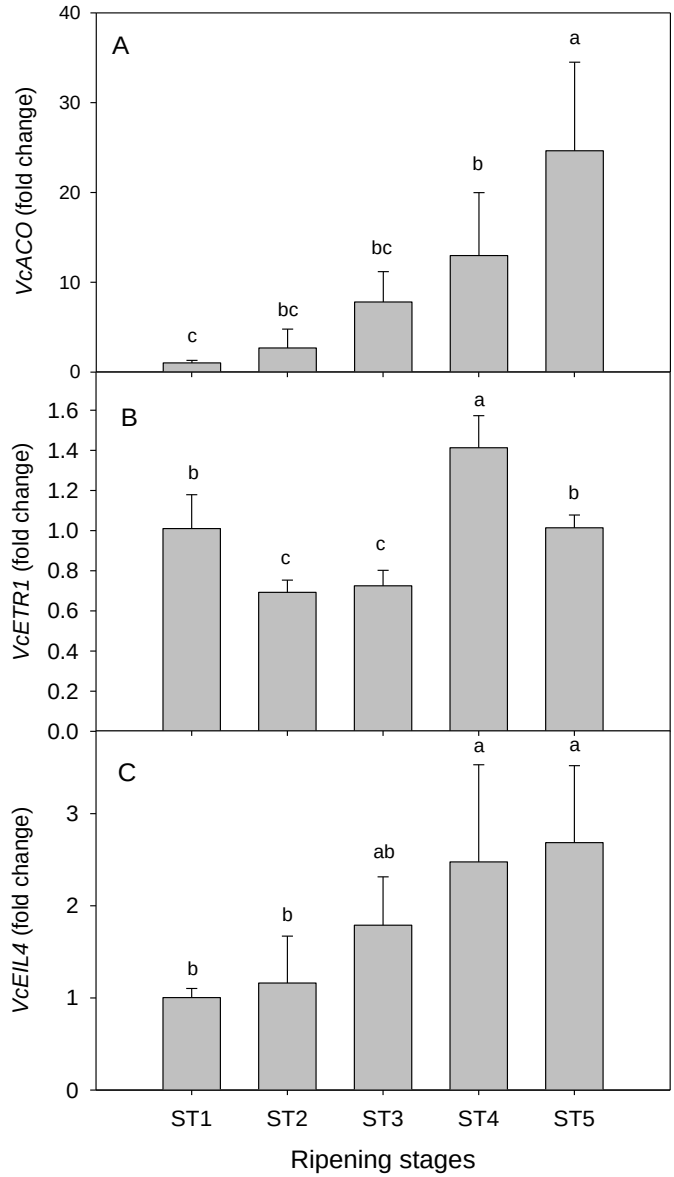

Fig. 3-6. Changes in the transcript levels of ethylene related genes (*VcACO*, *VcETR*1, and *VcEIL4*) in 'Nelson' blueberry fruit during ripening stages. The values are expressed as means ±SD of triplicate samples. Data in columns with different letters at the same stage are significantly different according to Duncan's multiple range test at $p < 0.05$.

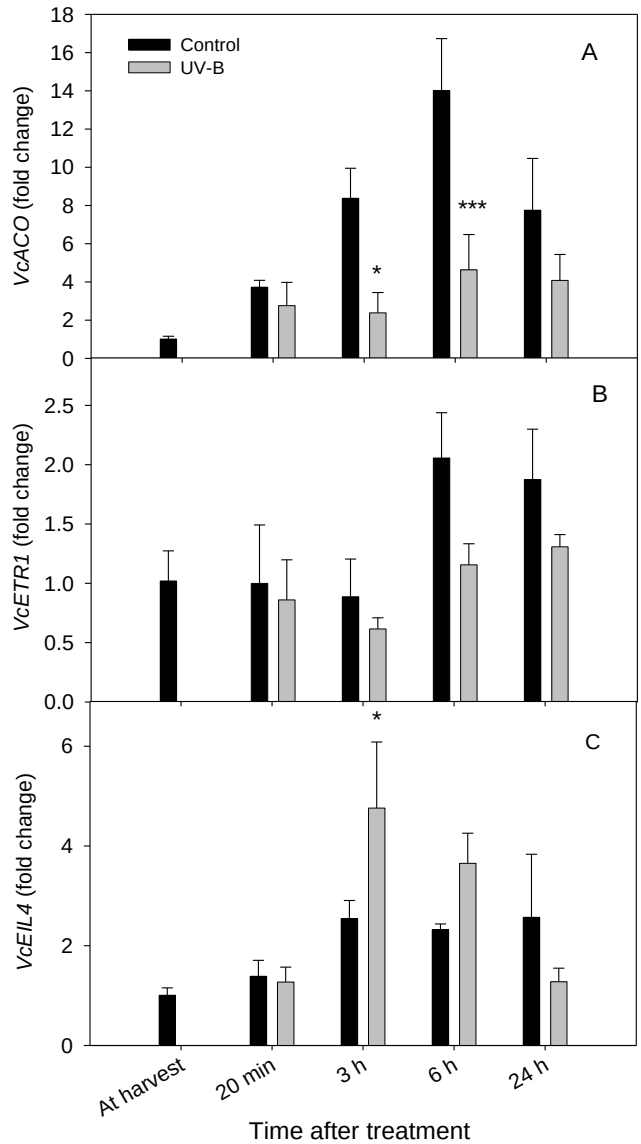

Fig. 3-7. Changes in the transcript levels of *VcACO*, *VcETR*1, and *VcEIL4* after UV-B treatment in the peel of 'Duke' blueberry fruit at room temperature. The values are expressed as means ±SD of triplicate samples. *$p < 0.05$; ***$p < 0.001$ based on Duncan's multiple range test between the Control and UV-B radiation treated at each point.

Expression of *VcTDR4* transcription factor

The expression of *VcTDR4* also was checked during 5 stage of ripening (Fig. 3-8). The data showed that the expression levels of this TF reduced significantly during ripening, from 1 fold at ST1 to 0.21 at ST5 in 'Nelson' blueberry fruit. It was interesting that the expression levels of *VcTDR4* was also significant reduced after UV-B treatment at peel tissue in 'Duke' blueberry fruit compared to control (Fig. 3-9) and at both of peel and whole tissues in 'Nelson' blueberry fruit (Fig. 3-10).

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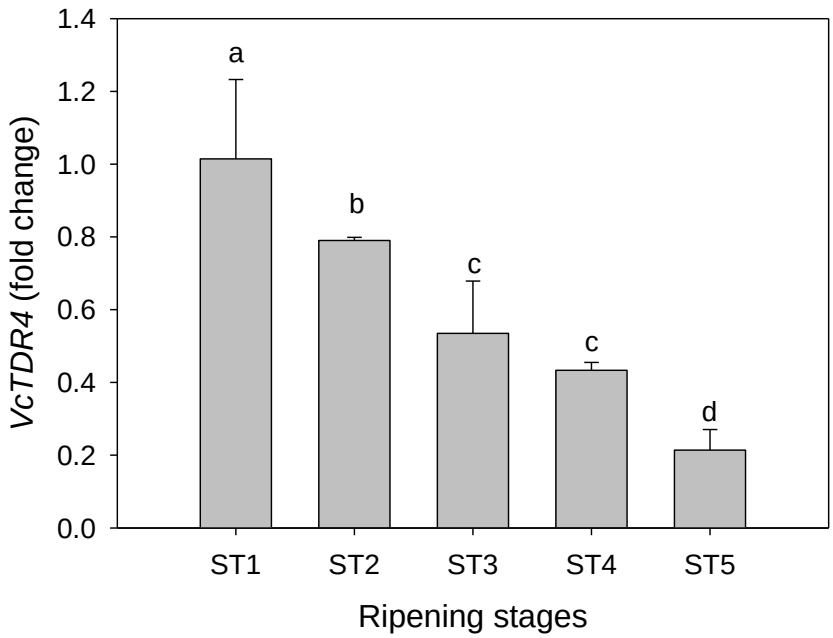

Fig. 3-8. Changes in the transcript levels of *VcTDR4* during ripening of 'Nelson' blueberry fruit. The values are expressed as means ±SD of triplicate samples. Data in columns with different letters at the same stage are significantly different according to Duncan's multiple range test at $p < 0.05$.

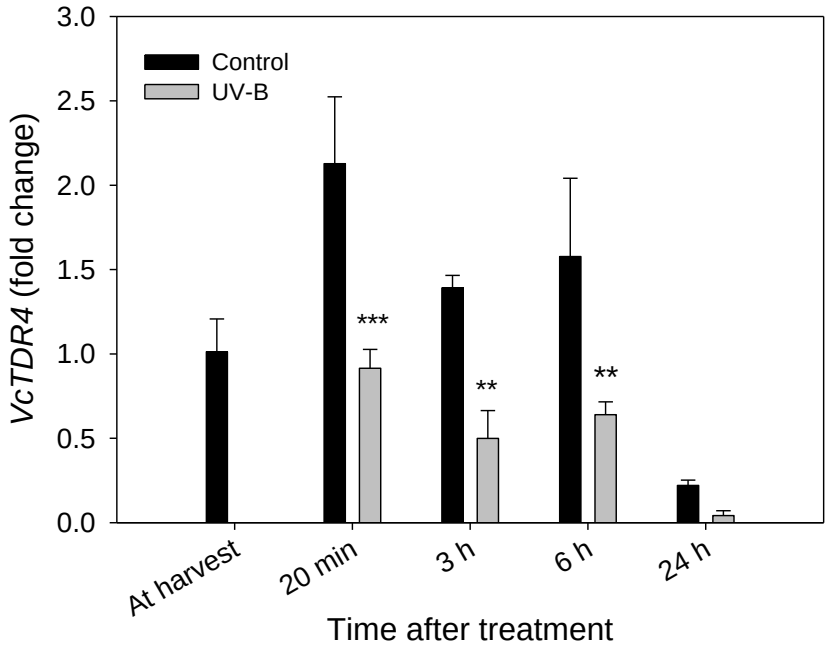

Fig. 3-9. Changes in the transcript levels of *VcTDR4* transcription factor after UV-B radiation in 'Duke' blueberry fruit. The values are expressed as means ±SD of triplicate samples. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ based on Duncan's multiple range test between the Control and UV-B radiation treated at each point.

