ROLES OF PHYTOHORMONES ON 'SHINE MUSCAT' GRAPE (*VITIS LABRUSCANA* BAILY ×*VITIS VINIFERA* L.) MATURATION

July 2018

LIN HONG

Graduate School of Horticulture

CHIBA UNIVERSITY

(千葉大学学位申請論文)

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LIST OF ABBREVIATIONS

1-MCP	1-methylcyclopropene
AAO	Abscisic-aldehyde oxidase
ABA	Abscisic acid
ABA-GT	ABA glucosyltransferase
ABF	ABA responsive elements-binding factor
Abz-E3M	Abscinazole-E3M
ACO	1-aminocyclopropane-1-carboxylate oxidase
ACS	1-aminocyclopropane-1-carboxylate synthase
ADH	Alcohol dehydrogenase
AI	Acid invertase
AP2/ERF	APETALA2/Ethylene Responsive Factor
BG	β-glucosidase
cDNA	Complementary deoxyribonucleic acid
CYP707A	ABA 8'-hydroxylase
DAFB	Days after full bloom
DEGs	Differentially expressed genes
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
GA	Gibberellin
GC-MS	Gas chromatography-mass spectrometry
GEM	GLABRA2 Expression Modulator
GPPS	Geranyl diphosphate synthase
HPLC	High performance liquid chromatography

HT	Hexose transporter
IAA	Indole-3-acetic acid
IPT	Isoprothiolane
LOX	Lipoxygenase
NAA	Naphtalenacetic acid
NCED	9-cis-epoxycarotenoid dioxygenase
NDGA	Nordihydroguaiaretic acid
NGS	Next-generation sequencing
NIFTS	Institute of Fruit Tree and Tea Science, NARO
PPH	Pheophytin pheophorbide hydrolase
RABT	Annotation Based Transcript
RCCR	Red chlorophyll catabolite reductase
SDR	Short-chain dehydrogenase/reductase
SPS	Sucrose phosphate synthase
SUS	Sucrose synthase
TPS	Terpene synthase
VDE	Violaxanthin de-epoxidase
ZEP	Zeaxanthin epoxidase

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

1.1.1 Introduction

Fruits are plant organs which evolved to protect the ovule and seed in time of embryo growth and ensure seed diffusing after maturation. The term of fruits refers to fleshy and edible parts which are the major component of fruits, such as grape, tomato, peach, citrus, strawberry, apple, melon, and banana. All of these fruits species are widely cultivated all over the world (Azzi et al., 2015).

In general, fruits are divided into two groups according to the characteristic of fruits ripening, which include climacteric and non-climacteric fruits. The climacteric fruits show obviously ethylene increases during the ripening phase with a peak in respiration. On the contrary, fruits in which the production of ethylene don't show dramatic change during the maturation and remain at very low level are classified as non-climacteric fruits.

Grapevine (*Vitis vinifera* L.) is a non-climacteric fruit species (Coombe and Hale, 1973) which has been widely used as fresh or dry fruit, wines and liquor. During grape berries development, a major transcriptomic reprogramming occurring in softening and pigment changes, such as anthocyanin accumulation in red grape cultivars (Terrier et al. 2005) and the decrease of chlorophyll level in white grape cultivars (Young et al. 2012). 'Shine Muscat' grape (*Vitis labruscana* Baily × *Vitis vinifera* L.) is a new popular cultivar in Japan with a strong flavor and high sugar concentration (Yamada et al. 2008). In general, consumers prefer green 'Shine Muscat' grapes over those with a yellow color. If grape maturation can be delayed, the harvest period will be prolonged and the grape berries may be more marketable. Therefore, it is necessary to explore the mechanism of delaying 'Shine Muscat' grape maturation.

In recent years, our knowledge of the molecular basis of ripening regulation has

improved. Hormones appear to play a central role, as the concentrations change prior to and during ripening and in response to several environmental (Kuhn et al., 2014). Grape berry maturation involves several regulatory steps, which include many significant changes to the metabolic and physiological traits in maturation berries. On one side, ABA can be considered as one of the main maturation control factors, due to the content of ABA is very low in young berry fruits but increases and peak at véraison (Davies et al., 1997; Deluc et al., 2009; Giribaldi et al., 2010) or two weeks after véraison (Wheeler et al., 2009). Exogenous ABA treatments also cause an increase in berry weight (Peppi et al., 2008) and a decrease in organic acid levels (Zoccatelli et al., 2013). The ABA inhibitor nordihydroguaiaretic acid (NDGA) blocks ABA biosynthesis by suppressing the activities of 9-cis-epoxycarotenoid dioxygenase (NCED) (Creelman et al., 1992) and delays maturation in grapes (Zhang et al., 2009). In addition, a novel ABA 8'-hydroxylase (CYP707A) inhibitor Abscinazole-E3M (Abz-E3M) has been shown to increase endogenous ABA production in plant (Takeuchi et al., 2016), but there is no information in grape maturation. Furthermore, auxin and ethylene have been reported to influence grape maturation (Davies et al., 1997; Chervin et al., 2004). ABA may promote grape maturation through complex interactions with auxin and ethylene metabolism (Kuhn et al., 2014). However, the interaction between ABA, auxin and ethylene as promoters of maturation in grape are not fully understood and need more study to elaborate.

Isoprothiolane (diisopropyl-1,3-dithiolan-2-ylidenemalonate; IPT) has been suggested as a new plant growth regulator (Ohtsuka, 2017). Previous study found that IPT remarkably promoted the growth of rice (*Oryza sativa* L.) seedlings through the enhancement or modification of auxin activity, cytokinin activity, and ethylene synthesis (Ohtsuka and Saka, 1988); similar effects were also reported in beans (*Phaseolus vulgaris* L.) (Ohtsuka et al., 1990) and date palm (*Phoenix dactylifera* L.) (Okawara et al., 2003).

These studies suggest that IPT may be able to regulate phytohormones such as auxin and ethylene. However, there is little information on the effects of IPT in fruit in general.

1.1.2 Objectives are as follows

1.1.2.1 To study the effects of IPT or NDGA application on ABA metabolism and maturation in 'Shine Muscat' grape berries.

1.1.2.2 To study the effects of Abz-E3M (abscinazole-E3M) or ethephon application on ABA metabolism and maturation in 'Shine Muscat' grape berries.

1.1.2.3 To investigate the possible relation between ethylene and ABA on grape berry maturation.

1.1.3 Scope of studies

1.1.3.1 The effects of 3.44 mM IPT or 100 μM NDGA application on the changes of endogenous ABA concentration, chlorophyll degradation, sugar increasing, and volatile contents enrichment in 'Shine Muscat' grape berries.

1.1.3.2 The effects of IPT or NDGA application on the maturation-related genes analyzed by next-generation sequencing (NGS), which involved in sugar increasing, volatile contents enrichment, and ABA, auxin and ethylene metabolism.

1.1.3.3 The effects of 50 μM Abz-E3M or 500 μM ethephon application on the changes of endogenous ABA concentration, ethylene production, chlorophyll degradation, and sugar increasing in 'Shine Muscat' grape berries.

1.1.3.4 The effects of Abz-E3M or ethephon application on the maturation-related genes including ABA metabolism genes (*VvNCED1* and *VvCYP707A1*), ethylene related genes (*VvACO1* and *VvERF2*), chlorophyll related genes (*VvPPH* and *VvRCCR*), and sugar related genes (*VvSPS* and *VvAI*) in 'Shine Muscat' grape berries.

1.1.4 Expected Outcomes

1.1.4.1 Clarifying the effects of IPT or NDGA application on delaying ‘Shine Muscat’ grape berry maturation.

1.1.4.2 Clarifying the effects of IPT or NDGA application on ABA metabolism and maturation in grape berries. Understanding the effects of IPT or NDGA application on the maturation related genes in ‘Shine Muscat’ grape berry maturation.

1.1.4.3 Clarifying the effects of Abz-E3M or ethephon application on ABA metabolism and maturation in grape berries. Understanding the effects of Abz-E3M or ethephon application on the maturation related genes in ‘Shine Muscat’ grape berry maturation.

1.1.4.4 Clarifying the relationship between ABA and ethylene biosynthesis in ‘Shine Muscat’ grape berry maturation.

1.2 LITERATURE REVIEW

1.2.1 Grape

1.2.1.1 Grapevine

Grapevines are classified into the genus *Vitis*, which is belong to the family *Vitaceae* (Myles et al., 2011). The genus *Vitis* L. includes about 25 species in North America, 40-60 species in Asia, and one European species—*Vitis vinifera* L. (This et al., 2004). Although previous study reported that there are eight to ten thousand cultivars exist in the whole world (Alleweldt and Possingham, 1988). *Vitis vinifera* L. is the principal species cultivated meanwhile other *Vitis* species are mostly used for fungus-resistant breeding or rootstocks cultivars (This et al., 2004). Worldwide, the grapes (*Vitis* species) are used as fresh or dry fruit, wines and liquor, and in 2016. Around 77 million tons of grapes were produced globally and 179200 tons were produced in Japan (Figure 1.1).

