INVESTIGATING MECHANISMS THAT REGULATE RIPENING AND POSTHARVEST FRUIT QUALITY IN BLUEBERRIES

by

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(Under the Direction of Savithri U. Nambeesan)

ABSTRACT

Blueberry (*Vaccinium* spp.) is an important crop for the United States, and the consumption of this fruit has been increasing due to its popularity as a nutraceutical. In spite of the increase in blueberry production, there exists a gap in knowledge regarding factors that regulate ripening and postharvest fruit quality. Blueberry ripening is non-uniform during the growing season, and requires multiple harvests resulting in higher production costs. Besides, the postharvest shelf-life of blueberry is only between 1-6 weeks after harvest. In order to 1) synchronize and manipulate blueberry ripening and 2) generate information for maintaining postharvest fruit quality, we would like to investigate the mechanisms involved in ripening and postharvest fruit quality in the four studies below. The first study investigated the respiration and ethylene production patterns during fruit ripening and the response of ethephon (ethylene releasing agent) application on ethylene biosynthesis in southern highbush and rabbiteye blueberry. Our data has shown that southern highbush and rabbiteye blueberry display an increase in the rate of respiration and ethylene evolution characteristic of climacteric behavior. However, blueberry fruit did not display auto-catalytic ethylene regulation during ripening. The second study explored the effect of three plant growth

regulators, ethephon, abscisic acid (ABA), and methyl jasmonate (MeJA), on rabbiteye blueberry ripening and postharvest fruit quality. The results indicate that ethephon but not ABA or MeJA promotes fruit ripening by increasing the proportion of blue (ripe) fruit with limited effect on postharvest fruit quality. The third study further investigated the mechanism of ethylene-induced ripening in blueberry by RNA-seq, a technique that reveals global changes in gene expression. The results show that ethylene initiates ripening mainly by downregulating photosynthesis-related genes and regulating hormone and cell wall modification genes. In the last study, to understand the mechanism that controls postharvest fruit quality in blueberry, RNA-seq was conducted using southern highbush cultivars with superior and inferior postharvest fruit quality during storage. The data reveal several processes that may play essential roles during postharvest storage, such as cytoskeletal and cell wall changes, accumulation of important amino acids, and water loss. In sum, the research has generated information that can be applied to the blueberry industry immediately and expanded the basic knowledge in blueberry ripening and postharvest fruit quality.

INDEX WORDS: blueberry, ripening, respiration, ethylene biosynthesis, plant growth regulators, ethephon, postharvest, fruit quality, RNA-Seq

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DEDICATION

This work is dedicated to my beloved parents, Chun-Yung Wang and Kuei-Chiao Liu, and my dear sister, Yi-Lan Wang.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Blueberry is native to North America and belongs to the *Ericaceae* family which also includes crops such as cranberry, lingonberry, and azalea [1]. Blueberries are considered as “super fruits” due to thier high antioxidant capacity [2]. Some health benefits of blueberry consumption include decreased cardiovascular risk, improved cogitative performance, and decreases in aging-related damage [3]. Thus, the demand for blueberries is increasing. The total utilized production of blueberries in the United States was 21% higher in 2019 compared with 2018 at 673 million pounds. In 2019, area harvested was around 102,700 acres [4]. The top producing states include Oregon, Georgia, California, Washington, Michigan, New Jersey, and Florida. In Georgia, blueberries are among the leading fruit crops with a farm gate value of over $300.4 million [5] and with current cultivation in over 30,000 acres.

The blueberry species of commercial importance include lowbush (*Vaccinium angustifolium* Ait.) and northern highbush (*Vaccinium corymbosum* L.), these are cultivated in the northern parts of the United States, and rabbiteye (*V. virgatum* Ait.) and southern highbush (hybrids of *V. corymbosum, V. virgatum, and V. darrowii* Camp.) cultivated mainly in the southern states including Georgia [6]. The adaptability of blueberry cultivars to certain regions is dependent on their chilling requirements for floral bud development and cold hardiness. Typically, lowbush, northern highbush, rabbiteye and southern highbush require around 1000, 800-1000, 600, and 550 chilling hours respectively [7]. Whereas lowbush types can tolerate

temperatures below -30 °C, and northern highbush up to -20 °C, both rabbiteye and southern highbush cannot tolerate temperatures much below freezing [7].

Blueberry fruit display double sigmoidal growth, which can be divided into three stages. This is similar to growth patterns observed in stone fruits such as peaches [8], and in grapes [9]. Fruit growth in Stage 1 begins after fruit set and mainly occurs due to cell division. Stage II, which is a lag phase does not involve substantial increase in fruit size. During this stage, although growth is retarded the endosperm and embryo continue to mature within the seed. This is followed by Stage III were fruit growth resumes mainly due to cell expansion. Towards the end of this stage ripening is initiated. Depending on cultivar and environmental factors fruit development and ripening can take 42–90 days in northern highbush, 55-60 days in southern highbush and 60-135 days in rabbiteye [7,10,11]. During ripening, individual berries within clusters do not ripen very uniformly. Therefore berries are usually harvested during a two to three week window during the growing season [12].

Ripening behavior in fruits

The ripening physiology in fruits is typically classified into two types. Some fruits such as tomato, banana, and apple are classified as climacteric fruit, whereas grape and strawberry are classified as non-climacteric fruit. In climacteric fruit, during the ripening phase a peak in respiration and a burst in ethylene production are observed. Ethylene production in climacteric fruits is necessary for triggering ripening-related changes. However in non-climacteric fruits, the climacteric respiratory peak is not discernable and the role of ethylene is not clearly understood [13]. Further some recent studies suggest the role of other phytohormones such as abscisic acid (ABA) in facilitating ripening in climacteric and non-climacteric fruits [14]. The ripening

physiology in blueberries is controversial. A few studies have indicated an increase in respiration and ethylene during ripening in blueberry suggesting a potential climacteric nature to the ripening process [12,15,16]. However, another study has classified blueberry as a non-climacteric fruit that does not show a substantial climacteric rise in respiration or ethylene evolution [17]. In spite of two different ripening characteristics in fruit, all fruit undergo similar biochemical changes during ripening. These changes include, fruit softening, accumulation of sugars and pigments, decrease in fruit acidity and susceptibility to postharvest pathogens. At harvest these biochemical changes are important determinants of fruit quality attributes. Further these biochemical changes continue after harvest and lead to decrease in fruit quality during postharvest storage. Below are discussed some ripening related changes that affect fruit quality attributes and may also be important during postharvest storage.

Fruit softening related changes

Fruit softening mainly occurs due to re-organization and disassembly of the cell wall and middle lamella. Cell walls of fleshy fruit tissues are composed of three main components which include cellulose, matrix glycans, pectins, and structural proteins. The middle lamella is mainly composed of pectins [18,19]. Cellulose is made up of chains with repeating units of D-glucose linked together via β-1,4-glycosidic bonds. Several such chains are connected to each other via hydrogen bonding to form the cellulose microfibril [20]. Matrix glycans are also referred to as hemicellulose or cross-linking glycans. The structure of hemicellulose is more complex with multiple sugars such as glucose, xylose, fucose and mannose participating in its structure. One of the more common hemicelluloses, xyloglucans that consist of D-glucose and xylose units, interacts with cellulose microfibrils, and together the cellulose-hemicellulose network imparts

tensile strength to the cell wall [21]. Pectins are the most complex polymers mainly made up of D-galacturonic acids. They consist of three types, homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II, with the former being the most predominant [19,22]. During ripening several cell wall related changes occur which include depolymerization of matrix glycans, and solubilization and depolymerization of pectins with some neutral sugars that are stripped from the side chains associated with pectins [18,23]. As such only slight changes occur in cellulose content during ripening. On the other hand, hemicellulose depolymerization is thought to play an important role in fruit softening. In fruits such as peach, this process plays a significant role in ripening compared with apple or other fruits such as strawberries [24-26]. In addition to depolymerization of matrix glycans, solubilization of pectin is an important event during ripening. In fact, fruits that have a higher crisp texture such as apple or watermelon show less pectin solubilization compared with fruit that display a softer melting texture such as tomato, peach, blackberry, strawberry, avocado and kiwi fruit [27]. Later during fruit ripening, depolymerization of pectin is evident, however the extent of these changes again differs among fruits. Fruit such as tomato and peach undergo greater depolymerization compared to fruit such as strawberry and apple [23,24,28].

The coordinated action of many enzymes that are involved in cell wall degradation bring about the above changes. These enzymes belong to large gene families with certain members displaying temporal regulation during fruit ripening [18,23]. Some of these enzymes are involved specifically in xyloglucan depolymerization. Of these, xyloglucan transglycosylase/hydrolase (XTH) enzymes may aid in maintaining cell wall integrity (transglycosylase activity which involves joining of xyloglucan) or in its loosening (xyloglucan degradation). These two classes of XTH enzymes have been shown to prevail during fruit ripening in peach and apples.

Nevertheless, the role of XTH is evident in fruit softening in persimmon [29,30]. Another enzyme, β-Mannosidase plays a role similar to XTH during fruit ripening and displays high activity during fruit ripening [31,32]. Similarly, expansins (EXP) facilitate cell wall modifications by loosening hydrogen bonding between cellulose and xyloglucans. Silencing of the tomato *SlEXP1* results in a slight increase in fruit firmness. However, overexpression of this gene was associated with xyloglucan depolymerization and increased softening [33]. Several enzymes such as pectate lyase polygalacturonase (PG), pectin methyl esterase (PME) and β Galactosidase (βGAL) are involved in changes associated with pectin solubilization and depolymerization [22]. Downregulation of *Pectate Lyase* and *PME* in strawberries result in increased firmness [34,35]. Expression of *PME* has been reported during strawberry ripening and has plays a role in maintaining tissue integrity during postharvest storage in tomato [36,37]. Downregulation of *βGAL* gene, *TBG4* in tomato and the *βGal4* in strawberry resulted in firmer fruit [38,39]. However due to the differences in complexity of cell wall components among fruits, and the coordinated action of many enzymes associated with cell wall loosening during ripening, it is unlikely that a single enzyme or process serves as a key regulator of fruit softening across fruits.

The events of cell wall disassembly during ripening were investigated during ripening in the blueberry cultivars, Duke and Bluecrop. Solubilization of pectin occurs during early ripening stages and is associated with higher activity of enzymes that aid in pectin solubilization such as PG, α-arabinofuranosidase, and β-GAL [40,41]. Further depolymerization of pectin is moderate whereas depolymerization of hemicellulose occurs throughout ripening and is correlated with reduction in fruit firmness and higher endo-1,4-β-xylanase activity during this period [40,41]. When cell wall associated changes were investigated during postharvest storage of blueberries cultivar, Brilliant, cellulose and hemicellulose content were found to decline with increase in

pectin solubilization. Also, enzymes activities of cellulose, α-Mannosidase, β-galactosidase, and polygalacturonase decreased during storage. During postharvest cold storage, degradation of cell wall components and associated enzyme activities were lower at 5 °C compared with 10 °C, suggesting that cold storage may enhance postharvest shelf life by inhibiting of processes involved in cell wall loosening [42].

Accumulation of pigments

During fruit ripening accumulation of various pigments render the fruit visually attractive. Evolutionarily this change is thought to be important for attracting birds and insects that help with seed dispersal. However, in the recent past pigments have received greater attention owing to their antioxidant properties [43]. Commonly two pigments accumulate, fat soluble carotenoids in fruits such as tomatoes and watermelon and water-soluble anthocyanins in strawberries and blueberries [44,45]. Anthocyanins are polyphenolic compounds that are glycosylated and normally accumulate in the vacuole. These compounds can exhibit color ranging from blue, purple, orange and red depending on the vacuolar pH, the position and number of hydroxyl groups and their methylation status [43,45]. Generally, there are six main anthocyanin compounds of which pelargonidin, cyanidin, delphinidin are the primary anthocyanidins. Peonidin is derived from cyanidin, whereas petunidin and malvidin are derived from delphinidin [46]. Further, anthocyanins can be linked to various sugars such as glucose, galactose, and arabinose, and acyl groups. These conjugations increase the diversity of the types of anthocyanins [45]. In blueberries, the predominant anthocyanin types include delphinidin, cyanidin, petunidin, peonidin and malvidin, conjugated to galactose, glucose and arabinose [47]. In general, anthocyanin content can vary with environmental conditions, with high light and low

temperature increasing anthocyanin accumulation [48]. However, some studies suggest that anthocyanin content in blueberry varies mainly due to cultivar differences rather than yearly fluctuations associated with the climatic conditions [47]. A study involving 42 blueberry cultivars in which 36 of them were rabbiteye, three hybrid derivatives of rabbiteye and three northern highbush suggested variation in various anthocyanin content and antioxidant activities [49].

The biosynthesis of anthocyanins occurs via the phenylpropanoid pathway [48]. The pathway is well conserved in angiosperms. A complex of three transcription factors, MYB-bHLH-WD40 play a role in anthocyanin biosynthesis [50]. In blueberries, 11 MYB, seven bHLH and sixWD40 members of these gene were identified, and the expression patterns for some of them correlated with anthocyanin biosynthesis [51]. Further, in blueberry cv. Bluecrop, RNA sequencing identified, 42 transcripts encoding 12 genes involved in the anthocyanin biosynthesis pathway. Of these, 25 transcripts belonging to the anthocyanin biosynthesis pathway were upregulated during ripening [52]. Further, enzymes involved in the anthocyanin biosynthesis pathway were upregulated after abscisic acid application [52].

Sugar metabolism during ripening

Fruit flavor is an important fruit quality attribute and relies on compounds that are water soluble which include sugars and organic acids [53]. Generally total soluble solids content measured using a refractometer is a proxy for total soluble sugars, however different sugars impart a different index of sweetness. For example, the sweetness index of fructose is 1.7-fold, glucose is 0.8-fold and sorbitol is 0.6-fold compared with that of sucrose [54]. In ripe blueberry fruit, among 15 carbohydrates that were detected, glucose, fructose, and galactose were present in high abundance [55]. During early fruit development, fleshy fruit are capable of

photosynthesis, however the level of photosynthesis is not sufficient to sustain fruit carbon requirements. Generally, fruit photosynthesis is capable of providing 10-20% to total fruit carbon [56]. However, when photosynthesis was downregulated in tomato fruit it did not result in decrease of fruit weight or primary metabolites indicating that under normal growth conditions, fruit photosynthesis may be dispensable [57]. Thus, most of the fruit carbon requirement is supported by leaf photosynthesis. In many species the major translocated carbon is sucrose, although in certain Rosaceae species sorbitol is translocated in the phloem [58]. In the blueberry leaf, glucose (~4-8 $mg \cdot g^{-1}$), fructose (2-7 $mg \cdot g^{-1}$), galactose (0.6-3 $mg \cdot g^{-1}$), and sucrose (~0.4-2 $mg \cdot g^{-1}$) were among the most abundant sugars present [55]. Translocated sucrose is broken down by two enzymes, sucrose synthase involved in the reversible conversion to fructose and UDP glucose, and invertase which is irreversible and generates glucose and fructose. These broken-down sugars in fruit are used to fuel several metabolic pathways including production of primary metabolites and complex carbohydrates as well as in respiration-related pathways involved in energy production. Further, UDP-glucose can be utilized to synthesize starch in developing fruits [54,59,60]. During ripening, starch degradation results in sugar accumulation and provides a source of energy for the ripening process. Starch breakdown is thought to contribute to the climacteric rise in respiration [61]. However, in climacteric fruit such as papaya and melon, storage of starch is not evident during fruit growth and therefore other carbon sources may be associated with the respiratory peak [62]. In comparison to climacteric fruit, fewer studies have addressed respiratory metabolism associated with non-climacteric fruit. Some commonalities have been observed between climacteric and non-climacteric fruit in relation to various intermediates in the glycolysis and TCA pathway, which are linked to respiration [63,64]. However, there is a more recent theory that starch accumulation in developing fruit is more

characteristic of climacteric fruit. This starch reserve is broken down into sugars during ripening and may allow for early harvest in climacteric fruit. In non-climacteric fruit, starch content decreases after anthesis and only soluble sugars accumulate during fruit growth and development, and therefore can be harvested only when fully ripe [65]. Particularly in blueberries, carbohydrate import into the fruit is dependent on leaves since there is no buildup of starch for continuous ripening. Further fruit may not be a major sink tissue since labelled carbon studies showed similar distribution in stem, roots and fruit [66]. Thus, in future it may be interesting to explore molecular events involved in starch and sugar metabolism during blueberry fruit growth and development.

Acid metabolism in fruit ripening

During fruit ripening there is a decrease in organic acid accumulation, thus displaying an inverse relationship with sugar levels [67]. Fruit titratable acidity is measured with a titrator using a base such as NaOH and is usually a composite measurement of all acids present in fruit. Some organic acids that are present in higher quantities in fruit include oxalic, quinic, malic, citric, isocitric, galacturonic, and tartaric acid [68]. As such, depending on the species or cultivar, the total organic acid amount and the comparative abundance of various organic acids can differ. The organic acids can also differ in response to several environmental factors such as temperature, water supply and management practices such as pruning that affects source to sink ratio [68,69]. Further, depending on the fruit a characteristic acid predominates, for example in apples and pear it is malic acid, in grape the major acids include malic and tartaric, and in citrus fruit it is citric acid [69,70]. In the blueberry cultivar Bluecrop, the predominant acids were citric acid (77-87%), quinic acid (11%) and malic acid (4%). In addition, minute quantities of succinic,

tartaric and shikimic acids were also detected [71]. In spite of the predominance of one particular kind of acid in fruit, generally malate and citrate are present in high amounts in both climacteric and non-climacteric fruit [69,70]. During the peak of respiration, some climacteric fruit use malate as a substrate, whereas in others, malate accumulation continues during ripening [72]. The organic acids can serve as intermediates in many metabolic pathways. These include amino acid synthesis, plant hormone synthesis such as that of auxins, gibberellins and salicylic acids, fatty acid synthesis, generation of various secondary metabolites and components of cell walls [68]. Further, certain intermediates in the sugar and acid metabolism such as galactinol, raffinose, trehalose and malate are associated with increasing postharvest shelf life in tomatoes and plum [73,74].

Plant hormones in fruit ripening

As mentioned earlier, ethylene is an important hormone mediating biochemical changes that occur in climacteric fruit. Further, more recent data has indicated ABA to be a positive regulator and auxin to be negative regulator of ripening in climacteric and non-climacteric fruit. Below, the role of some hormones that are important in ripening are discussed.

Ethylene in fruit ripening

Ethylene is synthesized from S-adenosyl methionine in a two-step process. S-adenosyl methionine is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS). ACC is then converted to ethylene via ACC oxidase (ACO) [75]. In tomato, both these enzymes are encoded by genes that belong to large gene families. Two systems of ethylene synthesis have been indicated [76]. In system 1, which occurs in leaves and young developing

fruit, ethylene inhibits its own production by inhibiting the ethylene biosynthesis enzymes ACS and ACO. During the transition phase to fruit ripening there is a switch from system 1 to system 2, where ethylene biosynthesis is autocatalytic by inducing expression of both biosynthesis enzymes. For example, in tomato fruit, the expression of genes coding for ACS, *ACS1A*, *ACS3*, *ACS6* is associated with system 1 and *ACS2*, *ACS4* are associated with system 2. Transcripts coding for ACO, *ACO1* and *ACO4* are associated with system 1 and 2 [77].

Once ethylene is produced it is sensed by ethylene receptors which are negative regulators of ethylene signaling. In the absence of ethylene, ethylene receptors present on the membrane of the endoplasmic reticulum activate CONSTITUTIVE TRIPLE-RESPONSE1 (CTR1). The CTR1 gene encodes a protein kinase that inhibits the action of ETHYLENE INSENSITIVE 2 (EIN2) by signaling its degradation. EIN2 belongs to a family of metal transporters and has two domains, a N-terminal transmembrane domain and a C-terminal cytoplasmic domain. When EIN2 is inactivated by CTR1, downstream ethylene signaling proteins, EIN3/ethylene insensitive3-like (EIN3/EIL) transcription factors are also degraded. Thus, in the absence of ethylene, the ethylene receptors turn off ethylene signaling. When ethylene is synthesized, it binds to its receptors which then is thought to inactivate CTR1. Thus, the inhibition of EIN2 by CTR1 is relieved. EIN2 undergoes processing with its C-terminal cytoplasmic domain being cleaved. This domain of EIN2 has two roles: 1. it binds to proteins that inhibit EIL transcription factors, thereby stabilizing EILs; 2. The C-terminal of EIN2 also posseses a nuclear localization signal, which helps it to translocate to the nucleus and positively regulates EIN3 and related EIL1 transcription factors which in-turn activate another set of transcription factors known as ETHYLENE RESPONSE FACTORS (ERFs). ERFs can now activate ethylene response genes [78,79]. In the tomato genome, there are 77 ERFs that can be

divided into various subfamilies. Some of these transcription factors respond to ethylene and may be positive or negative regulators in the ethylene signaling pathway [79,80].

Ethylene can induce the expression of cell wall loosening enzymes such as polygalacturonase in tomatoes and apples [81-83]. In non-climacteric fruit such as strawberry, ethylene can regulate PG and βGAL activities, but ethylene may not influence the expression of the EXPANSIN coding gene *FaEXP2* [84,85]. Further ethylene promotes fruit color in climacteric fruits such as apple and in non-climacteric fruit such as strawberry [85,86]. In apple, MdEIL1 increased the transcript abundance of *MdMYB1* that regulates anthocyanin biosynthesis. Further MdMYB1 increased the expression of *MdERF3*, that positively regulates ethylene synthesis, suggesting a positive loop between anthocyanin accumulation and ethylene biosynthesis [86]. In pears, ERFs promote the interaction of two transcription factors MYB and bHLH to promote anthocyanin biosynthesis [87]. In tomato, one member of the ERF subfamily, SlERF6 is a negative regulator of ethylene production and lycopene accumulation in tomato [88]. As such there is little evidence of a direct link between ethylene and changes in soluble sugars and organic acids (Gao et al., 2020). In grape, treatment with ethylene inhibitor, 1-methylcyclopropene (1-MCP) inhibited expression of genes related to sugar transport [89]. Further, ethylene can positively influence aroma development in banana and strawberry [76,80].

ABA in fruit ripening

ABA is another important plant hormone that plays a role in seed dormancy and during abiotic stress responses. More recently an important role for this hormone in fruit ripening is emerging [14]. Especially in non-climacteric fruits where the importance of ethylene in ripening is not clear, ABA has been shown to facilitate the ripening process. External application of ABA

has shown to enhance red coloration of fruit in grape. In one case, application of ABA after veraison promoted color development, however it did not affect fruit firmness, pH, TSS or TA at harvest [90]. Further ABA application did not change the concentrations of primary metabolites such as glucose, fructose, arginine, and alanine, suggesting the role of ABA in mainly influencing the anthocyanin metabolism related genes [90]. Similarly, in another study, ABA application improved fruit color in grapes without affecting fruit quality attributes such as firmness and fruit weight, 30 and 60 days after postharvest storage [91]. These results suggest that early harvest in grapes is possible with ABA application. In strawberries, when the ABA biosynthesis gene 9-cis-epoxy-carotenoid dioxygenase, NCED was down regulated, fruit color development in strawberries was inhibited [92]. Further, the authors also found that sucrose can stimulate ABA production and may be involved in facilitating ABA-regulated ripening in strawberries [92]. More recently ABA has been shown to play a role in climacteric fruit ripening. In tomato, ABA concentration increased and preceded the climacteric rise in ethylene (Zhang et al., 2009). Further, application of ABA accelerated ripening and increased the transcript abundance of certain members of the ethylene biosynthesis gene families which included *ACS* and *ACO,* suggesting that ABA may play a positive role in coordination with ethylene during tomato fruit ripening [93,94]. These data suggest that ABA plays a role upstream of ethylene in facilitating ripening. Further when ethylene signaling was blocked using 1-MCP, ABA accumulation was delayed compared to that in the control and ABA treatments alone, suggesting that ethylene may regulate ABA accumulation [93]. Thus, there seems to be a crosstalk between these two hormones, at least in a climacteric fruit to influence progression of ripening. In bilberry, which is closely related to blueberry, ABA concentration increases during ripening, and application of ABA promotes pigment development by increasing the expression of anthocyanin

biosynthesis related genes [95,96]. Similar results with increase in ABA concentration during blueberry ripening have been reported [97]. External foliar application of 600 and 100 ppm of ABA increased the proportion of pink fruit (surface color was 75 to 100% pink) but not ripe (blue) fruit. However, at the rate of 1000 ppm, leaf phytotoxicity was noted [98]. When fruit clusters were dipped in 1000 ppm ABA, it promoted pigment accumulation along with increase in the transcript abundance of genes related to the anthocyanin biosynthesis pathway [52]. These authors suggested that ABA positively regulates anthocyanin accumulation [52].

Auxin in fruit ripening

In both climacteric and non-climacteric fruit auxin concentration decreases during ripening. In grape, free IAA is high at anthesis and declines dramatically during ripening initiation. At the same time the conjugated form of IAA-Asp increases during ripening in grapes. Conjugated IAA is thought to reduce free IAA levels [99]. In grapes, auxin content is higher in seeds compared with the pericarp and auxin content is higher in fruit with high seed weight to berry weight ratio (indicating less ripe fruit) and low auxin content with low seed weight berry weight ratio (more ripe fruit) [100]. Free IAA levels decreased during fruit ripening in strawberry when whole fruits were used [101]. When IAA levels were measured in the two tissues, IAA concentration was higher in the achenes compared to the receptacle, however the levels remained similar with ripening [102]. Removal of achenes can accelerate ripening, which has been attributed to decrease in auxin and auxin application delays ripening suggesting that auxin is a negative regulator of fruit ripening [103]. In tomatoes both IAA and IAA-Asp are high during early fruit development; whereas free IAA decrease during fruit ripening, IAA-Asp increase during ripening [99]. In banana, application of IAA delayed starch degradation. Even with normal

ethylene levels breakdown of starch levels occurred when free IAA concentrations reached about 4 $ng \cdot g^{-1}$ with an increase in IAA-amide conjugation [104]. In strawberry, a fruit transcriptome study indicated that auxin and ethylene were the two main groups of hormones for which associated transcripts were over-represented during ripening in the achenes and receptacles [105]. Transcriptome analysis in tomato revealed that application of auxin delays ripening by delaying ethylene production [106]. Thus, the crosstalk between auxin and ethylene is also important to regulate fruit ripening.

Brassinosteroids and jasmonic acid in fruit ripening

Overall there is limited information on brassinosteroids and jasmonic acid in blueberry fruit ripening. Brassinosteroids positively influence ripening in tomato, strawberry and grape [107-109], but their role in blueberry ripening remains unknown. Jasmonic acid has been indicated to be involved in ripening initiation *via* cross talk with ethylene in apple and tomato [110]. At later ripening stages, the effect of jasmonates on ripening has been demonstrated to be inhibitory [110]. But, methyl jasmonate induces ripening-related genes in strawberries [111]. In blueberry, consistent effects of methyl-jasmonate application on ripening were not observed [98].

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CHAPTER 2

PHYSIOLOGY OF RIPENING IN BLUEBERRIES[1]

[1] Yi-Wen Wang, Tej Prasad Acharya, Hsuan-Ju Tsai, Anish Malladi, D. Scott NeSmith, and Savithri U. Nambeesan. To be submitted to Journal of Experimental Botany.

Abstract

Fruit ripening is a highly coordinated developmental process involving several biochemical changes such as softening, changes in sugar and acid metabolism, and flavor that render the fruit palatable. Typically, fruit can be classified as climacteric or non-climacteric fruit. Only climacteric fruit display a dramatic increase in respiration and ethylene production during ripening. In addition, there is an auto-catalytic ethylene regulation in climacteric fruit, which means ethylene induces its own biosynthesis. However, whether blueberry is a climacteric or non-climacteric fruit is still unclear. This study investigated the respiration and ethylene production patterns during fruit ripening in southern highbush and rabbiteye blueberry. Furthermore, we also investigated the concentration of ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the expression of ethylene biosynthesis genes: *ACC synthase* (*ACS*) and *ACC oxidase* (*ACO*) during ripening. To determine if ethylene production is auto-catalytic, ACC concentration and the expression of ethylene biosynthesis enzymes were determined after ethephon treatment in immature/green fruit in two rabbiteye blueberries 'Premier' and 'Powderblue'. Our data showed that southern highbush and rabbiteye blueberry display changes in the rate of respiration and ethylene evolution characteristic of climacteric behavior. Increase ethylene evolution during fruit development was associated with increased *ACS1* and *ACO2* transcript abundance. However, blueberry fruit did not display auto-catalytic ethylene regulation during ripening, likely due to the limitation in ACC availability as its concentration as well as *ACS* transcript abundance were not enhanced upon treatment with external ethylene. Together, these data suggest uncoupling of climacteric responses from auto-catalytic ethylene regulation during blueberry fruit ripening.

KEYWORDS: Respiration, ethylene, 1-aminocyclopropane-1-carboxylic acid synthase, 1-aminocyclopropane-1-carboxylic acid oxidase

Introduction

Fruit ripening is one of the final stages of fruit development. During fruit ripening, coordinated physiological and molecular events influence texture, flavor, aroma, color, and susceptibility to pathogens. Progression of ripening is associated with changes in cell wall disassembly, decrease in acidity, accumulation of sugars, pigments such as anthocyanins and/or carotenoids, and alterations in the volatile profile [1,2]. Although all fruits display these ripening characteristics, they are often grouped into one of two categories: climacteric and non-climacteric, based on specific changes in respiration and ethylene production during ripening. Climacteric fruits display a discernable increase in the rate of respiration and ethylene evolution during ripening such as that observed in banana (*Musa paradisica*) [3,4] and tomato (*Solanum lycopersicum*) [5,6]. On the other hand, non-climacteric fruits do not display a discernable change in the rate of respiration or ethylene production during ripening, such as that noted in grape (*Vitis vinifera*) [7] and strawberry (*Fragaria* × *annanasa*) [8].

Two systems of ethylene production have been proposed in plants (Barry et al., 2000; Liu et al., 2015). In System I, ethylene production is autoinhibitory indicating that ethylene self-regulates a decrease in its production. In system II, which is functional in climacteric fruits during ripening, ethylene production is autocatalytic. Here, ethylene induces its production by upregulating the expression of key biosynthesis genes, *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE* (*ACS*) and *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE* (*ACO*) [9]. In climacteric fruits, the autocatalytic increase in ethylene production is essential to coordinate physiological and molecular events that occur during ripening. However, the role of ethylene in regulating progression of ripening is less clear in non-climacteric fruit [10].

In blueberry (*Vaccinium* sp.), conflicting evidence on changes in respiration rates and ethylene evolution during ripening have been documented. While a few studies documented climacteric responses during fruit ripening, other studies were unable to find consistent evidence for such changes during the progression of ripening in blueberry [11-14]. A respiration peak at the initiation of change in fruit color were reported in northern highbush (*V. corymbosum*) and rabbiteye blueberry (*V. virgatum*) [11]. Further, increase in ethylene evolution was reported during this period in northern highbush and rabbiteye blueberries [11,13]. In contrast, the rate of respiration decreased gradually during fruit development without a notable change during ripening in lowbush blueberry (*V. angustifolium*) [15]. Similarly, in G-90, a selection of northern highbush blueberry, the rate of respiration and ethylene evolution were stable during ripening [16]. Additionally, the nature of ethylene responses, System I and System II, have not been adequately investigated in blueberry. The main objective of the current study is to determine if blueberry fruit display climacteric responses during ripening. To address this objective, respiration and ethylene evolution in blueberry were investigated. In addition, changes in transcript abundance of ethylene biosynthesis-related genes, *ACS* and *ACO*, during fruit ripening and in response to ethylene-releasing compound (ethephon) treatments were investigated.

Materials and Methods

Fruit collection and determination of the rate of respiration

Fruit from six southern highbush and six rabbiteye cultivars were used for measurement of respiration rate over two years of study. In 2017, rate of respiration was determined in five southern highbush cultivars (‘Emerald’, ‘Miss Alice Mae’, ‘Miss Lilly’, ‘Rebel’, and ‘Suziblue’) and six rabbiteye cultivars (‘Alapaha’, ‘Brightwell’, ‘Krewer’, ‘Powderblue’, ‘Premier’, and

‘Titan’). In 2018, measurements were repeated for all cultivars evaluated in 2017 with two exceptions for the southern highbush cultivars, Suziblue was excluded and ‘Miss Jackie’ was included. Fruit from above cultivars were collected from three commercial blueberry farms. In 2017, fruit from all cultivars were harvested at Cornelius Farm, Manor, GA, except for ‘Powderblue’ and ‘Premier’ which were harvested at the Durham Horticulture Farm, Watkinsville, GA. In 2018, ‘Emerald’, ‘Miss Alice Mae’, and ‘Rebel’ fruit were collected from Cornelius Farm, Manor, GA; ‘Miss Jackie’, ‘Miss Lilly’, ‘Alapaha’, ‘Brightwell’, ‘Krewer’, and ‘Titan’ were collected from UGA Blueberry Research Farm, Alapaha, GA; and ‘Powderblue’ and ‘Premier’ from Durham Horticulture Farm, Watkinsville, GA.

