

**Molecular characterization of stage-specific fruit ripening in rabbiteye
blueberry 'Tifblue'**
-Insights into the blueberry cluster ripening characteristics-

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Blueberry (*Vaccinium* spp.) fruit has been gaining popularity due to its potential health benefits, leading to an increased consumer demand. In most cultivars, multiple rounds of hand harvesting are required because the fruits within a single cluster do not ripen simultaneously. To enable cluster harvesting, a better understanding of the mechanisms underlying fruit ripening progression and artificial manipulation of ripening is needed. However, fundamental knowledge of ripening regulation in blueberries, including rabbiteye blueberry (*V. virgatum*), remains limited. Among phytohormones involved in fruit ripening, ethylene and abscisic acid (ABA) are considered key regulators. Because blueberries have long been classified as non-climacteric fruit, ABA rather than ethylene has been considered the predominant phytohormone promoting ripening. Consequently, the role of ethylene in blueberry ripening has remained unclear. In this study, I focused on ethylene to 1) investigate the relationship between endogenous phytohormone metabolism and ripening uniformity, and 2) identify key factors underlying ethylene-associated regulation of ripening uniformity.

In chapter 1, the effect of ripening related hormone ABA and ethephon (ETH, an ethylene-releasing compound) treatment on middle green fruits (diameter in 0.9 - 1 cm) of rabbiteye blueberry 'Tifblue' were investigated. Additionally, recent reports suggested DNA demethylation is involved in fruit ripening, thus the effect of DNA methylation inhibitor, 5-AzaC, on fruit ripening was also tested. Furthermore, the effect of combine treatment, ABA + 5-AzaC and ETH + 5-AzaC were also tested. Compared to the control treatment, all treatments increased the proportion of ripe fruit, as evidenced by enhanced coloration, sugar accumulation, fruit softening, and decreased acidity. Both combine treatments had better effect than single treatments. These results demonstrated that both ABA and ethylene promoted fruit pigmentation in 'Tifblue'. Although blueberry is generally classified as a non-climacteric fruit in which ABA, rather than ethylene, is assumed to regulate the ripening process, my results suggested that ethylene also contributed to fruit ripening and increased the proportion of ripened fruits, thereby enhancing the uniformity of fruit maturity. Additionally, 5-AzaC treatment promoted ripening, and combined treatments of 5-AzaC with ABA or ETH showed even stronger effects than single treatments. ABA has long been thought to be the main hormone controlling blueberry ripening and has been well studied. However, these results show that ethylene also helps blueberry fruits ripen. This is interesting and means that the role of ethylene and DNA demethylation in blueberry ripening deserves more study.

In chapter 2, the factors leading to higher uniformity of fruit ripening in a cluster of rabbiteye blueberry 'Tifblue' were investigated. Two-year phenology observations of fruit development and stage-specific phytohormone accumulation measurements

were conducted in two highbush (*V. corymbosum*) ('O'Neal', 'Sharpblue') and two rabbiteye blueberry cultivars ('Tifblue', 'Homebell') with particular focus on the relationship between ripening uniformity and phytohormone metabolism. Fruit developmental stages were defined based on fruit expansion ratios and coloration levels. Ripening parameters, including sugar contents and fruit firmness, exhibited similar stage-dependent patterns across cultivars. Two-year phenology observations showed that 'Tifblue' maintained continuous and uniform ripening throughout cluster development compared with others. The sensitivity to ripening related hormone and the pattern of ripening related hormone accumulation of four studied blueberry cultivars were investigated. All four cultivars responded similarly to exogenous phytohormones treatments, suggesting that their ripening responses to ABA and ethylene are broadly comparable, which imply that the sensitivity to ripening related hormone is similar and this is not the factor that decide the uniform ripening characteristic of 'Tifblue'. The results of the pattern of ripening related hormone in four studied cultivars suggest that 'Tifblue' exhibited a distinctive ethylene emission pattern, characterized by suppressed emission during early stages followed by a sharp increase to higher levels in late stages. ABA content in 'Tifblue' fruit also showed a marked late-stage accumulation. Taken together, it was hypothesized that early-stage suppression of both ABA accumulation and ethylene emission may inhibit premature and rapid ripening, which may contribute to the enhanced ripening uniformity observed in 'Tifblue'.

In chapter 3, the effects of artificial ethylene and DNA demethylation treatment on fruit ripening uniformity of 'O'Neal' which showed relatively lower ripening uniformity level among fruits in a cluster among four studied cultivars were investigated. ETH treatment increased the proportion of ripen fruits in a cluster. Furthermore, when ETH treatment is combined with 5-AzaC treatment, ripening uniformity was higher. To explore the molecular regulations underlying this effect, RNA-seq analysis was conducted. GO enrichment analysis of up-regulated DEGs from the control versus ETH comparison indicated that anthocyanin biosynthesis related GO terms were significantly enriched, whereas up-regulated DEGs identified from the control versus ETH + 5-AzaC comparison were mainly enriched in GO terms associated with ABA signaling. ETH and combined ETH and 5-AzaC treatment up-regulated anthocyanin biosynthesis genes and ABA biosynthesis-related genes. These results suggested ETH treatment had potential to promote cluster ripening through the modification of hormone signal transduction and metabolism.

In chapter 4, Molecular regulation underlying the late-stage-specific ethylene biosynthesis in 'Tifblue' was studied. RNA-seq analysis of fruits at five developmental stages was performed to investigate the regulation mechanisms underlying endogenous ethylene biosynthesis in 'Tifblue' (a high-uniformity cultivar) and 'O'Neal' (a low-uniformity cultivar). Expression of the ethylene biosynthesis-related gene *VcACO* exhibited a pronounced late-stage-specific increase in 'Tifblue', whereas this pattern was not observed in 'O'Neal'. To identify transcription factors that may regulate the stage-specific expression of *VcACO* and *ETHYLENE RESPONSE FACTOR (ERF)* genes involved in ethylene signal transduction were focused. Recent

studies have shown that tomato *ERFs* can either activate or repress the expression of *ACS* and/or *ACO* genes, thereby modulating ethylene biosynthesis. A total of 276 *VcERFs* were identified in the blueberry 'Draper' genome. Of these, 155 *VcERFs* expressed in fruit tissues (TPM > 1) were subjected to further analysis to identify putative activator- and repressor-type *ERFs*. Twenty *ERFs* exhibited increasing expression patterns correlated with fruit ripening progression. Conversely, 11 *VcERFs* containing an EAR motif (a transcriptional repression motif) showed elevated expression during the early stage in 'Tifblue', corresponding to the period characterized by low ethylene emission. In contrast, the orthologs of these genes displayed low expression in 'O'Neal' during the same stage. Transient overexpression of specific *VcERFs* in fruit demonstrated that overexpression of two EAR-containing *VcERFs* significantly reduced ethylene emission, whereas overexpression of one *VcERF* lacking the EAR motif significantly increased ethylene emission. These findings suggest that elevated expressions of repressor-type *VcERFs* during early fruit development may contribute to the suppression of the expressions of *VcACO* in 'Tifblue', thereby leading to its lower ethylene emission at early stages.

In conclusion, this study provides important insights into ripening dynamics within blueberry clusters, indicating that the modulation of ethylene may have potential for enhancing both ripening progression and ripening uniformity. Furthermore, the findings suggest that the stage-specific molecular regulation of ethylene emission is associated with the uniformity of ripening among fruits within a cluster.