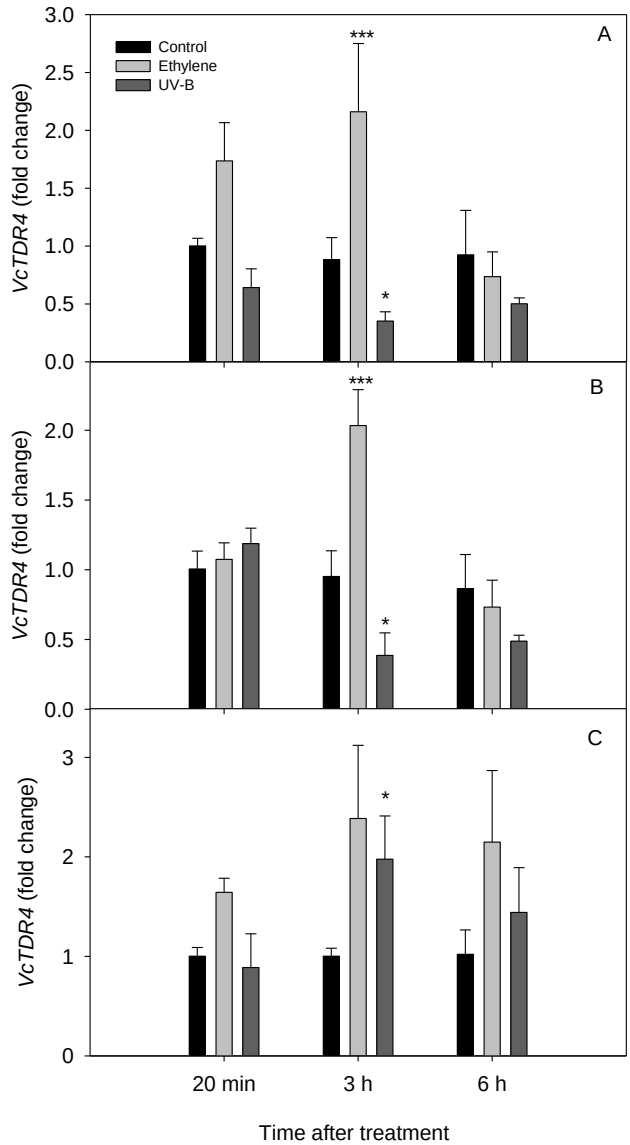

Fig. 3-10. Changes in the transcript levels of *VcTDR4* in 'Nelson' blueberry fruit after UV-B radiation and ethylene treatment at peel tissue (A), whole fruit tissue (B) and flesh tissue (C). The values are expressed as means ±SD of triplicate samples. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ based on Duncan's multiple range test between the Control and UV-B radiation treated at each point.

Changes of ethylene production

During ripening, the ethylene production was detected from ST1 to ST5 (Fig. 3-11). The LC equipment could not detect the ethylene production at ST1. The amount of ethylene was highest at ST3 when the fruit turns pink from green. It reached to 2.33 µL $kg^{-1} h^{-1}$ at ST3 then decreased to 1.87 µL $kg^{-1} h^{-1}$ and 1.26 µL $kg^{-1} h^{-1}$ at ST4 and ST5 respectively.

To understand ethylene production more clearly after UV-B treatment, treated samples at full ripening stages were analyzed. Fig. 3-12 showed that there was not a significant difference between control and UV-B after treatment immediately (20 min), but there was a significant difference between control and UV-B after 3 h. The amount of ethylene was inhibited by UV-B (0.67 µL $kg^{-1} h^{-1}$) compare to the control (3.44 µL $kg^{-1} h^{-1}$).

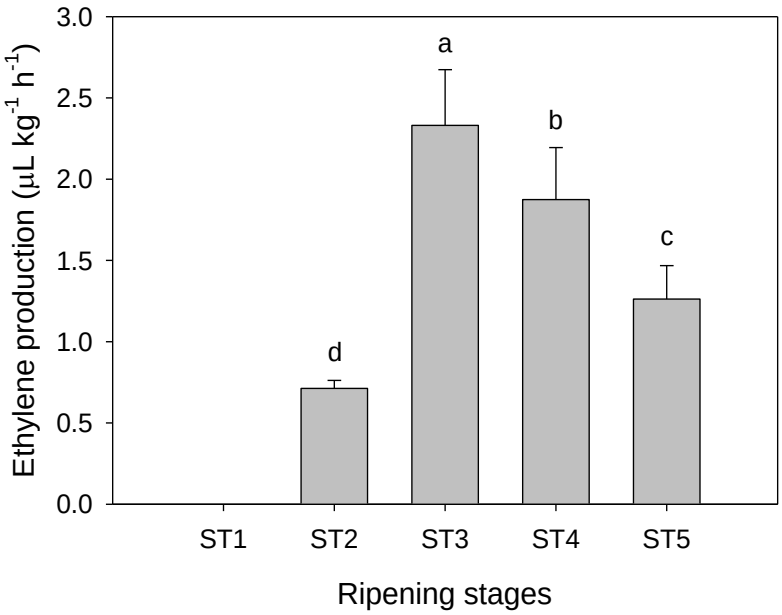

Fig. 3-11. Changes in the ethylene production of 'Nelson' blueberry fruit during ripening. The values are expressed as means ±SD of triplicate samples. Data in columns with different letters at the same stage are significantly different according to Duncan's multiple range test at $p < 0.05$.

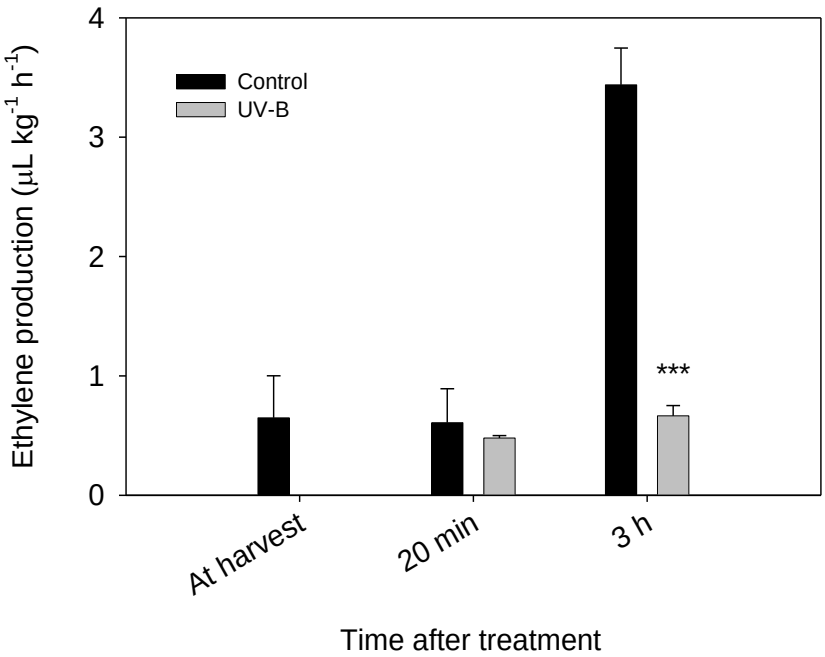

Fig. 3-12. Changes in the ethylene production of 'Nelson' blueberry fruit after UV-B radiation. The values are expressed as means ±SD of triplicate samples. $^{**}p < 0.01$; $^{***}p < 0.001$ based on Duncan's multiple range test between the Control and UV-B radiation treated at each point.

Analysis of individual anthocyanin compounds

Changes in the three individual anthocyanins in blueberry fruit at natural ripening stages are shown in Fig. 3-13 and after UV-B treatment are in Figs. 3-14 and 3-15.

The three kind of main individual anthocyanin (Delphinidin-3-Galactoside, Malvinidin-3-Galactoside and Malvinidin-3-Arabioside) increased during ripening (Fig. 3-13). The content of Del-3-Gal was higher than Mal-3-Gal and Mal-3-Ara. It reached to 16.42 µg g^{-1} F.W, 66.46 µg g^{-1} F.W and 109.92 µg g^{-1} F.W at ST3, ST4 and ST5 respectively. The individual anthocyanin content of Mal-3-Gal increased from 4.33 µg g^{-1} F.W at ST3 to 20.21 µg g^{-1} F.W at ST4 and 53.0 µg g^{-1} F.W at ST5. The anthocyanin content of Mal-3-Ara also increased from 4.59 at ST3 to 37.93 at ST5.

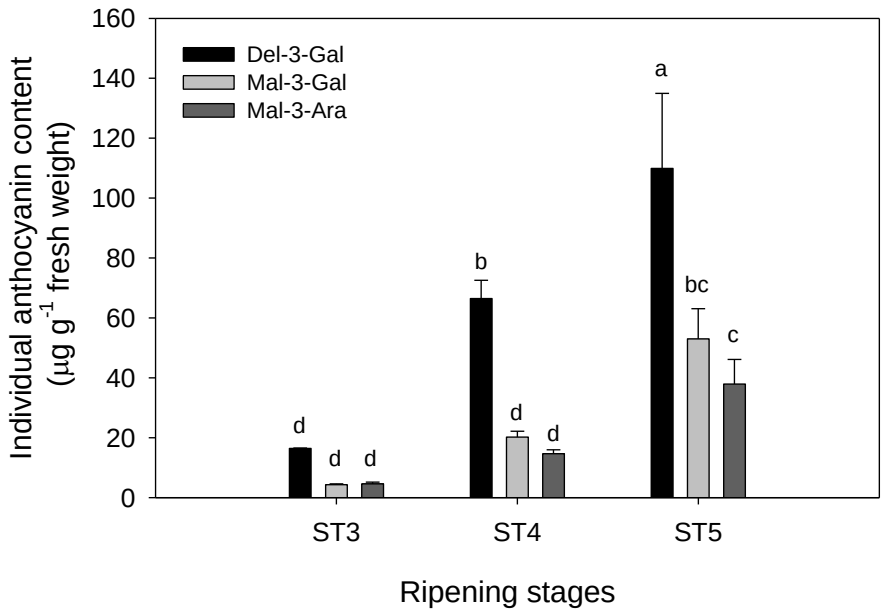

Fig. 3-13. Changes in the major individual anthocyanin of 'Nelson' blueberry fruit during natural ripening stages. The values are expressed as means ±SD of triplicate samples. $^{**}p < 0.01$; $^{***}p < 0.001$ based on Duncan's multiple range test between the Control and UV-B radiation treated at each point.