Fruit were collected at various developmental, ripening, and postharvest stages. Early growth stages included S2, S3, immature green (green fruit with increasing diameter: 6-8, 8-10, 10-12 mm respectively); while the ripening stages included green, pink and ripe (fruit predominantly green with $< 10\%$ pink; 100% pink; and completely blue skin respectively). Early growth stages collected were S3 for ‘Premier’, and S2 and S3 for ‘Powderblue’. For fruit collected from the Cornelius farm, Manor, GA and the UGA Blueberry Research Farm, Alapaha, GA, all fruit were brought to the laboratory and stored overnight in a walk-in cooler set at 4 °C under ~90 % relative humidity. The following day, fruit from all stages were sorted and kept at room temperature for several hours after which respiration and ethylene measurements were performed as described below. Additional ripe fruit were also re-sorted into clamshells and placed back in the walk-in cooler for measurements during postharvest stages between 8 and 26 days of storage. Fruit harvested from the Durham Horticulture Farm, Watkinsville, GA were analyzed on the same day for respiration and ethylene measurements. Ripe fruit were sorted into clamshells and stored in the walk-in cooler as described earlier. Fruit respiration rates were

determined by measuring CO_2 production at various fruit developmental stages using a closed system. Approximately 10 g of fruit were placed in an air-tight 495 mL glass jar fitted with a septum in the lid for 1 h at room temperature (23 °C). Total fruit weight (g) was recorded before measurements. Headspace samples (60 mL) were extracted using a syringe and analyzed with a CO_2 analyzer (Quantek, MA, United States, Model 902P). Rate of respiration was calculated as CO_2 evolution ($\mu L \cdot g^{-1} \cdot h^{-1}$). Each stage of fruit had 4 biological replicates.

Determination of ethylene evolution

Five southern highbush and four rabbiteye cultivars were used for ethylene measurement in two years at the fruit development postharvest stages described above. Two southern highbush ('Miss Lilly' and 'Suziblue') and four rabbiteye cultivars ('Brightwell', 'Powderblue', 'Premier', and 'Titan') were measured in 2017. Four southern highbush ('Emerald', 'Miss Jackie', 'Miss Lilly', and 'Rebel') and four rabbiteye cultivars ('Brightwell', 'Powderblue', 'Premier', and 'Titan') were measured in 2018.

Ethylene evolution from fruit was measured using a closed system. Approximately 25 g of fruit were placed in an air-tight 135 mL glass jar with a lid fitted with a rubber septum, for 4 h. Headspace samples (1 mL) were analyzed by GC-17A gas chromatography (GC 17A, Shimadzu, Japan) equipped with a 2 m micropacked column (Hayesep N, Restek, PA, United States) and a flame ionization detector. The temperature of the injection port and the detector of GC were set at 200 °C. The temperature program was 60 °C for 4 min; increased by 20 $°C \cdot min^{-1}$ to 150 °C; and hold at 150 °C for 1 min. The peak area from the resulting chromatograph and a standard curve generated using various concentrations of ethylene were used to determine ethylene evolution from the fruit sample, and expressed as $nL \cdot g^{-1} \cdot h^{-1}$ ($n = 4$).

Determination of ACC content

Fruit from rabbiteye blueberry 'Premier' and 'Powderblue' collected in 2018 during fruit ripening (as described above) were used for these analyses. Fruit were transported to the laboratory, frozen in liquid nitrogen and stored at -80 ºC. In addition, control and ethephon treated fruit of 'Premier' and 'Powderblue' in 2020 were collected from the same location. Spray applications were performed on individual blueberry plants when about 30 - 40 % of fruit on the plant were ripe (blue). All pink and ripe fruit were removed and subsequently foliar application of control (surfactant only) and ethephon (treatment with surfactant, 0.15% Latron B-1956, was performed after removing pink and ripe fruits on each plant. Fruit were collected at 0, 1, 2, 3 days after the treatment from a single branch. Fruit were a mix of all developmental stages. This would allow to distinguish between ethylene-induced and ripening-related changes. Fruit were immediately frozen in liquid nitrogen and stored at -80 ºC freezer. There were 4 biological replications for each stage and treatment.

Fruit was ground to fine powder using mortar and pestle in liquid nitrogen. Approximately 2 g of finely ground sample was placed in a 15 mL Falcon tube and sample weight was recorded. The sample was resuspended in 4 mL of 5% sulfosalicylic acid buffer and thoroughly vortexed. Samples were gently mixed using a rocker for 30 minutes at 4 °C. Next, samples were centrifuged at 4500 rpm for 30 min and supernatant was transferred into a separate tube. Subsequently 1.4 mL of the supernatant was transferred into a 20 mL vial (Restek Corporation, Bellefonte, PA) followed by the addition of 0.4 mL of mercuric chloride to the vial. The vial was immediately closed with a cap containing a septum. Next 0.2 ml of 2:1 sodium hypochlorite: sodium hydroxide mixture was introduced into to the vial by injecting with a

needle (and syringe). Samples were vortexed for 5 sec and placed in ice for 4 min for the reaction to continue. During this time ACC is non-enzymatically converted to ethylene. Samples were vortexed again for exactly 5 sec. Ethylene was measured by drawing out 1 ml of headspace gas using a syringe with needle and injected into the GC as described above for ethylene measurements. To calculate the recovery of ACC (reaction efficiency), every sample was also spiked with 20 µl of 50 µM ACC and ethylene released was measured. On each day three blank samples were also prepared, by using 20 µl of 50 µM ACC and measuring ethylene evolution after addition of mercuric chloride and 2:1 sodium hypochlorite: sodium hydroxide mixture. The reaction in the blank solution was assumed to have 100% reaction efficiency for conversion of ACC to ethylene. The concentration of ACC was calculated according to the protocol described by [17].

Quantitative RT-PCR

The samples used for quantitative RT-PCR was the same as that used for determination of ACC content. Transcript abundance of *ACS* and *ACO* were measured by quantitative PCR. Total RNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) -based protocol [18]. The synthesis of complementary DNA (cDNA) was conducted using 1 ug of RNA. After removing the potential DNA from the RNA sample by DNase, cDNA was synthesized by reverse transcription. Quantitative reverse transcription PCR (qRT-PCR)
analysis was performed using PowerUp SYBR Green Master Mix (ThermoFisher, USA) reagent and with Stratagene Mx3005P quantitative real-time PCR instrument (Agilent Technologies, USA). Transcript abundance of five *ACS* genes identified from the 'Draper' genome and two *ACO* genes from the 'Powderblue' transcriptome was quantified (Table 2.1).

Three reference genes, *UBIQUITIN-CONJUGATING ENZYME* (*UBC28*), *RNA HELICASE-LIKE*(*RH8*), and *CLATHRIN ADAPTER COMPLEXES MEDIUM SUBUNIT FAMILY PROTEIN* (*CACSa*) [18], were used to normalize the expression of the target gene. Changes in transcript abundance were quantified as described previously [17].

Results

Respiratory climacteric during blueberry ripening.

Increase in the rate of respiration was detected during fruit ripening in both southern highbush and rabbiteye blueberry. In 2017, in Powderblue the rate of respiration was highest during the very early stages of fruit development at S2 and S3 and decreased during the IMG and Green stages (Fig. 2.1B). Subsequently, it increased by over 1.5-fold at the Pink stage after which it declined and remained lower during the Ripe stage and postharvest (PH) periods. In 'Premier', the rate of respiration was higher during early fruit development but declined at the Green stage. It increased at the Pink stage by > 1.5-fold but continued to remain higher, thereafter (Fig. 2.1B). In four other cultivars where the rate of respiration was measured from the IMG stage onwards, it increased from the IMG stage reached a maximum value at the Pink stage and declined subsequently (Fig. 2.1B). Similar patterns of change in rate of respiration were observed during 2018 in 'Powderblue' and 'Premier', except that it did not decline significantly after the Pink stage in 'Powderblue' (Fig. 2.1D). The other cultivars tested in 2018 also displayed the highest rate of respiration at the Pink stage which declined at the Ripe stage (Fig. 2.1D). In 2017, four of the southern highbush blueberry cultivars exhibited a sharp increase in the rate of respiration by almost 2-fold between the IMG and Green stages and remained higher until the Pink stage (Fig. 2.1A). 'Rebel' displayed a gradual increase in respiration rate between IMG and

Pink stages. All cultivars displayed a decline in the rate of respiration at the Ripe stage and remained low or decreased further thereafter (Fig. 2.1A). In 2018, in southern highbush blueberry the highest rate of respiration was observed at the Pink stage and subsequently declined at the Ripe stage in all cultivars (Fig. 2.1C). Increase in the rate of respiration was evident between the Green and Pink stages in 'Miss Jackie' and 'Miss Lilly' (up to 1.5-fold), and between the IMG and Green stages in Miss Alice Mae (Fig. 2.1C). A gradual increase was noted in Rebel while generally higher values during early fruit development were observed in Emerald. All cultivars displayed a decrease in the rate of respiration between the Pink and Ripe stages (up to 3-fold) (Fig. 2.1C).

In general, differences in respiration rate during ripening among cultivars were marginal (Fig. 2.1). Further, no consistent pattern in rate of respiration was observed among cultivars between years (Fig. 2.1). At the pink stage, when respiration rate was the highest between cultivars and years, no cultivar differences were observed in southern highbush blueberry, in both years (Fig. 2.1A, C). In rabbiteye cultivars in 2017, at the Pink stage, the cultivars Alapaha, Brightwell, Krewer displayed higher rates of respiration than that in 'Powderblue' and 'Premier' (Fig. 2.1B). In 2018, 'Brightwell' displayed a higher rate of respiration than that in 'Powderblue' at the Pink stage (Fig. 2.1D). Between the blueberry types, higher rates of respiration were evident in southern highbush than in rabbiteye blueberry in 2017. The highest rate of respiration noted at the Pink stage ranged between 110-150 $\mu L \cdot g \cdot h^{-1}$ in southern highbush blueberry and between 50-80 $\mu L \cdot g \cdot h^{-1}$ in rabbiteye blueberry (Fig. 1A, B). In 2018, such differences were less evident although most of the southern highbush blueberry cultivars (except 'Miss Jackie') appeared to display higher rates of CO_2 evolution at the Pink stage than the rabbiteye cultivars (Fig. 1C, D).

Increase in the rate of ethylene evolution during blueberry ripening

Over the two years of study and across the different cultivars of rabbiteye and southern highbush blueberry, the rate of ethylene evolution ranged between 0 – 6 $nL \cdot g^{-1} \cdot h^{-1}$ during fruit development (Fig. 2.2). In 2017, ethylene evolution increased in 'Powderblue' rabbiteye blueberry between the IMG and Green stage by 20-fold and remained high for the rest of fruit development (Fig. 2.2B). In 'Premier', ethylene evolution rate increased between the Green and Pink stage by over 4-fold and remained high for the rest of fruit development (Fig. 2.2B). In 'Titan' and 'Brightwell', the rate of ethylene evolution increased by > 3-fold between Pink and Ripe stages. 'Titan' displayed higher rate of ethylene evolution than that in 'Powderblue' and 'Brightwell' at approximately 5 $nL \cdot g \cdot h^{-1}$ at the Ripe stage (Fig. 2.2B). In 2018, the rate of ethylene evolution in rabbiteye cultivars increased gradually over fruit development in all cultivars (Fig. 2.2D). The highest rate of ethylene evolution during fruit development was noted at the Ripe stage in all cultivars. It continued to increase during postharvest storage in 'Titan' (Fig. 2.2D). The two southern highbush cultivars tested in 2017 displayed large (> 5-fold) differences in ethylene evolution (Fig. 2.2A). In both cultivars, the rate of ethylene evolution increased between IMG and Green stages. But the magnitude of increase was greater in 'Miss Lilly' than in 'Suziblue' (Fig. 2.2A). It remained high during the rest of fruit development and declined during postharvest storage (Fig. 2.2A). In 2018, the rate of ethylene evolution increased by up to 3-fold between IMG and Green stages in 'Emerald', 'Miss Lilly' and 'Rebel', remained high during the rest of fruit development, and declined during postharvest storage (Fig. 2.2C). The highest rate was noted at the Ripe stage in these cultivars. In 'Miss Jackie', it increased between the Green and Pink stages by > 4-fold and declined during postharvest storage, with the

highest rate noted at the Pink stage (Fig. 2.2C). 'Rebel' displayed higher rate of ethylene evolution compared to the rest of the cultivars at the IMG and Green stages and compared to 'Emerald' at the Pink and Ripe stages (Fig. 2.2C).

Change in ACC concentration during rabbiteye blueberry fruit development and in response to ethephon

Concentration of ACC was determined in 'Premier' and 'Powderblue' rabbiteye blueberry cultivars (Fig. 2.3A). ACC concentration was variable, below 1 $nmol \cdot g^{-1}$ (FW), and did not change significantly during fruit development in either cultivar. However, in 'Premier' and 'Powderblue' the overall concentration value tended to increase between IMG and Pink and declined subsequently (Fig. 2.3A). Treatment with ethephon transiently reduced ACC concentration at 2 d after treatment in 'Premier' by approximately 3-fold. It did not influence ACC concentration in 'Powderblue' (Fig. 2.3B).

Transcript abundance of ACS and ACO during fruit development

Transcript abundance of various genes associated with ethylene biosynthesis during fruit development was investigated in the rabbiteye blueberry cultivars, Premier and Powderblue (Fig. 2.4). Among the four *ACS* genes analyzed, *ACS1* displayed highest abundance (based on Ct values). Transcript abundance of *ACS1* increased gradually over fruit development in 'Powderblue' being only slightly greater at the Ripe stage than that during early fruit development (Fig. 2.4A). However, it increased greatly between the IMG and Green stages and again between Pink and Ripe stages in 'Premier' (Fig. 2.4A). *ACS1* transcript abundance was greater by over 3-fold during several stages of late fruit development in 'Premier' compared to

that in ‘Powderblue’ (Fig. 2.4A). Transcript abundance of four other *ACS* genes, *ACS2-5* did not change significantly during fruit development in either cultivar and was not different between the cultivars (Fig. 2.4C, E, G, I).

Transcript abundance of *ACO1* was not altered during fruit development in ‘Powderblue’ but declined by approximately 5-fold between IMG and Green stages in ‘Premier’ (Fig. 2.5A). It was higher and lower in ‘Premier’ than in ‘Powderblue’ at IMG and Green stages, respectively. Transcript abundance of *ACO2* gradually increased during fruit development in ‘Powderblue’ by approximately 12-fold. In ‘Premier’, it increased slightly between IMG and Green and again between Pink and Ripe stages by approximately 10-fold (Fig. 2.5C).

Effect of ethephon on transcript abundance of ethylene biosynthesis-related genes

Effects of ethephon applications on *ACS* transcript abundance were determined in ‘Premier’ and ‘Powderblue’. Ethephon treatment reduced *ACS1* transcript abundance at 2 d after treatment in ‘Premier’ by approximately 3-fold (Fig. 2.4B). It did not alter *ACS1* transcript abundance in ‘Powderblue’ (Fig. 2.4B). Further, transcript abundance of *ACS2* and *ACS3* were unaffected by ethephon (Fig. 2.4D, F). *ACS4* transcript abundance significantly increased at 1 d after treatment in ‘Powderblue’ and that of *ACS5* was reduced at 3 d after treatment in ‘Premier’ (Fig. 2.4H, J). Interestingly, transcript abundance of *ACO1* and *ACO2* increased in response to ethephon treatment at 1 d after treatment in ‘Premier’ by up to 3-fold (Fig. 2.5B, D). Ethephon treatment in ‘Powderblue’ also resulted in increased transcript abundance on *ACO1* and *ACO2* at 2 and 3 d after treatment, and at 2 d after treatment, respectively, by up to 2-fold (Fig. 2.5B, D).

Discussion

High rates of respiration are often observed during early fruit development in many fruits such as tomato and apple [19]. Such elevated rates of respiration are thought to allow for generation of energy and carbon backbones required to support rapid growth often observed during this period. In this study, highest rates of fruit respiration were observed during early fruit development in the S2 and S3 stages. It may be speculated that high rates of respiration during this period support the rapid rate of growth in blueberry fruit. This period likely corresponds to the early phase of double sigmoid fruit growth observed in blueberry fruit. Subsequently, the rate of respiration decreased at later stages (IMG) in 'Powderblue' but continued to remain high in others such as 'Premier' indicating that considerable variation exits across blueberry genotypes in terms of the rates of respiration during fruit growth and development. This was also evident in southern highbush cultivars such as 'Rebel' which appeared to consistently display high levels of respiration during early fruit developmental periods. The significance of such differences in the rate of respiration during early fruit development across cultivars remains to be determined. It may be speculated that elevated rates of respiration are associated with higher rates of fruit growth and development.

Climacteric fruit ripening is characteristically associated with an increase in the rate of respiration during the onset of this process [20,21]. Although the cause for the climacteric respiration at the onset of ripening remain unclear [22], it is associated with extensive metabolic changes that are associated with the ripening syndrome, which include development and accumulation of pigments, fruit softening, accumulation of a range of secondary metabolites including volatiles. Presumably, these changes require substantial energy inputs as well as carbon backbones. The rate of respiration does not increase appreciably during the onset of ripening in non-climacteric fruit [22-24]. In this study, the rates of respiration increased during

later stages of fruit development in southern highbush as well as rabbiteye blueberry cultivars. Maximum rates of respiration were often observed around the Pink stage of fruit development, consistent with a ripening associated climacteric behavior. The maximum rates observed at these stages were often at least 2-fold higher than that noted during early fruit growth stages (IMG). The rates noted here were generally comparable and somewhat higher than that in noted in other climacteric fruits such as apple and tomato on a per gram fresh fruit weight basis [25,26].

Conversely, the rates of respiration during the ripening period were substantially higher than that sometimes noted in non-climacteric fruits such as grape [27]. Further, the extent of increase of around 2-fold is within the respiratory climacteric increase in comparison to the pre-climacteric period noted in other fruits [22]. Together, these data suggest that changes in respiration rate at the onset of ripening in blueberry are consistent with a climacteric classification. However, it should also be noted that the increase in respiration rates was sometimes gradual across different stages of fruit development leading up to the maximum rate. This pattern indicates a lack of a sharp rise in respiration in blueberry fruit. It should also be noted that the sharp rise in the rate of respiration has also be described as an artifact of harvesting and that fruit progressing through ripening on the plant may not display such an increase in the rate of respiration [22,28].

Another characteristic feature associated with climacteric ripening in fruits is the increase in ethylene evolution at the onset of ripening [23,24]. This increase in ethylene evolution is concomitant with the respiratory climacteric rise. In this study, increase in ethylene evolution was observed across multiple southern highbush and rabbiteye blueberry cultivars. Particularly, this increase was concomitant with the increase in the rate of respiration. Ethylene evolution increased by over 6-fold during this period. Further, the magnitude of ethylene evolution reached

up to 5 $nL \cdot g^{-1} \cdot h^{-1}$. These values are comparable to that noted in other climacteric species such as tomato, apple, plum and peach, and substantially greater than that noted in some studies in non-climacteric fruits such as grape, citrus and strawberry [29-32]. In fact, comparable levels were found during the onset of ripening (Turning stage) in tomato [33,34]. Further, it has been speculated that a large increase in ethylene may not be necessary owing to saturation of ethylene receptors at substantially lower ethylene concentrations. Hence, these data suggest increase in ethylene evolution during the onset of ripening in blueberry is consistent with a climacteric response.

The two types of blueberry tested did not display differences in the extent of ethylene evolution. However, considerable variation in the rate of ethylene evolution was noted across the blueberry cultivars evaluated. Within southern highbush blueberry, Miss Lilly and Suziblue displayed up to 6-fold difference in ethylene evolution during fruit development even if they both exhibited similar patterns. In the rabbiteye cultivars, 'Titan' and 'Premier' displayed generally higher rates of ethylene evolution compared to 'Powderblue' and 'Brightwell'. These data suggest that variation exists across cultivars in terms of ethylene evolution and related climacteric responses. Such differences across cultivars of a given fruit species in climacteric behavior has been reported previously in many fruit crops including melon and plum [35,36].

ACC synthase catalyzes the rate limiting step in ethylene biosynthesis, the conversion of S-adenosylmethionine to ACC. ACS is encoded by a multi-gene family in many plant species. In fruits such as tomato, *ACS1A* and *ACS6* are expressed during early fruit development and their gene products may aid in regulating autoinhibitory System 1 ethylene [33,34,37]. While *ACS1A* transcript abundance increases at the onset of ripening, that of *ACS6* declines rapidly [33,37]. During the transition from System 1 to autocatalytic System 2 ethylene, *ACS2* and *ACS4* display

an increase in transcript abundance that may have ethylene-independent (developmental) and ethylene-dependent components [34]. ACC oxidase catalyzes the terminal step in ethylene biosynthesis with the conversion of ACC to ethylene and does not appear to be rate limiting in fruits such as tomato. Transcript abundance of several *ACO* genes, *ACO1* and *ACO4*, is detectable during the pre-climacteric period but is also greatly enhanced at the onset of ripening as part of the System 2 ethylene response [33].

Transcript abundance of ethylene biosynthesis-related genes was evaluated in two rabbiteye blueberry cultivars which differed in the extent of ethylene evolution across at least two stages during the onset of ripening ('Premier' and 'Powderblue'). Among the *ACS* genes, transcript abundance of only *ACS1* displayed changes consistent with a role in mediating changes in ethylene evolution during ripening, suggesting that it may represent a key gene regulating changes in ethylene evolution during this period. In 'Powderblue' the cultivar with lower ethylene evolution, *ACS1* transcript abundance did not change substantially during most of fruit development but was still higher at the Ripe stage compared to the IMG stage. In 'Premier', a large increase in *ACS1* transcript abundance was noted between IMG and Green stages, preceding the increase in ethylene evolution, suggesting that ACS activity is limiting. Although ACC concentration was not significantly altered during fruit development, their patterns of change in abundance suggest that ACC increases transiently during the onset of ripening. Further, considering the low concentrations of ACC observed here, it is likely that ACC is limiting for the increase in ethylene during blueberry fruit ripening. Similarly, ACC has been found to be limiting for ethylene production in immature tomato fruit tissues and during ripening in non-climacteric strawberry [38]. The two *ACO* genes evaluated here displayed substantially different transcript abundance patterns. Transcript abundance of *ACO1* declined around the onset

of increase in ethylene in 'Premier' but that of *ACO2* increased in both cultivars during this period and continued to increase during the rest of fruit development. These data suggest that along with ACS1, ACO2 mediates increase the large increase in ethylene evolution during ripening in blueberry.

In tomato, transition from System 1 to System 2 ethylene has been associated with climacteric responses. In fruits such as strawberry, lack of an auto-catalytic ethylene response due to negative feedback has been associated with its non-climacteric behavior [38]. In this study, application of the ethylene-releasing compound ethephon resulted in a transient decrease in ACC concentration in 'Premier'. This was accompanied by a decrease in *ACS1* transcript abundance. Also, transcript abundance of *ACS4* was slightly enhanced in response to ethephon, while that of *ACS5* was reduced in 'Powderblue'. Considering the generally lower transcript abundance of *ACS4* and *ACS5*, the significance of this increase may be limited. Further, transcript abundance of *ACO1* and *ACO2* were enhanced in response to ethephon within 1 d in 'Premier' and 2-3 d in 'Powderblue'. These data indicate a rapid transcriptional response to ethephon application in blueberry fruit. However, they also indicate the lack of an auto-catalytic response to external ethylene availability in blueberry. The transcript abundance data along with the ACC concentration data suggest that external ethylene rapidly increases ACO abundance leading to a decline in ACC concentration. However, external ethylene also decreases *ACS1* abundance thereby resulting in a partial and transient auto-inhibitory response which limits further production of ACC. Together, these data suggest that ACS activity and ACC production are limiting in blueberry and that a System-2 ethylene response is lacking during blueberry ripening. Further, these data indicate that the increase in ethylene levels during blueberry fruit ripening is not auto-catalytic (ethylene-dependent) but is rather dependent on a developmental program.

Such a developmental program may preclude the need for an auto-catalytic rise in ethylene biosynthesis during blueberry fruit ripening thereby uncoupling the two processes.

Conclusions

Data presented in this study demonstrate that southern highbush and rabbiteye blueberry display changes in the rate of respiration and ethylene evolution characteristic of climacteric behavior. The pattern of increase in the parameters and their values in comparison to other climacteric fruits support this conclusion. Increase ethylene evolution during fruit development was associated with increased *ACS1* and *ACO2* transcript abundance. Blueberry fruit did not display auto-catalytic System-2 ethylene regulation during ripening likely due to limitation in ACC availability as its concentration as well as *ACS* transcript abundance were not enhanced upon treatment with external ethylene. Together, these data suggest uncoupling of climacteric responses from auto-catalytic System-2 ethylene regulation. These data further indicate that developmental programs regulate climacteric responses during blueberry ripening.

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Table 2.1. List of the blueberry ACS and ACO genes and the primer sequences used in the quantitative PCR analyses

Gene	Accession number	Primer orientation	Primer sequence (5' → 3')
ACS1	VaccDscaff13-augustus-gene-346.18-mRNA-1	Forward	ATTCAGCAATCCGTCGGGAG
		Reverse	AGAAGGCTTATTCCCCCAGC
ACS2	VaccDscaff31-augustus-gene-297.16-mRNA-1	Forward	CCTCTCATGGCCAAGACTCTTCC
		Reverse	ACATCATGGTAGGGGTTTTTCTCGT
ACS3	VaccDscaff16-snap-gene-134.14-mRNA-1	Forward	CGTACTTCCTCGGATGGCAGG
		Reverse	ACAGCTGATTCTCAGCGAGACC
ACS4	VaccDscaff28-snap-gene-37.26-mRNA-1	Forward	CACGGCAGAAGGAGCAAATG
		Reverse	GGGTCGAACCTCGCTCTATC
ACS5	VaccDscaff22-augustus-gene-15.20-mRNA-1	Forward	ACCAACGATGAACATGGGGAAGA
		Reverse	GCTGGTTTTCAGCAAGACCCATT
ACO1	PB.12198, PB4087	Forward	AGATTGGAGAATCTTGCAGAGGAGC
		Reverse	TCTGGCTTGGGACATGGTGG
ACO2	PB1185, PB4260, PB10761	Forward	CCACACTGATGCCGGTGGTC
		Reverse	CCTTGAGCAGCTGGAGTCCG

Sequence source: Draper genome [36] (*ACS1*-*ACS5*) and Powderblue transcriptome (*ACO1* and *ACO2*)

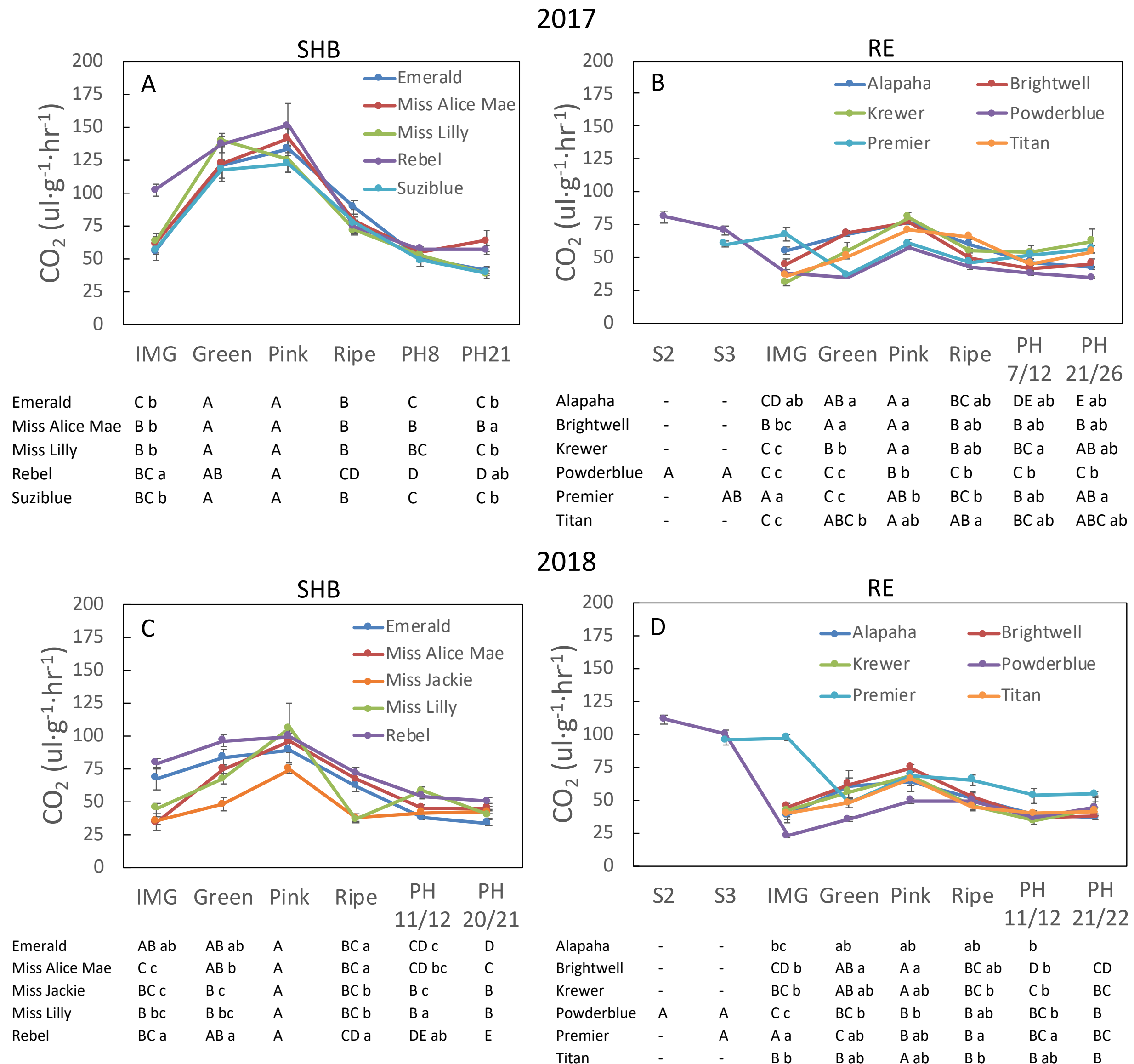

Figure 2.1. Respiratory rate of southern highbush (SHB) and rabbiteye (RE) blueberries during ripening and postharvest stages in 2017 and 2018. SHB in 2017 (A), RE in 2017 (B), SHB in 2018 (C), RE in 2018 (D). I: Immature green, PH: postharvest (days), S2 and S3: Stage 2 and 3. Data shown are means ± standard errors. The different uppercase and lowercase letters indicate the means are significant different in each fruit stages and in different cultivars respectively according to ANOVA and Tukey's HSD (α = 0.05).

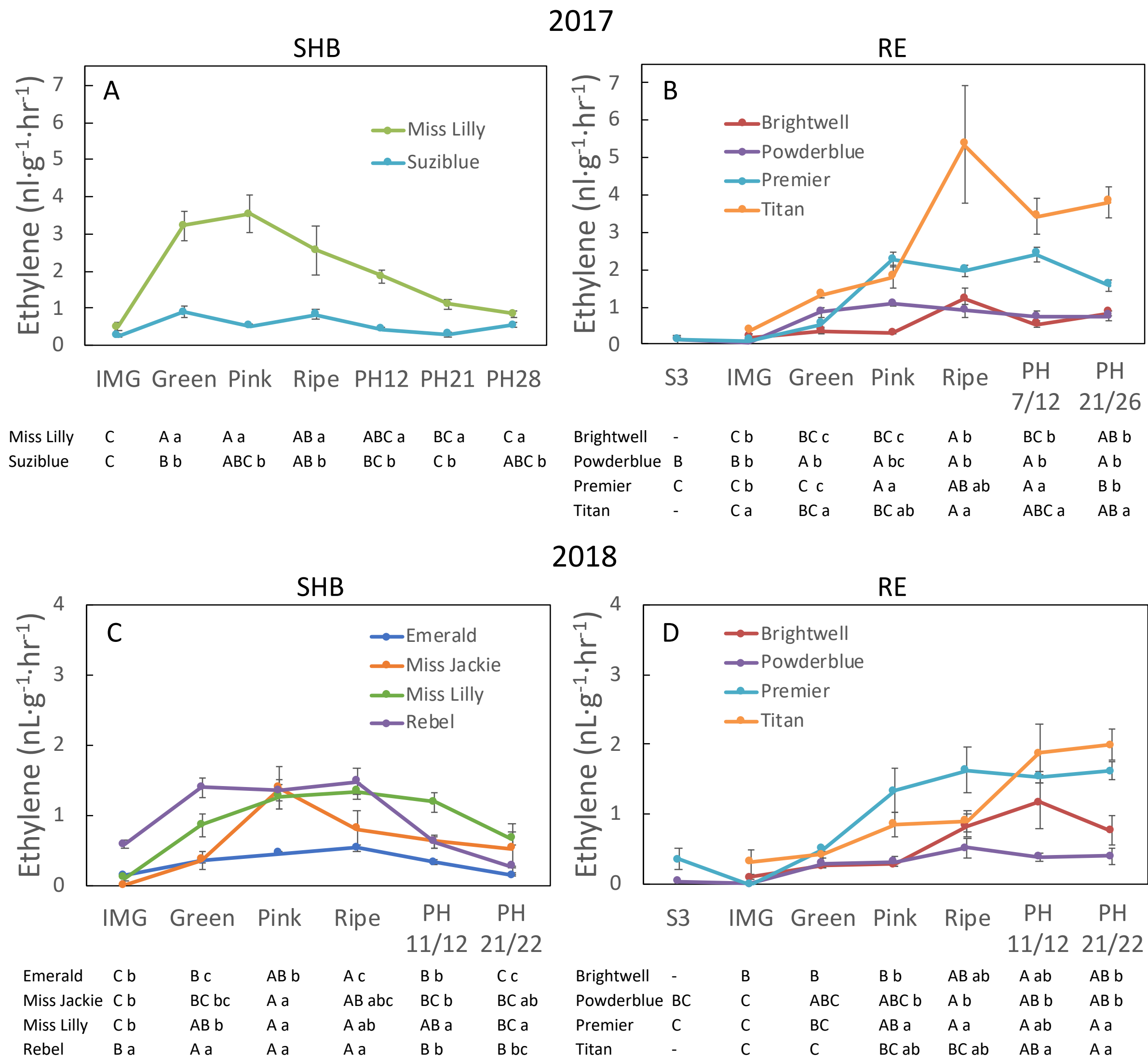

Figure 2.2. Ethylene production of southern highbush (SHB) and rabbiteye (RE) blueberries during ripening and postharvest stages in 2017 and 2018. SHB in 2017 (A), RE in 2017 (B), SHB in 2018 (C), RE in 2018 (D). I: Immature green, PH: postharvest (days), S3: stage 3. Data shown are means ± standard errors. The different uppercase and lowercase letters indicate the means are significant different in each fruit stages and in different cultivars respectively according to ANOVA and Tukey's HSD (α = 0.05).