1.2.1.2 'Shine Muscat' grape

'Shine Muscat' grape (*Vitis labruscana* Baily × *Vitis vinifera* L.) is a new yellow–green skin grape cultivar with a strong flavor and high sugar concentration released by NIFTS (Institute of Fruit Tree and Tea Science, NARO) in Japan (Yamada et al., 2008). After it was released in 2006, 'Shine Muscat' grape was quickly spread nationwide to become the leading cultivar in Japan. In 2013, the cultivation area of 'Shine Muscat' grape was 570.2 ha which was account for 3.82 percentage of total fresh grape area in Japan (Figure 1.2). Surprisingly, the cultivation area has drastically increased from 2 ha in 2007 to 992.3 ha in 2015, even though the total area of grapes cultivation has decreased from 2007 to 2015 consistently (Figure 1.3). The cultivation area increasing also reflects market demand increase (Figure 1.4), due to the popularity of 'Shine Muscat' grape known for its large size, strong aromatic taste, sweet and seedless berries which can be

eaten without peeling after gibberellin (GA) treatment.

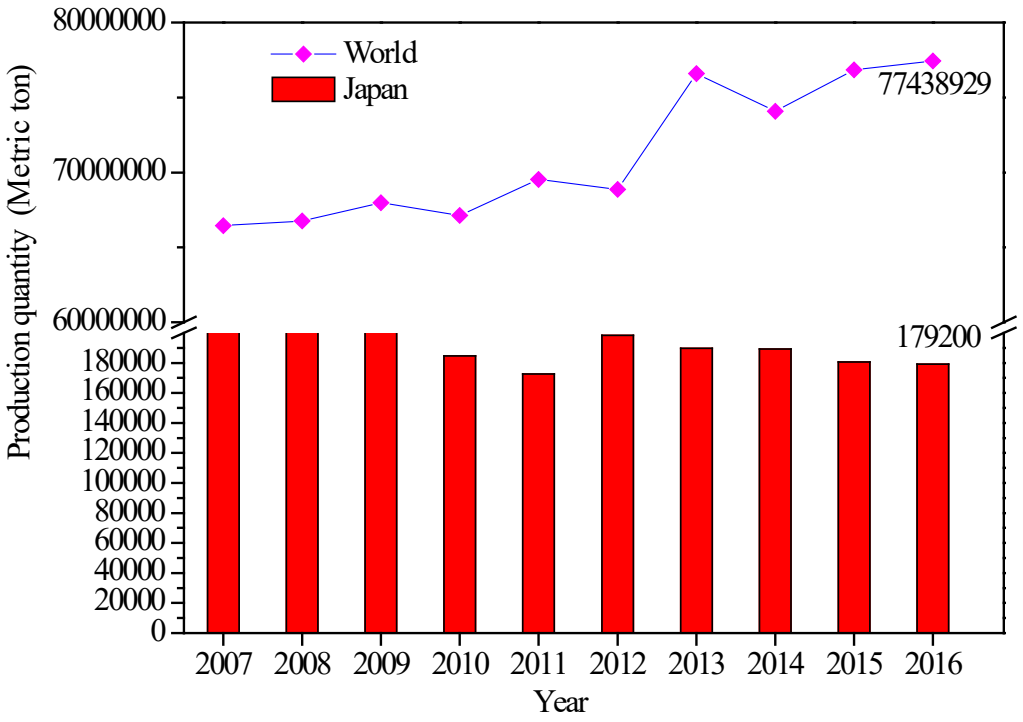

Figure 1.1 The quantity of grape produced in the whole world and Japan from 2007 to 2016 (Source: FAOStat.org, 2016).

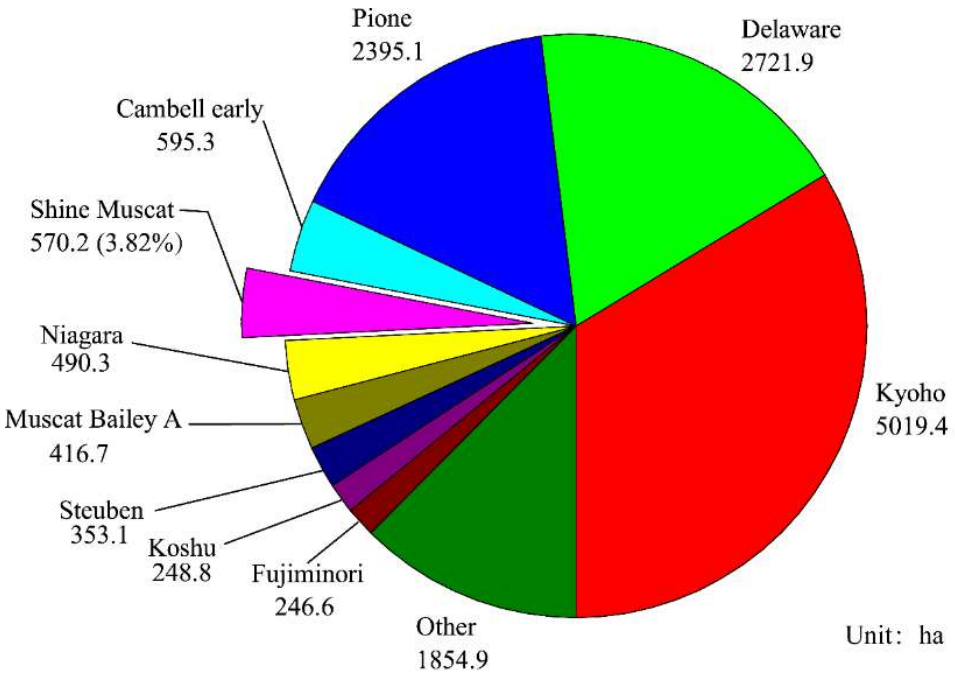

Figure 1.2 The cultivation area of table grape in Japan in 2013 (Source: Ministry of Agriculture, Forestry and Fisheries).

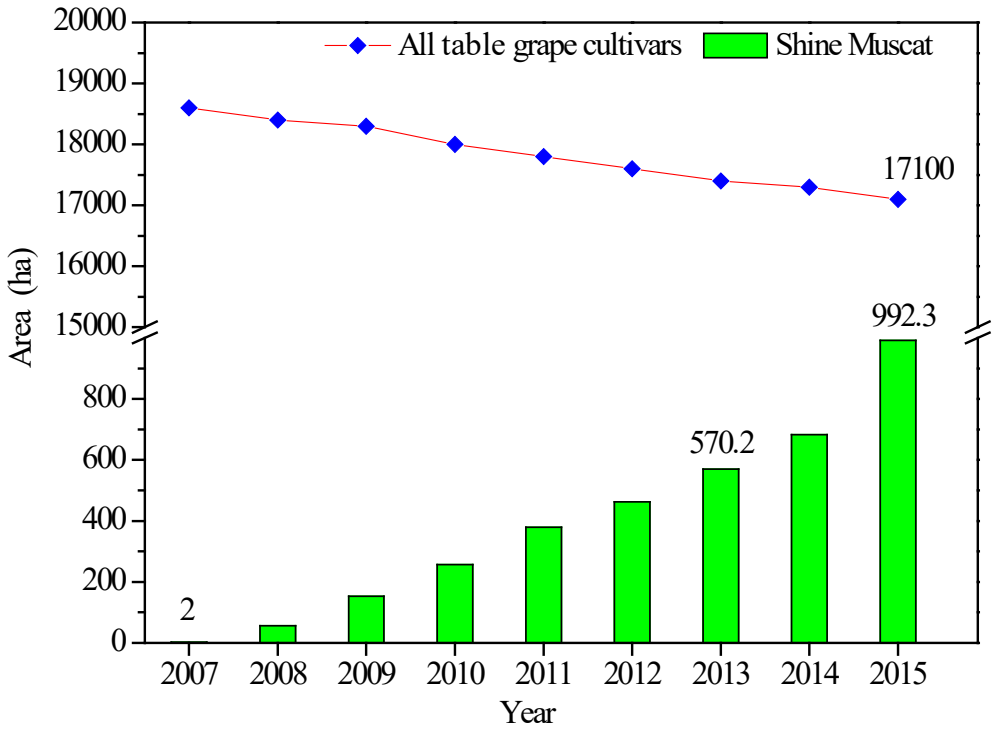

Figure 1.3 The cultivation area of all table grape cultivars and 'Shine Muscat' grape in Japan from 2007 to 2015 (Source: Ministry of Agriculture, Forestry and Fisheries).