To confirm the previous study, after ethylene and UV-B treatment, the individual anthocyanin in both peel and whole fruit tissues in treated samples were analysis (Figs. 3-14 and 3-15). The data showed all conducted individual anthocyanin (Del-3-Gal, Mal-3-Gal and Mal-3-Ara) were increased significantly after treatment with ethylene and with UV-B after 3 h in peel tissues (Fig. 3-15A, B, C). These anthocyanins reached highest values at samples treated by UV-B after 3 h. They reached to 177.7 µg g^{-1} F.W, 199.2 µg g^{-1} F.W and 179. µg g^{-1} F.W at Del-3-Gal, Mal-3-Gal, and Mal-3-Ara respectively.

There is a little significant difference after UV-B treatment on whole fruit tissues (Fig. 3-14). After 20 min of treatment immediately, the anthocyanin content of Mal-3-Gal and Mal-3-Ara increased significantly to 143.8 µg g^{-1} F.W (Fig. 3-14B) and 127.1 µg g^{-1} F.W (Fig. 3-14C). After 3 h of UV-B treatment, there was a significant increase in content of anthocyanin in Del-3-Gal. It reached to 113.3 µg g^{-1} F.W (Fig. 3-14A). Treatments with ethylene also lead to the significant increase of three above major anthocyanin after 3 h.

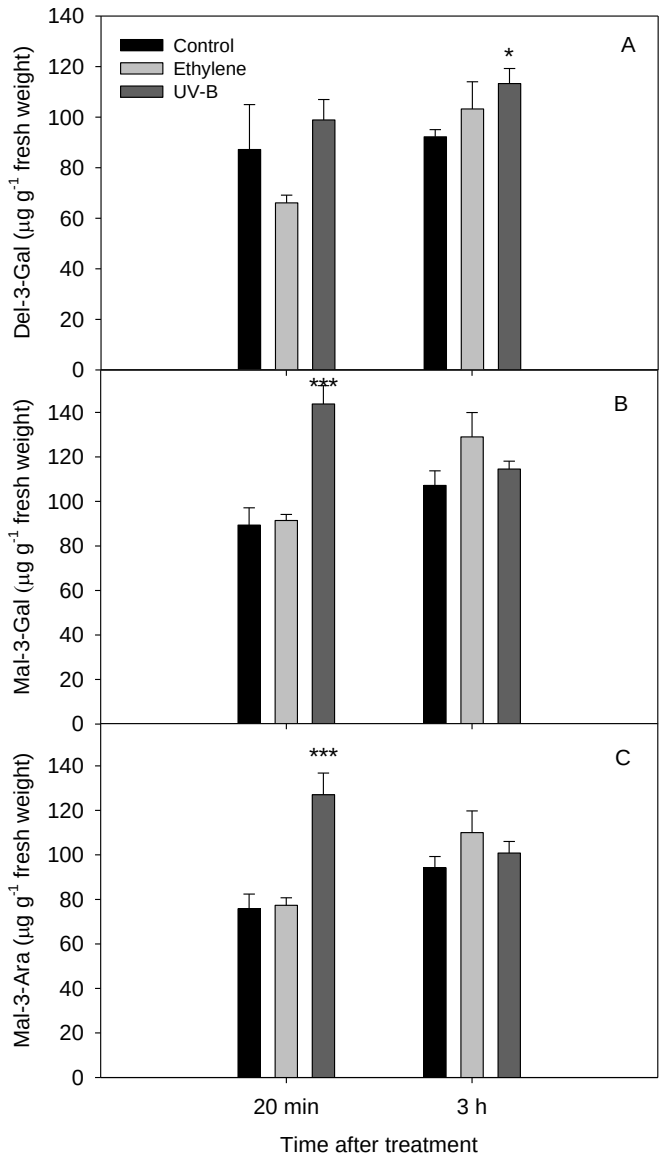

Fig. 3-14. Changes in the major individual anthocyanin at 'Nelson' blueberry fruit after UV-B radiation in whole tissue. The values are expressed as means ±SD of triplicate samples. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ based on Duncan's multiple range test between the Control, Ethylene and UV-B radiation treated at each point.

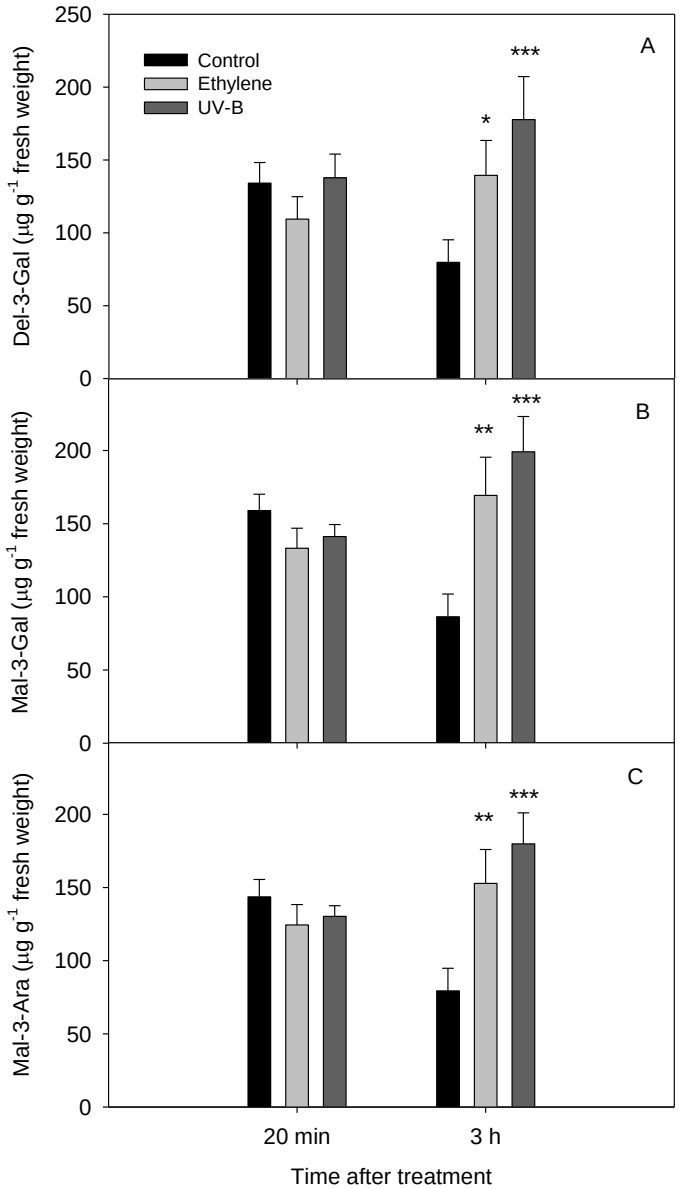

Fig. 3-15. Changes in the major individual anthocyanin at 'Nelson' blueberry fruit after UV-B radiation in peel tissue. The values are expressed as means ±SD of triplicate samples. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ based on Duncan's multiple range test between the Control, Ethylene and UV-B radiation treated at each point.

DISCUSSION

Kalt et al. (1999) reported that delphinidin was a main anthocyanidin in highbush blueberry fruit. In the chapter 1, the three major anthocyanindin of Delphinidin, Petunidin and Malvinidin was detected and Del-3-Gal, Mal-3-Gal and Mal-3-Ara were three of major individual anthocyanin in 'Duke' blueberry fruit (Nguyen et al., 2014). In this chapter, Del-3-Gal, Mal-3-Gal and Mal-3-Ara also were identified and they appeared from ST3 then they increased dramatically to ST5 during ripening in 'Nelson' blueberry fruit. The data also found a similar to previous study on blueberry fruit of Zifkin et al. (2012) which delphinidin was the main anthocyanidin at full ripe of blueberry fruit (Zifkin et al., 2012). Moreover, after UV-B radiation treatment, the three of above anthocyanin also increased in the peel and whole tissues in 'Nelson' blueberry fruit which found the similar to previous study (Nguyen et al., 2014) that anthocyanin accumulation took place after 3 h by UV-B radiation in 'Duke' blueberry fruit.

According to the review of Zoratti et al. (2014), the accumulation of anthocyanin caused by the increase in expression levels of anthocyanin biosynthesis genes. In this study, the increase of anthocyanin contents and transcript levels of genes related to anthocyanin (structural genes and

regulated genes) were characterized at different ripening stages in ‘Nelson’ blueberry fruit. The data found the similar to previous studies, that *VcMYB21* and *VcR2R3 MYB* are key transcription factors related to regulation of anthocyanin biosynthesis genes in apples (Takos et al., 2006), in berries (Primetta et al., 2015), and in grapes. In addition, *VcMYB21* and *VcR2R3 MYB* had a significantly positive correlation with structural genes of anthocyanin biosynthesis pathway in ‘Duke’ blueberry fruit (Supplementary Table 2-2). Interestingly, *VcMYB21* and *VcR2R3 MYB* had a negative correlation with *VcTDR4* during ripening in ‘Nelson’ blueberry fruit (Supplementary Table 3-2) and also by UV-B radiation in ‘Duke’ blueberry fruit (Supplementary Table 2-2). Thus, the hypothesis of this chapter is whether *VcTDR4* relates to negative regulation in the accumulation of anthocyanin.