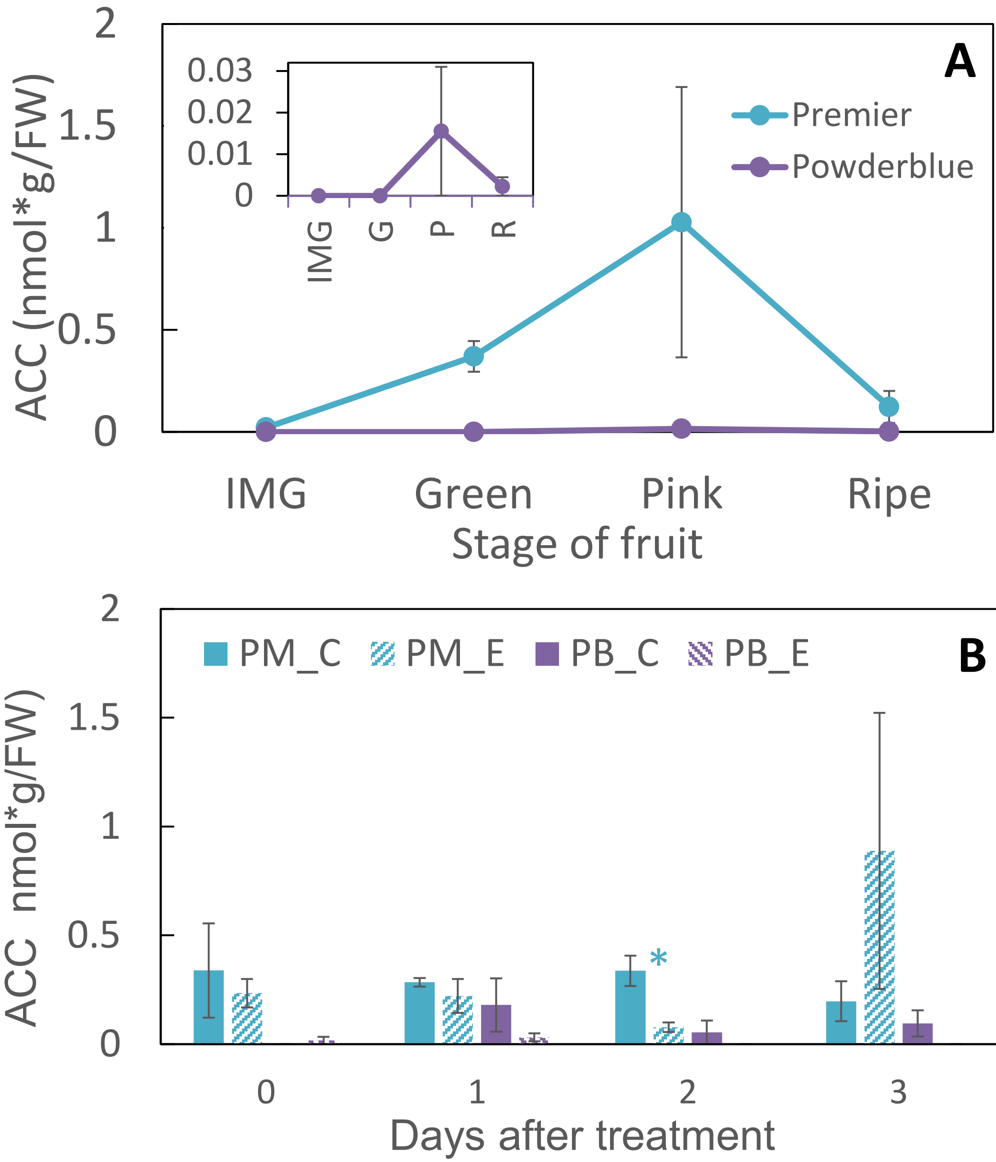

Figure 2.3. Concentration of 1-aminocyclopropane-1-carboxylic acid (ACC) during ripening (A) and in immature/green fruit after treatments with water and ethephon in Premier and Powderblue (B). Inset shows ACC concentration in only Powderblue (A). IMG: immature green, G: Green, P: Pink, R: Ripe, PM_C: Premier control, PM_E: Premier ethephon, PB_C: Powderblue control, PB_E: Powderblue ethephon. Asterisk indicates the means are significant different between cultivars/treatments according to t-test ($\alpha = 0.05$).

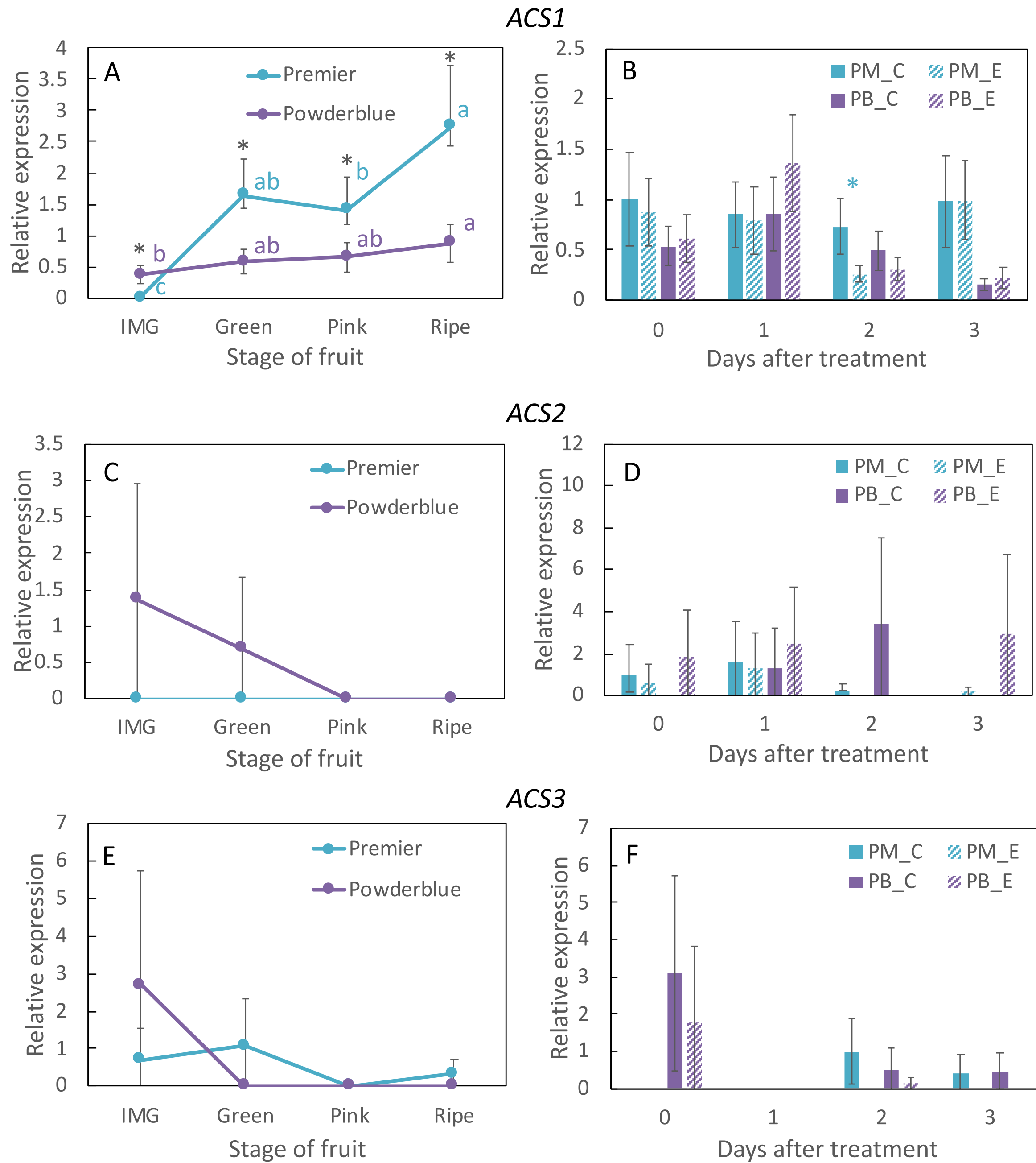

Figure 2.4. The gene expression of *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS*) during ripening (left) and in immature/green fruit after treatments with water and ethephon (right). IMG: immature green, PM_C: Premier control, PM_E: Premier ethephon, PB_C: Powderblue control, PB_E: Powderblue ethephon. Different letters indicate the means are significant different in each fruit stages according to ANOVA and Tukey's HSD (α = 0.05). Asterisk indicates the means are significant different between cultivars/treatments according to t-test (α = 0.05).

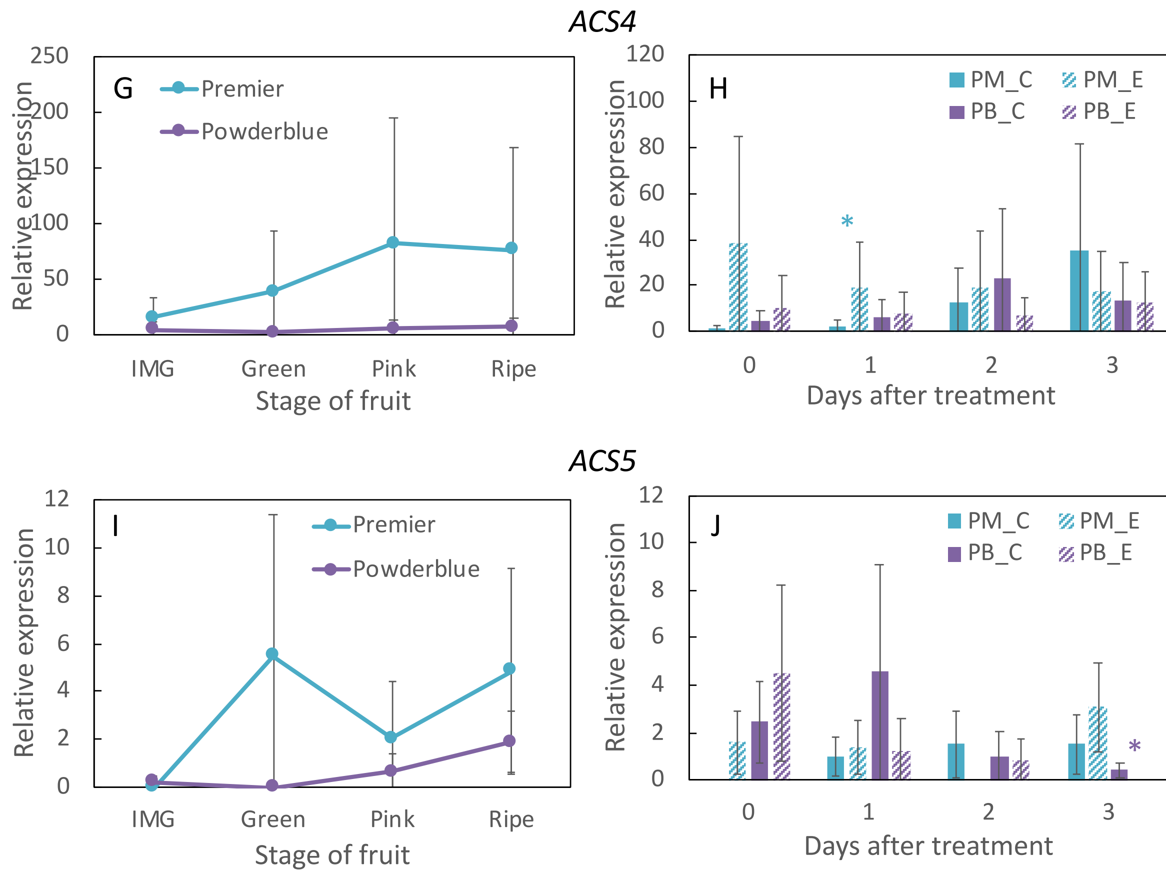

Figure 2.4. Continued. The gene expression of 1-*aminocyclopropane-1-carboxylic acid synthase* (ACS) during ripening (left) and in immature/green fruit after treatments with water and ethephon (right) (cont.). IMG: immature green, PM_C: Premier control, PM_E: Premier ethephon, PB_C: Powderblue control, PB_E: Powderblue ethephon. The different letters indicate the means are significant different in each fruit stages according to ANOVA and Tukey's HSD (α = 0.05). Asterisk indicates the means are significant different between cultivars/treatments according to t-test (α = 0.05).

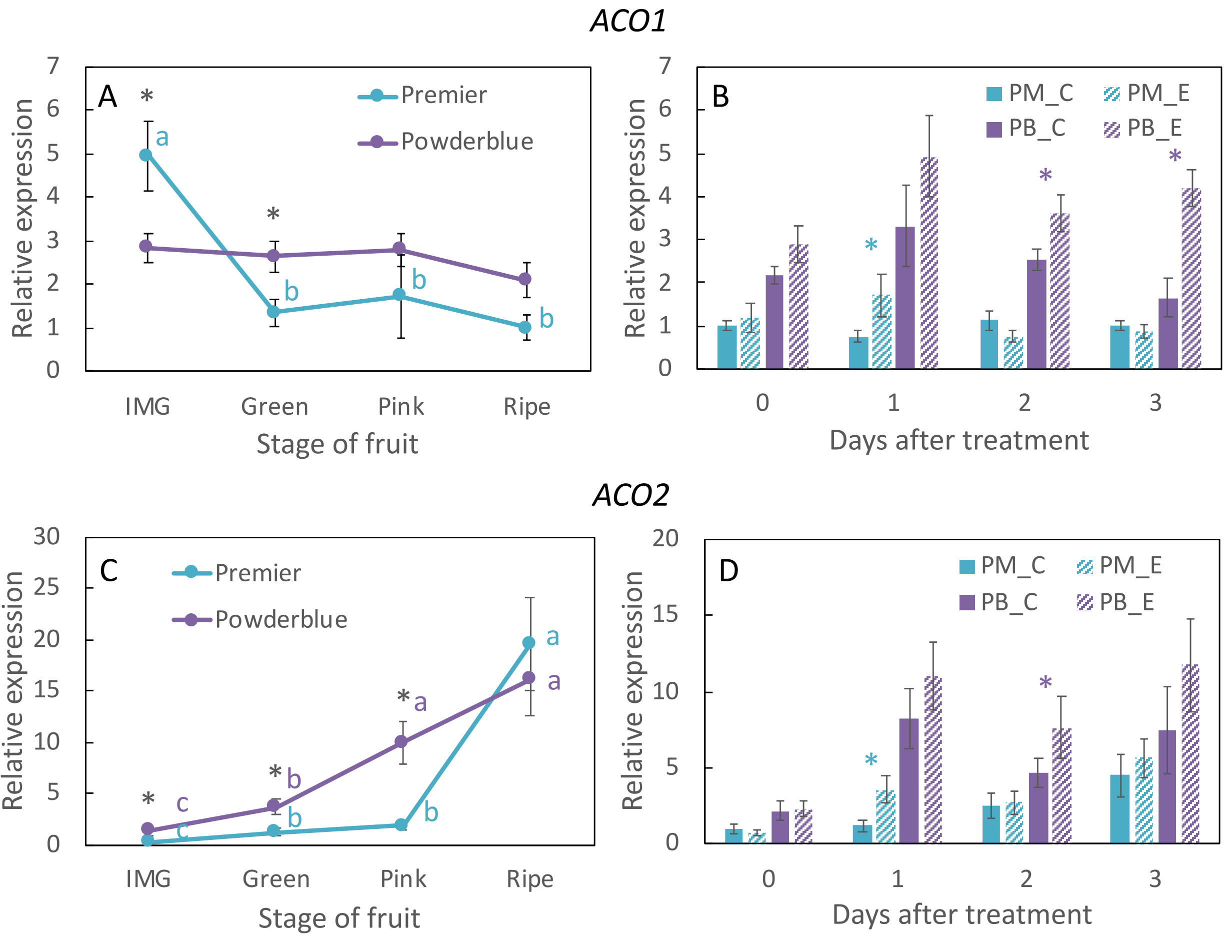

Figure 2.5. The gene expression of *1-aminocyclopropane-1-carboxylic acid oxidase* (*ACO*) during ripening (left) and in immature/green fruit after treatments with water and ethephon (right). *ACO1* (A, B); *ACO2* (C, D). IMG: immature green, PM_C: Premier control, PM_E: Premier ethephon, PB_C: Powderblue control, PB_E: Powderblue ethephon. The different letters indicate the means are significant different in each fruit stages according to ANOVA and Tukey's HSD (α = 0.05). Asterisk indicates the means are significant different between cultivars/treatments according to t-test (α = 0.05).

CHAPTER 3

THE EFFECT OF ETHEPHON, ABSCISIC ACID, AND METHYL JASMONATE ON FRUIT RIPENING IN RABBITEYE BLUEBERRY[2]

[2] Yi-Wen Wang, Anish Malladi, John W. Doyle, Harald Scherm, and Savithri U. Nambeesan. 2018. Accepted by Horticulturae 3:24. Reprinted here with permission of the authors (all articles published in this journals, copyright is retained by the authors)

Abstract

Ripening in blueberry fruit is irregular and occurs over an extended period requiring multiple harvests, thereby increasing the cost of production. Several phytohormones contribute to the regulation of fruit ripening. Certain plant growth regulators (PGRs) can alter the content, perception, or action of these phytohormones, potentially accelerating fruit ripening and concentrating the ripening period. The effects of three such PGRs—ethephon, abscisic acid, and methyl jasmonate—on fruit ripening were evaluated in the rabbiteye blueberry (*Vaccinium virgatum*) cultivars 'Premier' and 'Powderblue'. Application of ethephon, an ethylene-releasing PGR, at 250 mg L^{-1} when 30–40% of fruit on the plant were ripe, accelerated ripening by increasing the proportion of blue (ripe) fruit by 1.5–1.8-fold within 4 to 7 days after treatment in both cultivars. Ethephon applications did not generally alter fruit quality characteristics at harvest or during postharvest storage, except for a slight decrease in juice pH at 1 day of postharvest storage and an increase in fruit firmness and titratable acidity after 15 days of postharvest storage in Powderblue. In Premier, ethephon applications decreased the proportion of defective fruit at 29 days of postharvest storage. Abscisic acid (600–1000 mg L^{-1}) and methyl jasmonate (0.5–1 mM) applications did not alter the proportion of ripe fruit in either cultivar. These applications also had little effect on fruit quality characteristics at harvest and during postharvest storage. None of the above PGR applications affected the development of naturally occurring postharvest pathogens during storage. Together, data from this study indicated that ethephon has the potential to accelerate ripening in rabbiteye blueberry fruit, allowing for a potential decrease in the number of fruit harvests.

Keywords: plant growth regulators; ethephon; abscisic acid; methyl jasmonate; postharvest fruit quality

Introduction

Blueberries (*Vaccinium* spp.) contain bioactive compounds which offer potential health benefits and have witnessed a large increase in production over the last two decades [1,2]. Blueberries are native to North America and some common cultivated species include lowbush (*Vaccinium angustifolium* Ait.), northern highbush (*Vaccinium corymbosum* L.), rabbiteye (*V. virgatum* Ait.), and southern highbush (hybrids of *V. corymbosum*, *V. virgatum*, and *V. darrowii* Camp.) [3–5]. During fruit development, blueberry fruit on a branch mature at different rates, resulting in a non-uniform ripening period extending over 2 to 3 weeks [6]. As a result, blueberries intended for the fresh fruit market are hand harvested three to five times depending on the variety. This makes harvesting a labor intensive and expensive component of blueberry production, requiring up to 520 h of labor/acre and costing up to $0.70 per pound of harvested fruit [7–9]. Concentrating the period of ripening could help reduce the required number of harvests and reduce costs associated with production. Ripening is regulated by multiple plant hormones such as ethylene, abscisic acid, auxins, and jasmonates [10]. External applications of plant growth regulators (PGRs) that influence the levels or activity of these plant hormones may alter the progression of ripening and thereby help in concentrating the period of fruit ripening for efficient harvesting. Therefore, understanding the progression of ripening and developing tools such as PGR applications can help in improving the efficiency of blueberry harvesting.

Fruit ripening is a coordinated process involving changes in fruit texture, color, flavor, and susceptibility to biotic and abiotic factors [11,12]. Although all fruit display these changes during ripening, fruits can be generally classified into one of two types depending on physiological and biochemical changes accompanying the initiation and progression of ripening: climacteric and non-climacteric. In climacteric fruits such as tomato (*Solanum lycopersicum*),

banana (*Musa* spp.), and apple (*Malus × domestica*), ripening is accompanied by a peak in respiration and ethylene production [11–14]. In such fruits, once ethylene production is triggered at ripening, it is autocatalytic and is one of the key factors that regulate changes associated with ripening. Non-climacteric fruits, such as strawberry (*Fragaria × ananassa*) and grape (*Vitis vinifera*), do not exhibit an increase in respiration and ethylene in association with ripening. In these fruits, the role of ethylene and other signals in regulating ripening are not completely understood [12,14–16]. The roles of climacteric respiration and ethylene in the progression of fruit ripening in blueberry are unclear. Some previous studies observed an increase in respiration and ethylene during blueberry ripening, suggesting a potential climacteric nature to the ripening process [6,17,18]. Also, external application of the ethylene-releasing compound ethephon accelerated the progression of ripening and reduced the harvest time in blueberry [19–21]. However, several other studies have classified blueberry as a non-climacteric fruit that does not display a substantial climacteric rise in respiration or ethylene evolution [22]. Hence, further studies are required to better understand the contribution of ethylene in blueberry ripening, and to determine if manipulation of this plant hormone offers a viable option for controlling ripening.

Abscisic acid (ABA), another plant hormone, plays an important role in many developmental processes such as adaptation to stress and seed dormancy. In addition, recent work has suggested a role for ABA during ripening in climacteric as well as non-climacteric fruit. Abscisic acid concentration increases during fruit ripening in apple [23], orange (*Citrus sinensis*) [24], cherry (*Prunus avium*) [25], strawberry [26,27], and grape [28]. In strawberry, decreased expression of 9-cis-epoxycarotenoid dioxygenase (*FaNCED1*), a gene coding for an enzyme involved in ABA biosynthesis, lowered ABA levels and prevented fruit from ripening normally [27]. In grape, ABA applications improved red color and helped achieve early harvest,

underlining its potential for accelerating ripening [29–31]. Further, in tomato, ABA may function upstream of ethylene and induce the expression of ethylene biosynthesis genes to regulate ripening [32,33]. Similarly, in banana, ABA applications may enhance ethylene sensitivity and coordinate ethylene-regulated ripening [34]. In bilberry (*V. myrtillus* L.), which is closely related to blueberry, ABA has been implicated in the regulation of ripening [35]. In highbush blueberry (*V. corymbosum*), ABA levels increase at the onset of ripening and may be involved in regulating the production of flavonoids [36]. However, external applications of ABA delayed ripening and increased fruit firmness in southern highbush blueberry (*V. corymbosum* interspecific hybrids) [37]. Although these studies suggest a potential role for ABA in regulating blueberry ripening, it requires further investigation, especially to determine if external ABA applications can be used to reliably manipulate the progression of this process across different blueberry species.

Jasmonates are another group of phytohormones with well-characterized roles in defense responses and developmental processes such as senescence [38]. Jasmonates have been implicated recently in the regulation of fruit ripening [14,39]. In tomato and apple, jasmonates promoted ethylene biosynthesis by inducing the expression of genes involved in its biosynthesis [40]. In apple, methyl jasmonate (MeJA) applications influenced the production of aromatic volatiles, an integral component of fruit flavor, in a cultivar-dependent manner [41]. In peach (*Prunus persica*), jasmonates delayed ripening [42]. Although MeJA had a negative effect on ethylene biosynthesis during ripening in peach, it still promoted anthocyanin biosynthesis [43]. In non-climacteric fruits such as cultivated strawberry and Chilean wild strawberry (*Fragaria chiloensis*), application of MeJA increased ethylene evolution and respiration, and promoted color development thereby accelerating ripening [44,45]. In raspberry (*Rubus idaeus*), MeJA

application increased flavonoid content, total soluble solids (TSS) content, and total sugars, and lowered titratable acidity (TA), thus influencing multiple ripening characteristics [46]. Together, these emerging data suggest that the effect of jasmonates on fruit ripening may be species-specific, requiring further evaluation in the species of interest. Further, preharvest and postharvest applications of MeJA may not only improve fruit quality but also offer a protective role by limiting pathogen growth as seen in strawberry and peach [39]. MeJA applications on highbush blueberry resulted in changes in total sugar content, total anthocyanin content, and expression of anthocyanin biosynthesis genes in a cultivar-dependent manner [47]. However, the specific role of MeJA in blueberry ripening and its effect on postharvest fruit quality attributes is not clear and has not been investigated previously.

While the effects of multiple PGRs on fruit ripening have been evaluated in various fruit crops, these have not yet been tested extensively in blueberry. Blueberry production could greatly benefit from the use of PGRs that help manipulate the time of ripening. Hence, the main goal of this research was to evaluate three PGRs, ethephon, ABA, and MeJA, for their ability to alter the progression of ripening in two rabbiteye blueberry cultivars. These three PGRs were selected for further study due to previous research suggesting their potential as described above. Furthermore, as preharvest applications of these PGRs can influence postharvest fruit quality and storage characteristics including disease symptom development, the effects of their application on postharvest fruit quality and disease incidence were also evaluated.

Materials and Methods

Plant Material and PGRs

Two rabbiteye blueberry cultivars, Premier and Powderblue (both at 5 years since planting), grown at the Durham Horticulture Farm in Watkinsville, GA were used for this study. All applications were performed when around 30–40% of fruit on the plant were ripe. Whole plants were sprayed using a hand-held sprayer until run-off. For the early-maturing Premier, the treatments consisted of: control (water), 250 mg L^{-1} ethephon, 600 mg L^{-1} ABA, and 0.5 mM MeJA. All treatments were applied on 20 June 2016 along with an adjuvant (0.15% Latron B-1956; Simplot, Lathrop, CA, USA). The doses were determined based on preliminary studies. Applications on Premier were made in the evening close to sunset to minimize photo-destruction of ABA. For the later-maturing Powderblue, the same treatments were applied on 9 July 2016 except that the concentration of ABA and MeJA were increased to 1000 mg L^{-1} and 1 mM, respectively. Due to potential rainfall in the late afternoon, all applications on Powderblue were made early in the morning. For each treatment, four replicates consisting of four individual plants were used in both cultivars.

Rate of Ripening

Prior to PGR application, three 50 to 100-cm-long shoots, each consisting of a total of approximately 50–100 fruit, were tagged per replicate. Very small immature as well as ripe fruit were removed from the tagged branches. The number of green, pink, and ripe fruit was counted prior to and after PGR applications at regular intervals (2–4 days) up to 11 days and 13 days for Premier and Powderblue, respectively. Fruit counted as pink ranged from having around 25% pink color (75% green) to around 75% pink (25% blue) on the fruit surface. Fruit was considered

ripe when the color of the entire fruit was blue. The percentage of green, pink, and ripe fruit was calculated from these data for each assessment date.

Postharvest Fruit Quality and Disease Incidence

Two additional shoots containing around 300 fruit (total) were tagged on each replicate to study the effect of PGR applications on postharvest fruit quality and disease incidence. Very small immature and ripe fruit at the time of application were removed. Ripe fruit were hand-harvested approximately 10 days after application of PGRs and split into three groups for postharvest fruit quality analyses. These groups were randomly assigned to one of the following treatment periods for postharvest evaluation: PH + 1 (postharvest + 1 day); PH + 15 (postharvest + 15 days); and PH + 29 (postharvest + 29 days). For postharvest storage, fruit were placed in a walk-in cooler set to 4 °C and a relative humidity of 90–95%. For each storage period and replicate, around 40 fruit were used for fruit quality evaluation and around 60 fruit were used for disease incidence evaluation. For fruit quality analysis, visual evaluation of quality was conducted and weight, texture, pH, TA, TSS content, and berry color were measured. For visual evaluation of fruit quality, 30 fruit per replicate were scored for symptoms of bruising such as tears, dents, leakiness, and appearance of mold to determine the percent defective fruit. Fruit weight was measured on 20 fruit, using a balance (Quintix® Precision Balance, Sartorius, Bohemia, NY, USA). Fruit texture measurements were made using a fruit texture analyzer (GS-15, Güss Manufacturing, Strand, South Africa). Two tests, compression and skin puncture, were performed on 12 fruit per replicate for determining fruit texture by orienting the fruit on the equatorial plane. For compression analyses, a probe with a 15-mm diameter end plate was used with parameters set at a measure speed of 5 mm s^{-1} and measure distance of 1 mm. To measure

skin puncture force, a 1.5-mm probe was used with parameters set at a measure speed of 5 mm s^{-1} and measure distance of 3 mm.

For measuring pH, TA, and TSS, juice from around 30 g of fruit was extracted using a blender followed by centrifugation for 10 min at 3901× g on a benchtop centrifuge (Allegra X-22, Beckman Coulter Life Sciences, Indianapolis, IN, USA). The supernatant was filtered through cheesecloth. Around 0.3 mL of supernatant was used to determine TSS using a digital handheld refractometer (Atago USA, Belleveue,WA, USA). To determine pH and TA, the supernatant was titrated using an automatic mini titrator (Hanna Instruments,Woonsocket, RI, USA) and alkaline titrant. The titrator has a pH electrode which provided an initial pH value of the supernatant before titration is initiated. For TA, the data are expressed as percent citric acid (CA) equivalents. Fruit color was determined on 20 fruit using a handheld colorimeter (3nh Technology Co., Shenzhen, China).

To determine natural postharvest disease incidence, fruits were maintained at 23–25 ^{o}C for 4 days after removing them from cold storage at the three postharvest intervals described above. Fruit displaying symptoms of disease and/or signs of plant pathogens were recorded using around 60 fruit per replicate. The associated pathogens were identified microscopically as described in Mehra et al. [48].

Statistical analysis (one-way analysis of variance for a randomized complete block design) was performed separately for every time-point after harvest using JMP Pro 12 (SAS Institute, Cary, NC, USA). Means were separated using Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

Results

Effect of PGR Application on Fruit Ripening

In both cultivars, the proportion of green fruit decreased while that of ripe fruit increased over the duration of the experiment (Figure 3.1). In Premier, ethephon-treated fruit had a lower proportion of green fruit than that in the control from 4 days after treatment (Figure 3.1A). At this stage, the proportion of pink fruit was higher in ethephon-treated fruit (Figure 3.1B). The proportion of ripe fruit was significantly higher in the ethephon treatment from 7 days after treatment compared with the control (Figure 3.1C). At 7 days after treatment, 42% of the fruit were ripe in the control compared with 61% in the ethephon treatment. In contrast, treatment with ABA did not affect the proportion of green or ripe fruit but transiently increased the proportion of pink fruit at 7 days after treatment, compared with the control (Figure 3.1B). Similarly, treatment with MeJA did not alter the proportion of green and ripe fruit compared with the control, but increased the proportion of pink fruit at 2 days and 7 days after treatment (Figure 1B).

In Powderblue, the doses of application of ABA and MeJA were higher (see Materials and Methods) as these PGRs did not appear to affect ripening in Premier at lower doses of application. The PGR applications generally resulted in similar effects on Powderblue fruit ripening as in Premier, with a few exceptions (Figure 3.1D–F). The proportion of green fruit was lower (Figure 3.1D), and ripe fruit was higher (Figure 3.1F) than in the control in ethephon-treated fruit starting from 4 days after treatment until the end of evaluation; and, the proportion of pink fruit was higher at 4 days after treatment (Figure 3.1E). At 4 days after treatment, while only 33% of the fruit were ripe on the control plants, around 58% were ripe in response to the ethephon treatment (Figure 3.1F). Application of ABA did not affect the proportion of green or

ripe fruit compared with the control but increased the proportion of pink fruit from 4 days after application (Figure 3.1E). At the rate of ABA used in this study (1000 mg L^{-1}), phytotoxicity symptoms were observed in leaves (data not shown). Application of MeJA did not alter the proportion of green, pink, or ripe fruit at any stage after treatment in comparison with the control (Figure 3.1D–F).

Effect of PGR Application on Fruit Color

None of the fruit color-related parameters were significantly different among the PGR treatments in Premier at 1 day after harvest (Table 3.1). In Powderblue, treatment with ethephon and ABA also did not alter any of the fruit color-related parameters with respect to the control treatment at 1 day after harvest. In response to MeJA treatment, however, the parameters L^*, which measures the lightness, and b^*, which measures yellow/blue color, were higher and lower respectively, indicating lighter and greater blue fruit color than in the control (Table 3.1).