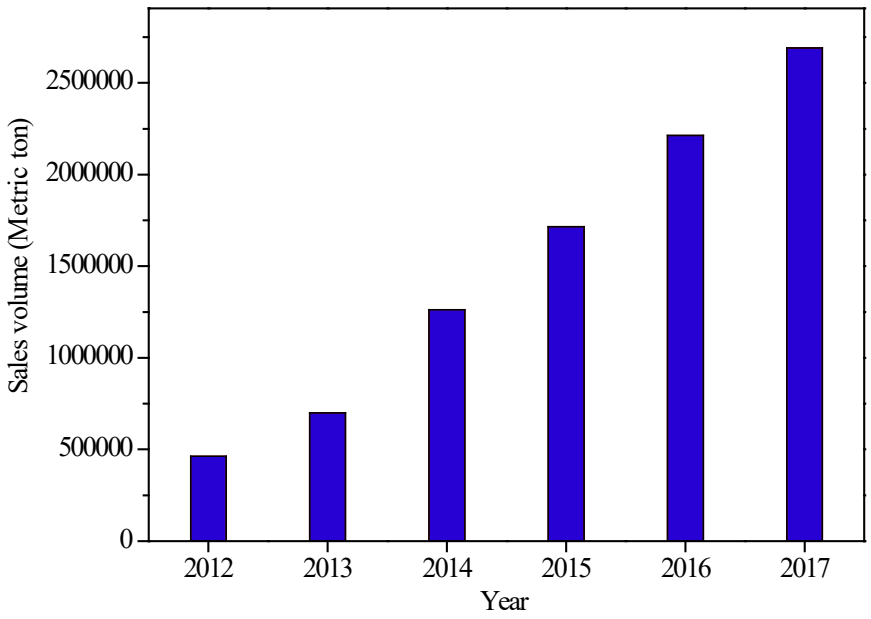

Figure 1.4 The sales volume of 'Shine Muscat' grape in Tokyo Metropolitan Central Wholesale Market from 2012 to 2017 (Source: Tokyo Metropolitan Central Wholesale Market).

1.2.1.3 Stage of grape development

The grape berry development displays a typical double sigmoidal growth pattern with three main phases (Figure 1.5), encompassing a series of physical and biochemical changes such as cell division and elongation (Ojeda et al., 1999; Coombe and Mccarthy, 2000), primary and secondary metabolism (Dal Santo et al., 2013). The initiation time of grapes maturation, which is called véraison by viticulturists, showed a major transcriptomic reprogramming occurs during the abrupt drop in berry firmness and color changes. The skin changes from green to red in berries of red grape cultivars by the transition between the lag phase and maturation (Terrier et al., 2005). In contrast, the chlorophyll degradation lead to berries skin changes from green to yellow in white grape cultivars (Young et al. 2012; Massonnet et al., 2017). The maturation sets in with high concentrations of glucose and fructose accumulate after véraison along with a decrease in elasticity (Castellarin et al., 2016) and accompanied by berry softening and accumulation of anthocyanin content (Duchene et al., 2014; Ferrara et al., 2015). In addition, it is known that the aromatic volatiles emit in grapes after maturation (Fenoll et al., 2009).

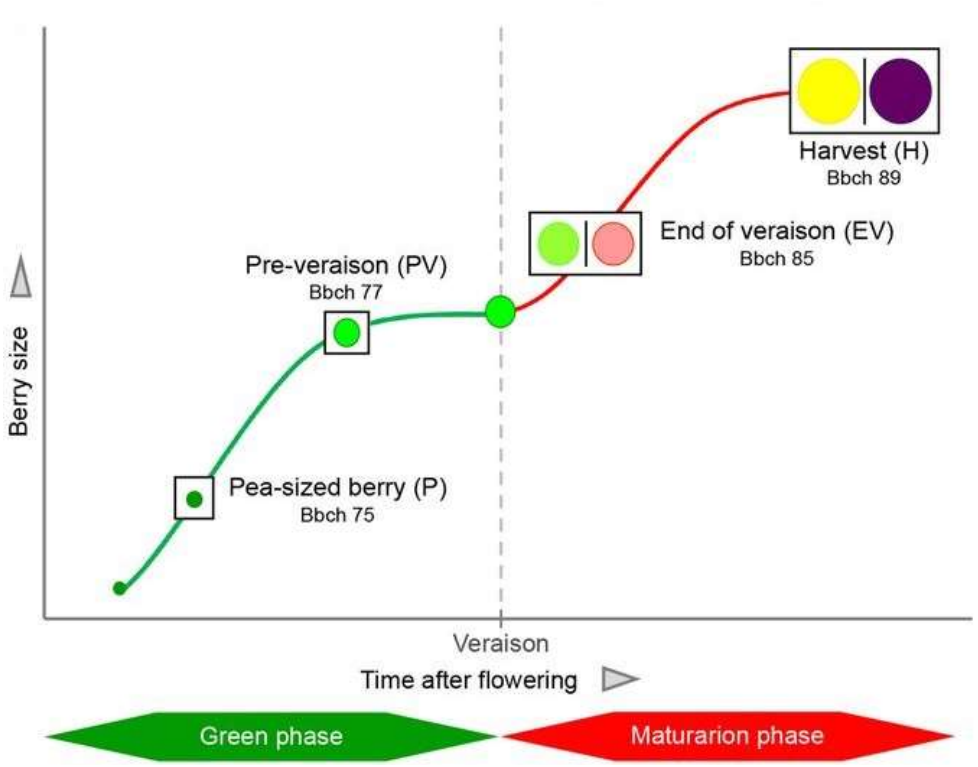

Figure 1.5 The schematic graph for grape development from flowering to maturation (Massonnet et al., 2017).

1.2.2 Abscisic acid (ABA)

1.2.2.1 ABA biosynthesis

The phytohormone abscisic acid (ABA) plays many important aspects of plant development including seed maturation and germination, embryo growth, and stomatal closure (Tan et al., 1997; Tian and Brown, 2000; Cai et al., 2017). Its role is also responses to environmental stresses, such as low temperature, thermal or heat stress, heavy metal stress, high level of salinity, drought, and UV-B stress (reviewed by Vishwakarma et al., 2017; Kondo et al., 2012). As shown in Figure 1.6, nine-*cis*-epoxycarotenoid dioxygenase (NCED) is the key rate-limiting enzyme for ABA biosynthesis in plants (Tan et al., 1997; Burbidge et al., 1999). Abscisic-aldehyde oxidase (AAO) is the enzyme that catalyzes the final step of ABA biosynthesis (Seo et al., 2000). ABA 8'-hydroxylase (CYP707A) is the key enzyme in ABA catabolism, which plays a major regulatory role in controlling the level of ABA in plants (Kushiro et al., 2004).

1.2.2.2 ABA and grape maturation

During grape maturation, some significant changes occur in maturation berries including berry weight and sugar concentration increasing, anthocyanin accumulation. These changes occurring from véraison onwards simultaneously increase the ABA concentration (Wheeler et al., 2009). ABA has been considered as one of the main maturation control factors in grape, due to ABA concentration is very low in the young berries but increased around véraison (Davies et al., 1997; Deluc et al., 2009; Giribaldi et al., 2010) or two weeks after véraison (Wheeler et al., 2009). Exogenous ABA application to grape stimulated berry softening and skin anthocyanin accumulation (Cantín et al., 2007). In addition, exogenous ABA treatments also cause an increasing in berry weight (Peppi et al., 2008) and a decreasing in organic acid levels (Zoccatelli et al., 2013).