The accumulation of anthocyanin is also related to ripening and depends on the cultivar of blueberry fruit. There are still having argument that blueberry is belongs to non-climacteric fruits (Zifkin et al., 2012) or climacteric fruits (El-Agamy et al., 1982). The mechanism of ethylene action in inducing the ripening of highbush blueberry fruit has not been resolved, especially after UV-B light treatment. Moreover, *VmTDR4* was reported that it related to adjusting anthocyanin accumulation in bilberries (Jaakola et al., 2010). In order to understand more clearly about the

molecular level of anthocyanin accumulation with ethylene, ripening and after UV-B radiation treatment, the changes of *VcTDR4* transcription factor and ethylene production in that conditions have been investigated. In addition, TDR4 is also involved in the ripening of tomato (Bemer et al., 2012). In this study, effect of UV-B radiation treatment on ethylene production, *VcTDR4* transcription and individual anthocyanin contents were compared by changing of them in natural ripening stages. *VcTDR4* was designed based on the sequence of *VmTDR4* from bilberry fruit in the report of Jaakola et al. (2010) to get the accession number of KX263323 with 99.2% identity (Supplementary Table 3-3).

In general, like other fruits, 'Nelson' blueberry fruit also required ethylene to ripe. Ethylene production was maintained in all ripening stages (ST2 to ST5) at much higher level than that in the unripe stage (ST1) (Fig. 3-11). However, after getting the peak at stage 3 (ST3), the ethylene level got decreased at later stages (ST4 and ST5). This suggests that after ST3, the synthesis of ethylene might be induced anymore while the degradation of ethylene was still happening, explaining why the ethylene level was reduced in ST4 and ST5. In other words, the level of ethylene at the ST3 might be enough to stimulate the ripening process in 'Nelson' blueberry fruit. Interestingly, the ethylene production was declined after 3 h by UV-B radiation in full-ripe 'Nelson' blueberry fruit (Fig. 3-12).

According to Jaakola et al. (2010), *VmTDR4* is an important transcription factor in the accumulation of anthocyanin in bilberry fruits. This chapter expected that whether *VcTDR4* also acts in a similar way in blueberry fruit like *VmTDR4* in bilberry fruit. Interestingly, the study found that *VcTDR4* was significantly reduced while anthocyanin contents were increased in the ripening process of 'Nelson' blueberry fruit. The similar phenomenon was observed in treated fruit with UV-B radiation. In the other word, the study found that UV-B radiation accelerated the down-regulation of *VcTDR4* at peel tissue of both 'Duke' (Fig. 3-9) and 'Nelson' (Fig. 3-10A) blueberry fruit where anthocyanin is present (Riihinen et al., 2008). This suggests that TDR4 might not positively regulate anthocyanin in this chapter as reported.

In order to understand better about ripening mechanism in blueberry fruit, the study investigated the changes in expression levels of *VcACO*, *VcEIL4*and *VcETR1* in the peel tissues of 'Duke' blueberry fruit (Fig. 3-7) and 'Nelson' blueberry fruit at different ripening stages (Fig. 3-6). The data showed the transcript levels of *VcACO*, *VcETR1* and *VcEIL4* increased during ripening as a natural change of fruits. However, the expression of *VcACO* decreased but *VcEIL4* increased at 'Duke' blueberry fruit tissue after UV-B radiation treatment. EIL4 is consider as a transcription factor that regulated ethylene gene expression (Guo and Ecker, 2004). The results

found the similar to a previous study that EIL4 was arranged into the group of up-regulated genes while ACO was arranged into the group of down-regulated genes in harvested papaya (Zou et al., 2014).

According to Takada et al. (2005), ETR1 is an ethylene receptor gene which is related to accumulation of anthocyanin. The contrast data in this study showed the expression of *VcETR1* decreased at ST5 (Fig. 3-6) but there was no a significant decrease about this gene after UV-B radiation in 'Duke' blueberry fruit (Fig. 3-7B). This chapter's results also showed ethylene production also declined after ST3 of ripening and after UV-B radiation in 'Nelson' blueberry fruit. Moreover, the content of individual anthocyanin significantly increased while ethylene production was reduced after 3 h by UV-B radiation treatment at 6 kJ m^{-2}. This phenomenon is similar to what was found that the accumulation of anthocyanin was regulated by increase of light and decrease by ethylene in Arabidopsis (Jeong et al., 2010) or in red cabbage under red light (Kang and Burg, 1973). So, this study noticed that whether the reduction of ethylene production induces the increase of anthocyanin contents after UV-B radiation in highbush blueberry fruit. The hypothesis is whether UV-B radiation might causes inhibition factors to remove ethylene production and reduced ethylene regulated genes.

Moreover, TDR4 and EIL4 are transcription factors involve in the

ripening process. While ethylene production had negative correlation with *VcEIL4* at ST4 of ripening (Supplementary Table 3-2) but there was not a significant correlation between ethylene production and *VcTDR4*. After UV-B radiation the contrast change in the decrease of *VcTDR4* and increase of *VcEIL4* (Fig. 3-7B) can be explained that at full ripening stage, UV-B might continue induced the decrease of *VcTDR4* but it is not effect on *VcEIL4*.

In conclusion, UV-B accelerated the down-regulation of *VcTDR4* in peel of 'Duke' blueberry fruit and in the peel and whole tissues of 'Nelson' blueberry fruit. UV-B also induced the reduction of ethylene production in 'Nelson' blueberry fruit. Although *VcTDR4* had negative correlation with *VcMYB21*, *VcR2R3 MYB* and structural genes in anthocyanin biosynthesis pathway but it was still unclear whether *VcTDR4* involved in accumulating anthocyanin. Further research is required to determine whether the *VcTDR4* transcription factor plays a role in the anthocyanin accumulation in highbush blueberry fruit.

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CONCLUSIONS

Highbush blueberry fruit contain a high amount of anthocyanin which is playing an important role for improving health benefit of human. After harvesting the quality of blueberry reduced by microbial growth, bruising or enzymatic browning. This study was focused on the molecular mechanism of the accumulation of anthocyanin induced by UV light in highbush blueberry fruit that improved postharvest quality of fruits.

Treatment with UV-B and UV-C light at 6 kJ m^{-2} for 20 min decreased in decay, increase total anthocyanin, individual anthocyanin, antioxidant activity as well as quality of fruits during cold storage at 0^{o}C. The increase of anthocyanin after 3 h by UV-B in 'Duke' and 'Nelson' highbush blueberry fruit might be related to the molecular genetic mechanism of anthocyanin biosynthesis genes. UV-B induced the up-regulation of anthocyanin biosynthesis genes and some transcription factors related to anthocyanin accumulation, such as *VcMYB21, VcR2R3 MYB*, *VcBBX (KX300037)* and *VcDW40* (TTG1: *KX447762*) in the peel of 'Duke' blueberry fruit. Similar to the report about the relationship between ethylene and anthocyanin accumulation in *Arabiopsis*, the increase in accumulation of anthocyanin in 'Duke' blueberry fruit might involve in the

reduction of ethylene biosynthesis genes (*VcACO*), ethylene receptor (*VcETR1*), and the reduction of ethylene production. In addition, UV-B accelerated the down-regulation of *VcTDR4* in peel of 'Duke' and in the peel and whole tissue of 'Nelson' blueberry fruit which suggests that TDR4 might not positively regulate anthocyanin in the two cultivars. Moreover, the decrease of ethylene production by UV-B radiation in 'Nelson' blueberry fruit suggested that UV-B radiation and reduction of ethylene maybe related the increase of anthocyanin in highbush blueberry fruit. However, it is necessary to gain more evidences to support the hypothesis. Further research is required to determine whether *VcTDR4* transcription factor play the role in the anthocyanin accumulation in highbush blueberry fruit.

UV-B radiation at low dose around 6 kJ m^{-2} can be applied as postharvest technology in order to improve the quality and anthocyanin of harvested blueberry fruit. Question of whether UV-B radiation might remove ethylene production in blueberry fruit or not should be answered. In addition, relationship between ethylene and anthocyanin should be confirmed in highbush blueberry fruit.

ABSTRACT IN KOREAN

블루베리는 안토시아닌이 풍부한 과일로 알려져 있고, 이러한 안토시아닌은 과일의 색소로 작용하여 대표적으로 청색, 보라색, 빨간색을 보이는 과일들이 있다. 이러한 과일의 안토시아닌이 가지는 항산화 기능은 인간의 건강 증진에 중요한 역할을 한다. 하지만 블루베리는 저장성이 약한 과일로 수확후 저장기간 동안에 품질 저하뿐만 아니라 기능성을 갖는 안토시아닌 함량이 급격하게 감소한다. 수확후 처리 기술 중 하나인 자외선 조사 기술은 일반적으로 과실의 품질과 안토시아닌 함량을 증진시키는 것으로 보고되고 있다. 하지만 수확한 블루베리에서 자외선 조사에 의해 유도되는 안토시아닌 함량 증가에 대한 분자적 수준의 기작 연구는 거의 알려져 있지 않다. 본 연구의 목표는 수확된 블루베리에 20분간 6.0 kJ m^{-2} 수준의 자외선을 조사하여 품질, 안토시아닌 함량, 에틸렌 생성량을 분석하여 변화를 관찰하고 이러한 변화 기작을 구명하고자 안토시아닌 생합성 및 이를 조절하는 인자에 대한 분자적 연구를 수행하였다.