Effect of PGR Application on Fruit Quality during Postharvest Storage

Visual assessment of postharvest fruit quality using variables such as bruises, dents, and mold incidence during postharvest storage indicated a 20% increase in the percentage of defective fruit from 1 day until 29 days after storage in Premier (Table 3.2). There were no significant effects of the PGR treatments until 29 days after harvest in Premier (Table 3.2). At 29 days after harvest, ABA application resulted in a higher proportion whereas ethephon application resulted in a lower proportion of defective fruit compared with the control. In Powderblue the percentage of defective fruit increased by 15% from 1 day until 29 days after storage in control

fruit; none of the PGR applications significantly affected the visually assessed variables for fruit quality (Table 3.2).

In Premier, fruit compression and puncture declined in the control by 18 and 20%, respectively, at 29 days after storage compared with 1 day after storage (Table 3.3). In Premier, ABA applications reduced the force required for fruit compression at 29 days of postharvest storage by ~17%, suggesting a decrease in fruit firmness relative to the control (Table 3.3). None of the other treatments affected fruit texture characteristics or the other fruit quality characteristics such as fruit weight, TSS, TA, and juice pH, evaluated during postharvest storage with respect to the control (Tables 3.3 and 3.4). In Powderblue, fruit compression and puncture declined in the control by 17% and 26%, respectively, at 29 days after storage compared with 1 day after storage (Table 3.3). In Powderblue, fruit firmness as measured by compression was higher by 16% in ethephon-treated fruit compared with the control at 15 days of postharvest storage (Table 3.3). Fruit weight did not differ among various treatments during postharvest storage with respect to the control (Table 3.3). Ethephon treatment resulted in higher TA values than that in the control at various times after storage, although this was significant only at 15 days of postharvest storage (by 21%) (Table 3.4). TSS was lower in the ABA treatment than in the control at 15 days after harvest by ~11%. Also, juice pH was lower in response to ethephon and MeJA treatments than in the control at 1 day after harvest (Table 3.4).

Effect of PGR Application on Postharvest Disease Incidence During Storage

The major postharvest pathogens indicated by disease symptoms and signs in this study were *Colletotrichum acutatum* (causal agent of anthracnose fruit rot), *Phomopsis vaccinii*, *Botrytis cinerea* (gray mold), *Alternaria* spp., and *Pestalotia* spp. Postharvest disease incidence

in both Premier (typically < 5%) and Powderblue (typically < 10%) was low, despite the 4-day incubation period at room temperature following various postharvest storage periods. Due to low pathogen counts, only overall postharvest disease incidence was analyzed; no significant differences among treatments at different time intervals of storage were observed (Figure 3.2).

Discussion

Data from this study clearly indicate that both of the rabbiteye blueberry cultivars responded rapidly to ethephon applications. The time taken for 50% of fruit to ripen was advanced by up to 3 days after ethephon treatment compared with the control (Figure 3.1). Ethephon also increased the number of ripe fruit; ripe fruit increased from 42% to 61% in Premier at 7 days after application and 46% to 83% in Powderblue 6 days after treatment, indicating that the application of this PGR can accelerate the progression of ripening and reduce the time to harvest in blueberry. Several previous studies demonstrated that ethephon accelerates ripening and can reduce the number of required harvests in blueberry [19–21,49]. Results from the current study further expand these findings of acceleration of fruit ripening by ethephon to rabbiteye blueberry.

For a PGR to be effective as a ripening aid, in addition to accelerating ripening it should display minimal negative effects on postharvest fruit quality. Ethephon treatment resulted in a substantial decrease in the proportion of defective fruit after postharvest storage (29 days), at least in Premier. However, fruit texture was not affected by ethephon application in Premier. In Powderblue, compression force at 15 days after ethephon treatment was slightly higher but was not different by 29 days after storage, indicating that ethephon had minimal and temporary effects on fruit firmness characteristics during postharvest storage. These data are generally

consistent with those of Dekazos [20] who used rabbiteye blueberry. However, Ban et al. [21] reported substantial reduction in firmness in another rabbiteye blueberry cultivar, Tifblue, in response to ethephon. In that study, fruit slices were used for analysis of firmness rather than intact fruit as used in the current study as well as several others [20,50,51], which may explain the different observations. In the current study, no change in fruit weight in response to ethephon treatment was observed, consistent with results from a study on Tifblue treated with 200 mg L^{-1} ethephon [21], and in two highbush blueberry genotypes treated with similar doses of ethephon (240 mg L^{-1}) [19]. With similar or comparable (500 mg L^{-1}) doses of application, Eck [19] and Howell et al. [52] reported that ethephon did not affect TSS in highbush blueberry, consistent with results from the current study. In rabbiteye blueberry, Dekazos [20] reported no effect of ethephon on TSS even with repeated 500 mg L^{-1} applications or a single 1000 mg L^{-1} application. However, Ban et al. [21] reported an increase in TSS with ethephon applications at 8 days after treatment in Tifblue, although the effects of this application on postharvest storage were not evaluated in this study. Overall, it appears that ethephon applications do not generally alter TSS content in blueberry fruit during postharvest storage. Several studies have reported a decrease in TA after ethephon applications (ranging from 200 to 3840 mg L^{-1}) in highbush and rabbiteye blueberry [19–21]. In the current study, TA levels were unaffected by ethephon in Premier and slightly increased in Powderblue during postharvest storage (15 days). It is possible that the genotypes used here responded differently for this ripening parameter. Juice pH was generally not affected by ethephon treatment as has been seen previously [19,20], except at 1 day after storage. Dekazos [20] reported changes in fruit color parameters in response to ethephon, in contrast to that reported here. As indicated above, Dekazos [20] used repeated and higher doses of ethephon, which may explain the different results observed. Overall, data from this study

suggest that ethephon application at 250 mg L^{-1} may have minimal effects on rabbiteye blueberry fruit quality during postharvest storage.

The role of ethylene in regulating blueberry ripening and postharvest quality is not completely clear [50]. Although a peak in respiration and ethylene production has been observed in blueberry in some studies [6,17,18], this was not conclusive in others [22]. Treatment of mature fruit with the ethylene perception inhibitor 1-MCP enhanced ethylene production, accelerated loss of fruit firmness, and had little effect on fruit quality characteristics in rabbiteye blueberry cultivars [50], underlining the complex and unclear role of ethylene in regulating blueberry ripening. Recently, based on the analysis of transcriptomics data during various stages of fruit development in highbush blueberry, Gupta et al. [53] indicated that genes associated with ethylene biosynthesis were abundant during the initiation of ripening, suggesting that ethylene may in fact play specific role(s) in modulating the progression of this process in blueberry fruit. The data presented in the current study demonstrating the effect of an ethylene-releasing compound on the progression of ripening further support a potential role for ethylene in the regulation of the ripening program in rabbiteye blueberry. These data indicate that blueberry fruit are responsive to external ethylene. Further studies evaluating the climacteric/non-climacteric nature of blueberry ripening are needed to better understand the potential role of ethylene in the regulation of this fruit developmental process. This information will also be critical for fine-tuning the timing of ethephon application in relation to fruit development.

In the current study, ABA generally did not affect the rate of ripening in blueberry fruit, even when applied at a rate of 1000 mg L^{-1}, although it increased the proportion of pink fruit. It may be likely that ABA (1000 mg L^{-1}) was able to stimulate the synthesis of anthocyanin pigments associated with pink color in the fruit. Phytotoxicity symptoms were observed in leaves

when ABA was applied at 1000 mg L^{-1} (data not shown). Application of ABA did not consistently affect any of the fruit quality characteristics measured across the two cultivars except for compression in Premier at 29 days and TSS in Powderblue at 15 days after harvest. Furthermore, ABA applications appeared to increase the proportion of defective fruit at 29 days after storage in Premier. Overall, external ABA applications did not influence the progression of ripening in rabbiteye blueberry, in contrast to some previous reports with highbush blueberry and closely-related bilberry, where ABA concentration was found to increase during ripening leading to the hypothesis that it may regulate anthocyanin biosynthesis and other ripening related characteristics [35,36]. It may be that rabbiteye blueberry is less responsive to ABA or that the genotypes studied exhibited limited ABA responsiveness. Further analysis involving comparison of different blueberry genotypes and species may be needed to clarify any potential roles of ABA in blueberry ripening.

While MeJA applications have been noted to alter the progression of ripening in several fruits such as strawberry, raspberry, peach, apple, and tomato [14,39,43], in the current study MeJA application did not result in any consistent effects on the progression of ripening even when the application doses were at 1 mM. Further evaluation may be required to determine whether higher doses of MeJA can affect ripening in blueberry. However, previous studies have indicated that MeJA application at 10 mM and higher accelerate fruit detachment and result in extensive fruit drop in blueberry [54–56]. Hence, if higher doses of MeJA are successful at accelerating the progression of ripening, this needs to be optimized such that fruit detachment responses are not induced. Additionally, quantification of jasmonates during fruit development and specifically during ripening may help provide further insights into their potential roles in blueberry ripening.

Conclusions

Data from this study indicated that ethephon applied at a relatively low dose of 250 mg L^{-1} accelerated the progression of ripening in rabbiteye blueberries without altering many of the fruit quality characteristics. The other two PGRs tested, ABA and MeJA, did not appear to alter the progression of ripening in these cultivars. Further studies are needed to determine whether ethephon can consistently alter the ripening process in other types of blueberry, particularly southern highbush blueberry. In such studies, it may be essential to evaluate multiple doses of application, stages of application in relation to fruit development, and the time of day of application to determine the optimum application parameters for this PGR. Additionally, considering the response to ethephon, mechanisms involved in ethylene-mediated alteration of fruit ripening warrant further evaluation in blueberry.

Table 3.1. Effect of preharvest treatment with water (control), ethephon, abscisic acid (ABA), and methyl jasmonate (MeJA) on fruit color after 1 day of cold storage at 4 ^{o}C in Premier and Powderblue blueberry.

Cultivar/Treatment [z]	L^*	a^*	b^*	c^*	h^*
Premier					
Control	38.0	−1.1	−6.3	6.4	260.0
Ethephon	38.3	−1.0	−6.3	6.4	261.2
ABA	37.9	−1.0	−5.9	6.1	260.2
MeJA	38.1	−1.0	−6.3	6.5	260.9
Significance	NS	NS	NS	NS	NS
Powderblue					
Control	40.9b	−1.2	−6.37a	6.6ab	260.5
Ethephon	43.3ab	−1.3	−6.44ab	6.6ab	258.1
ABA	40.7b	−1.1	−6.16a	6.3b	260.1
MeJA	44.0a	−1.4	−6.77b	6.9a	258.6
Significance	0.0078	NS	0.0066	0.0063	NS

[z] Means followed by the same letter within a column are not significantly different, according to Tukey's HSD ($\alpha = 0.05$).

Table 3.2. Percent defective fruit determined at various times after harvest in Premier and Powderblue blueberry following preharvest treatment with water (control), ethephon, abscisic acid (ABA), and methyl jasmonate (MeJA).

	Defective Fruit (%) [z]		
Cultivar/Treatment	**1 Day**	**15 Days**	**29 Days**
Premier			
Control	3.3	19.2	23.3b
Ethephon	2.2	7.8	10.0c
ABA	3.3	16.7	43.3a
MeJA	11.7	15.0	28.3ab
Significance	***NS***	***NS***	***0.0003***
Powderblue			
Control	5.0	13.3	20.0
Ethephon	5.0	6.7	19.2
ABA	4.2	8.3	18.3
MeJA	5.8	11.7	21.7
Significance	***NS***	***NS***	***NS***

[z] Means followed by the same letter within a column for a given time-point after storage are not significantly different, according to Tukey's HSD ($\alpha = 0.05$).

Table 3.3. Effect of preharvest treatment with water (control), ethephon, abscisic acid (ABA), and methyl jasmonate (MeJA) on fruit texture and weight sampled at 1, 15, and 29 days of cold storage at 4 °C in Premier and Powderblue blueberry.

	Berry Texture [z]								
	Compression (kgF)			Pressure (kgF)			Berry Weight (g) [z]		
Cultivar/Treatment	**1 d**	**15 d**	**29 d**	**1 d**	**15 d**	**29 d**	**1 d**	**15 d**	**29 d**
Premier									
Control	0.22	0.20	0.18a	0.15	0.15	0.12	0.81ab	0.82ab	0.81
Ethephon	0.23	0.20	0.19a	0.15	0.15	0.12	0.77ab	0.80ab	0.76
ABA	0.20	0.19	0.15b	0.14	0.14	0.11	0.86a	0.88a	0.79
MeJA	0.23	0.21	0.19a	0.15	0.16	0.13	0.70b	0.72b	0.70
Significance	***NS***	***NS***	***0.0166***	***NS***	***NS***	***NS***	***0.0229***	***0.0067***	***NS***
Powderblue									
Control	0.23	0.19 b	0.19ab	0.19	0.15	0.14	0.87	0.83	0.82
Ethephon	0.26	0.22 a	0.21 a	0.18	0.15	0.15	0.64	0.68	0.69
ABA	0.24	0.20 b	0.18 b	0.18	0.15	0.14	0.84	0.83	0.89
MeJA	0.24	0.21ab	0.17 b	0.19	0.16	0.15	0.80	0.77	0.78
Significance	***NS***	***0.0077***	***0.0120***	***NS***	***NS***	***NS***	***NS***	***NS***	***NS***

[z] Means followed by the same letter within a column for a given time-point after storage are not significantly different, according to Tukey's HSD (α = 0.05).

Table 3.4. Effect of preharvest treatment with water (control), ethephon, abscisic acid (ABA), and methyl jasmonate (MeJA) on fruit quality sampled after 1, 15, and 29 days of cold storage at 4 °C in Premier and Powderblue blueberry.

	Total Soluble Solids (Brix) [z]			**Titratable Acidity (%)** [z]			**Juice pH** [z]		
Cultivar/Treatment	**1 d**	**15 d**	**29 d**	**1 d**	**15 d**	**29 d**	**1 d**	**15 d**	**29 d**
Premier									
Control	11.2	10.6	10.6	0.40	0.34	0.27	3.48	3.60	3.70
Ethephon	9.7	9.8	9.4	0.46	0.37	0.35	3.47	3.60	3.53
ABA	10.9	9.6	9.9	0.37	0.34	0.29	3.53	3.60	3.60
MeJA	10.5	9.8	9.9	0.44	0.36	0.30	3.43	3.58	3.70
Significance	***NS***	***NS***	***NS***	***NS***	***NS***	***NS***	***NS***	***NS***	***NS***
Powderblue									
Control	12.7	13.1a	13.2	0.48	0.45b	0.36	3.48a	3.48a	3.45
Ethephon	12.0	12.1ab	12.6	0.56	0.54a	0.41	3.35b	3.38a	3.45
ABA	11.4	11.6b	12.4	0.49	0.50ab	0.36	3.40ab	3.40a	3.48
MeJA	12.1	13.0a	13.2	0.60	0.53ab	0.41	3.35b	3.38a	3.43
Significance	***NS***	***0.0166***	***NS***	***NS***	***0.0490***	***NS***	***0.0150***	***0.0486***	***NS***

[z] Means followed by the same letter within a column for a given time-point after storage are not significantly different, according to Tukey's HSD (α = 0.05).

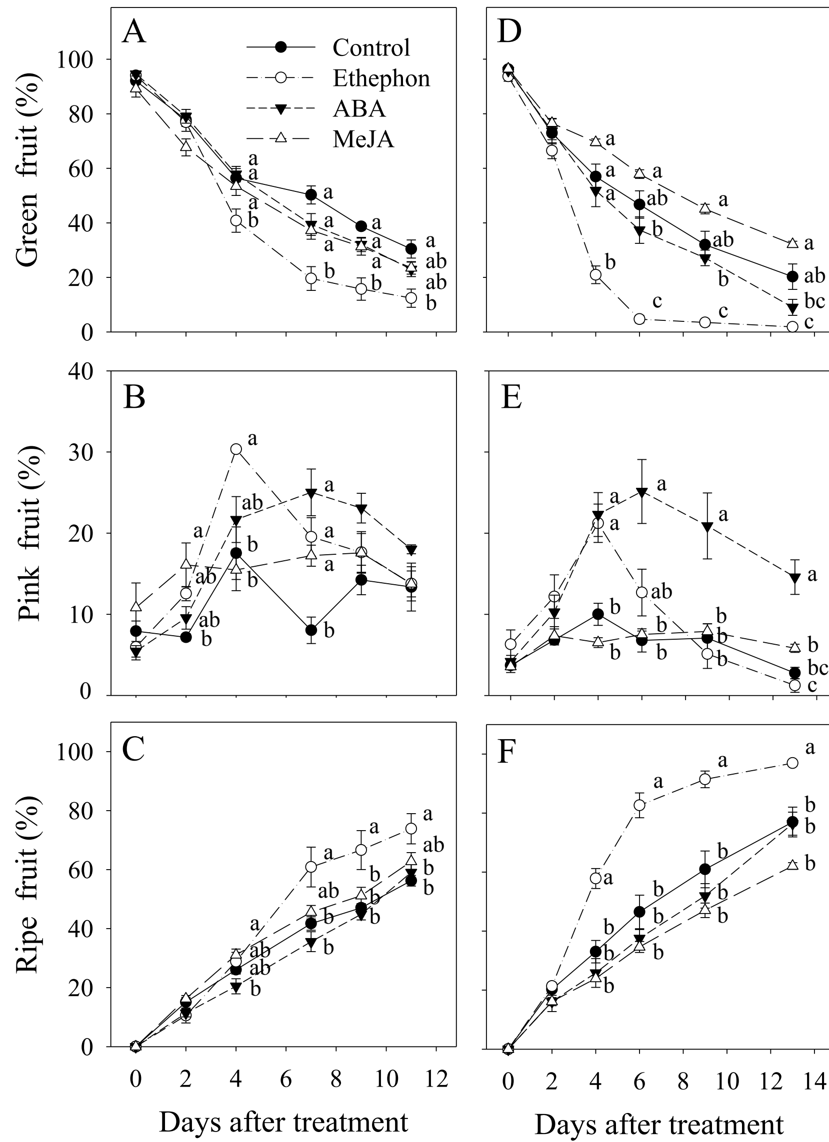

Figure 3.1. Effect of preharvest treatments with water (contol), ethephon, abscisic acid (ABA), and methyl jasmonate (MeJA) on ripening of rabbiteye blueberry, Premier (A–C) and Powderblue (D–F). Values are means and standard errors of four replicates. Within each assessment period, means with the same letter are not significantly different according to ANOVA and Tukey's HSD (α = 0.05).

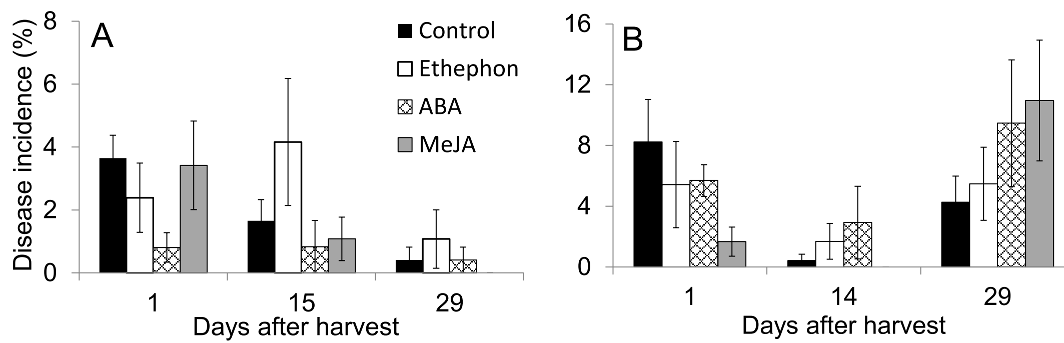

Figure 3.2. Percent postharvest disease incidence determined at various times after harvest in Premier (A) and Powderblue (B) rabbiteye blueberry following applications of water (control), ethephon, abscisic acid (ABA), and methyl jasmonate (MeJA). Values are means and standard errors of four replicates, and 40 to 60 fruit per replicate. No significant differences ($\alpha = 0.05$) were detected among the treatments.

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CHAPTER 4

TRANSCRIPTIONAL REGULATION OF ETHYLENE-REGULATED RIPENING IN BLUEBERRIES[3]

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Abstract

Fruit ripening is a highly coordinated developmental process involving several biochemical changes such as softening, changes in sugar and acid metabolism, and flavor that render the fruit palatable. Previous studies have shown that ethephon, a plant regulator that releases ethylene, can promote blueberry ripening. However, the role of ethylene in regulating blueberry ripening is still unclear. Therefore, in this study, we use transcriptome sequencing to investigate the mechanisms of ethylene-regulated ripening in blueberry fruit. RNA extracted from four fruit developmental stages, immature green, advanced green, pink and ripe, along with control and ethephon-treated fruit, from rabbiteye blueberry 'Powderblue' was used for Illumina RNA-seq. Reads generated by the Illumina sequencing were mapped to a full-length fruit specific-transcriptome generated from 'Powderblue' and quantified by featureCounts program. Next, differentially expressed genes were analyzed using edgeR package. These results identified ripening-related genes such as genes involved in aromatic compound and flavonoid biosynthesis. In addition, a subset of ethephon-induced ripening genes was identified. These results suggest that ethylene promotes fruit ripening by mainly downregulating genes associated with photosynthesis and upregulating genes related to cell wall, auxin and abscisic acid metabolism. The results from this study provided basic information on ethylene-regulated ripening mechanisms in blueberry fruit. In the future, results from this study can be used for blueberry breeding to generate fruit with altered ripening and fruit quality traits.

KEYWORDS: ethylene, ripening, blueberry, RNA-seq

Introduction

Ripening is the final stage of fruit development that imparts flavor and renders the fruit palatable. Ripening includes coordinated changes of multiple biochemical pathways such as increase in sugar to acid ratio, changes in cell wall metabolism, and accumulation of pigments and flavor volatiles [1]. Although all fruits display the above changes, fruit can be classified according to their ripening physiology into two types: climacteric or non-climacteric fruit. Climacteric fruit display a rise in respiration during ripening, whereas such an increase is not discernable in non-climacteric fruit. Further, in climacteric fruits, the levels of ethylene increase during ripening and facilitate ripening-related changes [2]. The production of ethylene can be categorized into System 1 or System 2 [3]. In System 1, ethylene inhibits its own biosynthesis via downregulation of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO), key terminal enzymes in ethylene biosynthesis. System 1 is present in leaves and young developing fruits. During ripening of climacteric fruit, there is a switch from System 1 to System 2 ethylene regulation. In System 2, ethylene production is autocatalytic, and ethylene increases the expression of both biosynthesis enzymes, *ACS* and *ACO*. In tomato (*Solanum lycopersicum*), the differential expression of certain members of the *ACS* and *ACO* gene family are associated with system 1 and system 2 regulation [4]. In tomato, when ethylene biosynthesis was downregulated, ripening was inhibited and was restored by exogenous ethylene application [5]. In addition, ethylene insensitive mutants of tomato, *Green-ripe* and *Never-ripe2*, display inhibited ripening characteristics [6-8]. The importance of ethylene in ripening has also been demonstrated in other climacteric fruits such as banana [9-11], peach [12,13], and apple [14-16]. Ethylene can induce several ripening-related changes in climacteric fruits. It induces the expression of cell wall loosening enzymes such as polygalacturonase in tomatoes [17], apples

[18], and kiwifruits [19]. Further in apple, a gene involved in ethylene signaling, EIN3-LIKE 1 (MdEIL1), increased the transcript abundance of *MdMYB1*, a transcription factor that positively regulates anthocyanin biosynthesis [20]. In pears, ethylene response factors (ERFs) regulated transcription factors, MYB and bHLH to promote anthocyanin biosynthesis [21]. Even though the role of ethylene is not clear in non-climacteric fruits, it is involved in promoting specific aspects of ripening. For example, in strawberries, ethylene can regulate the activities of cell wall degrading enzymes, polygalacturonase, and β-galactosidase, but not *EXPANSIN2* gene expression [22,23]. Further, ethylene promotes fruit color in strawberry [23]. In grape, 1-methylcyclopropene (1-MCP) treatment, which inhibits ethylene signaling also inhibits the expression of sugar transport related genes [24].

Ripening physiology in blueberries is not very well understood. Blueberry fruit develop in clusters and exhibit non-uniform ripening. Thus, during the growing season, fruit are harvested multiple times [25]. Further the ripening behavior of blueberries have not been well studied and are controversial. Some studies indicated enhanced respiration and ethylene during ripening which suggest a potential climacteric nature to the ripening process [25-27]. However, another study suggested that blueberry may exhibit non-climacteric behaviour since it does not display a substantial climacteric rise in respiration or ethylene evolution [28]. Data presented here indicates that blueberry fruit exhibit climacteric respiration and increase in ethylene evolution during ripening. However, ethylene production in blueberries is not autocatalytic (Chapter 2). Further application of ethephon, a PGR that releases ethylene, increased the proportion of blue fruit in blueberries [29]. To understand the role of ethylene in facilitating ripening, transcriptome sequencing analyses were performed following ethephon applications. This study is expected to reveal ethylene regulated mechanisms that are important for initiating ripening in blueberry.

Materials and methods

Ethephon treatment and phenotypic data collection

Rabbiteye blueberry 'Powderblue' grown at the Durham Horticulture Farm in Watkinsville, GA were used to conduct the ethephon treatments on June 23rd, 2017. Ethephon (250 mg L^{-1}) and control treatments were applied following the method in [29]. Each treatment had 4 biological replicates.

Phenotypic data collected included ethylene production of fruit and rate of ripening. Ethylene production of fruit was measured by a closed system. Approximately 25 g of fruit were placed in an air-tight 135 mL glass jar with a lid fitted with a rubber septum, for 4 hours. Headspace samples (1 mL) were analyzed by GC-17A gas chromatography (GC 17A, Shimadzu, Japan) equipped with a 2 m micropacked column (Hayesep N, Restek, PA, United States) and a flame ionization detector. The temperature of the injection port and the detector of GC were set at 200 ^oC. The temperature program was 60 ^oC for 4 min; increased by 20 $^oC \cdot min^{-1}$ to 150 ^oC; and held at 150 ^oC for 1 min. The peak area from the resulting chromatograph and a standard curve generated using various concentrations of ethylene were used to determine ethylene evolution from the fruit sample and expressed as $nL \cdot g^{-1} \cdot h^{-1}$. On the other hand, the rate of ripening was determined by visual color assessment, following the method in [29].

RNA-sequencing

Two sets of fruit samples were collected for RNA-sequencing: 1) fruit at 4 developmental/ripening stages: Immature green (IMG), Green, Pink and Ripe 2) control and ethephon treated fruit at 1 and 2 days after treatment. Green, Pink, and Ripe fruit had 2 biological

replicates, while the remaining samples had 3 replicates. Fruit from control and ethephon treatment were comprised of a random sample with fruit at varying developmental time-points. Each sample was frozen in liquid nitrogen and then were stored at -80 $^{\circ}$C until further processing. Fruit were ground into fine powder and the RNA was isolated from the powder by a cetyltrimethyl ammonium bromide-based method described in [30]. Afterward, high quality RNA (RIN > 8.0) from each sample was used for RNA-Seq library construction with KAPA Stranded mRNA-Seq Kit (KAPA Biosystems, USA) for Illumina platform, following the instructions provided in the manufacturer's manual. Then the libraries were sequenced at the Georgia Genomics and Bioinformatics Core at UGA using an Illumina NexSeq500 platform with 75 bp paired-end sequencing.

RNA-seq data analyses

The reads generated by RNA-sequencing were trimmed, aligned to the full-length fruit transcriptome, with further downstream analyses to determine differential gene expression. First, adapters and low-quality reads were trimmed from the raw reads by Trimmomatic version 0.36 [31] with the parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. Afterwards, the clean reads were aligned to a full-length transcriptome of 'Powderblue' (generated previously in our laboratory using PacBio Sequencing) by STAR version 2.7.1 [32], and the aligned reads were counted according to the genomic feature by FeatureCounts under Subread version 1.6.2 [33]. The raw counts were used for identifying differentially expressed genes (DEGs) and normalized expression level, transcripts per million (TPM), by EdgeR [34]. All DEGs during ripening were clustered based on the expression patterns by a soft clustering of

time series gene expression data, Mfuzz [35]. Furthermore, GO enrichment analysis was conducted by OmicShare tools (http://www.omicshare.com/tools).

Results

Effect of ethephon application on fruit ripening in blueberries

Ethephon application resulted in significant (6-fold) increase of ethylene production compared to the control at 2 days after treatment (Fig. 4.1). Ethephon promoted fruit ripening and the effect on ripening could be observed as early as 3 days after treatment. Ethephon treated fruit displayed a lower proportion of green fruit than that in the control from 3 days after treatment (Fig. 4.1 A). The proportion of Pink fruit increased by 3- and 2-fold in the ethephon treatment in comparison to the control at 3 and 5 days after treatment, while the proportion of ripe fruit was significantly higher (by 1.25-2.5-fold) in the ethephon treatment from 5 days after treatment (Fig. 4.1 B, C).

To understand the mechanisms of how ethephon regulates blueberry ripening, fruit from 4 developmental/ripening stages, and from ethephon and control treated fruit were collected for RNA-seq analysis (Fig. 4.2). Genes that regulate fruit ripening (ripening-related genes) could be identified by comparing the gene expression level among 4 stages of fruits, including IMG, Green, Pink, and Ripe fruit. Genes that are regulated by ethephon/ethylene (ethylene-regulated genes) could be identified by the gene expression levels between ethephon and control treated fruit. Since underlying gene expression differences precede phenotypic differences (evident 3 days of treatment in percentage of Green and Pink fruit), ethephon and control treated fruit at 1 and 2 days were used in this study. By comparing the ripening-related genes and the ethylene-regulated genes, ripening genes that are regulated by ethylene were identified.

RNA-seq reveals global gene expression patterns during blueberry ripening and ethylene-regulated ripening genes

To obtain an overview of the transcriptomes among all samples, transcript profiles using leading log-fold-changes between each pair of samples were visualized by a multidimensional scaling (MDS) plot. The higher the distance between each pair of samples the greater the dissimilarity of their transcriptomes. Overall, all replicates within a given ripening stage, and control and ethephon treated fruit at each day were clustered together (Fig. 4.3), which indicates the quality of the samples used for the analyses was good. Furthermore, the separation of each ripening stage was distinct, while the variation within control and treated fruit in day 1 and 2 were smaller. In general, IMG, Green, Pink and Ripe fruit were separated from left to right along the x-axis (Fig. 4.3). Ethephon treated fruit on day 1 was on the right side of control day 1, which was also closer to green fruit than IMG fruit. This suggests ethephon promotes the initiation of ripening on day 1 of treatment. Besides, ethephon treated fruit on day 2 was also on the right side of control day 2, but it was not as close as day 1 to the green fruit. This implies that ripening initiation induced by ethephon occurred mostly on day1.

Ripening related genes

The differentially expressed genes (DEGs) between each pair of ripening stages have been identified with the cutoff of < 0.01 false discovery rate and >= 2-fold change. There were 2,019, 1,009, and 1,663 DEGs in the IMG versus green, Green versus Pink, and Pink versus Ripe respectively (former stage as control). The numbers of upregulated and down-regulated genes were similar between immature and green fruit (1,108 and 911, respectively). There were more

down-regulated genes (601) than upregulated genes (408) between Green and Pink fruit. However, there were about 2-fold more upregulated genes (1,156) than down-regulated genes (508) between Pink and Ripe fruit.

Further, to investigate the temporal expression patterns during ripening, all DEGs were clustered based on the standardized expression level. In other words, if genes have similar expression patterns, they would be classified into the same cluster. After clustering analysis by Mfuzz, nine clusters with various expression patterns been identified (Fig. 4.5). Each line indicates a gene, with colors from purple to orange showing the fitness of the gene in the cluster from high to low. For example, the gene expression in cluster 1 decreased from IMG to Pink and then maintained similar levels between Pink and Ripe; while the expression in cluster 9 increased from IMG to Pink, then maintained similar levels between Pink and Ripe. The numbers of the genes in each cluster from 1 to 9 were 1,095, 564, 451, 326, 883, 677, 698, 329, and 603.

To understand the functions of genes in each cluster, gene ontology (GO) enrichment analysis for each cluster was conducted. The top 20 of GO enrichment terms in biological process aspect based on Q value in each cluster are shown in Fig. 4.6 - 4.14. In cluster 1, photosynthesis related terms were the most common, for example, photosynthesis (GO:0015979), photosynthesis, light reaction (GO:0019684), photosynthesis, light harvesting (GO:0009765) (Fig.4.6), which indicates that the expression of photosynthesis related genes decreases intensely between IMG to Green stage. In cluster 2, amide biosynthesis process (GO:0043604), peptide biosynthesis process (GO:0043043), and translation (GO: 0006412) were enriched (Fig. 4.7), which indicates amide biosynthesis and translation decreases during ripening. In cluster 3, terms involved in cell well modification, such as plant-type cell wall organization (GO:0009664), pectin catabolic process (GO:0045490), and polysaccharide

catabolic process (GO:0000272) (Fig. 4.8), which suggests cell wall modification keeps increasing between IMG to Pink stage. In cluster 4, abiotic stress-related terms were enriched, such as response to hydrogen peroxide (GO:0042542), response to heat (GO:0009408), response to temperature stimulus (GO:0009266) (Fig. 4.9), indicates abiotic stress-related genes also play a role in ripening. In cluster 5, protein folding (GO:0006457) was enriched (Fig. 4.10). In cluster 6, chloroplast RNA processing (GO:0031425) and homogalacturonan biosynthetic process (GO:0010289) were enriched, but there were only a few genes under each term (Fig. 4.11). In cluster 7, aroma-related terms, such as aromatic amino acid family catabolic process (GO:0009074), were enriched (Fig. 4.12), which indicates biosynthesis of aromatic compounds keep increasing during ripening. In cluster 8, protein folding (GO:0006457) was enriched (Fig. 4.13). In cluster 9, flavonoid and aroma-related terms, such as flavonoid biosynthesis (GO:0009813) and aromatic amino acid family metabolic process (GO:0009072), were enriched (Fig. 4.14), which suggest biosynthesis of flavonoid and aroma increases between IMG to Pink and maintain their expression between Pink and Ripe.