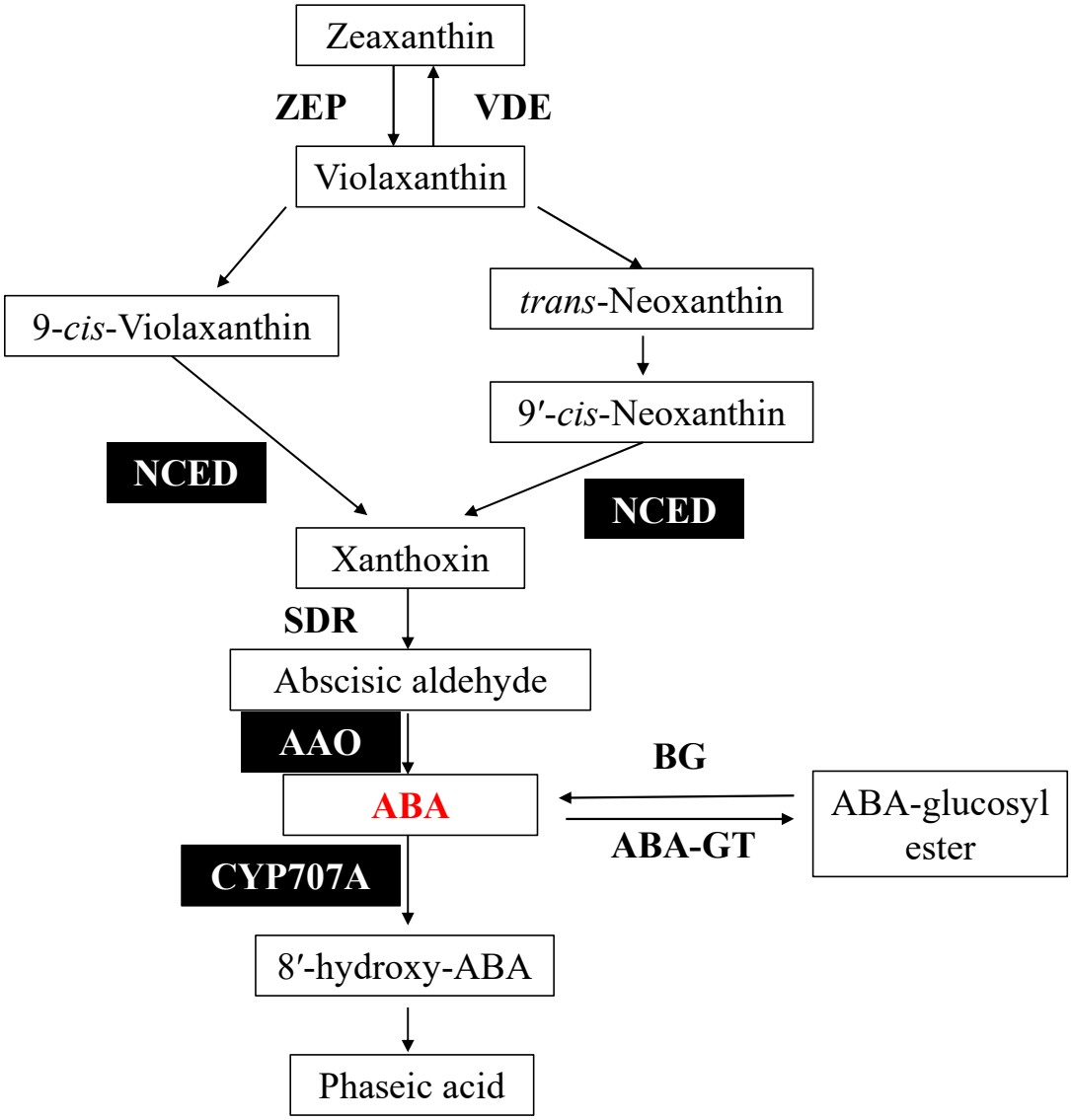

Figure 1.6 The pathway of ABA biosynthesis (Schwartz et al., 2003; Lee et al., 2006). NCED, 9-cis-epoxycarotenoid dioxygenase; AAO, abscisic-aldehyde oxidase; CYP707A, ABA 8'-hydroxylase; ZEP, zeaxanthin epoxidase; SDR, short-chain dehydrogenase/reductase; VDE, violaxanthin de-epoxidase; ABA-GT, ABA glucosyltransferase; BG, β-glucosidase.

1.2.2.3 Nordihydroguaiaretic acid (NDGA)

It is well known that the nordihydroguaiaretic acid (NDGA) can inhibit the NCED enzyme effectively regard to its permeation speed and capability to block ABA biosynthesis (Creelman et al., 1992). Previous studies showed that NDGA application delayed 'Pione' and 'Kyoho' grape coloration (Zhang et al., 2009; Jia et al., 2018). Mou et al. (2015) reported that NDGA treatment inhibited the transcription of genes involved in pigments metabolism, including chlorophyll degradation and carotenoids biosynthesis in tomato. According to these studies, it is suggested that grape maturation inhibited by NDGA may relate to chlorophyll degradation.

1.2.2.4 Abscinazole-E3M (Abz-E3M)

Abscinazole-E1 (Abz-E1) and Abscinazole-E2B (Abz-E2B) are specific inhibitor of abscisic acid (ABA) 8'-hydroxylase (Okazaki et al., 2011; 2012). Previous study found that Abz-E2B application obviously increased the endogenous ABA levels at 4 days after treatment in apple (*Malus domestica*) seedlings and then increased salt tolerance (Sales et al., 2017). Abscinazole-E3M (Abz-E3M), a more practical and effective inhibitor of CYP707A than Abz-E2B, was shown to increase endogenous ABA production (Takeuchi et al., 2016). Therefore, this novel chemical tool may be of benefit to fruit maturation study by adjusting the ABA concentration.

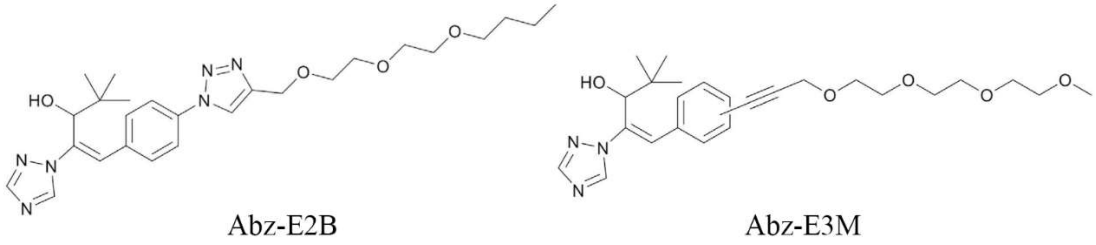

Figure 1.7 Chemical structures of Abscinazole-E2B and Abscinazole-E3M.

1.2.3 Ethylene

1.2.3.1 Ethylene biosynthesis

Ethylene is a simple gaseous hormone which plays various roles of plant normal growth and development, and fruit ripening and senescence. It also serves as a key modulator between plant response to environmental stresses (Yang and Hoffman, 1984; Abeles et al., 2012). The ethylene biosynthesis starts from the conversion of SAM (S-adenosyl-L-methione) into ACC (1-aminocyclopropane-1-carboxylic acid) by ACS (1-aminocyclopropane-1-carboxylate synthase). Then ACC can be converted to MACC [1-(malonylamino) cyclopropane-1-carboxylic acid], by ACC ***N***-malonyl transferase, or to the end product ethylene by ACO (1-aminocyclopropane-1-carboxylate oxidase) as shown in Figure 1.8 (Bulens et al., 2011).

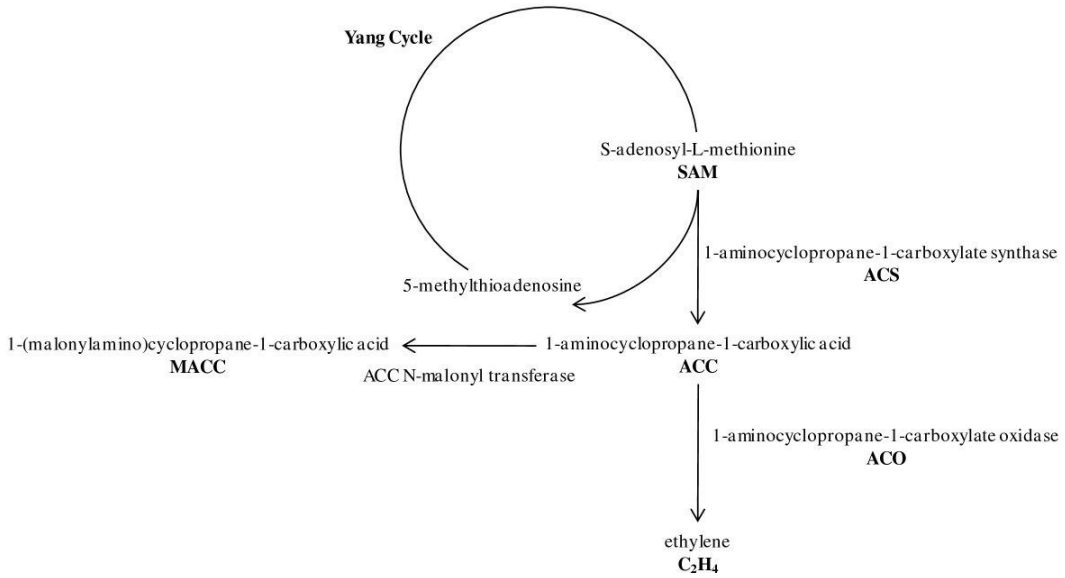

Figure 1.8 The pathway of ethylene biosynthesis (Bulens et al., 2011).

1.2.3.2 Ethylene and grape maturation

In general, ethylene is considered as climacteric fruits regulator, such as tomato, apple, peach, and banana. However, several studies suggest that ethylene plays a role in non-climacteric fruit (Barry and Giovannoni, 2007; Kuhn et al., 2014). In grape, Chervin et al. (2004) suggested that ethylene is required for increasing of berry diameter, decreasing acidity content and anthocyanin accumulation around véraison. Ethylene application at véraison led to berry diameter increase and the expression pattern of various aquaporins (AQUA) genes and "cell wall structure" genes changed (Chervin et al., 2008). In contrast, treatment with 1-methylcyclopropene (1-MCP) decreased grape berries diameter, anthocyanin accumulation and acidity decreasing (Chervin et al., 2004).