자외선은 크게 UV-A, B, 그리고 C로 세 가지 종류로 구분하는데 이들은 각각의 파장과 강도가 다르다. 따라서 본 연구에서는 자외선 종류 중 블루베리의 품질과 안토시아닌 함량을 증진시키는데 가장 효과적인 자외선을 구명하기 위해 선행적으로 UV-A, B, 그리고 C를 모두 처리하였고, 이 중 UV-B와 C조사가 블루베리의 저장성과 안토시아닌 함량을 증진시킨 것으로 밝혔다. 이러한 결과를 토대로 UV-B를 조사한 블루베리 'Duke'와 'Nelson' 품종들을 대상으로 에틸렌 생성, 안토시아닌 정량 확인과 함께 안토시아닌 생합성 유전자와 자외선 조사 및 착색에 관련된 에틸렌 생합성 유전자, 그리고 이들의 전사인자들 (*VcBBX, VcMYB21, VcWD40 VcR2R3 MYB, VcEIL4, VcTDR4)*의 발현량을 분석하였다. 'Duke' 품종에서는 안토시아닌 생합성 유전자 중 *VcPAL, VcCHS, VcF3H, VcDFR, VcANS, VcUFGT* 와 그들을 조절하는 전사인자 중 *VcR2R3 MYB, VcMYB21, VcBBX*와 에틸렌 반응과 연관된 *VcEIL4*은 UV-B 조사에 의해 유의적으로 발현이 유도되었고, 반면에 에틸렌 생성량과 이를 조절하는 *VcTDR4, VcACO, VcETR1*의 발현은 감소된 것으로 나타났다. 특히 UV-B조사에 의한 블루베리의 전자인자 발현 유도 효과는 처리 직후

혹은 처리후 3시간 이내에 최고수준을 보이다가 급격히 감소되는 것을 보아 ‘Duke’ 품종에서 UV-B조사가 안토시아닌 및 관련된 유전자의 발현을 조절하는 것으로 사료된다. 하지만 UV-B 조사가 ‘Duke’ 품종에서 *VcTDR4, VcACO, VcETR1* 의 발현은 억제시키는 것으로 나타났으며 ‘Nelson’ 품종에서도 역시 에틸렌 생성량이 감소된 것을 확인하였다. 안토시아닌 함량은 이들의 종류와 블루베리 조직에 따라 다양하나 블루베리의 주요 안토시아닌인 Del-3-Gal, Mal-3-Gal, Mal-3-Ara 성분들은 UV-B조사에 의해 처리 직후 혹은 처리 후 3시간이 된 시기의 블루베리에서 가장 높은 함량이 유의하게 검출되었다. 따라서 수확후 블루베리의 UV-B 조사는 안토시아닌의 생합성과 이들을 조절하는 전사인자의 발현을 유도하고 에틸렌 생합성 혹은 반응에 관련된 유전자의 발현은 억제하여 안토시아닌 함량을 증가시키고 에틸렌에 의한 후숙을 지연시켜 선도유지 효과를 도출하였다고 판단된다. 이러한 연구는 향후 UV-B 조사에 의한 수확후 처리기술로써 상용화하여 완숙된 블루베리의 저장성과 기능성을 유지시키는 효과를 기대할 수 있을 것으로 사료된다.

Supplementary Table 2-1. Primers used in qRT-PCR.

Gene	Accession number	Forward primer (5' to 3')	Reverse primer (5' to 3')
VcGAPDH	AY123769.1	GGTTATCAATGATAGGTTTGGCA	CAGTCCTTGCTTGATGGACC
VcPAL	AY123770.1	TTACAACAATGGGTTGCCCT	CCTGGTTGTGTGTCAGCACT
VcCHS	JN654702.1	CTTGACTGAGGAAATCTTGAAGG	AGCCTCTTTGCCCAATTTG
VcF3'H	AB694901.1	CGAGATTCGATGCGTTTCTGAGTG	GATTTCGGTATCGGTGAGCTTCC
VcDFR	KF960989.1	CACTGAGTTTAAGGGGATTCCTAAGG	CCCTTCTCCCTACAAGTGTCAATGG
VcANS	JN654701.1	CTTCATCCTCCACAACATGGT	GCTCTTGTACTTCCCATTGCTC
VcUFGT	AB694900.1	AGTTTGCTTTGAAGGCTGTTG	ATGTGCTGGTGTGCATTTG
VcBBX	KX300037.1	GAGGCCAACGTKCTRTGYTG	ACCCTCGGTGYTTBCYSGC
VcR2R3MYB	JQ085966	CGAGACCAAGGAACCGACCCG	ACTTTCTGCTTTCGGGCCTCCA
VcMYB21	KT225483.1	ACGGTGAAGGCTGTTGGCGT	TCTTCGGCAAAGTTGCCCCT
VcTTG1	KX447762	GAGCTGGAGAGGCACAGGGC	ATTGGGACCTGCCACCGTCG

Supplementary Table 2-2. Correlation between anthocyanin related genes in ‘Duke’ blueberry fruit after UV-B radiation

	VcR2R3MYB	*VcMYB21*	*VcTDR4*	*VcPAL*	*VcCHS*	*VcF3’H*	*VcDFR*	*VcANS*	*VcUFGT*	*VcBBX*
VcR2R3MYB	1									
VcMYB21	0.68^{ns}	1								
VcTDR4	-0.99^{**}	-0.99^{*}	1							
VcPAL	0.98^{ns}	0.55^{ns}	-0.98^{ns}	1						
VcCHS	0.99^{**}	0.67^{ns}	-0.99^{**}	0.98^{ns}	1					
VcF3’H	0.97^{ns}	0.99^{*}	-0.97^{ns}	0.92^{ns}	0.97^{ns}	1				
VcDFR	0.58^{ns}	0.99^{ns}	-0.57^{ns}	0.43^{ns}	0.57^{ns}	0.74^{ns}	1			
VcANS	0.28^{ns}	-0.96^{ns}	-0.29^{ns}	0.45^{ns}	0.30^{ns}	0.08^{ns}	-0.61^{ns}	1		
VcUFGT	0.99^{*}	0.61^{ns}	-0.99^{*}	0.99^{ns}	0.99^{*}	0.95^{ns}	0.50^{ns}	0.37^{ns}	1	
VcBBX	0.68^{ns}	0.99^{**}	-0.99^{*}	0.54^{ns}	0.67^{ns}	0.82^{ns}	0.99^{ns}	-0.54^{ns}	-0.99^{**}	1

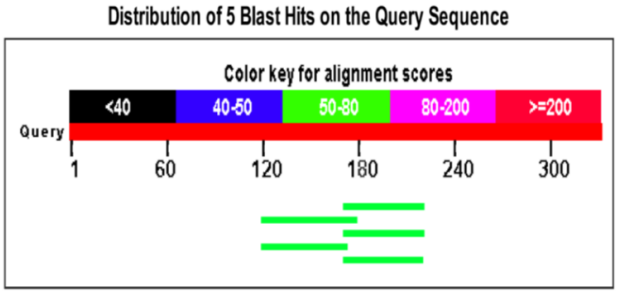
Supplementary Table 3-1. Primers used in qRT-PCR.

Gene	Accession number	Forward primer (5' to 3')	Reverse primer (5' to 3')
VcGAPDH	AY123769.1	GGTTATCAATGATAGGTTTGGCA	CAGTCCTTGCTTGATGGACC
VcPAL	AY123770.1	TTACAACAATGGGTTGCCCT	CCTGGTTGTGTGTCAGCACT
VcCHS	JN654702.1	CTTGACTGAGGAAATCTTGAAGG	AGCCTCTTTGCCCAATTTG
VcF3H	KP334104	GGGTTTTCCAGGTCGTCGAT	TATCGAACCGCAGCTTCTCC
VcDFR	KF960989.1	CACTGAGTTTAAGGGGATTCCTAAGG	CCCTTCTCCCTACAAGTGTCAATGG
VcANS	JN654701.1	CTTCATCCTCCACAACATGGT	GCTCTTGTACTTCCCATTGCTC
VcUFGT	AB694900.1	AGTTTGCTTTGAAGGCTGTTG	ATGTGCTGGTGTGCATTTG
VcR2R3MYB	JQ085966	CGAGACCAAGGAACCGACCCG	ACTTTCTGCTTTCGGGCCTCCA
VcMYB21	KT225483.1	ACGGTGAAGGCTGTTGGCGT	TCTTCGGCAAAGTTGCCCCT
VcTTG1	KX447762.1	GAGCTGGAGAGGCACAGGGC	ATTGGGACCTGCCACCGTCG
VcTDR4	KX263323.1	GGGGAGAGGGAGGGTKCAGH	AGCAACCTCAGCATCACANARVA
VcETR1	EU170628.1	GGTGCTTGTGCAGTTCGGCG	TGCACATGACACCACGGCAG
VcEIL4	KF319045.1	GCCCAACGGGCTGCAGAGAA	ACGGGCTTTGCACACCTCCA
VcACO	JQ062390.1	ACTACCCTCCGTGTCCCCGC	GCCGTCCTTGAGCAGCTGGA

Supplementary Table 3-2. Correlation between major genes in ‘Nelson’ blueberry fruit during ripening

	MYB21	*R2R3MYB*	*EIL4*	*TDR4*	*ANS*	*UFGT*	Ethylene production
MYB21	1						
R2R3MYB	0.99*	1					
EIL4	0.98^{ns}	-0.84^{ns}	1				
TDR4	-0.99*	-0.99*	-0.62	1			
ANS	0.99**	0.54^{ns}	-0.99*	-0.95^{ns}	1		
UFGT	0.99**	0.99^{ns}	0.98^{ns}	0.48^{ns}	0.95^{ns}	1	
Ethylene production	-0.54^{ns}	-0.83^{ns}	-0.99*	0.46^{ns}	-0.99*	0.99*	1

GAPDH

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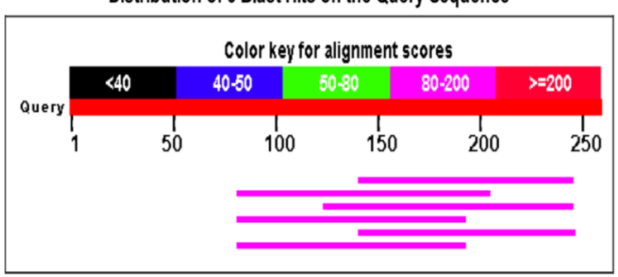
                121  GGTTATCAATGATAGGTTTGGCATCGTTGAGGGTCTTATGACTACCATCCACTCTATCAC  180
VcGAPDH_FW1      15                                                  .........   23
VcGAPDH_RV1      66  ................-......................A...G.T.....C.C...   8
VcGAPDH_FW2      17                                                  .........   25
VcGAPDH_RV2      65  ................-.....T.................A...G.-.....       14
VcGAPDH_FW3      18                                                  .........   26