Ethephon regulated genes

There were 625 and 260 DEGs induced by ethephon on day 1 and day 2 respectively (Fig. 4.15), which indicates that the most regulation induced by ethephon occurred at 1 day after treatment. In addition, within the DEGs, there were 107 and 70 upregulated genes and 518 and 190 down-regulated genes on day 1and day 2 respectively after ethephon treatment (Fig. 4.15). This indicates that ethephon regulate ripening mainly through downregulation of genes.

GO enrichment analysis for DEGs induced by ethephon were also conducted to find the represented function of genes. On day 1 after treatment, cell wall macromolecule catabolic

process (GO:0016998), aminoglycan catabolic process (GO:0006026), and chitin metabolic process (GO:0006030) were enriched upregulated genes. On day 2 after treatment, galactose metabolic process (GO:0006012) and endoplasmic reticulum unfold protein response (GO:0030968) were enriched upregulated genes. On the other hand, the enriched terms in down-regulated genes on both days were similar. Photosynthesis-related genes, such as photosynthesis (GO:0015979) and photosynthesis, light reaction (GO:0019684), downregulated by ethephon on both day 1 (Fig. 17) and day 2 (Fig. 4.19).

Ethelene-regulated ripening

The ripening genes regulated by ethylene were identified by overlapping the ripening-related genes in each cluster and the upregulated/downregulated genes by ethephon (includes both day 1 and day 2). The top 2 clusters overlapping with ethephon upregulated genes were cluster 3 and cluster 8, which had 38 and 34 overlapping genes respectively (Table 4.1). Genes in the clusters were upregulated between IMG to Green (Fig. 4.5). These ripening-related genes upregulated by ethylene included auxin conjugation genes (*INDOLE-3-ACETIC ACID-AMIDO SYNTHETASE*), abscisic acid (ABA) signaling genes, and cell wall modification genes, such as pectate lyase and expansin. On the other hand, cluster 1 and cluster 6 were the top 2 clusters overlapping with ethephon downregulated genes, with 356 and 95 overlapping genes respectively (Table 4.1). Genes in the clusters were downregulated between IMG to green (Fig. 4.5). These ripening-related genes downregulated by ethylene were mainly photosynthesis-related genes. The gene expression levels of these showed very similar patterns, which were highly expressed in IMG fruit, then decreased between Green to Ripe fruit. Besides, the

expressions of these genes were inhibited by ethephon on both day 1 and day 2 after treatment (Fig. 4.20).

Discussion

In this study, genes that were differentially regulated between the IMG and Ripe stages of blueberry fruit development were identified and classified into nine clusters based on their expression patterns. Further, genes differentially expressed following ethephon application were identified. After ethephon application, ethylene levels increase within 2 d after treatment, and a significant decrease in percent Green fruit and increase in percent Pink fruit is observed at 3 d after treatment. Therefore, fruit sampled at 1 and 2 d after ethephon treatment were used to capture the initial ethylene-induced ripening-related changes. Following ethephon application, there was a higher number of differentially expressed genes in 1 d (625) than 2 d (260). There were 4.8- and 2.7-fold more downregulated than upregulated genes at 1 and 2 d after ethephon treatment, respectively. These data suggested that ethephon mainly downregulated gene expression and that these changes were more pronounced very early on after application.

Within the fruit development-related transcriptome, clusters 1, 2, 4 and 6 contained genes that displayed dramatically downregulated transcript abundance between IMG and Green stages and continued decline until the Pink Stage. Beyond the Pink stage, expression patterns differed across the four clusters. In cluster 1, transcript abundance remained largely similar from the Pink to the Ripe stage; it steadily declined until the Ripe stage in cluster 2; greatly increased between Pink and Ripe stages in cluster 4; and was slightly upregulated between Pink and Ripe stages in Cluster 6.

In Cluster 1, the GO enriched categories were related to photosynthesis, suggesting downregulation of photosynthesis related transcript abundance during the onset of ripening. During early stages of fruit development, fruit photosynthesis can provide 10-20% of the total fruit carbon, although this is not sufficient to completely support fruit carbon requirements [36,37]. A bi-phasic decline (one during early fruit development and a second at later stages) in photosynthesis-related transcript abundance has been noted previously during fruit development in fruits such as tomato [38]. In tomato, downregulation of fruit photosynthesis affected the timing of seed development, but had little effect on fruit growth, ripening and accumulation of primary metabolites. These authors suggest that fruit photosynthesis may be dispensable under normal growth conditions but of consequence during stress [39]. During ripening initiation some of the early changes include disassembly of the thylakoid and chlorophyll degradation. This is coupled with plastid transitions from chloroplast to chromoplast, especially in fruits such as tomato, watermelon, citrus, and persimmon [40-42]. When photosynthesis capacity increases in unripe fruit, it may lead to increased starch content which in-turn influences mature fruit sugar content [43]. Downregulation of photosynthesis prior to ripening is not only conserved in fruit that accumulate carotenoids during ripening but also in fruits such as grape and litchi which accumulate anthocyanins, suggesting that it is a conserved feature in fleshy fruit development [44,45]. Multiple plant hormones have been shown to influence photosynthesis. In litchi fruit, ABA applications trigger chlorophyll degradation during ripening [45]. In tomato, overexpression of an auxin response factor, S*lARF6A* in fruit increased rate of photosynthesis and inhibited and ethylene biosynthesis enzyme, S-adenosyl methionine synthetase and thus inhibited ethylene production and ripening [46]. Thus, auxin can positively regulate photosynthesis and delay ripening by inhibiting ethylene production. In the current study, 356 transcripts were regulated

by ethylene in Cluster 1, and many of these were associated with photosynthesis. These data further indicate that ethylene plays a role in regulating blueberry ripening by downregulating the transcript abundance of photosynthesis related genes. Additionally, ABA concentration increases during blueberry ripening [47,48], while that of auxin decreases during blueberry ripening (Wang and Nambeesan, unpublished data). These data suggest that increase in ethylene and associated changes in the onset of ripening are associated with concomitant changes in auxin and ABA. It remains to be determined if these changes are inter-related during the onset of ripening in blueberry.

In cluster 2, GO enriched categories included translation or peptide metabolic process suggesting these mechanisms were downregulated during the onset and progression of ripening. It may be speculated that part of the downregulation of mechanisms is associated with downregulation of photosynthesis as indicated earlier. Similarly, downregulation of translation in the chloroplast has been reported during tomato ripening [49]. Around 22 genes in this cluster were also downregulated in response to ethephon. This group included genes associated with water transport and cell wall modification, suggesting down-regulation of associated processes.

In cluster 6 the GO enriched categories were associated with chloroplast RNA processing suggested further that these transcripts are associated with downregulation of photosynthesis (Cluster 1) and translation (Cluster 2). Further, GO categories associated with homogalacturonan metabolic process and hexose transmembrane transport were also identified in this cluster. Downregulation of these transcripts leading up to the Ripe stage may be associated with changes in cell wall composition and sugar accumulation during fruit ripening. Approximately, 95 transcripts in this cluster were common with those downregulated in response to ethephon application. Some of these transcripts were related to sugar transport such as the polyol

transporter 5-like further indicating changes in sugar accumulation characteristics during ripening. Transcripts putatively coding for proteins associated with auxin metabolism were also identified in this group. Auxin biosynthesis is downregulated during ripening in tomato and strawberry [50,51]. As indicated earlier, downregulation of auxin biosynthesis during fruit ripening is also hypothesized in blueberry fruit (Wang and Nambeesan, unpublished). It may be speculated that ethylene cross talks with auxin metabolism during ripening in blueberry fruit. Such a possibility warrants further analyses.

Most of the transcripts in cluster 4 were assigned to GO categories associated with multiple abiotic stress responses. These transcripts were upregulated between the Pink and Ripe stages likely due to a developmental transition and may represent a part of late-ripening related changes. In tomato, small heat shock proteins that generally are upregulated during abiotic stress, may play specific roles in ripening by promoting color development during this period [52]. Similarly, it may be expected that some of the genes grouped in this cluster regulate final stages of ripening. Only a small fraction (33 genes) overlapped with ethephon-downregulated transcripts in this cluster. However, these were not related to abiotic stress tolerance mechanisms.

In cluster 8, transcripts displayed upregulation between IMG and Green stages and then down regulation between the Green and Ripe stages. This pattern suggests association with mediating a developmental transition from immature fruit to initiation of ripening. The GO enriched categories in this cluster included those associated with protein folding, and endoplasmic reticulum (ER) unfolded protein response. The ER unfolded protein response is generally triggered due to misfolded proteins when stress conditions prevail in the ER. These transcripts initiate a signal transduction response in the ER in response to stress to restore

homeostasis [53,54]. Especially during heat stress, the unfolded protein response in the ER can induce heat shock proteins [55]. In addition to abiotic stress, ER unfolded protein response is important during vegetative and reproductive development. The role of such events in progression of natural ripening is not currently known [53]. Such ER stress between IMG and Green stages is consistent with upregulation of transcripts associated with stress response noted earlier in cluster 4. There were 34 ethylene upregulated genes that overlapped with genes in cluster 8. These transcripts putatively coded for expansin-A4, sugar transporter *ERD6-LIKE 16*, and genes involved in ABA signaling (*ABSCISIC ACID RECEPTOR PYR1-LIKE*), auxin responses (short auxin-responsive protein - *SAUR76-LIKE*) and regulation of ethylene signaling (*ARGOS-like protein*). These data further suggest cross-talk among phytohormones, particularly ethylene, ABA and auxin during the progression of ripening.

The GO enrichment terms associated with cluster 3 genes were associated with plant cell wall organization and carbohydrate metabolic processes. During blueberry ripening increase in fruit size as well and fruit softening is observed. Solubilization of pectin occurs during early ripening stages, while hemicellulose depolymerization occurs throughout ripening and is correlated with reduction in fruit firmness [56,57]. Thus, it is not surprising that genes involved in plant cell wall organization, galacturonan and pectin metabolic processes were upregulated during this period. This cluster showed the highest overlap with those altered by ethephon application, 38 transcripts. Transcripts encoding *PECTATE LYASE 8* and *GALACTINOL SYNTHASE* were upregulated after ethephon application. The effect of ethylene in inducing expression of cell wall modification enzymes have been described in many fruits such as tomatoes, apples and strawberries [17,18,23,58]. Further galactinol synthase enzyme is involved in the biosynthesis of raffinose family oligosaccharides including raffinose. The carbohydrates may

play a role in various process which include storage of carbon, as a solute for osmotic adjustment and stabilization of macromolecules [59]. They may be important in aiding the fruit with oxidative stress responses during ripening in plum [60]. It may be hypothesized, that increased galactinol and potentially raffinose family oligosaccharides may similarly aid in stress response as well as carbohydrate storage in blueberry. Also, an auxin conjugating enzyme, *INDOLE-3-ACETIC ACID-AMIDO SYNTHETASE* that decreases free IAA levels was upregulated after ethephon treatment, further linking ethylene and auxin responses during blueberry fruit ripening.

Genes upregulated during ripening were mainly present in clusters 7, 9 and 5. In clusters 7 and 9, transcripts were upregulated from IMG to Green and then to the Pink stage. In cluster 7, transcript abundance increased further from Pink to Ripe stage whereas in Cluster 9 transcript abundance was similar between these stages. In both these clusters genes related to aromatic amino acids, tyrosine metabolism and flavonoid metabolism were present. The main aromatic amino acids, phenylalanine, tyrosine and tryptophan are derived from chorismite as part of the phenylpropanoid pathway and are precursors to many different metabolites involved in secondary metabolism related to fruit ripening [61]. For example, tyrosine can be converted into betalains and lignin. More than 8000 compounds ranging from lignin to volatiles, flavonoids and anthocyanins are derived from phenyl alanine. Among, the total polyphenols in blueberry fruit more than 60% are made of anthocyanin flavonoids [62]. During ripening, blueberry fruit accumulate anthocyanins mainly in the peel tissue [48]. The predominant anthocyanin types in blueberry include delphinidin, cyanidin, petunidin, peonidin and malvidin. These are conjugated to various sugar moieties including galactose, glucose and arabinose [63,64]. Further, during ripening many volatile organic compounds are synthesized and interaction among these contributed to blueberry aroma development. In rabbiteye blueberry around 50 volatile

compounds were detected during ripening [65,66]. However, there are genetic differences in aromatic profile among *Vaccinium* species [67,68]. Upregulation of transcripts related to aromatic amino acids during blueberry ripening noted in these cluster is consistent with these observations. Approximately 17 and 24 transcripts from clusters 7 and 9, respectively, overlapped with those in differentially expressed in response to ethephon. Upstream anthocyanin biosynthesis genes, and three transcription factors MYB-bHLH-WD40, play roles in anthocyanin biosynthesis regulation [69]. Only one transcript *bHLH93-LIKE*, a transcription factor, was identified within these overlapping genes. It would be interesting to determine if the *bHLH93-LIKE* gene identified in this study is involved in regulating anthocyanin biosynthesis. However, anthocyanin biosynthesis-related genes were not identified within these groups, likely owing to the early time of sampling following ethephon treatment and the downstream response-nature of the biosynthesis genes.

In cluster 5, transcripts associated with chitin and aminoglycan processes were enriched and were upregulated during ripening especially during the Pink and Ripe stages. Chitin is an important component of fungal cell wall along with other carbohydrates such as mannan and glucan. Thus, transcripts associated with chitin and aminoglycan metabolism suggest that they are involved in protection against fungal pathogens [70]. Increase in chitinase, an enzyme that can hydrolyze fungal cell walls was observed during ripening in banana and grape. Generally, these enzymes are expressed in response to pathogens [71-73]. It has also been suggested that these genes may be regulated developmentally independent of pathogen infection [73]. Twenty transcripts in cluster 5 overlapped with those differentially expressed after ethephon application. Among these, a zinc finger CCCH domain-containing protein 20-like, with potential role in biotic stress responses was detected. Developmental and ethephon-induced upregulation of this

transcript may aid in priming of the fruit for potential interaction with biotic factors during ripening.

Conclusions

Our data suggests that ethylene triggers ripening by mainly down-regulating transcript abundance, especially those associated with photosynthesis. Ethephon application upregulated *PECTATE LYASE 8* and *GALACTINOL SYNTHASE* genes suggesting regulation of cell wall and carbohydrate related metabolic processes by ethylene. Further, genes involved in auxin and ABA metabolism were differentially regulated after ethephon application suggesting cross talk among ethylene, auxin and ABA. The role of ethylene in regulating auxin and ABA concentration during ripening initiation and progression warrants further analyses. Overall, our data suggest that a rise in ethylene during ripening in blueberry fruit is important in the initiation phase of ripening.

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Table 4.1. Numbers of genes overlapping between ripening-related genes in each cluster and upregulated/downregulated genes by ethephon (includes day 1 and day 2 after treatment).

Cluster	Upregulated	Downregulated
Cluster 1	3	356
Cluster 2	1	22
Cluster 3	38	0
Cluster 4	1	33
Cluster 5	20	17
Cluster 6	0	95
Cluster 7	17	0
Cluster 8	34	2
Cluster 9	24	1

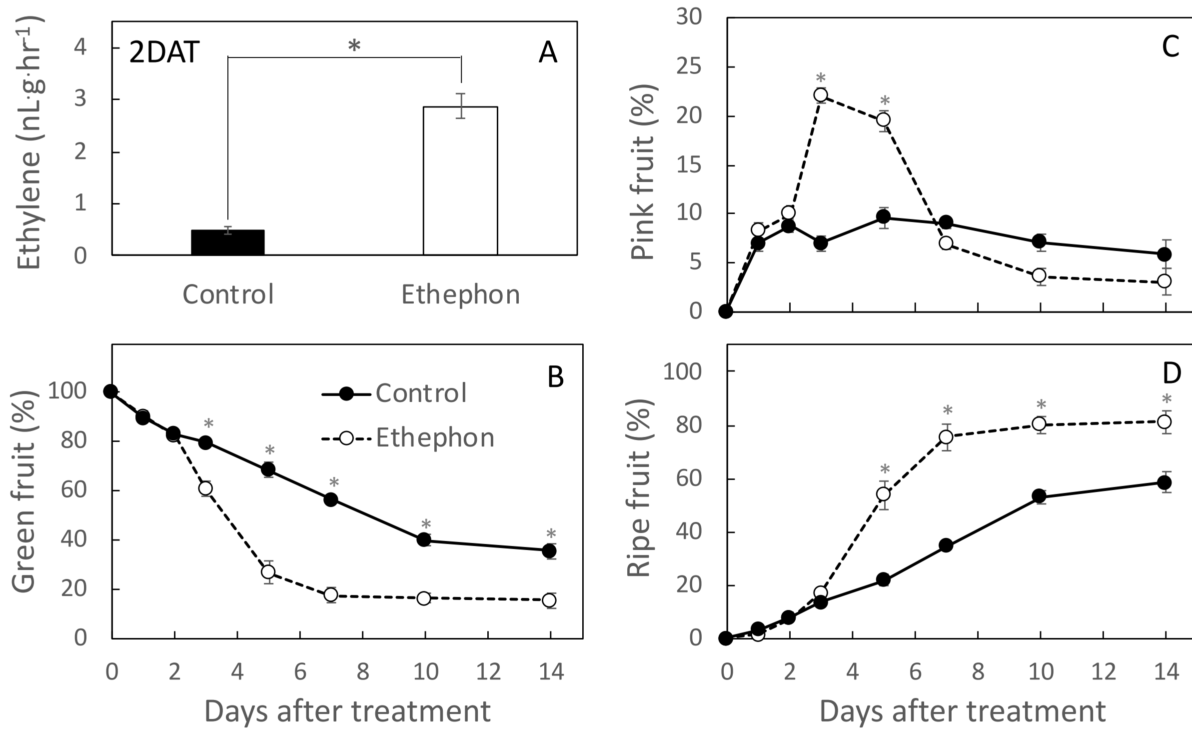

Figure 4.1. Phenotype of rabbiteye blueberry 'Powderblue' after preharvest treatments with water (control) and ethephon. Ethylene production of blueberry fruit on 2 days after treatment (A). Ripening based on the skin color of blueberry fruit after treatments (B-C).

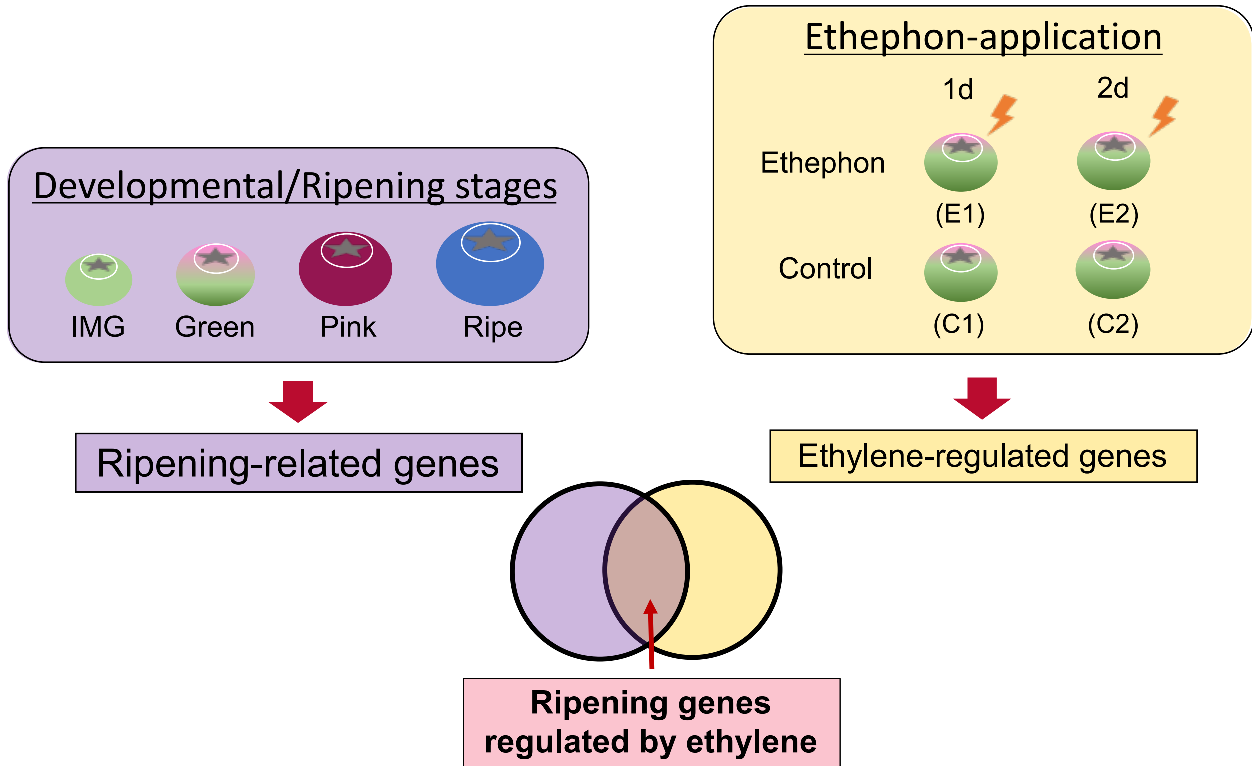

Figure 4.2. Experimental design for transcriptome analysis. IMG: Immature green

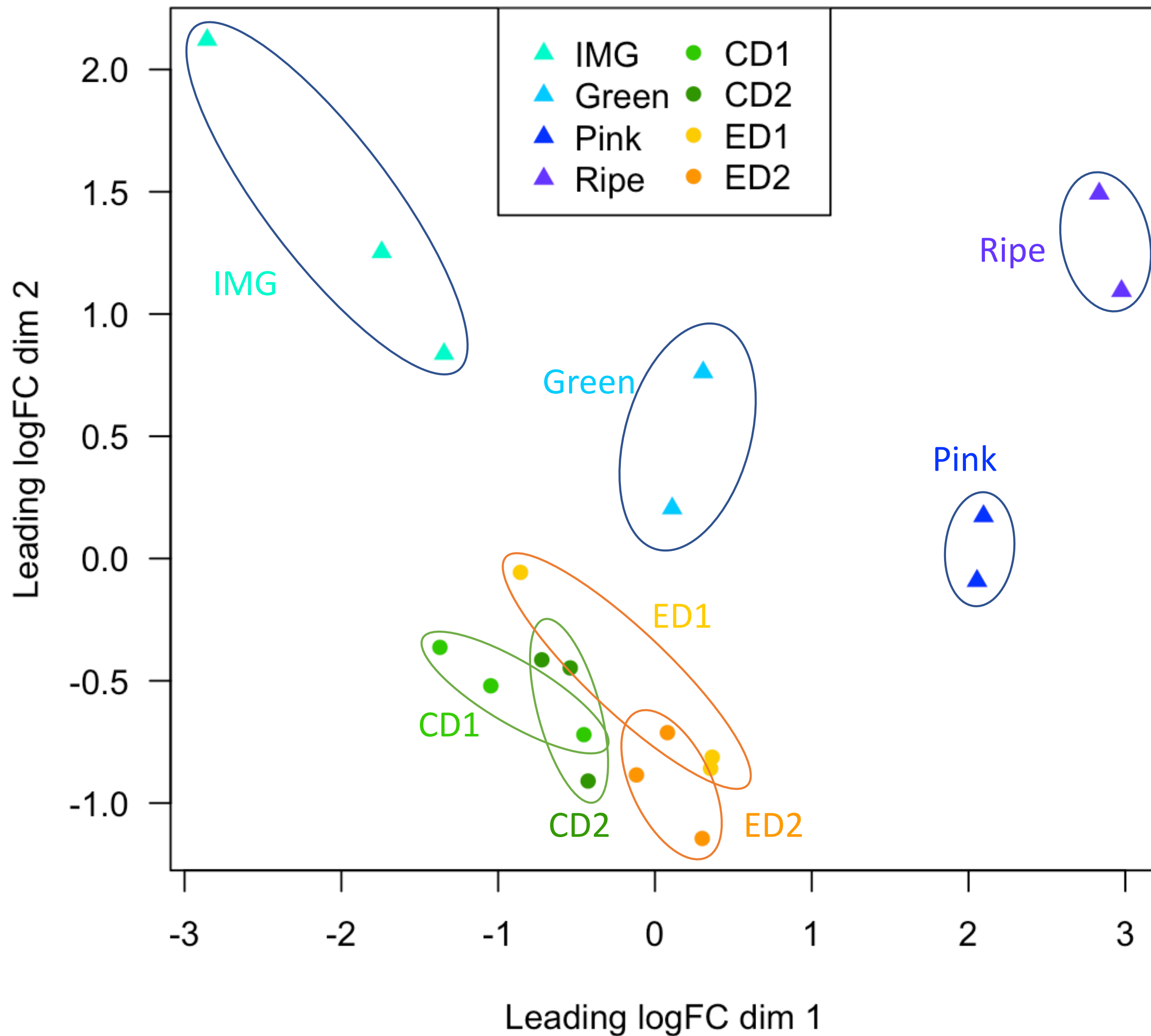

Figure 4.3. Multi-dimensional scaling (MDS) plot of rabbiteye blueberry 'Powderblue' transcript profile using leading log-fold-changes between each pair of samples. Samples include 4 ripening stages: immature green (IMG), Green, Pink, Ripe, and 4 samples after treatments: control day1 and day2 (CD1, CD2), ethephon day 1 and day2 (ED1, ED2). Each symbol represents one replicate.

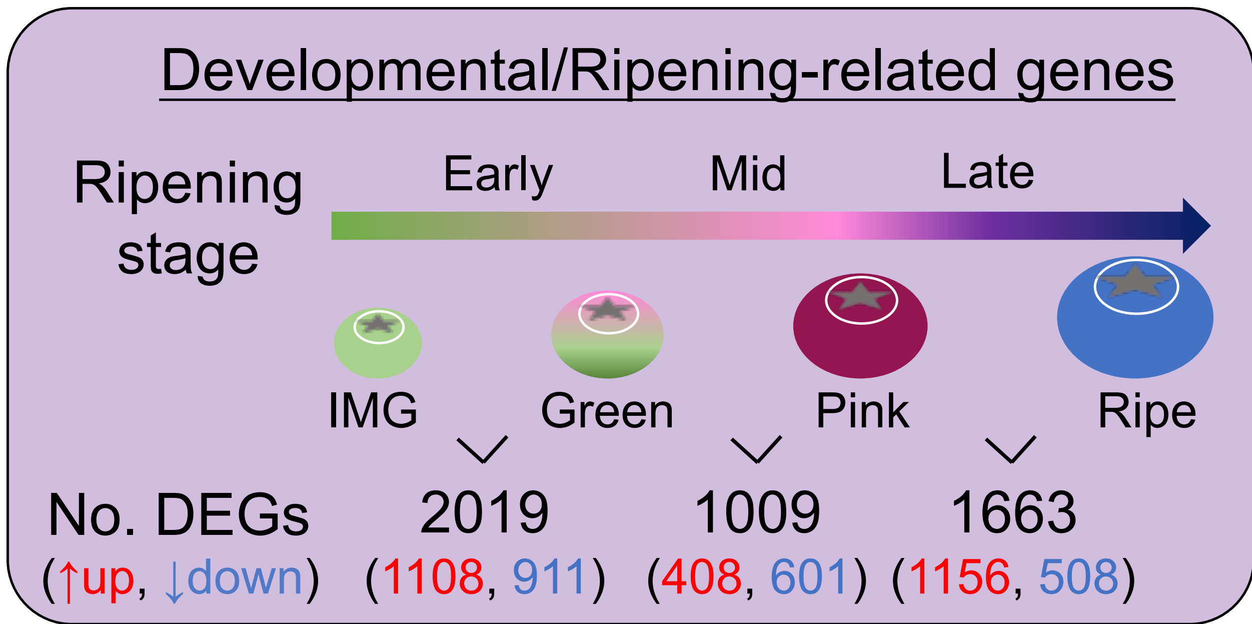

Figure 4.4. Differentially expressed genes (DEGs) between each pair of ripening stage of rabbiteye blueberry 'Powderblue'. IMG: immature green

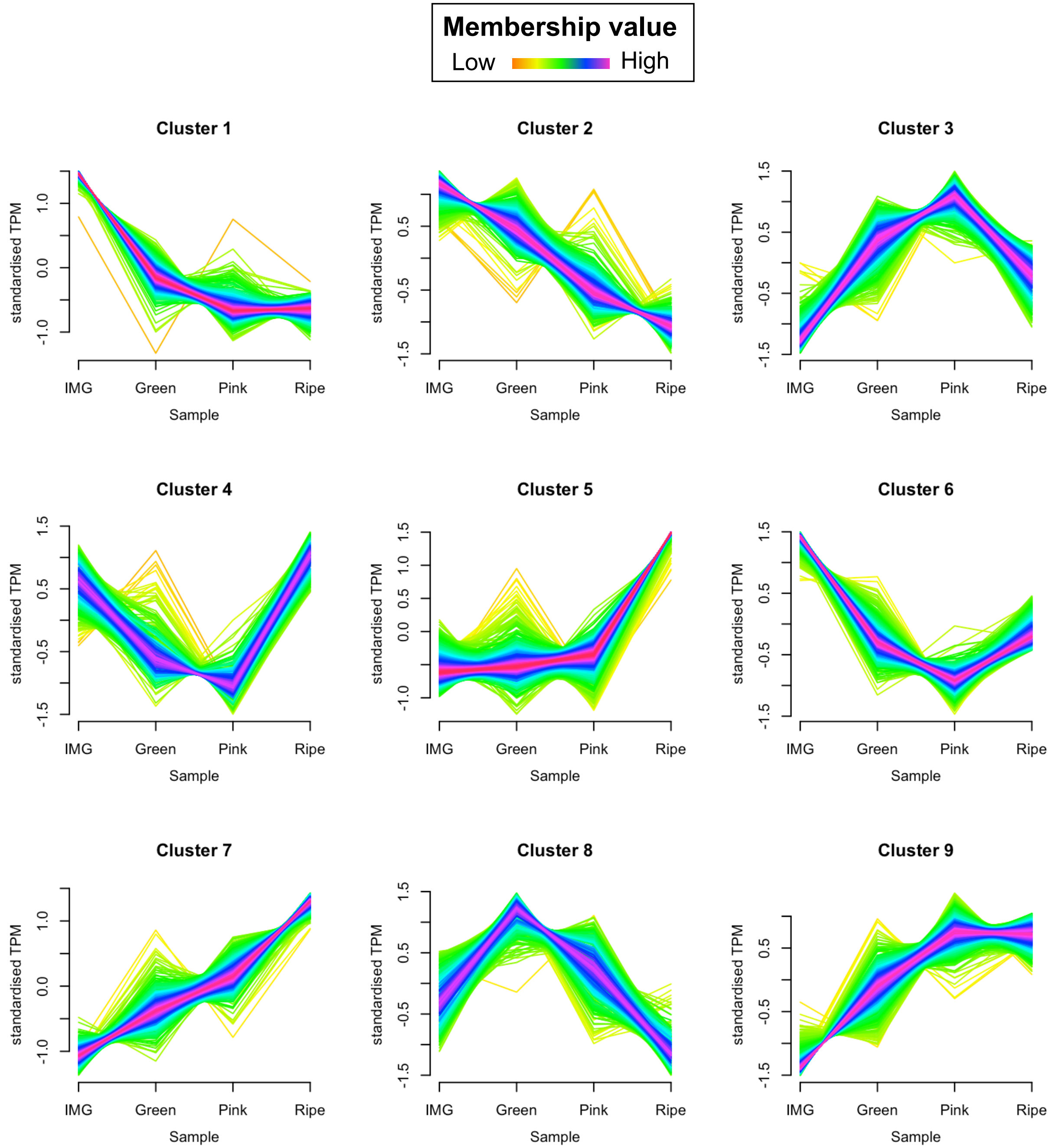

Figure. 4.5. Cluster analysis of differentially expressed genes during ripening by using Mfuzz.