1.2.3.3 Ethephon

Ethephon (2-chloroethylphosphonic acid) is a major plant growth regulator, which has been wildly used for decomposing to ethylene. Generally, ethephon is applied to accelerate the climacteric fruit ripening, such as increase of total soluble solids (TSS) and red color in tomato (Logendra et al., 2004), enhancement of red peel color and flavonoid compounds concentration in apple (Li et al., 2002). In grape, previous studies have shown that ethephon application can improve coloration in red cultivars (Leao et al., 2015), and promote the berry juice acidity decreasing (Chervin et al., 2002).

1.2.4 Auxin

1.2.4.1 Auxin biosynthesis

The plant hormone auxin, which is predominantly represented by indole-3-acetic acid (IAA), is a key regulator of many aspects of plant growth and development, such as cell division and elongation, apical dominance, differentiation, tropisms, flowering, abscission, and senescence (Woodward and Bartel, 2005; Teale et al., 2006). Even though

IAA was the first plant hormone identified, the biosynthetic pathway at the genetic level is not completely clear (Mano and Nemoto, 2012).

1.2.4.2 Auxin and grape maturation

Previous study showed that IAA observed a high concentration in the early developmental stages, thereafter declined and remained low throughout the rest of berry development (Cawthon and Morris, 1982), treatment with the synthetic auxin before véraison delays berry maturation (Davies et al., 1997; Ziliotto et al., 2012), as seen in several maturation related physiological processes changed (Bottcher et al., 2011).

1.2.5 Isoprothiolane (IPT) and plant growth regulation

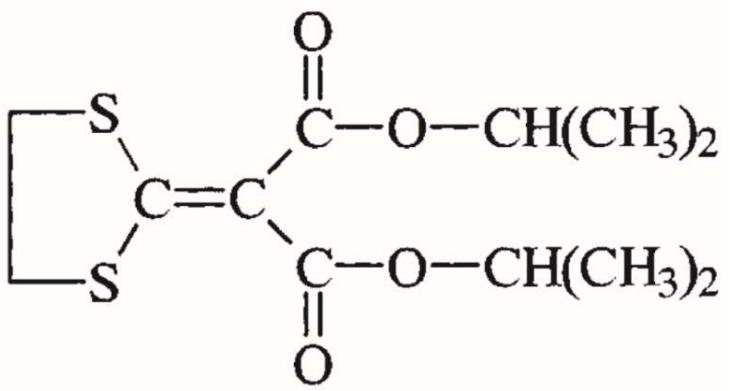

Figure 1.9 Chemical structure of isoprathiolane.

Isoprothiolane (diisopropyl-1, 3-dithiolan-2-ylidenemalonate; IPT) is a fungicide primarily used for the control of rice blast and planthopper (Taninaka, 1980). Up to today, IPT is still widely used to control the rice blast fungus *Magnaporthe oryzae*. On the other hand, IPT has been suggested as a new plant growth regulator (Ohtsuka, 2017). Previous study found that IPT could remarkably promote the growth of rice (*Oryza sativa* L.) seedlings through the enhancement or modification of auxin activity, cytokinin activity, and ethylene synthesis (Ohtsuka and Saka, 1988); similar effects were also reported in

beans (*Phaseolus vulgaris* L.) (Ohtsuka et al., 1990) and date palm (*Phoenix dactylifera* L.) (Okawara et al., 2003). Nevertheless, there is no knowledge of IPT effects on fruits until to now, especially the interaction of IPT and ABA in grape maturation.

1.2.6 Next-generation sequencing

Next-generation sequencing (NGS, also known as massively parallel sequencing) technology has revolutionized genomic, transcriptomic and epigenetic levels. Especially, it is easier to understand grape development and identify genetic factors affecting wine quality, with the published grapevine genome sequence (Jaillon et al., 2007). One side, NGS can improve the output/cost ratio of genome sequencing dramatically (Bolger et al., 2014), as the whole genome shotgun (WGS) is still expensive and time-consuming. On the other hand, the NGS technology enables the sequencing of total cDNA (RNA-Seq) to derive accurate measure of individual gene expression, differential splicing activity and novel regions of transcription discovery (Zenoni et al., 2010). Therefore, we used NGS strategy to measure genes expression after IPT and NDGA application to grapes in this study.

CHAPTER 2

EFFECTS OF IPT OR NDGA APPLICATION ON ABA METABOLISM AND MATURATION IN 'SHINE MUSCAT' GRAPE BERRIES

2.1 INTRODUCTION

The phytohormone abscisic acid (ABA) plays various roles in the regulation of plant growth and responses to environmental stresses (Tan et al., 1997; Kondo et al., 2012). A previous study found that ABA promoted embryo growth (Tian and Brown 2000). ABA has also been shown to induce stomatal closure in two terrestrial fern species, *Polystichum proliferum* and *Nephrolepis exaltata* (Cai et al., 2017). It has been shown that ABA is one of the crucial maturation control factors in grape development (Cantín et al., 2007; Zoccatelli et al., 2013). ABA concentrations are associated with sugar accumulation, chlorophyll degradation and volatile emissions in grapes (Fenoll et al., 2009; Young et al., 2012; Castellarin et al., 2016). The ABA inhibitor NDGA blocks ABA biosynthesis by suppressing the NCED enzyme activity (Creelman et al., 1992) and delays maturation in grapes (Zhang et al., 2009). Pan et al. (2005) found that ABA activates *AI* expression and related to soluble sugar content increasing. Furthermore, previous study has shown that the C_6 aroma volatiles hexanol and (*E*)-2-hexenal increase rapidly after véraison (Kalua and Boss, 2010). Linalool is the typical aromatic compound for Muscat flavor grape (Martin et al., 2010). However, little is known about how ABA promotes hexanol, (*E*)-2-hexenal and linalool concentration increase during grape development.

'Shine Muscat' grape (*Vitis labruscana* Baily × *Vitis vinifera* L.) is a popular cultivar in Japan with a strong flavor and high sugar concentration (Yamada et al., 2008). In general, consumers prefer green 'Shine Muscat' grapes over those with a yellow color. Therefore, 'Shine Muscat' grapes would be more marketable if the green color could be preserved during the maturation stage.

Isoprothiolane (diisopropyl-1, 3-dithiolan-2-ylidenemalonate; IPT) is a fungicide

used for the control of rice blast (Taninaka 1980). A previous study found that IPT remarkably promoted the growth of rice (*Oryza sativa* L.) seedlings through the enhancement or modification of auxin activity, cytokinin activity, and ethylene synthesis (Ohtsuka and Saka, 1988); similar effects were also reported in beans (*Phaseolus vulgaris* L.) (Ohtsuka et al., 1990) and date palm (*Phoenix dactylifera* L.) (Okawara et al., 2003). These studies suggest that IPT may be able to regulate phytohormones such as auxin and ethylene. In addition, it has been shown that auxin and ethylene influenced grape maturation (Davies et al., 1997; Chervin et al., 2004). ABA may promote grape maturation through complex interactions with auxin and ethylene metabolism (Kuhn et al., 2014). Although the function of IPT was reported in rice and other crops, its function in the berry maturation process has not been well-documented. However, we assumed that IPT could play a negative function in the berry maturation process because our preliminary study showed that IPT inhibited anthocyanin formation in grapes (data not presented). Therefore, we intended to clarify the IPT function by comparison with the widely-known function of ABA in berry maturation using NDGA.

In this study, IPT and NDGA treatments were applied to 'Shine Muscat' grapes one week before véraison [43 days after full bloom (DAFB)]. Transcriptome analysis was performed using the Illumina RNA-Seq method to clarify the role of IPT and NDGA on maturation in 'Shine Muscat' grapes.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Twenty-seven 3-year-old 'Shine Muscat' (*Vitis labruscana* Baily × *Vitis vinifera* L.) grape vines were grafted onto a 'Teleki-Kober 5BB' rootstock (*V. berlandieri* ×*V. ripariahybrids*), grown in a 45-L pot with well-drained soil and vertical shoot trellising in a greenhouse at Chiba University (35°N latitude, 140°E longitude, and elevation of 37 m). One week before véraison (43 DAFB), 27 clusters (9 clusters per treatment) were randomly given one of three treatments: 3.44 mM IPT, 100 μM NDGA, or distilled water only as the untreated control. Each treatment contained 0.1% (v/v) surfactant Approach BI (50% polyoxyethylene hexitan fatty acid ester; Kao, Osaka, Japan). The concentrations of 3.44 mM IPT and 100 μM NDGA were effective without toxicity in our preparatory experiment. Three clusters (35 berries per cluster) per treatment were randomly collected at 43 DAFB (one week before véraison), 70 DAFB (two weeks after véraison), and 111 DAFB (maturation stage; commercial harvest time). The flesh and skin were separated and the skin was frozen with liquid nitrogen and stored at -80°C until analysis.