VcGAPDH_FW1     181  CGCAACACAA-AAAACT-GTC-GAT-GGTCCATC-AAGCAAGGACTG  222
VcGAPDH_FW2      24  .....T...-....-.-...T...G........A............   67
VcGAPDH_FW3      26  G.....T...-....A.T..T-...-........--...........   67
                 27  ..........G..G.T.-...T...G........-...........   70
```

PAL

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VcPAL_FW1        82  TTTACAACAATGGGTTGCCCTCAAATCTCTCCGGCGGGCGCAACCCTAGCTTGGATTACG  141
VcPAL_RV1         2                                                          .    2
VcPAL_FW2       123  ...........................................G..T.........   64
VcPAL_RV2         7                                          ....G..T.........   24
VcPAL_FW3       125  ...........................................G..T.........   66
VcPAL_RV3                                                                   .
VcPAL_RV3       125  ...........................................G..T.........   66

VcPAL_FW1       142  GGTTCAAAGGCGCTGAAATAGCCATGGCGGCCTATTGCTCCGAGCTCCAGTTCCTGGCTA  201
VcPAL_RV1         3  ....T..G.....A.....................T.....................   62
VcPAL_FW2        63  ....T..G.....A.....................T..................-....   5
VcPAL_RV2        25  ....T..G.....A.....................T.....................   84
VcPAL_FW3        65  ....T..G.....A.....................T.....................   15
VcPAL_RV3         2  ....T..G.....A.....................T.....................   61
                 65  ....T..G.....A.....................T.....................   15

VcPAL_FW1       202  ACCATGTAACCAATCATGT-CAAAGTGCTGACACACAACCAGGAC  245
VcPAL_RV1        63  ...CA..............C.....................      106
VcPAL_FW2         4  ...                                            2
VcPAL_RV2        85  ...CA..............C.....................      128
VcPAL_FW3        62  ...CA..............C.....................      106
```
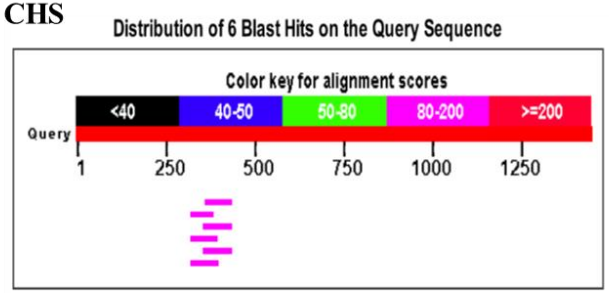

Supplementary Fig 2-1. Sequencing results of anthocyanin biosynthesis regulating genes

CHS

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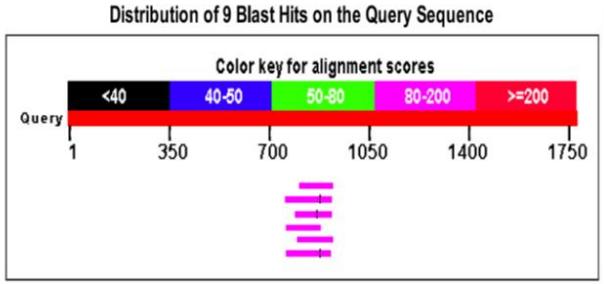
          320  CTTGACTGAGGAAATCTTGAAGGAGAACCCCAATGTGTGCGCGTACATGGCACCTTCCCT  379
VcCHS_FW1   8                                                 ......-.....  26
VcCHS_RV1  66  ......................A.....................................  7
VcCHS_FW2   4                                                 .........-...  26
VcCHS_RV2  77  ...C......T.T.........A.....................................  19
VcCHS_FW3   3                                                 .........-...  25
VcCHS_RV3  76  ......................A.....................................  17

          380  GGACGCTAGGCAGGATATGGTGGTTGTGGAAATTCCCAAATTGGGCAAAGAGGCT  434
VcCHS_FW1  27  ..................................................  81
VcCHS_RV1   6  ...                                                 4
VcCHS_FW2  27  ..................................................  81
VcCHS_RV2  18  .-..A...........                                    5
VcCHS_FW3  26  ..................................................  79
VcCHS_RV3  16  .-.....-.........                                   2
```

F3'H



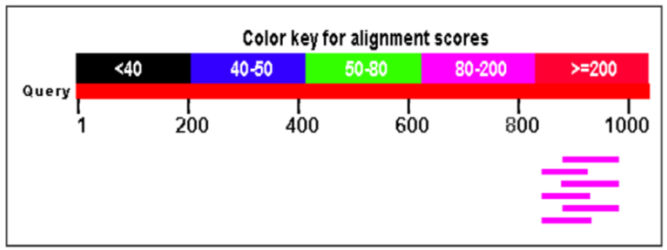

```
VcF3H_FW1  761  GCACTCGAGATTCGATGCGTTTCTGAGTGAGATTCTCGA-GGAGCATAAGGTGGGGTCCA  819
VcF3H_RV1    1                                                 .......-..  9
VcF3H_RV1  123  ..........................................-.....................  65
VcF3H_FW2  114                                     ......-.TGC.................  139
VcF3H_FW2    4                                                 ....-..  9
VcF3H_RV2  175                                     ..-.....................  154
VcF3H_FW3  120  ....................................-.....................  65
VcF3H_RV3    5                                                 ...C.......-..  17
VcF3H_RV3  114  ....................................-.....................  59
VcF3H_RV3  108                                     ......G..G.................  134

VcF3H_FW1  820  TTGGTGGTGGGGCCCAGAGTCACCACACTGATTTGTTGAGCACTTTGATTTCGCTCAAGG  879
VcF3H_RV1   10  ..........................---.....................................  66
VcF3H_RV1   64  ........................---.....................-.........  9
VcF3H_FW2  140  ..............C....CT.-.....---.....-.........-....C.A  191
VcF3H_FW2   10  ..........................---.....................................  66
VcF3H_RV2  153  ........................---...........A.........GT.....  104
VcF3H_RV2   64  ..........................---.....................-.........  9
VcF3H_FW3   18  ..........................---.....................................  74
VcF3H_RV3   58  ..........................---.....................-.........  3
VcF3H_RV3  135  ..................-.-.AT..-..T....--.-......GA.....-...C.A  186

VcF3H_FW1  880  AGGAGGATGATGGC-GAGGGAGGGAAGCTCACTGATACCGAAAT-CAAAG  927
VcF3H_RV1   67  ..............-..................C...........-.C...  114
VcF3H_RV1    8  ..                                                 7
VcF3H_RV1  192  .-..CA........-....A...-..-...C.-...-.......-...  232
VcF3H_FW2   67  ..............-..................C...........A...  113
VcF3H_RV2    8  .....                                              4
VcF3H_FW3   75  ..............-..................C...........-.C...  122
VcF3H_RV3    2  ..                                                 1
VcF3H_RV3  187  ..-...........C....A...-..-...C.-...-.......       225
```

Supplementary Fig 2-1. Continued

DFR

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VcDFR_FW1 843  CACTGAGTTTAAGGGGATTCCTAAGGATTTGCCAAATGTGTCGTTTTCGTCGAAGAAGTT  902
VcDFR_RV1   2                                              ..............-.-....  20
VcDFR_FW2  89  ..........................................................  31
VcDFR_RV2   3                                             ..............-.-....  23
VcDFR_RV2  90  ..........................................................  32
VcDFR_FW3   4                                             ..............-.-....  22
VcDFR_RV3  91  ..........................................................  32

VcDFR_FW1 903  GATAGGGATGGGGTTTCAGTTCAAGTACAGCTTGGAGGATATGTTCAGAGGAGCCATTGA  962
VcDFR_RV1  21  ..........................................................  80
VcDFR_FW2  30  ........................                                   7
VcDFR_RV2  24  ..........................................................  83
VcDFR_FW3  31  ........................                                   4
VcDFR_RV3  23  ..........................................................  82
VcDFR_RV3  31  ............................                               1

VcDFR_FW1 963  TACTTGTAGGGAGAAGGGA  981
VcDFR_FW2  81  C...................  99
VcDFR_FW3  84  C...................  102
VcDFR_RV3  83  C...................  101
```
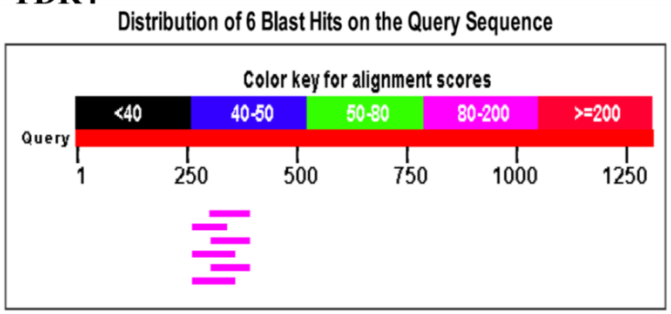
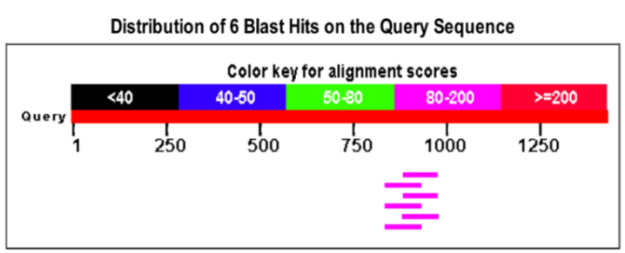