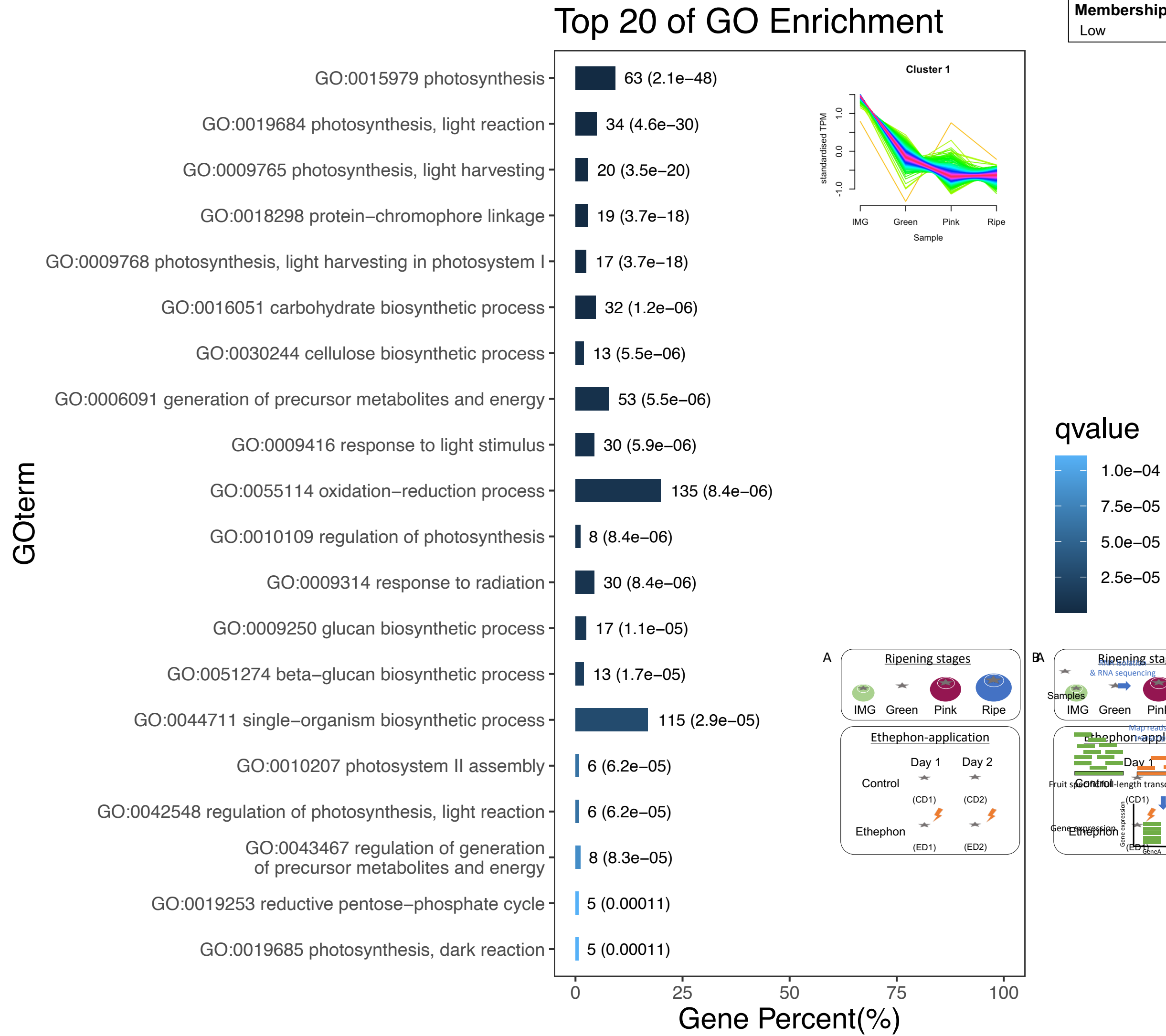

Figure 4.6. Top 20 enriched biological process gene ontology (GO) terms for ripening-related genes in cluster 1

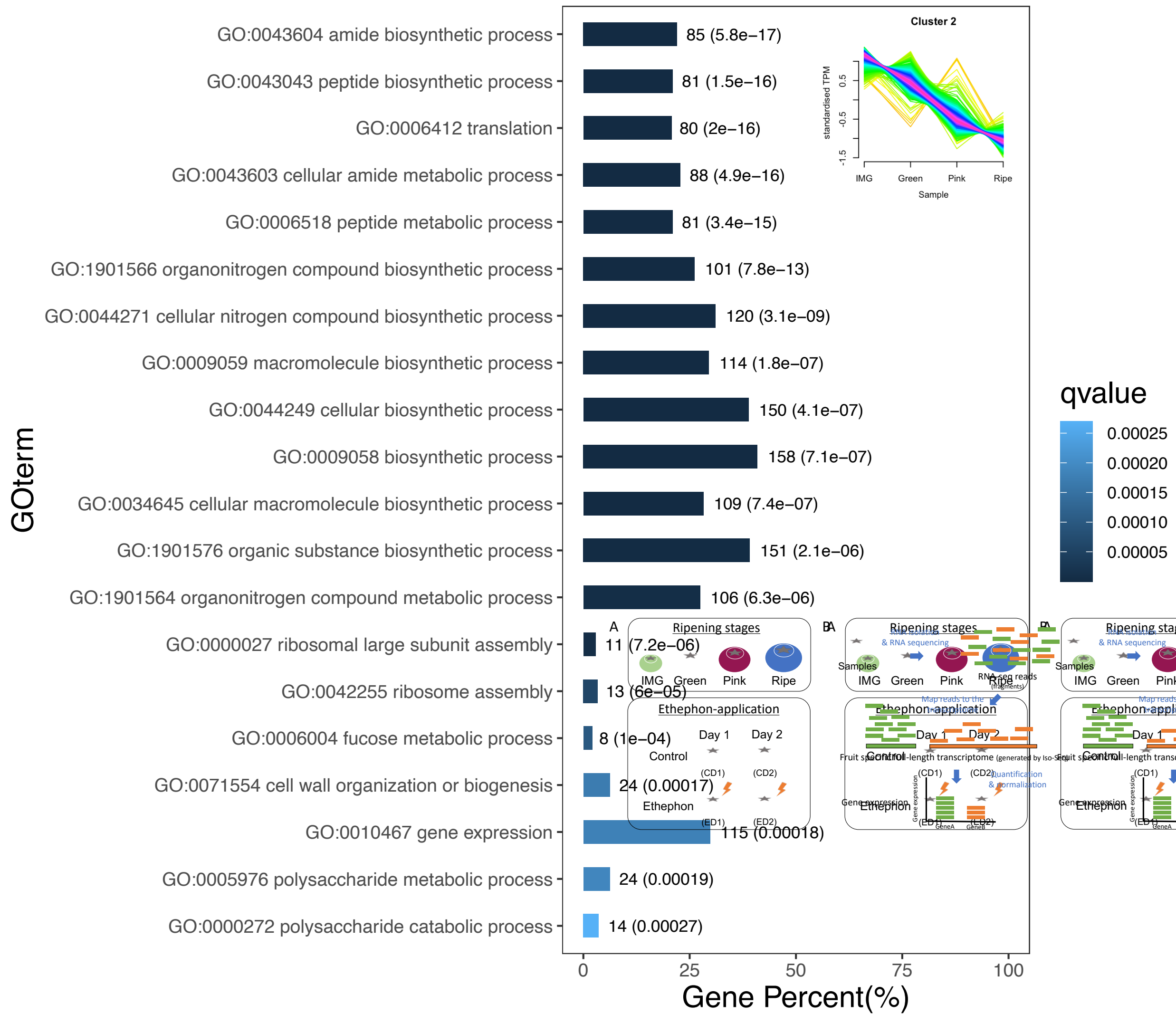

Figure 4.7. Top 20 enriched biological process gene ontology (GO) terms for ripening-related genes in cluster 2.

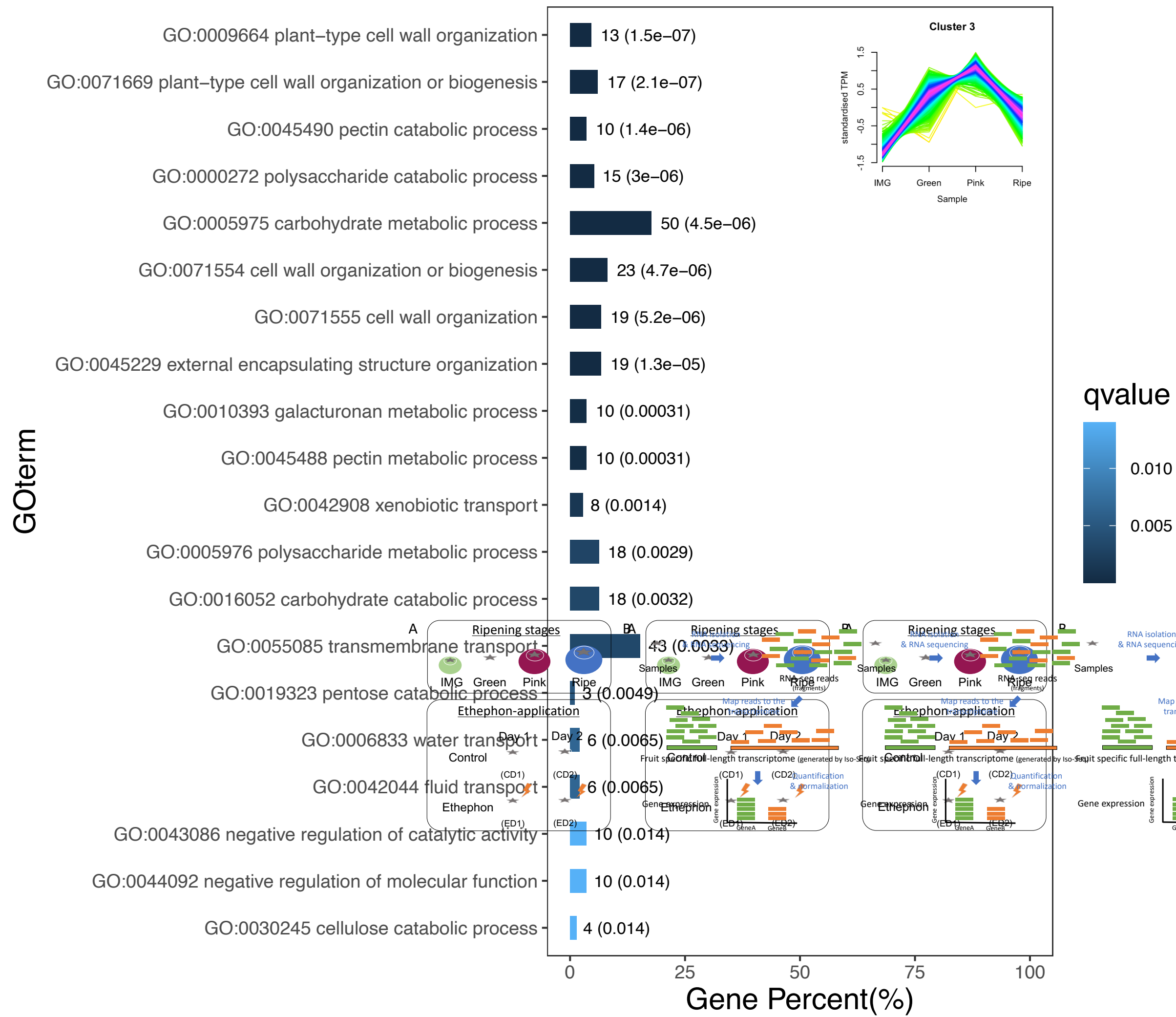

Figure 4.8. Top 20 enriched biological process gene ontology (GO) terms for ripening-related genes in cluster 3.

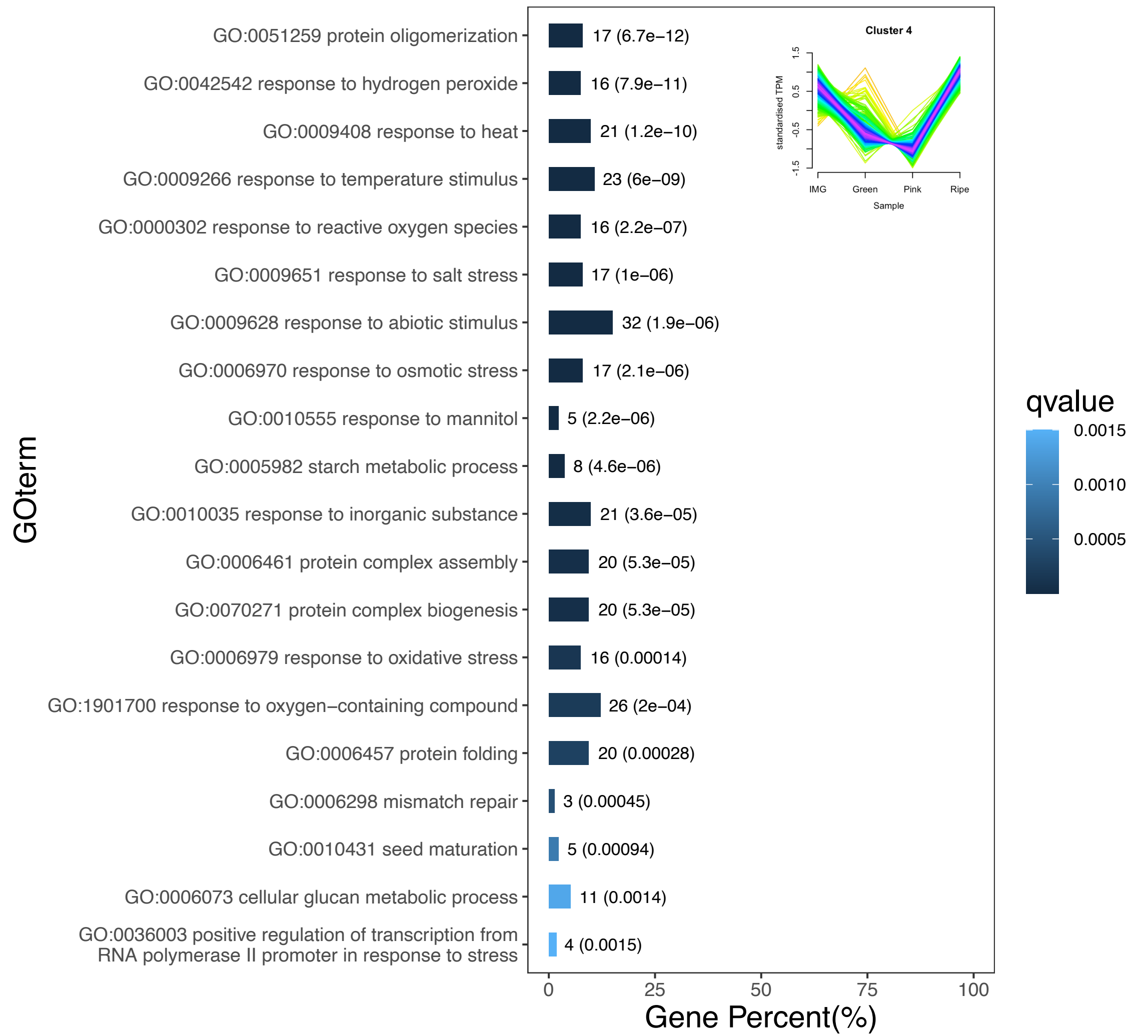

Figure 4.9. Top 20 enriched biological process gene ontology (GO) terms for ripening-related genes in cluster 4.

Low High

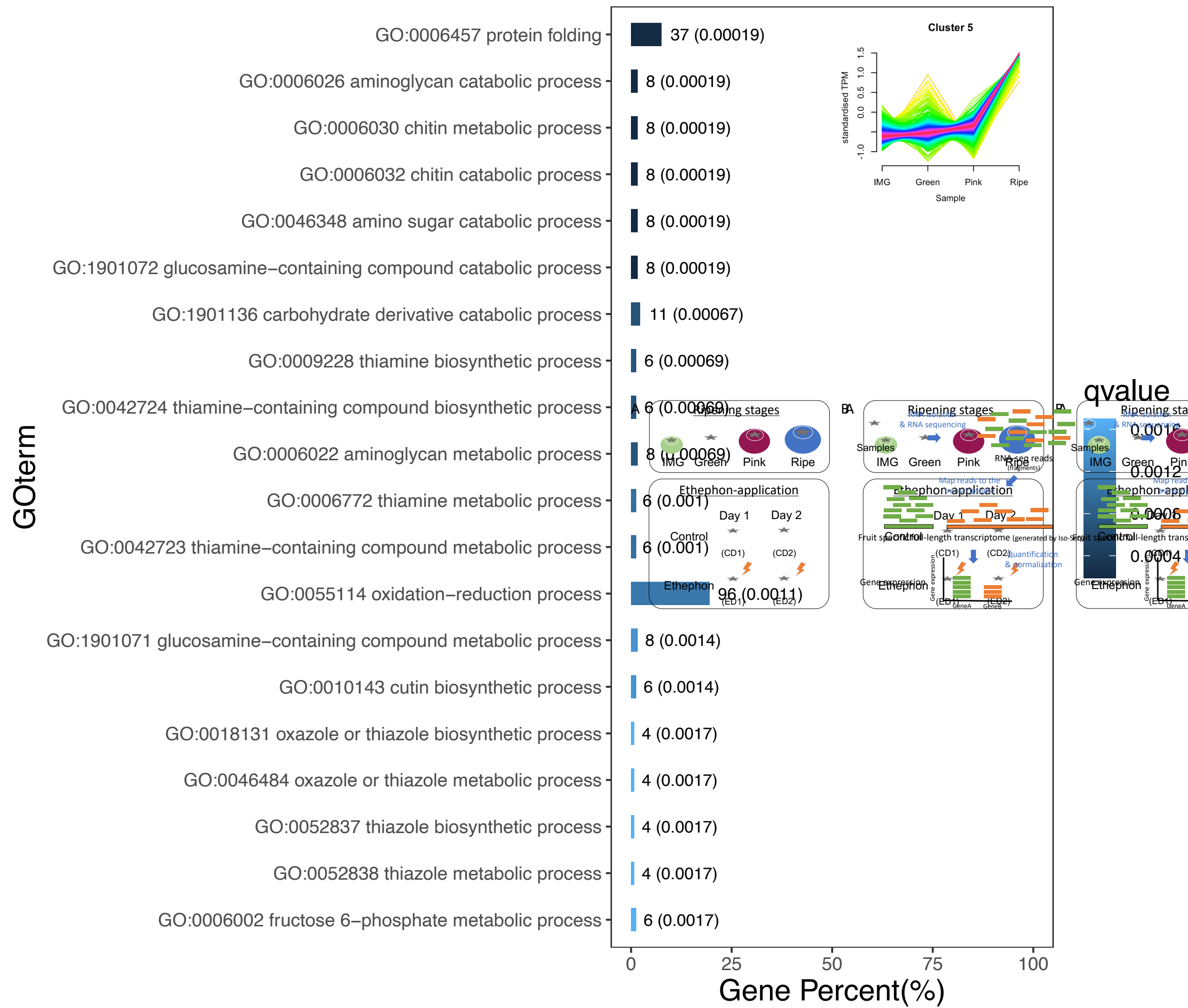

Figure 4.10. Top 20 enriched biological process gene ontology (GO) terms for ripening-related genes in cluster 5.

Low High

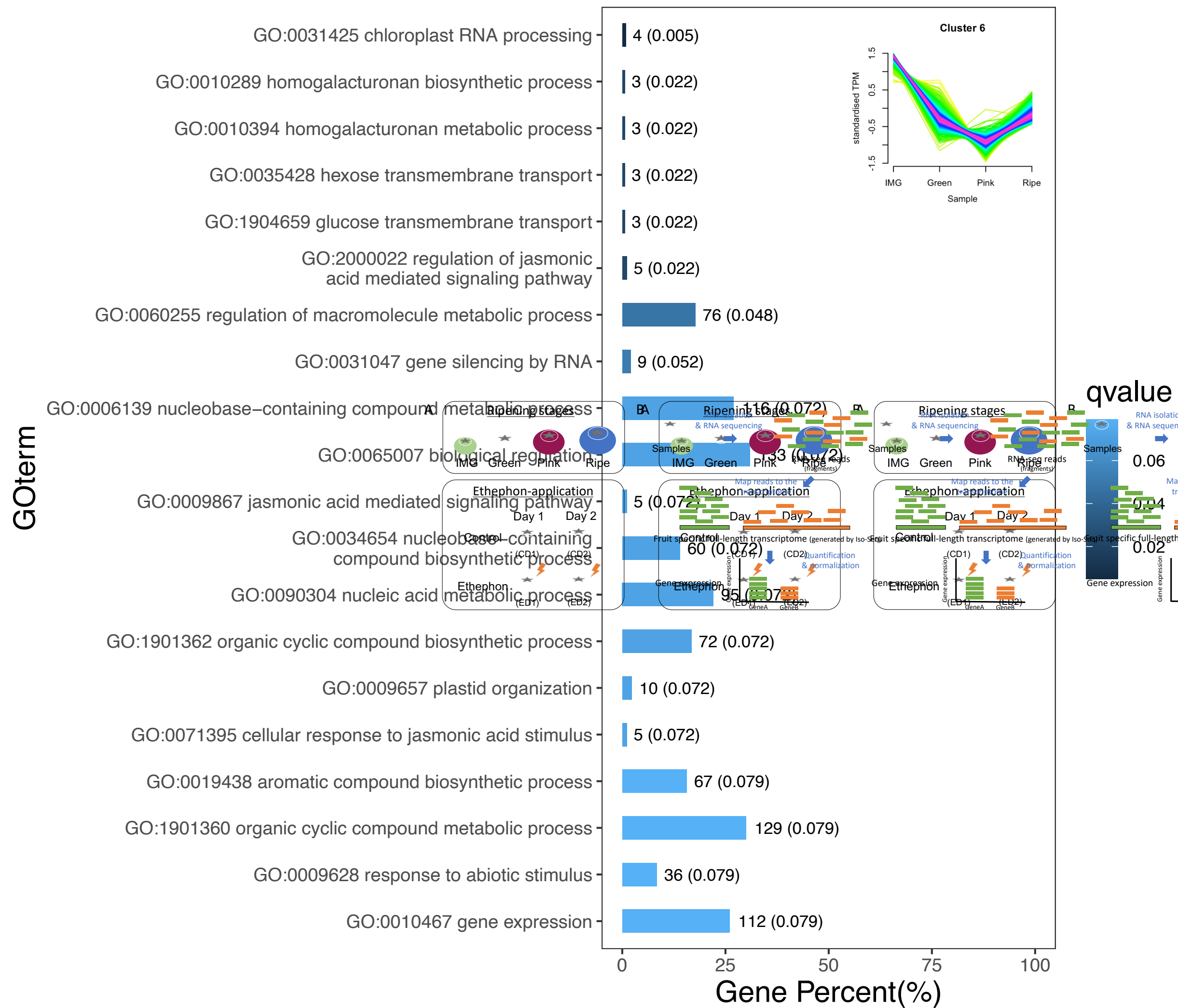

Figure 4.11. Top 20 enriched biological process gene ontology (GO) terms for ripening-related genes in cluster 6.

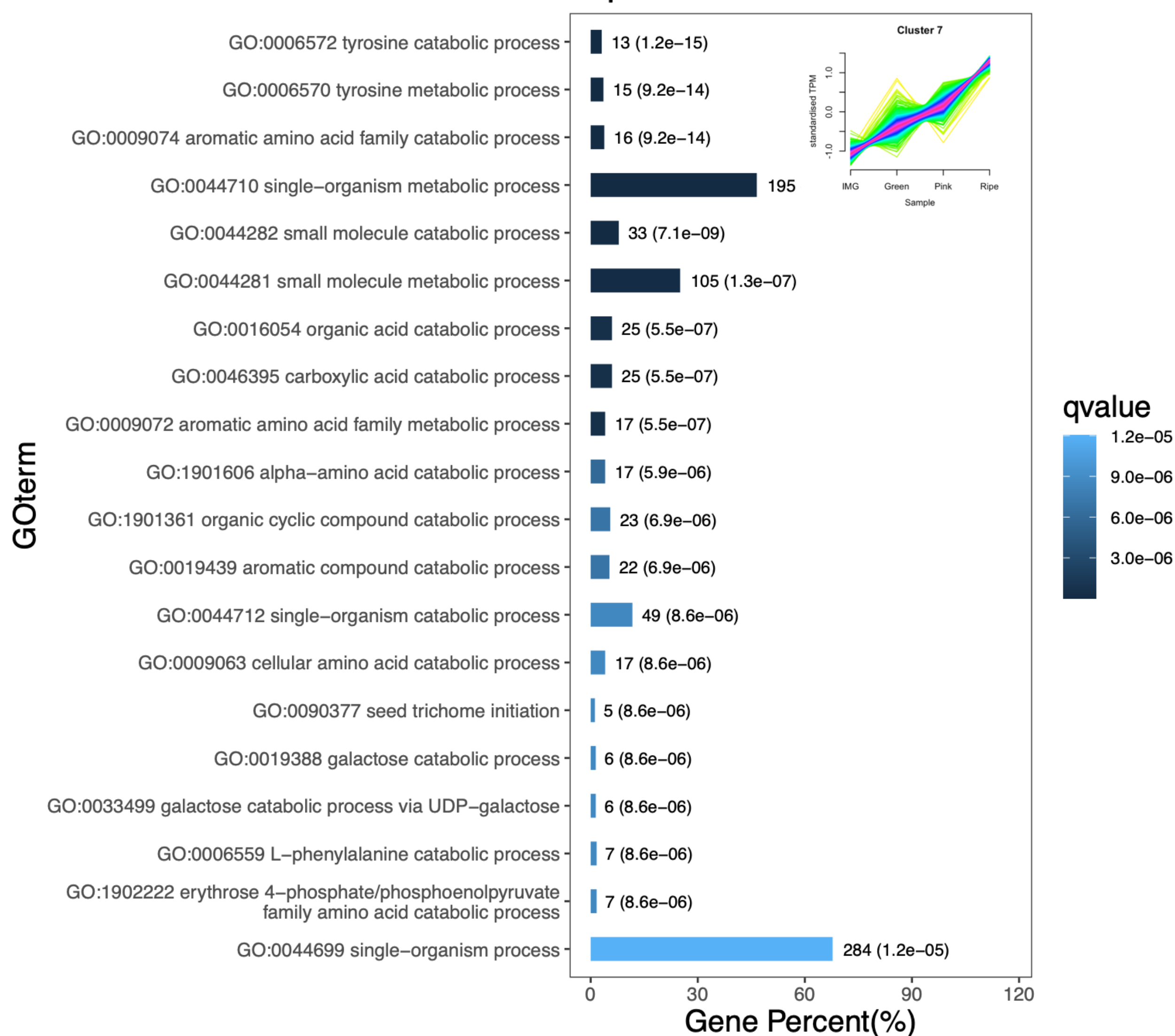

Figure 4.12. Top 20 enriched biological process gene ontology (GO) terms for ripening-related genes in cluster 7.

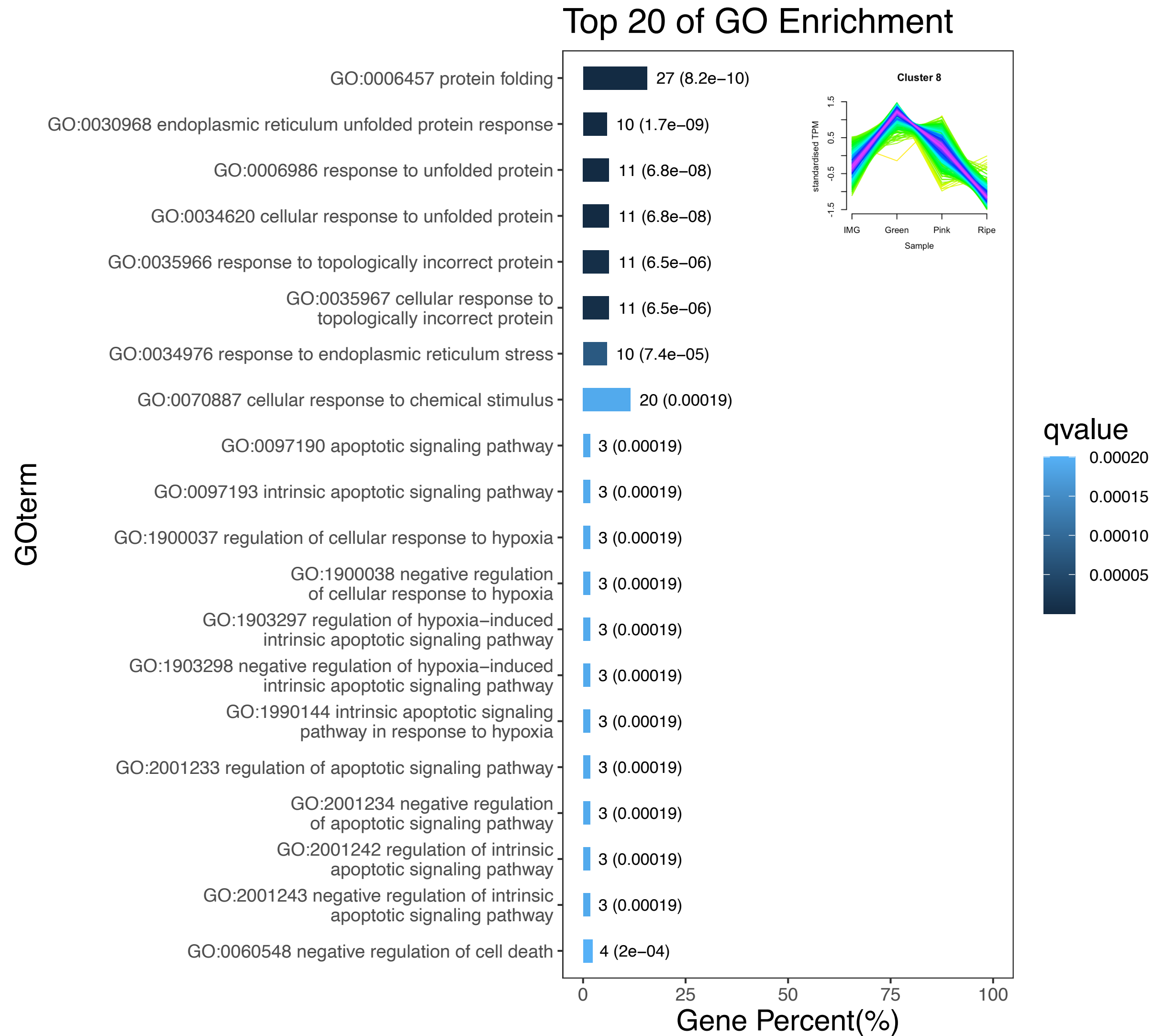

Figure 4.13. Top 20 enriched biological process gene ontology (GO) terms for ripening-related genes in cluster 8.

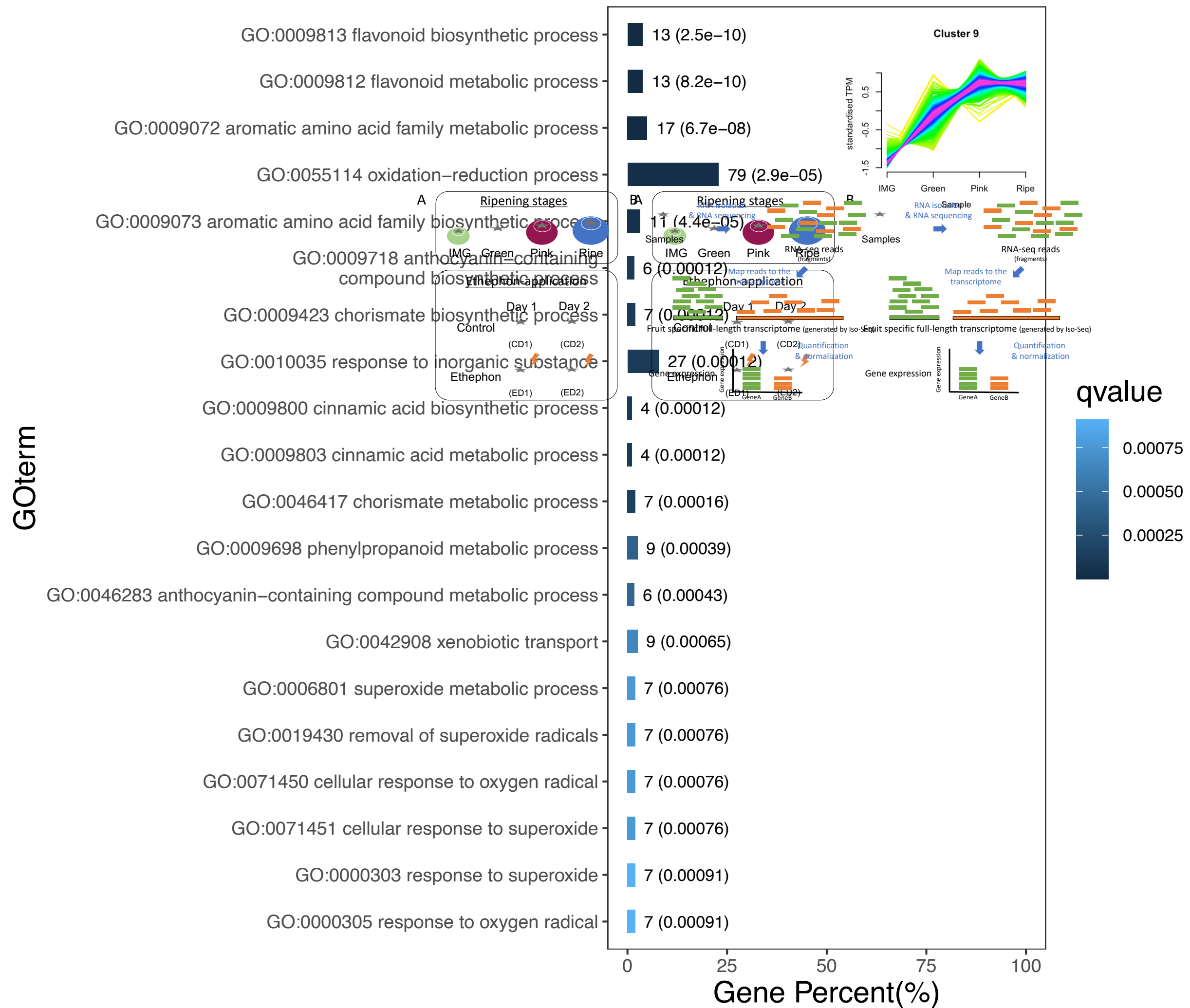

Figure 4.14. Top 20 enriched biological process gene ontology (GO) terms for ripening-related genes in cluster 9.