2.2.2 Determination of chlorophyll, ABA, sugar, and volatile concentrations

Total chlorophyll concentrations were analyzed using a previously reported method (Hu et al., 2013) with modifications. One gram skin (fresh weight; 3 replications of 35 berries) was extracted with 4 mL 80% acetone containing 20% (v/v) 0.2 M Tris–HCl pH 8, and incubated at 4°C in the dark for 12 h. The chlorophyll concentrations were measured at 645 nm and 663 nm, respectively, using a spectrophotometer (HITACHI U-2910; Hitachi, Tokyo, Japan). Extraction buffer was used as a blank. Endogenous ABA in the skin was measured with a gas chromatography–mass spectrometry (GC–MS:

QP5000; Shimadzu, Kyoto, Japan), and sugar concentrations were analyzed by high performance liquid chromatography (HPLC: L-6200; Hitachi) as previously reported by Kondo et al. (2014). Aromatic volatile compounds were extracted with headspace solid-phase micro-extraction and analyzed with a GC–MS (QP2010; Shimadzu) according to a previous report (Wang et al., 2015).

2.2.3 RNA extraction, preparation of RNA-Seq, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA extraction (1 g sample, 3 replications of 35 berries) for both RNA-Seq and qRT-PCR experiments was performed using the cetyltrimethylammonium bromide and silica column-based extraction method (Henderson and Hammond, 2013). Concentrations of total RNA were assessed using a spectrophotometer (SmartSpec™ Plus spectrophotometer; Bio-Rad, Hercules, CA, USA) and agarose gel electrophoresis. For RNA-Seq, the cDNA library preparation was performed using the TruSeq RNA Sample Prep Kit v2.0 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Prior to sequencing, RNA integrity values were evaluated using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and samples with an RNA integrity number (RIN) $\geq$ 7.0 were selected for deep sequencing. Sequencing was performed with an Illumina HiSeq 2000 platform (Illumina, Santa Clara, CA, USA).

For qRT-PCR validation, analysis was performed with a StepOnePlus™ system (Applied Biosystems, Foster City, CA, USA). The cDNAs were synthesized from 1 μg of total RNA using a cDNA Synthesis Kit (FSQ-201; Toyobo, Osaka, Japan) according to the manufacturer's instructions. The reaction mixture was prepared using a KAPA SYBR FAST Master Mix (Kapa Biosystems, Boston, MA, USA); the specific primers used in

qRT-PCR are listed in Table 2.1. The expression level was calculated as $2^{-\Delta\Delta Ct}$ and normalized to the Ct value of *VvActin* (Sun et al., 2010).

2.2.4 Analysis of the RNA-Seq data

Raw data (raw reads) of the fastq format were first processed through in-house Perl scripts, and the overall quality of the reads, total bases, total reads, GC content and basic statistics were calculated. Clean data (clean reads) were obtained by removing low-quality reads, adaptor sequences, contaminant DNA or PCR duplicates from raw data. At the same time, reads were trimmed by sequencing quality (Q30) and good-quality reads were aligned to the PN40024 12X grapevine reference genome (Jaillon et al., 2007) using the software package TopHat v2.0.13. The reference genome was downloaded from the NCBI (GCF_000003745.3), and gene model annotation files were downloaded from the Genoscope database (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/).

Differential transcript expression levels per sample were normalized as FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values by a Reference Annotation Based Transcript (RABT) in Cufflinks (v2.2.1) software. In order to analyze differentially expressed genes (DEGs) among samples, low-quality transcripts were filtered, then log2 transformation of FPKM+1 and quantile normalization were performed with the preprocess Core' R library to reduce systematic bias. In this study, a log2 fold change ($|FC| \geq 2$ was adopted as indicating significance of transcript expression differences using cuffdiff2 (Roberts et al., 2011).

A separate functional annotation of transcripts was visualized using MapMan BINs (Thimm et al., 2004). Heatmaps of gene expression patterns in ABA, sugar, and aroma volatile metabolic pathways were performed using the pheatmap R package. The RNA-

Seq data were deposited in the Short Read Archive at NCBI under accession number SRR7168903, SRR7168904, and SRR7168905.

2.2.5 Statistical analysis

Data are presented as mean values of three replicates ± standard errors (SE), subjected to analysis of variance (ANOVA) procedures, and separated by the Tukey-Kramer test at $p \leq 0.05$ using the SAS statistical analysis package (version 8.2; SAS Institute, Cary, NC, USA).

Table 2.1 Primers used for qRT-PCR in IPT or NDGA application assay.

Gene name		Forward/reverse primer (5'–3')	Gene number
VvAAO4	(F)	GCATGTCACTCTTCTCGGCT	GSVIVT01025879001
	(R)	GCCCTTTCCGCTTCAGAAAC	
VvGEM-like 4	(F)	CTACACGACACCAGACTCCC	GSVIVT01034412001
	(R)	GTCAGCCTTCTTCCCAAGCT	
VvNCED1	(F)	GGTGGTGAGCCTCTGTTCCT	AY337613
	(R)	CTGTAAATTCGTGGCGTTCACT	
VvCYP707A1	(F)	GGTCACTTGGAGGGTAATTAC	XM_002282197
	(R)	TGTTGTCGGCGATTTGATCCT	
VvAI	(F)	GCTGTGCCCAAAAATCTCTC	GSVIVT01016869001
	(R)	CCAAGCAGTCGTAGGGTCTC	
VvSUS4	(F)	AGATGGACCGTGTCAGGAAC	GSVIVT01015018001
	(R)	CAAATGTTGGCAATCCACAG	
VvLOX	(F)	CCCCAAGTCATCAAAGAGGA	GSVIVT01000084001
	(R)	TGACAACTGGGTTGAGTCCA	
VvADH	(F)	CATCTCAGGCGAAATGGAGT	GSVIVT01033919001
	(R)	CAAGAAGGCCCCATACACAT	
VvGPPS	(F)	CGCCACTTTGTTCTCTCTCC	GSVIVT01019299001
	(R)	GCCGAACACCACTCAAGAAT	
VvTPS	(F)	GGAAGCTGGAGGATGAAGTG	GSVIVT01000401001
	(R)	TGCCCTAAACCTAACCGTTG	
VvActin	(F)	GTGCCTGCCATGTATGTTGCC	AF369524
	(R)	GTCACGTCCAGCAAGGTCAAG	

2.3 RESULTS

2.3.1 Effects of IPT and NDGA application on chlorophyll concentration

Chlorophyll concentrations in the skin of all grapes decreased during the period from 43 DAFB to 111 DAFB (Fig 2.1A, B). Chlorophyll concentrations in IPT- and NDAG-treated berries were higher than those in the untreated control berries at 70 DAFB. However, there were no significant differences among treatments at 111 DAFB (Figure 2.1B).

2.3.2 RNA-Seq and mapping

Three libraries from the untreated control, IPT-treated and NDGA-treated grapes at 70 DAFB were sequenced and generated 32,129,014 reads, 29,676,640 reads, and 32,144,516 reads, respectively, with a read length of 101 bp (Table 2.2). After removing the low-quality reads and trimming the adapter sequences, approximately 30 million reads were successfully obtained from each library. The reads had a Q30 percentage over 96% (percentage of sequences with sequencing error rate lower than 1%), and the GC percentages were 47.06%, 47.04%, and 46.90%, respectively. These trimmed reads produced unique mapping reads ranging from 83.2% to 84.6%, while the total mapped reads ranged from 84.9% to 86.3%, depending on the samples considered (Tables 2.2 and 2.3).

Figure 2.1 Effects of IPT and NDGA applications on 'Shine Muscat' grape berries. (A) Grape development stages at 43 DAFB (one week before véraison), 70 DAFB (two weeks after véraison) and 111 DAFB (mature stage), and (B) Chlorophyll concentrations in berry skin. Different letters indicate significant differences by the Tukey-Kramer test at $p \leq 0.05$. An error bar is the standard error of the mean (n = 3).

Table 2.2 Summary of the sequencing data.

Sample ID	**Number of raw reads**	**Number of trimmed reads**	**GC content(%)**	**Q30(%)**	**Usable reads (%)**
Untreated control	32,129,014	31,903,716	47.06	96.76	99
IPT	29,676,640	29,487,490	47.04	97.17	99
NDGA	32,144,516	31,929,218	46.90	96.99	99

Table 2.3 Summary of the mapped data.