TDR4




```
VcTDR4_FW1 267  GGGGAGAGGGAGGGTGCAGATGAAGAGGATAGAGAACAAGGTGAGCAGACAAGTGACGTT  326
VcTDR4_RV1   9                                            ..A.........G........  30
VcTDR4_FW2  96  ..........................................................--  39
VcTDR4_RV2  12                                            ...........G........  31
VcTDR4_FW3  95  .....T............C.............................G......-.  37
VcTDR4_RV3   9                                            ...........G........  28
VcTDR4_FW3  98  ..........................................................--  41

VcTDR4_FW1 327  TTCGAAGCGGCGGAGCGGGCTGTTGAAGAAAGCGCATGAGATTTCAGTGCTGTGTGATGC  386
VcTDR4_RV1  31  ..........................................................T.....  90
VcTDR4_FW2  38  ...............                                              22
VcTDR4_RV2  32  ..............................................................  91
VcTDR4_FW3  36  ................-.A....--G....T.....                          4
VcTDR4_RV3  29  .....-........................................................  87
VcTDR4_RV3  40  ......................-....--G........                        7

            387  TGAGGTTGCT  396
VcTDR4_FW1  91  ..........  100
VcTDR4_FW2  92  ..........  101
VcTDR4_FW3  88  ..........  97
```

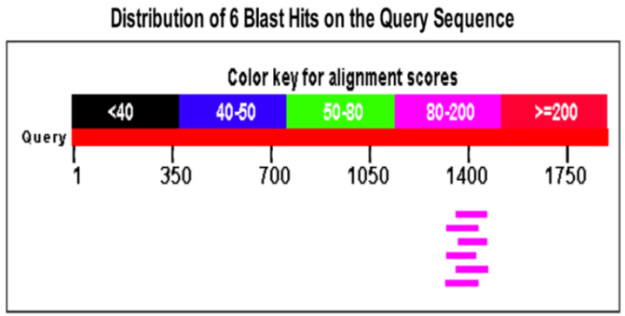
Supplementary Fig 2-1. Continued

ANS




```
VcANS_FW1   834  CTTCATCCTCCACAACATGGTCCCCGGCCTGCAACTCTTCTACGAGGGCAAATGGATCAC  893
VcANS_RV1    12                                                ...-.........   24
VcANS_FW2   103  ..........................................................   44
VcANS_RV2     5                                                ...-.........   17
VcANS_FW3   103  ..........................................................   44
VcANS_RV3     2                                                ..............   16

VcANS_FW1   894  AGCAAAATGTGTCCCTAACTCCATCATTATGCACATTGGCGACACGGTCGAGATTTTGAG  953
VcANS_RV1   103  ..........................................................   84
VcANS_FW2    43  ..............................-...........                    9
VcANS_RV2   103  ..........................................................   77
VcANS_FW3    43  ..............................-...........                    9
VcANS_RV3   103  ..........................................................   76
                 ..............................-...........                    9

VcANS_FW1   954  CAATGGGAAGTACAAGAGCA  973
VcANS_FW2    85  ....................  103
VcANS_FW3    78  ....................   96
VcANS_RV3    77  ....................   96
```

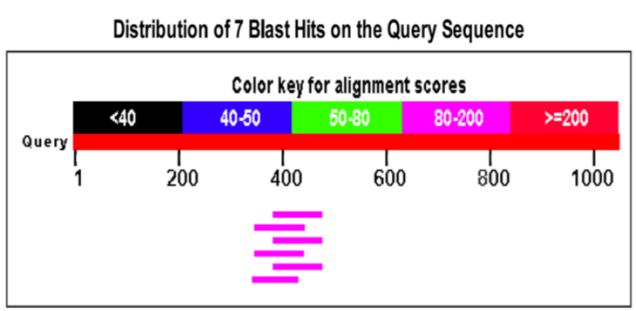
UFGT




```
            1319  GAGTTTGCTTTGAAGGCTGTTGGACCAAAAGGGAGATCAACTCAAAATTTAAACACATTG  1378
VcUFGT_FW1     4                                                .....T..--.....   23
VcUFGT_RV1   114  ..........................................................   57
VcUFGT_FW2    10                                                .......-.....   23
VcUFGT_RV2   113  ..........................................................   55
VcUFGT_FW3     4                                                .....T..--.....   23
VcUFGT_RV3   116  ..........................................................   57

            1379  CTGGAGTTAGTGAGAGGGTACAACATTTAGAAATGAACGCCGCTACGCGCACACCACATA  1438
VcUFGT_FW1    24  ..........................................................   83
VcUFGT_RV1    56  ..................................-.......................    3
VcUFGT_FW2    24  ..........................................................   83
VcUFGT_RV2    54  ........................................-....              8
VcUFGT_FW3    24  ..........................................................   83
VcUFGT_RV3    56  ..................................-...............C..       3

            1439  CGCGCACCCCAAATGCACACCAGCACATC  1467
VcUFGT_FW1    84  T.........................     110
VcUFGT_FW2    84  T.........................     110
VcUFGT_FW3    84  T.........................     112
```

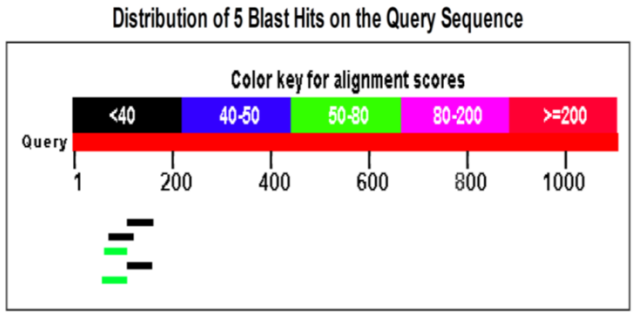
Supplementary Fig 2-1. Continued

R2R3MYB




```
                346  CTCCGAGACC-AA--GGAACCG-ACCCG-AGTACCC-ACAAAAAA-TTATCGGAA-TATC  397
VcR2R3MYB_FW1    10                                                 .......--....   20
VcR2R3MYB_RV1   101    .......-..--.......-.....-.......-........-.........-....   53
VcR2R3MYB_FW2     8                                                 .......--....   18
VcR2R3MYB_RV2   100    .......G..--.......-.....-.......-........-.........-....   51
VcR2R3MYB_RV3     8                                                 .......--....   18
                107  ...T......-G.AG.......G.....G.......T........A.........A....   49

                398  CTAACGACCAACCACCAAAGAAGAGGAGGAACAACAATAGAA-AGAAGAACAAGTCAAAT  456
VcR2R3MYB_FW1    21  ..........................................-..................   79
VcR2R3MYB_RV1    52  ........................-..............-.......               5
VcR2R3MYB_FW2    19  ...-.....................................-..................   76
VcR2R3MYB_RV2   623                                            ...........        613
VcR2R3MYB_FW3    50  ....................................-....G....                5
VcR2R3MYB_RV3    19  ..........................................-..................   77
                 48  .........T.................-........                          15

                457  TTGGAGGCCCGAAAGCAGAAAGTC  480
VcR2R3MYB_FW1    80  ........................  102
VcR2R3MYB_FW2    77  ........................  99
VcR2R3MYB_FW3    78  ........................  101
```

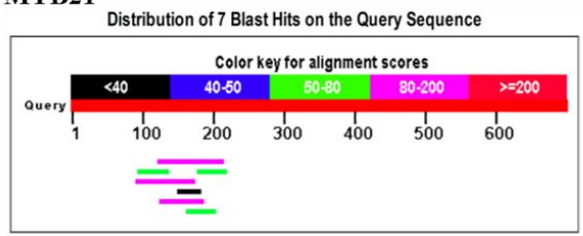
BBX




```
VcBBX_FW1    62  GCGGA-GGCCAACGTCCTCTGCTGCGCTGACGAGGCGGCGCTGTGCTGGGCGTGCGACGA  120
VcBBX_RV1    13                                                      ......T..   21
VcBBX_RV2    60              ..G..G.....T..G..T...........-.......-..-...-..   18
VcBBX_FW3    72     ..-.....-...G..G.....T..G..T...................            28
VcBBX_RV3     8                                                      ......T..   16
                74  .....T.........G..G.....T..G..T...................            25

               121  GAAGGTTCACAAAGCGAACAAGCTCGCGAGCAAACACCAGAGGGT  165
VcBBX_FW1    22  ..C--.....GCG..AT.T.....G..C.....-...........  63
VcBBX_RV1    17  ....                                           14
VcBBX_FW3    17  ..T.-.....GCG..-..T.....G..C..--...........    55
```