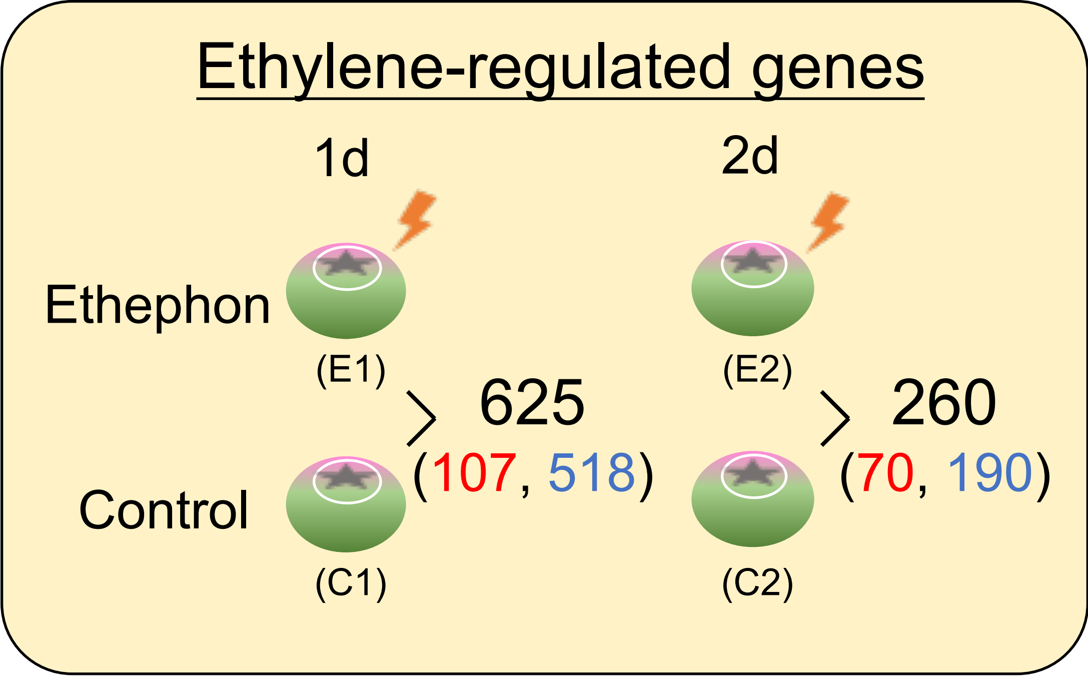

Figure 4.15. Differentially expressed genes (DEGs) between ethephon and control treated fruit on 1 day (1d) and 2 days (2d) after treatment in rabbiteye blueberry 'Powderblue'.

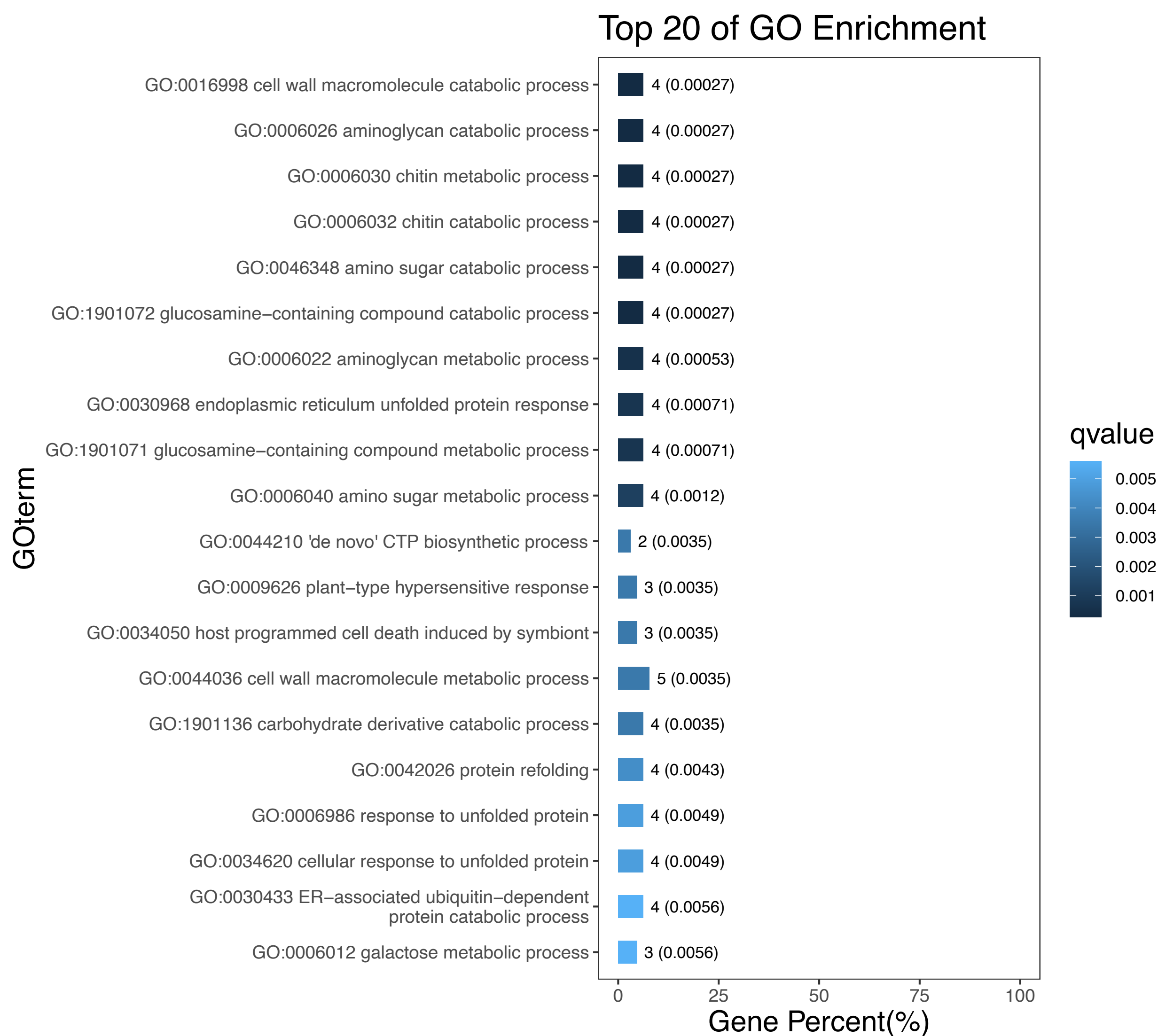

Figure 4.16. Top 20 enriched biological process gene ontology (GO) terms for genes upregulated by ethephon on day 1 after treatment.

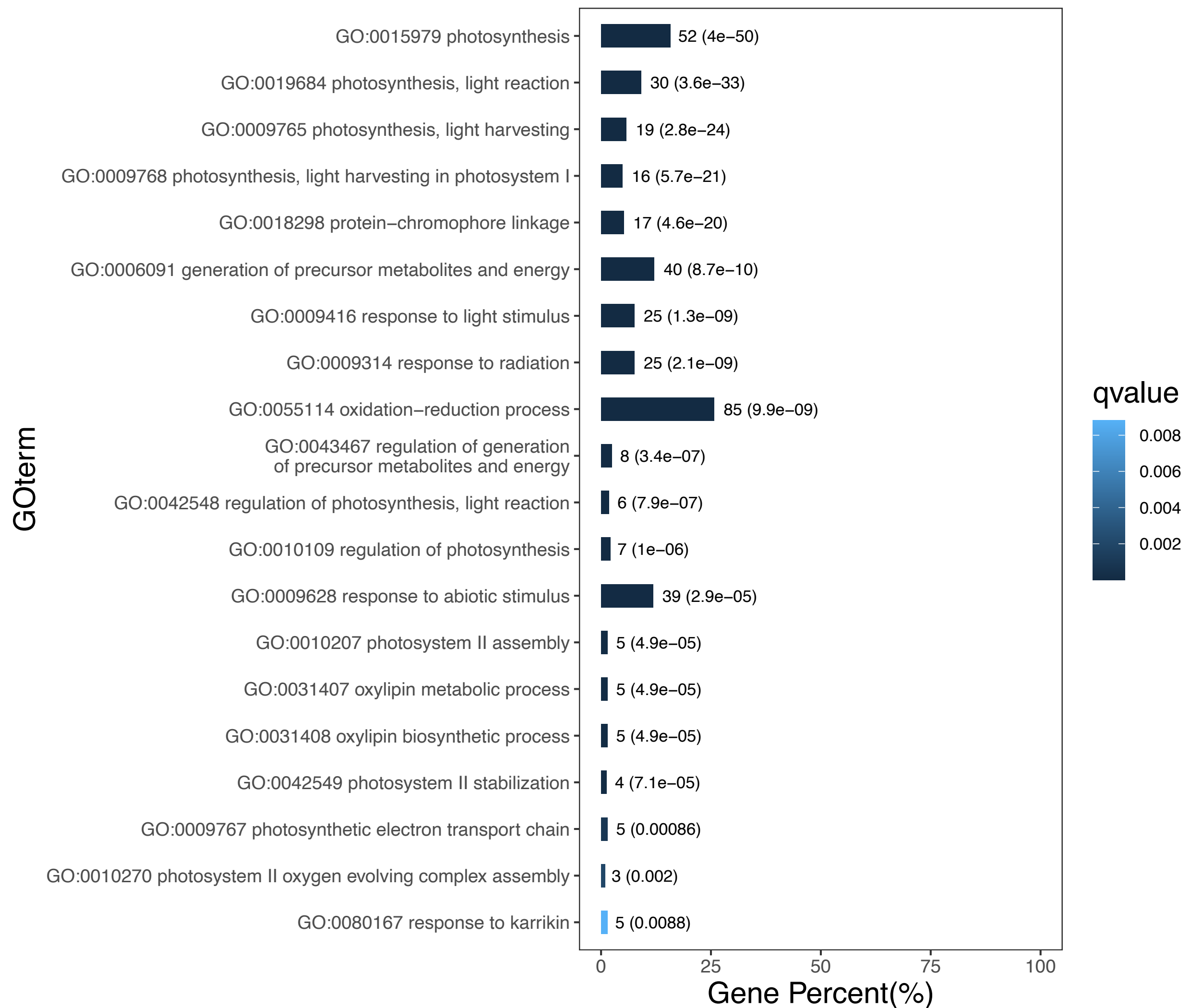

Figure 4.17. Top 20 enriched biological process gene ontology (GO) terms for genes downregulated by ethephon on day 1 after treatment.

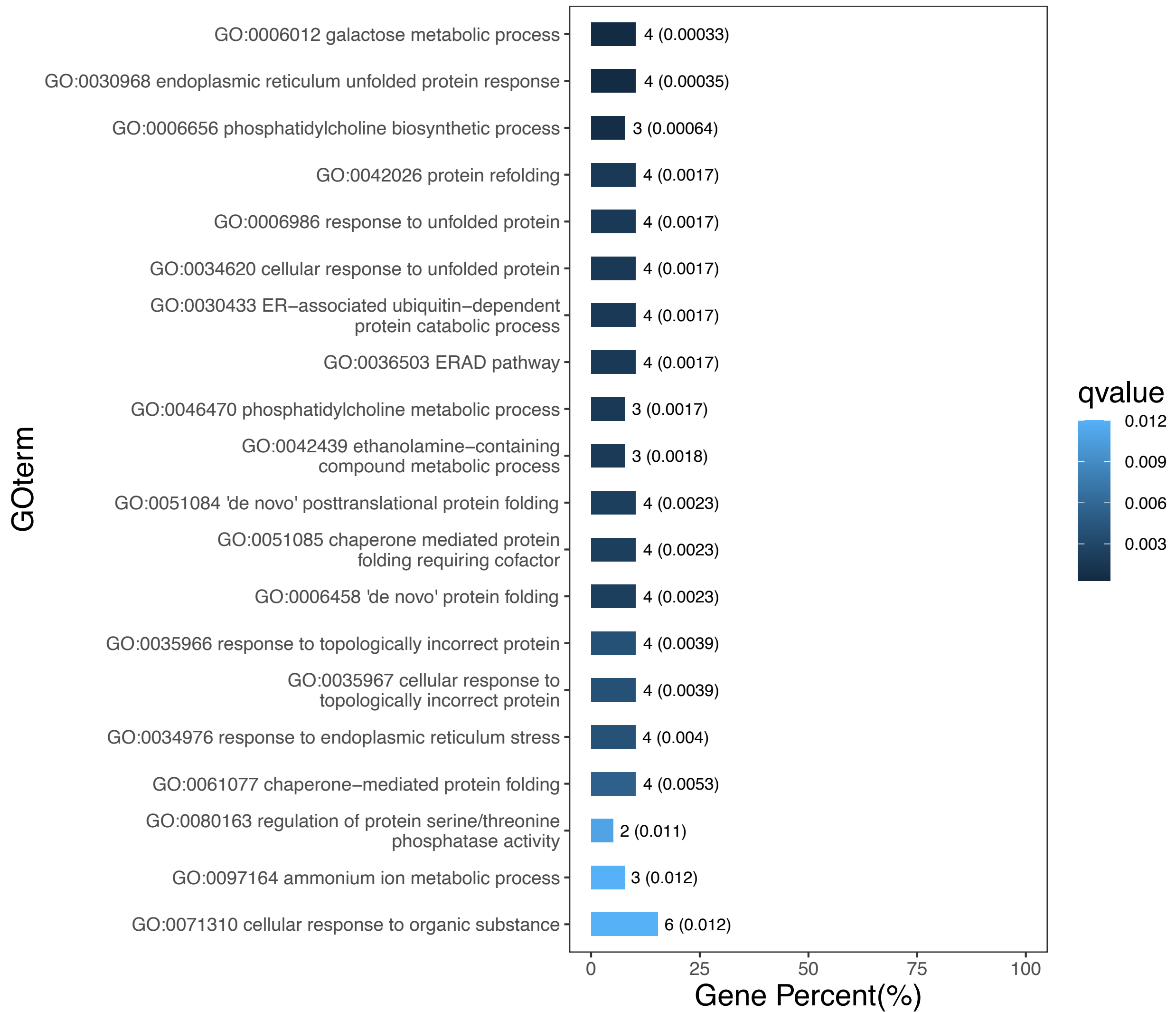

Figure 4.18. Top 20 enriched biological process gene ontology (GO) terms for genes upregulated by ethephon on day 2 after treatment.

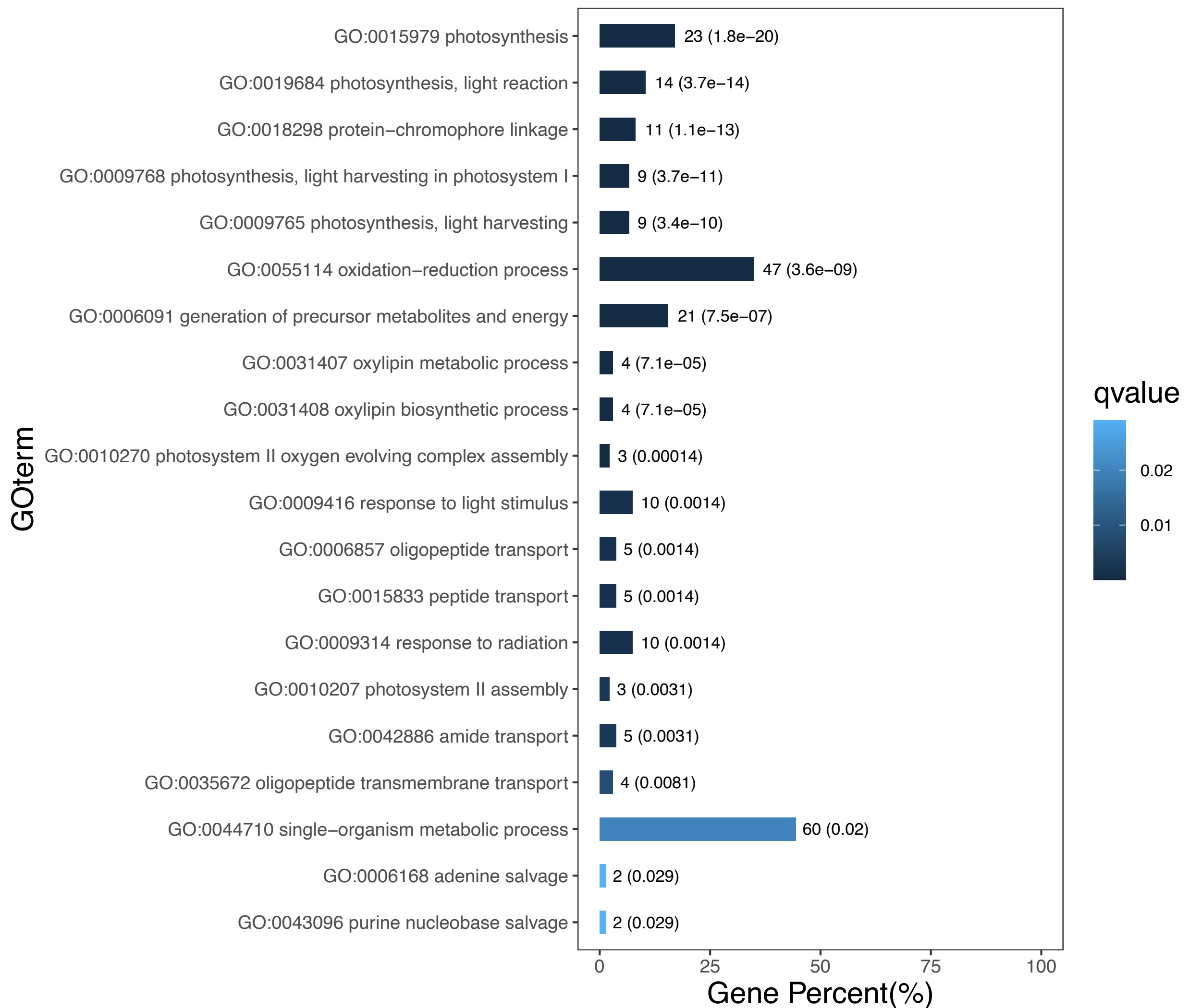

Figure 4.19. Top 20 enriched biological process gene ontology (GO) terms for genes downregulated by ethephon on day 2 after treatment.

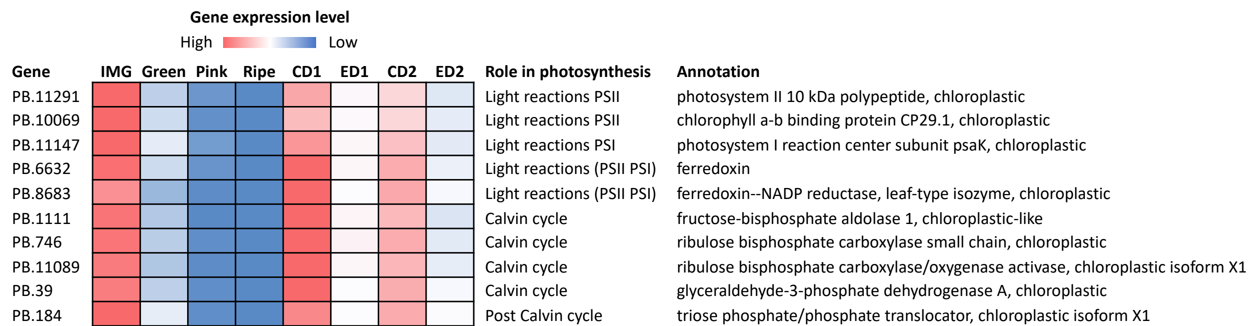

Figure 4.20. Gene expression levels of photosynthesis-related genes. IMG: immature green, CD1, CD2: control treated fruit after 1 and 2 days of treatment, ED1, ED2: ethephon treated fruit after 1 and 2 days of treatment. The gene expression level was normalized within each gene.

CHAPTER 5

TRANSCRIPTIONAL REGULATION OF POSTHARVEST FRUIT QUALITY IN BLUEBERRIES[4]

[4] Yi-Wen Wang, Shan Jing, Ann E Loraine, D. Scott NeSmith, and Savithri U. Nambeesan. To be submitted to Postharvest Biology and Technology.

Abstract

The popularity of blueberries is increasing due to consumer awareness of their health benefits. However, blueberries have a shelf-life of only about 1 to 7 weeks after harvest. To understand the mechanism that regulates postharvest fruit quality in blueberry, RNA-seq was conducted using southern highbush cultivars with superior and inferior postharvest fruit quality during storage, Suziblue and Rebel. By comparing the differentially expressed genes during postharvest storage in both cultivars, we identified several processes that may play important roles during postharvest storage. These include cytoskeletal and cell wall changes, accumulation of various amino acids, and water loss during storage. Further, these data revealed that many of the differentially expressed genes might also be triggered by various abiotic and biotic stress conditions. It is possible that once the fruit is harvested and stored under cold temperatures, they are subjected to multiple abiotic stressors such as fruit water loss, cold stress, and reduced light availability. Additionally, they are potentially exposed to multiple postharvest pathogens. These conditions may elicit a fruit transcriptional response during postharvest storage that allows the fruit to adapt to the stress conditions. The information generated by this study provides several hypotheses regarding factors that regulate postharvest fruit quality that can be tested in the future.

KEYWORDS: RNA-seq, postharvest storage, fruit quality, stress

Introduction

The popularity of blueberries is increasing due to consumer awareness of their numerous health benefits, including high antioxidant [1] and anti-inflammatory activities [2]. During postharvest storage, fruit quality loss can occur due to fruit softening [3] and postharvest diseases [4,5]. Previous studies have indicated that depending on the genotype, method of harvest, and storage regime, blueberries have a shelf-life of about 1 to 7 weeks after harvest [6,7]. Among various northern highbush cultivars, the shelf-life of 'Brigitta' and 'Legacy' is 7 weeks, 'Bluegold' is 3-5 weeks, and 'Bluecrop', 'Elliott', 'Nelson', 'Jersey', and 'Little Giant' can be stored for only 3 or fewer weeks under 5°C [7]. When conditions for long-term storage are used (0°C, 90% relative humidity, 2% O_2 and 8% CO_2), 'Bluegold', 'Brigitta' and 'Legacy' have higher storability for 4-7 weeks compared with 'Elliot', 'Little Giant' and 'Nelson' [6]. These studies suggest that genetic control of postharvest shelf-life exists among blueberry cultivars. However, mechanisms underlying the genetic control of blueberry postharvest shelf-life are unclear and warrant further research.

Transcriptome analysis is a robust approach to reveal the mechanisms of genetic variation in fruit quality. Compared to the table grape 'Kyoho', ROS and pathogenesis related genes are highly expressed and may play roles in its early-ripening mutant 'Fengzao' by RNA-seq [8]. Also, in woodland strawberry (*Fragaria vesca*), RNA-seq reveals that not only flavonoid biosynthesis genes but also transcription factors such as MYB, WDR, MADS are downregulated in yellow fruit 'Yellow Wonder' than red fruit 'Ruegen' [9]. Further, this approach has also been employed to identify candidate genes that may regulate postharvest fruit quality in blueberries and other fruits [10,11]. For example, in rabbiteye blueberry 'Garden blue', genes that regulate the common volatile compounds during postharvest storage, such as ethyl acetate and linalool, had been

identified [12]. In northern highbush blueberry 'Duke', transcription factors and genes that regulate membrane lipid and energy metabolism have been found involved in the mechanism of chilling injury during cold storage [13]. However, there are still limited studies that reveal the mechanism of genetic variation in blueberry postharvest storage. In this study, the genetic control of blueberry postharvest shelf-life of rabbiteye blueberry will be investigated using cultivars identified as with better and inferior postharvest fruit quality and transcriptomic analysis.

Methods

Plant materials

Fruits from southern highbush blueberry cultivars, 'Rebel' and 'Suziblue' at the ripe stage fully blue) were hand harvested at the UGA Blueberry Research farm in Alapaha, Georgia, and were transported to the laboratory at University of Georgia, Athens, Georgia for further analyses. Each cultivar had 4 biological replicates. After the fruits were brought to the laboratory, each replicate was sorted and distributed evenly into 4 clamshells, with at least 40 berries, respectively, for postharvest storage (4 ^{o}C under ~90% relative humidity). Clamshells from each replicate were randomly taken at 3, 8, 13, 21 days of storage for the fruit quality measurement or RNA-sequencing.

Fruit quality measurement

Defective fruit and fruit compression were measured following the method described in [14]. In short, defective fruit were evaluated by visual assessment with any fruit displaying bruising, dents, leakiness, or mold symptoms characterized as defective fruit. Thirty fruit were

used for each replicate. The ratio of fruit without any defect to fruit showing any visual damage was recorded as the percentage of defect-free fruit. On the other hand, compression, an indicator of firmness of fruit, was measured by 12 fruit per replicate with a fruit texture analyzer (GS-15, Güss Manufacturing, Strand, South Africa). Each berry's equatorial side was measured with a 15-mm diameter end plate and the setting of 5 mm s^{-1} and measure distance of 1 mm.

RNA-sequencing

Fruit at 4 stages were collected for RNA-sequencing: ripe (at harvest), 3, 8, and 13 days after postharvest storage (PH3, PH8, PH13) with 3 replicates in liquid nitrogen and then were stored at -80 ˚C. The extraction of RNA was performed using a cetyltrimethyl ammonium bromide-based method described in [15]. Afterward, libraries for RNA-Seq were prepared by using KAPA stranded mRNA-seq Kit (KAPA Biosystems, USA) for Illumina platform, following the instructions provided in the manufacturer's manual. After quality control, libraries were sequenced at the Georgia Genomics and Bioinformatics Core at UGA using an Illumina NexSeq500 platform with 75 bp paired-end sequencing.

RNA-seq data analyses

The reads generated by RNA-sequencing were trimmed, aligned to the full-length fruit transcriptome, with further downstream analyses to determine differential gene expression. First, adapters and low-quality reads were trimmed from the raw reads by Trimmomatic version 0.36 [16] with the parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. Afterward, the clean reads were aligned to a full-length transcriptome of 'Suziblue' (generated previously in our laboratory) by STAR version 2.7.1 [17], and the aligned reads were counted

according to the genomic feature by FeatureCounts under Subread version 1.6.2 [18]. The raw counts were used for identifying differentially expressed genes and normalized expression level, transcripts per million (TPM), by EdgeR [19]. Furthermore, GO enrichment analysis was conducted by Singular Enrichment Analysis (SEA) in agriGO v2.0 [20].

Results

Postharvest fruit quality attributes

Two southern highbush cultivars, Rebel and Suziblue, were used for postharvest fruit quality measurement. During postharvest storage, Suziblue displayed a higher percentage of defect-free fruit compared with Rebel. Overall, the percentage of defect-free fruit decreased after postharvest storage in both cultivars. In Rebel, the defect-free fruit decreased by 70% between 3 – 21 days, whereas in in Suziblue it declined by 29%. On the other hand, the decrease in compression (fruit firmness) was not severe as in defect-free fruit. In Rebel, the compression was maintained within the first 13 days and then decreased by 21% between 13-21 days after harvest. In Suziblue, compression increased by 21% between 3-13 days then reduced by 20% between 13-21 days. Besides, Suziblue had a higher compression than Rebel throughout the postharvest storage. Overall, Suziblue had a better fruit quality, while Rebel had inferior fruit quality.

RNA-sequencing

To understand the mechanism of postharvest fruit quality in blueberries, RNA-sequencing was performed on the two southern highbush cultivars, Suziblue (better fruit quality) and Rebel (inferior fruit quality). Samples from ripe (at harvest) and postharvest 3, 8 and 13 days (PH3, PH8, PH13) were used for RNA-sequencing. These stages were considered sufficient to

capture transcriptional differences during postharvest storage between the two cultivars since fruit quality decreased dramatically after PH13. Including PH21 may not differentiate gene expression differences. Each sample had 3 biological replicates.

The global expression patterns among samples were visualized by a multi-dimensional scaling (MDS) plot. The distances between each symbol indicates the leading log-fold-change between each pair of samples. In general, all replicates within a given stage and cultivar clustered together, which indicates their expression patterns were similar. Besides, the results showed that transcriptional changes in Rebel versus Suziblue were distinct. All the Rebel samples were located at the upper right, while Suziblue was located at the bottom left. Further, the ripe versus the 3 postharvest stages in each cultivar separated clearly. Ripe stages from both cultivars separated to the top left, while the postharvest stages were at the bottom right in Rebel, and bottom left in Suziblue (Fig. 5.2). These indicate that the main variations were cultivars and ripe versus postharvest stages, while the variations within the 3 postharvest stages were smaller. Furthermore, the separations among the 3 postharvest storage stages were more distinct in Rebel than in Suziblue. In Rebel, the 3 postharvest stages were close but could still be distinguished. However, in Suziblue, PH3 and PH8 partially overlapped (Fig. 5.2). These suggest the differences among postharvest stages in Suziblue were smaller than in Rebel, especially between PH3 and PH8, however the earlier postharvest stages were discernable compared with PH13.

Differentially expressed genes during postharvest storage

Differentially expressed genes (DEGs) were identified by the comparison of each pair of stages using the cutoff of 2-fold change and false positive rate of 0.01. Both Rebel and Suziblue have more DEGs between ripe and postharvest stages than between the postharvest stages.

Around 5,000 and 3,000 genes were differentially expressed between ripe and all postharvest stages in Rebel and Suziblue, respectively. However, only about 1,000 genes were differentially expressed among the three postharvest stages in Rebel and Suziblue. Within those DEGs, there were more down-regulated genes than up-regulated genes, with about 2-fold differences. Besides, Rebel had more DEGs in all comparisons between each stage than in Suziblue. Also, no DEG was identified with the current criteria between PH3 versus PH8 in Suziblue (Fig. 5.3). This may suggest that there were more metabolic changes during postharvest storage in Rebel than in Suziblue.

For further downstream analyses we only compared the similarity and the differences between Rebel and Suziblue during postharvest storage, the DEGs between PH3 and PH13 (the comparisons in the box with red line border in Fig. 5.3). These stages truly represented maximum changes occurring during postharvest storage. Comparisons between the ripe stage and postharvest stages may represent genes regulated due to harvest or cold-storage, hence were not included in the initial downstream analyses. The DEGs that were common and unique in Rebel and Suziblue between PH3 and PH13 were identified in Venn diagrams. The Venn diagrams show that there were a common set of 137 up-regulated genes and 523 down-regulated genes in both cultivars. In addition, there were 385 up-regulated genes and 484 down-regulated genes specific to Rebel; 164 up-regulated genes and 477 down-regulated genes specific to Suziblue (Fig. 5.4). The common set of DEGs in both the cultivars may be involved in postharvest storage. In addition, cultivar-specific genes may be important in maintenance of cultivar-specific fruit quality during storage.

In order to determine the feature of the DEGs, gene ontology (GO) enrichment analyses were performed. Each top 10 GO terms based on the false discovery rate are summarized in the

three aspects of ontology: biological process, molecular function, and cellular component (Fig. 5.5 - 5.7). In biological process, response to chitin, response to organonitrogen compound, and microtubule-based process were enriched in the common up-regulated DEGs in both cultivars; while water transport, fluid transport, and translation were enriched in the common down-regulated DEGs. The functional descriptions of DEGs in water transport and fluid transport were aquaporins. In addition, cell killing, killing of cells of other organism, disruption of cells of other organism were enriched in the Rebel specific, up-regulated DEGs. We further found all DEGs under these 3 GO terms were thaumatin-like proteins. In addition, genes related to auxin metabolism was also upregulated (auxin transporter-like protein 2 and auxin response factor 5/like). There were only three terms enriched in Suziblue upregulated genes. They all related to tropism with two main transcripts BTB/POZ domain-containing protein NPY4-like and protein SHOOT GRAVITROPISM 5/5-like. Further Rebel-specific downregulated transcripts also belonged to similar categories involved in translation and photosynthesis. In case of Suziblue, most of the downregulated genes were enzymes that were involved in oxido-reduction processes involved in glycolysis or respiration.

Discussion

Genes that were differentially regulated between PH13 and PH3 were selected to investigate postharvest mechanisms. Transcripts differentially regulated between Ripe and PH3 may show differential regulation due to detachment from the parent plant or due to initial storage under cold temperature conditions and therefore were not included in the analyses.

Common upregulated transcripts in 'Rebel' and 'Suziblue':

Genes commonly upregulated across both genotypes reflect common processes associated with postharvest storage. Many genes related to microtubules organization were found to be upregulated in this group. Microtubules are associated with the cytoskeleton and are composed of alpha- and beta-tubulins [21]. Although microtubules have been studied with respect to root hair and vascular development their role in fruit ripening or postharvest storage have not been evaluated [22]. As microtubules are associated with the organization of cell wall components, it is likely that cytoskeletal changes are associated with cell wall and plasma membrane changes during ripening [21,22]. In fact, downregulation of the tubulin β-chain has been noted during ripening in date palm and pear suggesting a role in cytoskeletal changes related to fruit softening [23,24]. Further microtubule reorganization during cold acclimation was associated with increased expression of alpha tubulin genes in wheat [25]. It has been suggested that increased expression of alpha tubulin genes may be associated with conferring resistance during cold acclimation [25,26]. Since fruit in this study were under cold-storage, it is also possible that upregulation of alpha tubulin genes may have been a consequence of cold acclimation during storage.

Zinc finger CCCH-domain containing protein 29-like and C2H2-domain containing zinc finger protein ZAT10 and ZAT10-like were also identified in this group. Zinc finger transcription factors belong to one of the largest families of transcription factors and members of this gene family are involved in flower and fruit development [27,28]. Overexpression of a rice CCCH-zinc finger transcription factor delayed leaf senescence [29]. A chilling resistant zucchini cultivar 'Natura' displayed increased expression of *ZAT10-like* when stored at 4° C, and the chilling sensitive cultivar showed decreased expression when stored at 20° C suggesting the

importance of this transcription factor during postharvest storage and chilling tolerance [30]. Further members of the CCCH and C2H2 zinc finger family transcription factor expression have been associated with osmotic, drought, salt, heat and biotic stress responses [31-33]. It is likely that postharvest water loss, cold storage or pathogen infection triggered changes in the abundance of these transcription factors in this study.