Sample ID	**Read type**[1]	**Processed reads**[2]	**Mapped reads**[3]	**Multiple mapping**[4]	**Uniquely mapped**	**Total mapped ratio**[5]
Untreated control	Read 1	15951858	13683226 (85.8%)	324401 (2.4%)	26556824	85.30%
	Read 2	15951858	13516659 (84.7%)	318660 (2.4%)	(83.2%)	
IPT	Read 1	15964609	13851564 (86.8%)	281678 (2.0%)	27007999	86.30%
	Read 2	15964609	13716185 (85.9%)	278072 (2.0%)	(84.6%)	
NDGA	Read 1	14743745	12571430 (85.3%)	258139 (2.1%)	24534866	84.90%
	Read 2	14743745	12477336 (84.6%)	255761 (2.0%)	(83.2%)	

[1] Paired-end reads, [2] Number of cleaned reads after trimming, [3] Number of reads mapped to reference, [4] Number of reads removed due to multiple mapping, [5] Total mapped reads / total processed reads.

2.3.3 Identification of differentially expressed genes (DEGs) and MapMan visualization

In total, 44,051 transcripts were predicted for the three treatments at 70 DAFB on the basis of the grape genome sequences (Jaillon et al., 2007). After excluding 14,969 transcripts with at least one zero fragments-per-kilobase-million (FPKM) value, 29,082 transcripts were entered into the analysis (Figure 2.2). A total of 2,968 DEGs with log2 fold changes (|FC|) greater than or equal to 2 compared to the untreated control at 70 DAFB were identified. Of these, 2,060 DEGs (765 up-regulated, 1295 down-regulated) were identified in the grapes subjected to NDGA treatment, and 2,110 DEGs (826 up-regulated, 1,284 down-regulated) were identified in those given the IPT treatment (Figure 2.3A). More specifically, at 70 DAFB, there were 384 and 474 up- and down-regulated DEGs in the NDGA-treated grapes, respectively, and 438 and 470 up- and down-regulated DEGs in the IPT-treated grapes, compared to the untreated control (Figure 2.3B).

MapMan analysis showed that DEGs involved in cell wall and secondary metabolism were mainly down-regulated in both the IPT- and NDGA-treated grapes compared to the untreated control. In contrast, a general up-regulation of DEGs in light reactions and the starch catabolic pathway was found in the IPT- and NDGA-treated grapes (Figure 2.4). In terms of the auxin biosynthesis and signal transduction pathway, the DEGs showed no tangible results in either IPT- or NDGA-treated grapes at 70 DAFB. Only the transcript encoding auxin-induced protein 6B-like (GSVIVT01024152001) was up-regulated in IPT- and NDGA-treated grapes. Moreover, six DEGs involved in the ethylene biosynthesis and signal transduction pathway were down-regulated in NDGA-treated grapes at 70 DAFB, and three such DEGs were down-regulated in IPT-treated grapes (Figure 2.5).

'Shine Muscat' grapes have a strong muscat flavor and high soluble solid concentration at maturation, and ABA has a pivotal role in grape maturation. Within the set of DEGs, we focused on the portions of the grape transcriptome related to ABA, sugar, and aroma volatile compound metabolism.

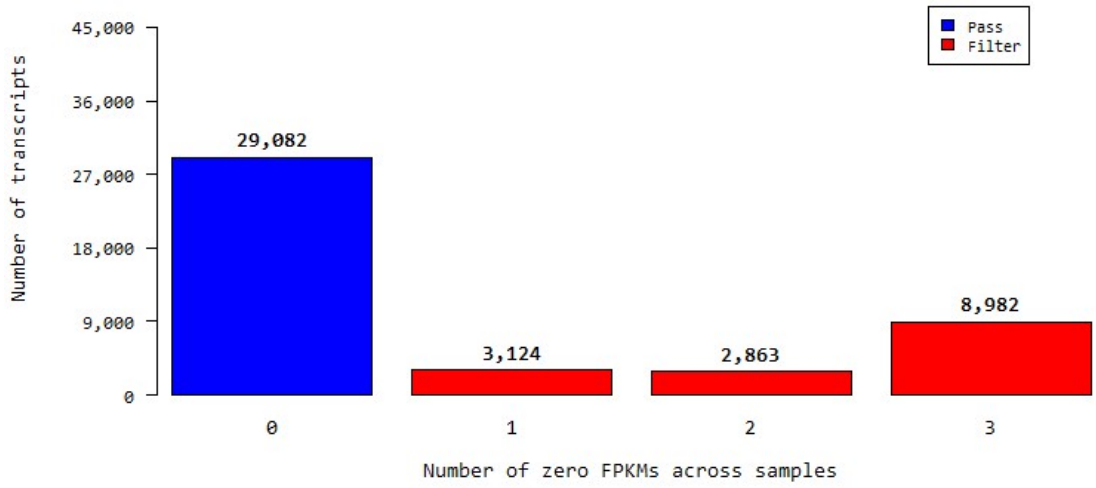

Figure 2.2 Distribution of transcripts with various numbers of zero FPKMs.

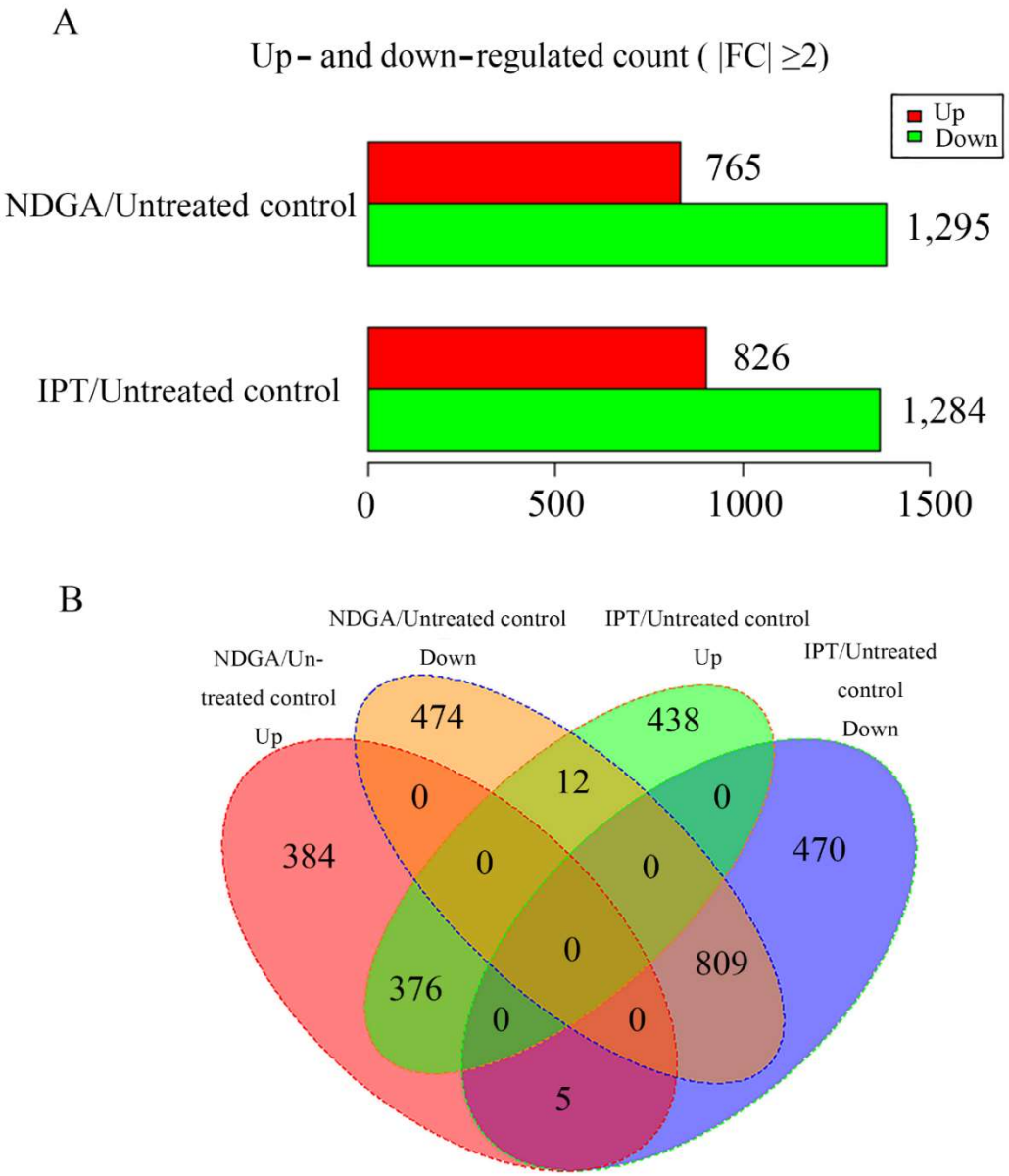

Figure 2.3 Analysis of DEGs in IPT- and NDGA-treated berries at 70 DAFB. (A) Numbers of up- and down-regulated DEGs, using log2 fold change (|FC|) ≥ 2. (B) Venn diagram of unique and common DEGs between IPT and NDGA treatment.