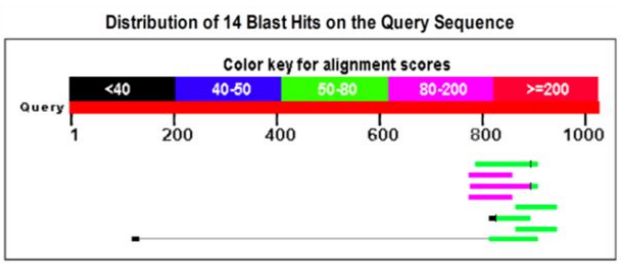
Supplementary Fig 2-1. Continued

MYB21



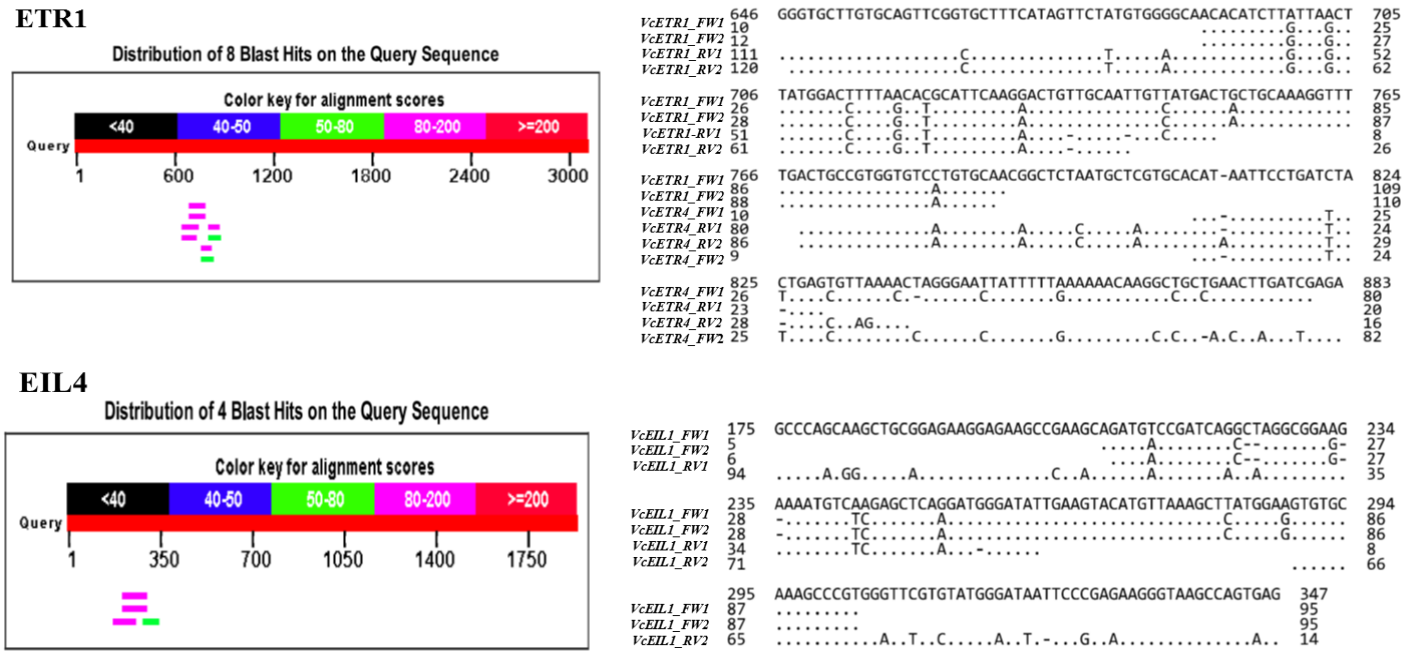

```
VcMYB1_FW1   92  ACGGTG-AAGGCTGTTGGCGTACCATCCCGCAGGCTGCAGGACTACTTCGTTGTGGTAAA  150
VcMYB1_FW1    8                                ..C........-..................   35
VcMYB1_RV1   48    ....T.........................................              3
VcMYB1_RV1  100  ......-.....................................................   42
VcMYB1_FW2   20                                                          .     20
VcMYB1_FW2   67                                        ........................  42
VcMYB1_RV2

             151  AGTTGTAGGCT-AAGATGGATTAATTACCTGAGACCAGACCT--CAAAAGGGGCAACTT-  206
VcMYB1_FW1    36  -----------.................................A-..............C  92
VcMYB1_FW1    33                                ..............--.............---  59
VcMYB1_RV2    41  ............A.............                                      17
VcMYB1_FW2    21  ............-.........--.A....G...                            50
VcMYB1_RV2    41  ............-.............-.....-......                       8
VcMYB1_FW2     9                  ..............................TC............   51

             207  TGCCGAAGATGA  218
VcMYB1_FW1    93  ........      101
VcMYB1_FW1    60  .C......T...  71
```

WD40 (TTG)



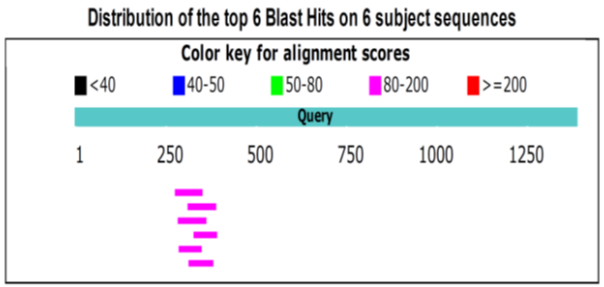

```
VcWD40_RV1  723  GGACAGCAATAAAGTTGTGATCTTGGATATCCGATCGCCGACGATGCCAGTGGCGGAGCT  782
VcWD40_FW2   97                                                        .....  92
VcWD40_FW2  199                                                        .....  195
VcWD40_RV2   97                                                        .....  92

VcWD40_FW1  783  GGAGAGGCACAGAGGTAGTGTGAATGCTATTGCTTGGGCCCCCCAGAGTTGTAGGCACAT  842
VcWD40_FW1   21                                                 .....A.CC....T..  37
VcWD40_RV1  185        .....G.CG.....C..C..G...........T..A......A.CC....T..  133
VcWD40_FW2   91  ...........G.CG.....C..C..G...........T..A......A.CC....T..  32
VcWD40_FW2  194  ...........G.CG.....C..C..G...........T..A......A.CC....T..  135
VcWD40_RV2   10                                 .........T.A.-.....A.CC....T..  38
VcWD40_RV2   91  ...........G.CG.....C..C..G...........T..A......A.CC....T..  32
VcTTG8_RV1   94                                          T..A.......CCC....T..  72
VcTTG8_RV1  323                                 .....................          311
VcTTG8_RV2  100                                 .......T..A.......CCC....T..  73

VcWD40_FW1  843  TTGCTCTGCTGGGGATGACACTCAGGCGCTTATTTGGGACCTGCCCACGGTCGCTGGGCC  902
VcWD40_FW1   38  C..T..............TG.G..A..A..C.........GT....G.....G..A..T..  97
VcWD40_FW1  132  C..T..............TG.G..-C.A..CGA..........-....C....          82
VcWD40_RV1   31  C..-..............                                             15
VcWD40_FW2  134  C..T..............TG.G.....A..C.A..........-....C....          83
VcWD40_FW2   39  C..T..............TG.G..A..A..C.........GT....G.....G..A..T..  98
VcWD40_RV2   31  C..T..............                                             14
VcTTG8_FW1   21                              ..A..C.........GT....G.....G..A..T..  55
VcTTG8_FW1   58                                   ..........-....C....C...       40
VcTTG8_FW1   71  C..T..............TG.G..A..A..C.........GT....G.....G..          18
VcTTG8_RV1   15                              ..A..C.........GT....G.....G..A..T..  49
VcTTG8_FW2   52                                   ..........-....C....           34
VcTTG8_FW2   72  C..T..............TG.G..A..A..C.........-G....G.....G..-A.T..  15

VcWD40_FW1  903  GAATGGAATCGACCCCATGTCGATGTACTCCGCAGGTGCGGAGA  946
VcWD40_FW2   98  C...C..                                      104
VcWD40_FW2   99  C...T..                                      105
VcTTG8_FW1   56  C.....G..T..T..A.................T..GT.C....  99
VcTTG8_FW2   50  C.....G..T..T..A.................T..GT.C....  93
VcTTG8_RV2   14  C......                                     8
```

Supplementary Fig 2-1. Continued



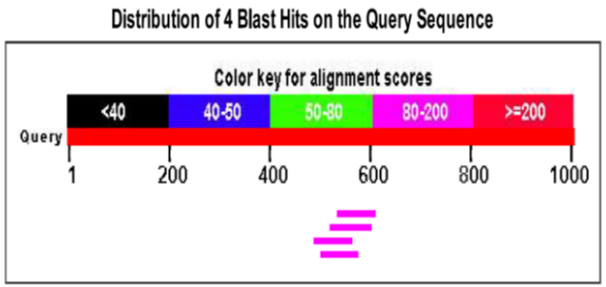
Supplementary Fig 3-1. Sequencing results of ripening related genes

F3H




```
                276  GGGTTTTCCAGGTCGTCGATCACGGCGTCGACGCCGGCCTTATTTCCGATATGACTCGAT  335
VcF3H_RV3       81  .....................................-.................-  24
VcF3H_FW3        9  ...................................T..G.A...T.............  34
VcF3H_RV2       72  ..................................-.................-  24
VcF3H_FW2       23  ..................................................-  31
VcF3H_RV1       70  ..............................-.................-  23
VcF3H_FW1       11  ..................................G.....T.............  32

                336  TGGCTCGGGAGTTCTTTGCCTTGCCGCCGGAG-GAGAAGCTGC-GGTTCGATA  386
VcF3H_RV3       23  .......A..                                            14
VcF3H_FW3       35  ................................-........C.AT......  84
VcF3H_RV2       23  ...........-.....C..                                  5
VcF3H_FW2       32  ....................C.......A...-........C.-.T.......  82
VcF3H_RV1       22  ........                                              15
VcF3H_FW1       33  ....................C.......A...AT......              72
```

ACO




```
                488  ACTACCCTCCGTGTCCTCGGCCAGAGCTGATCAAGGGTCTCCGAGCCCACACTGACGCCG  547
VcACO_FW1       13                                                .........T....  26
VcACO_FW2        6                                      .........-.T...-.......T....  31
VcACO_RV1      106  ................C..C..G.....................T...........T....  47
VcACO_RV2       76  ..............C..C..G.....................T...........T....  31

                548  GTGGCATCATCCTCCTCTTCCAAGACAACAAGGTCAGCGGACTCCAGCTGCTCAAAGATG  607
VcACO_FW1       27  ....TC.......T............G.............................G..C.  86
VcACO_FW2       32  ....TC.......T............G..............C......A.....      86
VcACO_RV1       46  ...T.-..-........                                          32
VcACO_RV2       30  ....TC.......-...-.G........                              5

VcACO_FW1      608  GC  609
               87   ..  88
```

Supplementary Fig 3-1. Continued

Supplementary Table 3-3. Identity of studied gene fragments from highbush blueberry fruit compared to published sequences.

Genes	Accession number	Identities
VcGAPDH	AY123769.1	95 %
VcPAL	AY123770.1	94 %
VcCHS	JN654702.1	99 %
VcF3H	KP334104	93 %
VcDFR	KF960989.1	100 %
VcF3'H	AB694901.1	95 %
VcANS	JN654701.1	100 %
VcUFGT	AB694900.1	98 %
VcR2R3 MYB	JQ085966	99 %
VcMYB21	KT225483.1	98 %
VcTTG1	KX447762.1	83 %
VcTDR4	KX263323.1	99 %
VcBBX	KX300037.1	87 %
VcETR1	EU170628.1	91%
VcEIL4	KF319045.1	88%
VcACO	JQ062390.1	88%