Another class of genes that encode chitinases were upregulated. Endochitinases are enzymes synthesized by plants that breakdown chitin, which is a major component of the phytopathogenic fungi, thus limiting infection [34,35]. In banana higher chitinase activity was observed after inoculation with *Mycosphaerella fijiensis* in a resistant compared to a susceptible genotype [36]. Some of the common postharvest pathogens in blueberry include *Alternaria tenuissima*, *Colletotrichum acutatum*, and *Botrytis cinerea* [37,38]. Typically, blueberry fruit are treated with preharvest fungicide to control the above postharvest pathogens. However, the two cultivars used in this study were harvested from a farm where no preharvest fungicides were used. Thus, upregulation of chitinases may have been a result of pathogen load which is common during postharvest storage.

Upregulation of a cell wall degrading enzyme xyloglucan endotransglucosylase/hydrolase was also observed in this study. These enzymes display xylogucan transglucosylase (XET) and/or hydrolase activity (XEH) [39]. In tomatoes, XET activity is involved in maintenance of cell wall integrity, while delayed softening is noted when XEH activity is increased [40]. Overexpression of persimmon *DkXTH8* in tomato led to increased fruit softening [41]. In this study higher expression of *XTH* during postharvest storage may suggest a role for it in cell wall modification (probable xyloglucan endotransglucosylase/hydrolase protein 23). Interestingly, Rebel showed a 5.9-fold increase whereas Suziblue a 2.5-fold increase from PH3 to PH13. In

addition, 5 more transcripts annotated as probable *XTH* also showed 2.4- to 5.1-fold upregulation only in 'Rebel' (between PH3 to PH13). Thus, in future it would be interesting to determine the role of XTH in fruit softening in blueberry fruits that differ in postharvest shelf-life.

Upregulation of Rebel-specific transcripts:

In Rebel it was not surprising that the top GO categories included transcripts associated with defense. As mentioned earlier Rebel showed higher visual incidence of postharvest pathogens. Further, Rebel-specific up-regulated transcripts also included thaumatin-like protein (TLP), which are members of the pathogenesis-related proteins and have been implicated in biotic and abiotic stress responses [42,43]. Accumulation of TLPs during cold storage have been noted in peaches. Although both chilling resistant and sensitive peach cultivars accumulated TLPs, earlier accumulation of these proteins in chilling resistant cultivars suggest that they may offer protection against cell wall [44]. Supporting this, upregulation of a brassinosteroid insensitive 1-associated receptor kinase 1 transcript that encodes a protein involved in brassinosteroid signaling was also found. Brassinosteroids have been implicated in protection against abiotic stress [45]. Application of brassinosteroids improves postharvest storage and decreases decay due to *Penicillium expansum* in jujube fruit and increases marketable fruit yield in strawberries [46,47]. Thus, the above transcripts are likely upregulated due to Rebel exhibiting higher rate of decrease in percent healthy fruit and possibly greater pathogen load compared to Suziblue.

Further, several GO category terms associated with amino and amine metabolism were upregulated in Rebel. After harvest, fruit continue to undergo respiration that fuels catabolic processes such as degradation of nucleic acids, lipids and proteins which can eventually lead to

loss in fruit quality [10,48,49]. This can lead to changes in the primary metabolic pathways, which include changes in the amino acid content during postharvest storage [48,50]. Amino acids also serve as precursors for volatile compounds in ripe fruit and contribute to flavor and defense [51,52]. Further certain amino acids function as osmoprotectants under abiotic stress responses. For example, proline accumulation has been associated with many abiotic stress responses where it functions to protect against membrane damage and in limiting oxidative stress [45,53,54]. Proline accumulation has been indicated to reduce symptoms of chilling injury in banana and pear [55,56]. In grapes, proline and glycine betaine were associated with enhanced postharvest shelf life through reduction of water loss and protection of membranes [57]. In addition, concentrations of multiple other amino acids such as Valine, Isoleucine, Threonine, Alanine, Tryptophan, Aspartate, Glutamate and GABA are altered during kiwi postharvest storage [58]. Similar changes in multiple amino acids have been noted during cold storage and defense responses [59,60]. In this study, transcripts belonging to the AFT family of amino acid transporters such as amino acid transporter AVT1I, auxin transporter-like protein 2, lysine histidine transporter-like 8, probable amino acid permease 7 isoform X1, probable GABA transporter 2, and proline transporter like were upregulated. While proline and GABA transporters specifically transport proline and GABA respectively, some of the other transporters such as the amino acid transporter AVT (involved in vacuolar transport), and lysine histidine transporter and amino acid permease transporter may transport multiple amino acids and display low specificity [61]. These data suggest that there may be accumulation of some amino acids such as proline and GABA during blueberry storage. Similarly, several amino acid transporters were also upregulated during postharvest storage in citrus [62]. Enrichment of these transporters were specifically observed in Rebel, likely as Rebel was displaying greater loss of fruit quality.

Further, auxin transporters usually transport auxin instead of amino acids [61]. In fact, auxin response factors (ARFs) displayed higher abundance in this data set. It would be interesting to see in future experiments if auxin plays a role in blueberry postharvest storage. In addition, transcripts associated with polyamine metabolism (arginine decarboxylase, probable polyamine oxidase, and S-adenosyl decarboxylase proenzyme-like) and nitrogen mobilization (glutamate dehydrogenase and asparagine synthetase) were identified. Genes related to both these processes are involved in senescence [63-66]. Collectively these transcripts suggest higher accumulation of senescence related genes in Rebel.

Upregulation of Suziblue-specific transcripts:

Many of the upregulated genes specifically in Suziblue were auxin-related with functions in shoot gravitropism. During root gravitropic response, sedimentation of starch filled granules called amyloplasts occurs in the columella cells, and auxin plays an important role in root bending along the gravity vector [67]. In this study, shoot gravitropism 5, that encodes a C2H2 finger transcription factor involved in gravitropic responses in the inflorescence stem in Arabidopsis, displayed higher expression [68]. Another gene, BTB/POZ domain-containing protein NPY4-like belonged to a family of genes among which one member NPY1 encodes a protein is involved in auxin regulated organogenesis in Arabidopsis [69,70]. Interestingly, an auxin transporter-like protein 4 was also identified among the enriched genes (this gene was enriched in the Molecular Function category), suggesting auxin involvement. We also found enriched genes in the Molecular Function Category that encode for 1,4-alpha-glucan-branching enzyme 1 and granule-bound starch synthase, enzymes involved in starch synthesis [71]. It is possible that these genes belong to the pathway where in auxin is involved in starch metabolism.

In banana, auxin related transcription factors altered the expression of starch branching enzymes which may modulate starch levels during fruit development [72]. In future, studies it may be interesting to determine the role of starch during postharvest storage as currently there is limited information regarding its metabolism during this period.

<u>Common downregulated transcripts in Rebel and Suziblue</u>:

Aquaporins are membrane proteins that facilitate water transport. In addition, they have been shown to transport small neutral molecules such as glycerol, urea, carbon dioxide, ammonia, and reactive oxygen species [73,74]. An important event during fruit softening is a decrease in cell turgor but the role of aquaporins in water loss and decrease in fruit firmness has not been very well studied [75]. In strawberry, a firmer cultivar displayed higher expression of aquaporin genes compared with cultivars that softened quicker. The authors concluded that loss of cell turgor may be related to aquaporins in the cultivar that displayed greater softening [76]. Characterization of all genes belonging to the aquaporin gene family in cherry indicated that transcript abundance of most aquaporin genes was higher in the flesh than in the skin. The study also showed with a few exceptions, most of the aquaporin genes decreased in expression in mature fruit [77]. Further, during fruit development one of the most abundant transcripts, *PIP1;4* was highly expressed both in the flesh and skin. The transcript abundance of this gene decreased in the skin during fruit development but remained consistent in the flesh, suggesting spatial regulation [77]. This aquaporin was shown to transport both water and hydrogen peroxide suggesting alteration of more than just water transport during storage [78]. In citrus, transcript abundance of many aquaporin genes were downregulated, while some members were upregulated especially in the flesh suggesting higher water loss in the flesh tissue [62]. However,

in contrast, in grapes aquaporin genes were upregulated during ripening mainly in the pulp [79]. In strawberry, plasma membrane associated aquaporin genes were down regulated by auxin, suggesting hormone regulation of these genes [80]. Two plasma membrane aquaporins genes were downregulated 3 days after ethylene treatment after kiwi fruit were harvested at the physiologically mature stage, suggesting decrease in water transport through the cell membrane [81]. In our study, most of the enriched aquaporin genes were downregulated. In the future, it would be interesting to compare the expression of these genes in the flesh and skin of blueberry to determine spatial expression patterns. Further, certain members of the aquaporin gene family are differentially regulated during abiotic stress conditions. Although different members can be up or down-regulated in cold and drought stress, in general most of them are down regulated during drought stress [73]. In the current study, since fruit were stored at low temperature with possibly some postharvest water loss, it is possible that these abiotic stress conditions may contribute to their altered transcript abundance. Therefore, in future it may be interesting to determine the effect of postharvest stress during storage on transcript abundance of aquaporin genes.

Besides, several photosynthesis and reactive oxygen species-related genes were also downregulated. A decrease in photosynthesis related gene expression during fruit maturation and ripening has been observed in many fruits such as apples, persimmon, grapes and strawberries [82-85]. In tomatoes, one of the changes that occur during ripening, involves chloroplast to chromoplast conversion. These changes are coupled to downregulation of photosynthetically active genes during ripening [86,87]. In blueberries downregulation of photosynthesis related genes may be an extension of ripening events. It is also possible that since these fruit were stored in cold and dark conditions following their harvest, downregulation of photosynthesis-related

gene expression may have ensued. Down regulation of genes involved in reactive oxygen species was surprising since generally their expression is expected to be upregulated under abiotic stress conditions, as such stressful conditions may prevail after postharvest. However, in this case, the downregulation of superoxide dismutase (SOD), *Cu/ZnSOD* and *MnSOD*, mostly prevalent in the chloroplast and mitochondria suggest removal of superoxide radical generated during photosynthetic and mitochondrial electron transport reactions [58]. Similarly during postharvest storage of kiwi under cold, there was downregulation of superoxide dismutase genes, and the authors suggested that this may be compensated by increase in amino acids [58]. It is possible that such a scenario exists during postharvest storage in blueberries since an upregulation of several amino acid transporters was noted in this study. Further, in the current data set many transcripts that encode proteins associated with ribosomes were downregulated which was similar to that observed in apples during fruit maturation [85].

Downregulation of Rebel-specific transcripts:

Rebel specific down-regulated genes included additional photosynthesis and translation related transcripts. It is possible that these processes are linked to the rate of deterioration in postharvest fruit quality, and are thus specific to Rebel which displays a greater decrease in percent healthy fruit during storage compared with Suziblue. In addition, there were also transcripts associated with auxin signaling that were down regulated. This included the auxin-responsive proteins SAUR21-like and SAUR50-like, and IAA27-like. Since multiple genes involved in auxin-related metabolism was differentially regulated (up and down-regulated) it would be essential to explore to role of auxin during postharvest storage in blueberry fruit in future studies.

Downregulation of Suziblue-specific transcripts:

The major GO term for Suziblue-specific upregulated genes belonged to the oxidation-reduction process. The transcripts in this category included genes related to the glycolysis and respiration pathways such as glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, cytochrome b-c1 complex, and ferredoxin. It is possible these genes were downregulated as a consequence of downregulation of photosynthesis related genes or due the redox state of the fruit during postharvest storage [88,89].

Conclusions

In this study several key processes such as cytoskeletal changes (upregulation of tubulin and XTH genes), accumulation of important amino acids (up regulation of amino acid transporters) and changes in water relations (down regulation of aquaporins) were identified as playing potentially important roles during postharvest storage. Further, these data revealed that many of the genes that are differentially regulated may also be triggered by various abiotic and biotic stress conditions. It is possible that once fruit are harvested, they are subjected to multiple abiotic stressors such as fruit water loss (desiccation stress), cold stress (low temperature storage) and reduced light availability (dark storage). Additionally, they are potentially exposed to multiple postharvest pathogens. These conditions may elicit a fruit transcriptional response during postharvest storage that allows for the fruit to adapt to the stress conditions. Such a response to biotic and abiotic stress during postharvest storage has also been indicated in citrus and persimmon [62,84,90].

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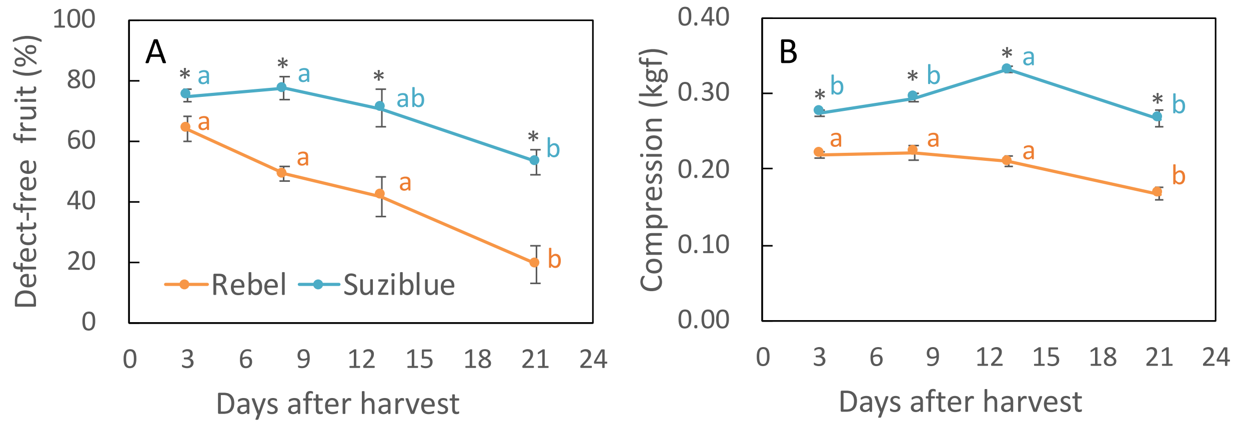

Figure 5.1. Postharvest fruit quality attributes of southern highbush blueberry Rebel and Suziblue. Defect-free fruit (A) and compression (B). Fruits were stored at 4°C under 90% humidity after harvest. Error bars represent standard errors. Asterisk indicates the means are significantly different between cultivars according to t-test (α = 0.05). Different letters indicate the means are significantly different among each day within each cultivar according to ANOVA and Tukey's HSD (α = 0.05).

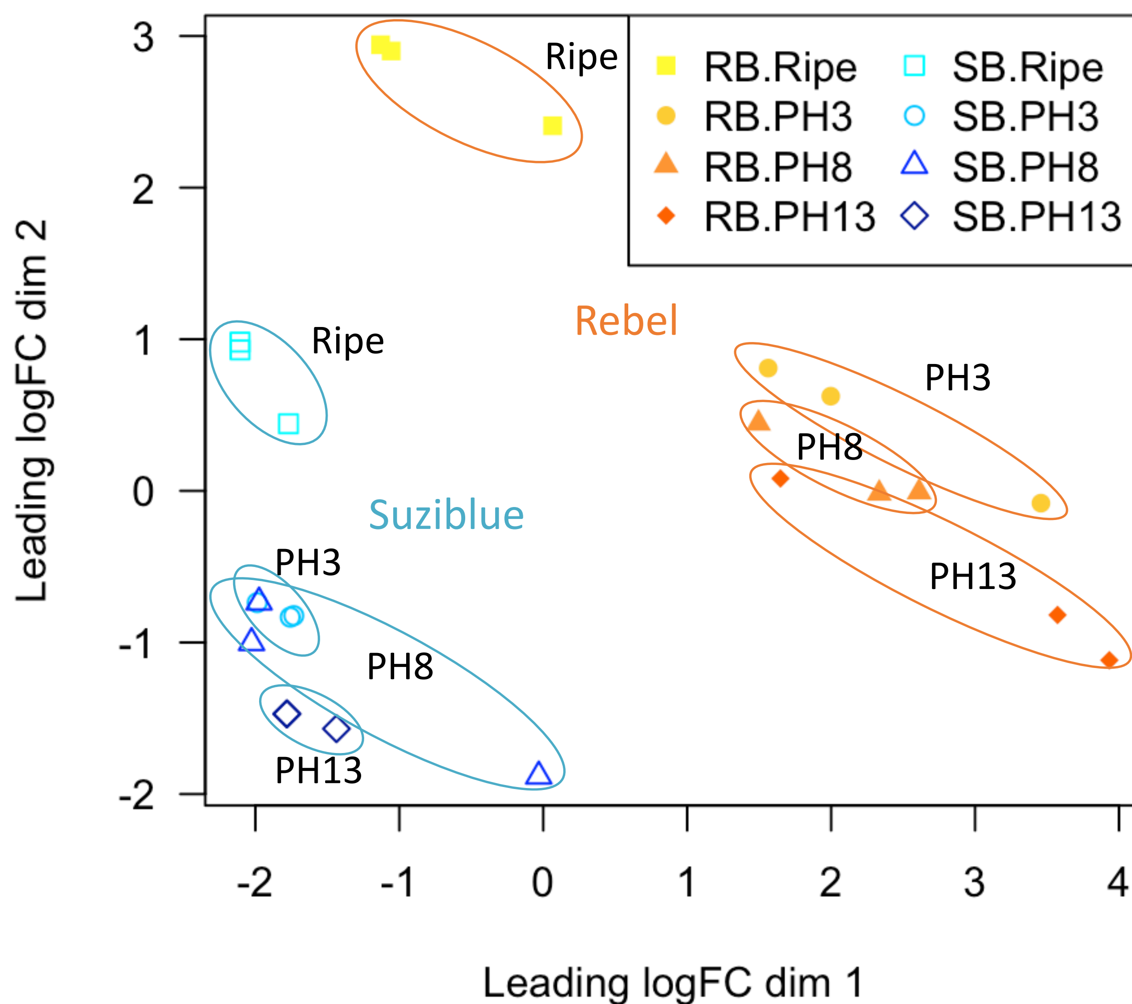

Figure 5.2. Multi-dimensional scaling (MDS) plot of southern highbush blueberry transcript profile using leading log-fold-changes between each pair of samples. Samples include Rebel (RB) and Suziblue (SB) from ripe, postharvest storage 3, 8, 13 days (PH3, PH8, PH13). Each symbol represents one replicate.

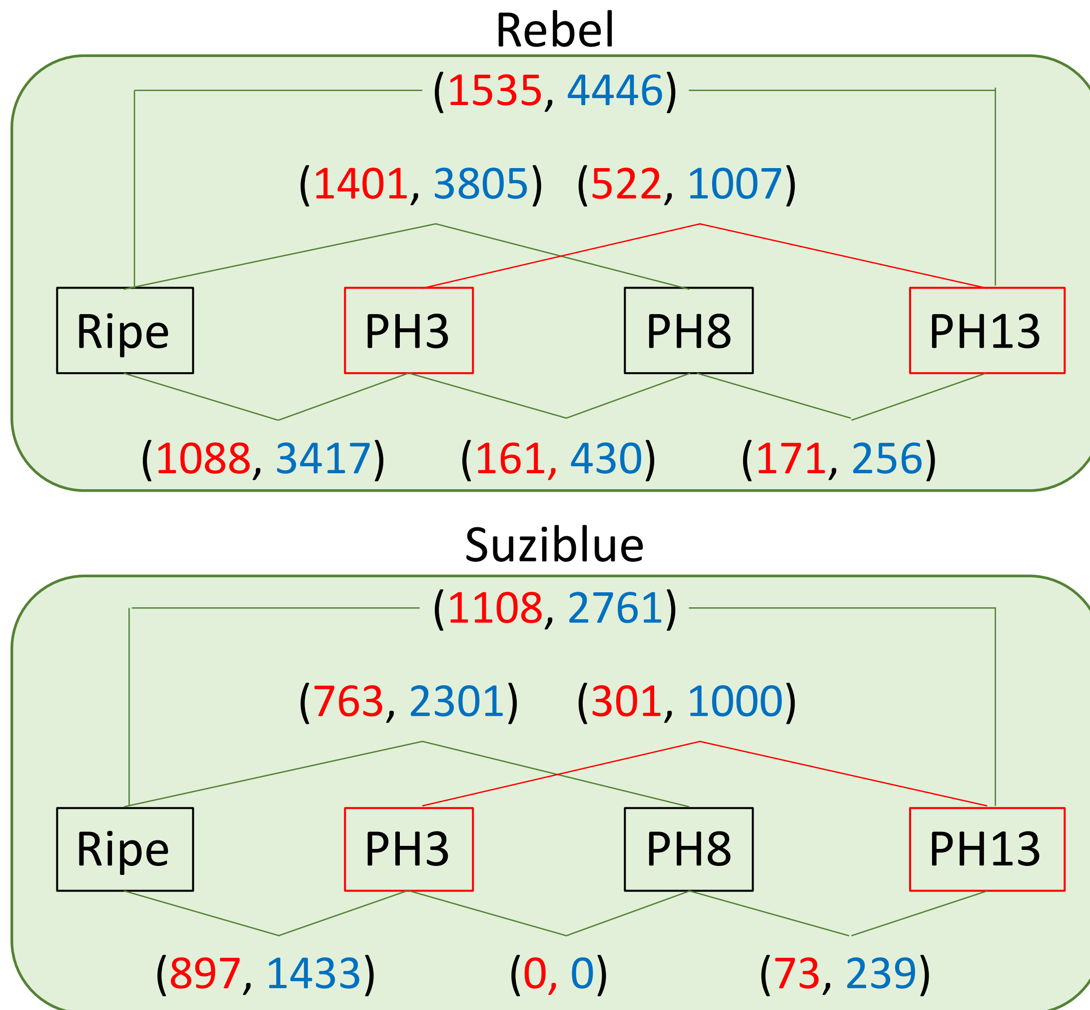

Figure 5.3. Differentially expressed genes (DEGs) between each pair of samples for southern highbush blueberry Rebel and Suziblue at ripe and during postharvest storage. PH3, PH8, PH13 indicate postharvest storage 3, 8, 13 days. Number in red and blue colors represents the number of up-regulated and down-regulated genes in the latter sample respectively. The criteria of DEGs are < 0.01 false discovery rate and ≥ 2-fold change. The comparison highlighted in red colors (PH3 vs PH13) will be used afterward.

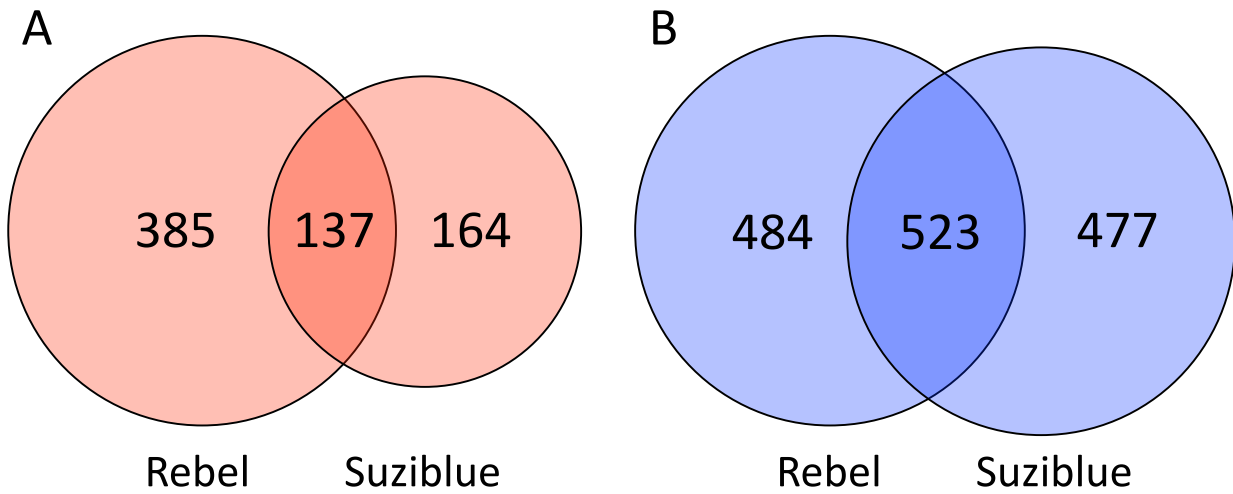

Figure 5.4. The differentially expressed genes (DEGs) between postharvest storage 3 and 13 days that unique or common in southern highbush blueberry Rebel and Suziblue. Genes up-regulated (A) and down-regulated (B) at the postharvest storage 13 days. The criteria of DEGs are < 0.01 false discovery rate and ≥ 2-fold change.

Figure 5.5 Top 10 enriched gene ontology in biological process from the differentially expressed genes between PH3 and PH13 in Rebel and Suziblue. PH3 and PH13 indicate postharvest storage 3 and 13 days.

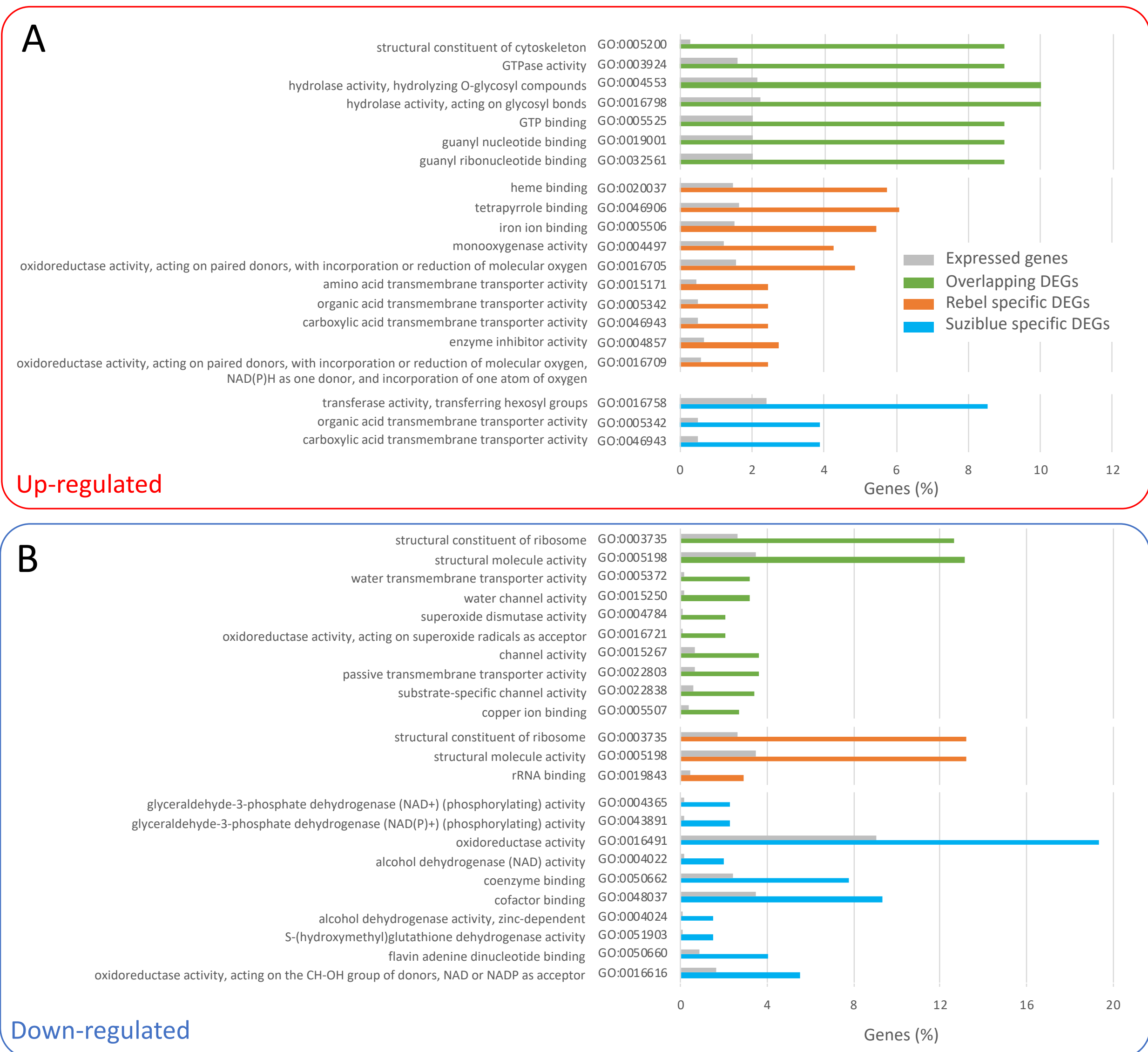

Figure 5.6 Top 10 enriched gene ontology in molecular function from the differentially expressed genes between PH3 and PH13 in Rebel and Suziblue. PH3 and PH13 indicate postharvest storage 3 and 13 days.

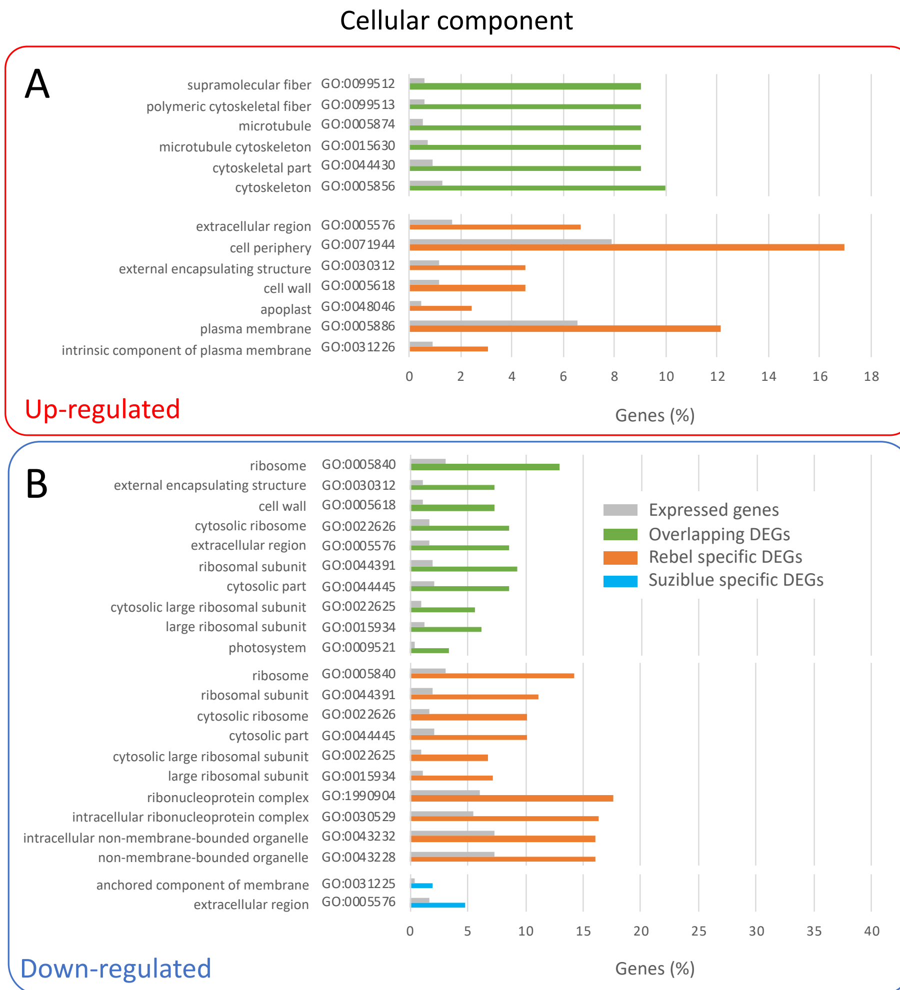

Figure 5.7 Top 10 enriched gene ontology in cellular component from the differentially expressed genes between PH3 and PH13 in Rebel and Suziblue. PH3 and PH13 indicate postharvest storage 3 and 13 days.

CHAPTER 6

CONCLUSIONS

The mechanisms that regulate ripening and postharvest fruit quality in blueberries were investigated in this study. Typically, fruits are classified as climacteric or non-climacteric fruit based on their ripening characteristics. In this study, climacteric rise in respiration and ethylene production during ripening were observed in southern highbush and rabbiteye blueberries, which support blueberry as a climacteric fruit. However, no autocatalytic ethylene production at the level of ethylene-biosynthesis related gene expression or ethylene precursor (ACC) was detected after exogenous ethephon (a plant growth regulator that releases ethylene) application in rabbiteye blueberries. Climacteric fruits display autocatalytic ethylene production, the absence of which would lead blueberry to be classified as an atypical climacteric fruit. Further, ethephon application can promote fruit ripening by increasing the proportion of ripe fruit with limited effect on postharvest fruit quality. To investigate the mechanisms of ethylene in regulating ripening, RNA-Seq analysis was performed. Ethylene initiates ripening mainly by downregulating photosynthesis-related genes and upregulating cell wall modification and carbohydrate metabolism-related genes. Further, crosstalk at the transcriptional level with other important hormones such as abscisic acid and auxin that facilitate ripening were identified. Blueberry fruits are harvested when completely blue, and their shelf-life can vary (1-7 weeks). Genotype differences have been shown to affect variation in shelf-life. In this study, blueberry cultivars with superior and inferior postharvest fruit quality, such as ‘Suziblue’ and ‘Rebel’

respectively, were identified. Using an RNA-Seq strategy, several key processes such as cytoskeletal changes (upregulation of tubulin and XTH genes), accumulation of important amino acids (upregulation of amino acid transporters), and postharvest water loss (downregulation of aquaporins) were identified as playing potentially important roles during postharvest storage. Together, the research has generated information that can be applied to the blueberry industry immediately and expanded the basic knowledge in blueberry ripening and postharvest fruit quality.