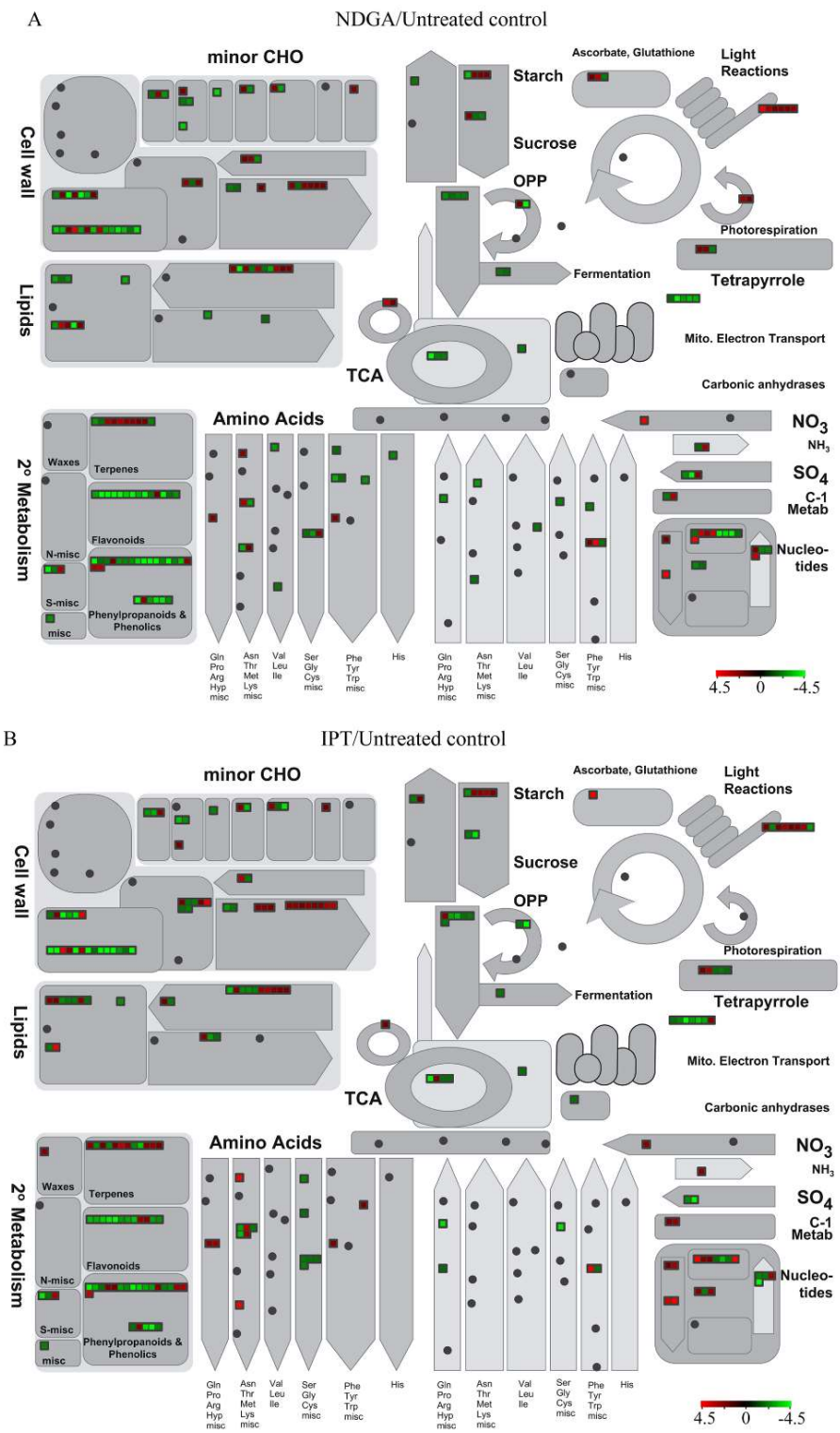

Figure 2.4 MapMan visualization of DEGs in response to the IPT and NDGA treatments at 70 DAFB berries. (A) DEGs in response to the NDGA treatment, and (B) DEGs in response to the IPT treatment. The conventional red-to-green scale was used to indicate up-regulation (red) or down-regulation (green).

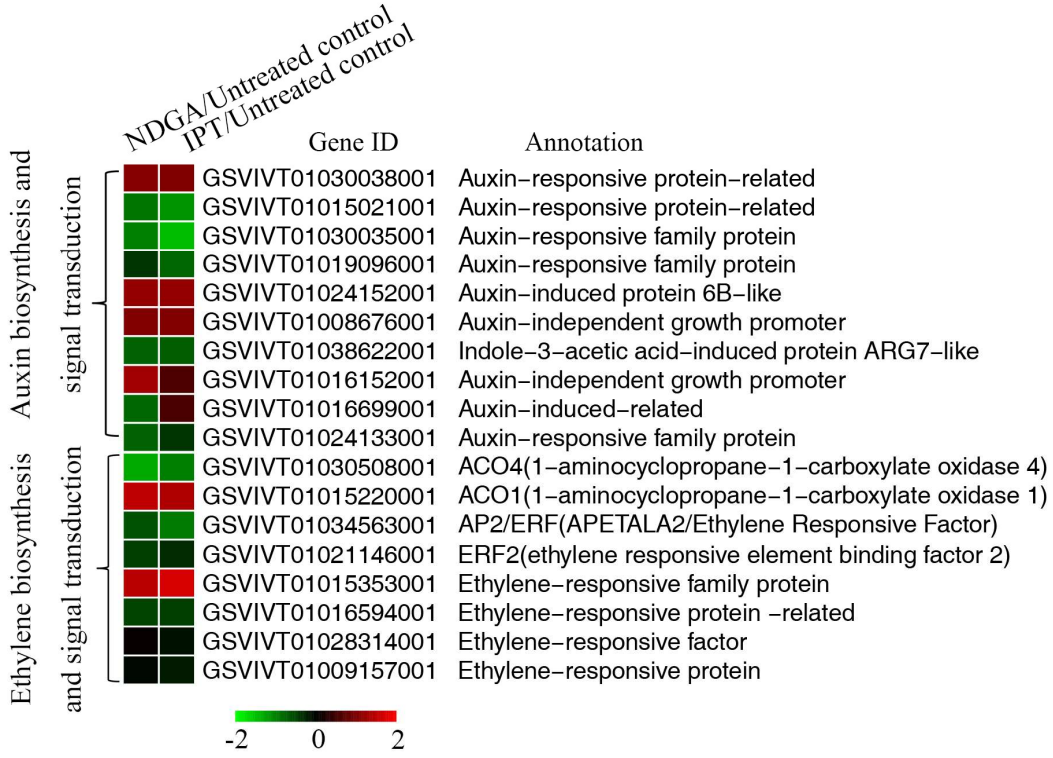

Figure 2.5 Heatmap diagram of relative gene expression levels for DEGs in auxin and ethylene metabolisms at 70 DAFB, using log2 fold change (|FC|) ≥ 2. The red-to-green scale was used to indicate up-regulation (red) or down-regulation (green).

2.3.4 Effects of IPT and NDGA treatment on ABA metabolism

Endogenous ABA in the IPT- and NDGA-treated berries were lower than those in the control berries at 70 DAFB, but ABA concentrations in IPT-treated berries at 111 DAFB were higher than those in either the untreated control or NDGA-treated berries (Figure 2.6A). We constructed a heatmap diagram of DEGs involved in the ABA pathway according to Mapman analysis (Figure 2.6B). The gene encoding *abscisic aldehyde oxidase 4* (*AAO4*, GSVIVT01025879001) was down-regulated in both IPT- and NDGA-treated grapes. Three transcripts encoding a *GEM (GLABRA2 Expression Modulator*)-like protein annotated as an ABA-responsive protein—namely, GSVIVT01034412001, GSVIVT01012919001 and GSVIVT01034410001—showed down-regulation in IPT- and NDGA-treated berries compared to the untreated control berries. However, *GEM*-like 5 *(GSVIVT01000958001*) and *ABF2* (*ABA responsive elements-binding factor 2, GSVIVT01009485001*) were up-regulated in the IPT-treated grapes at 70 DAFB (Figure 2.6B). *VvAAO4* and *VvGEM-like4* were down-regulated by IPT and NDGA treatment at 70 DAFB, which was consistent with the RNA-Seq data (Figure 2.6C). The expression levels of *VvNCED1* and *VvCYP707A1* were down-regulated in IPT- and NDGA-treated berries at 70 DAFB. In addition, *VvCYP707A1* was down-regulated by ITP treatment at 111 DAFB (Figure 2.6C).

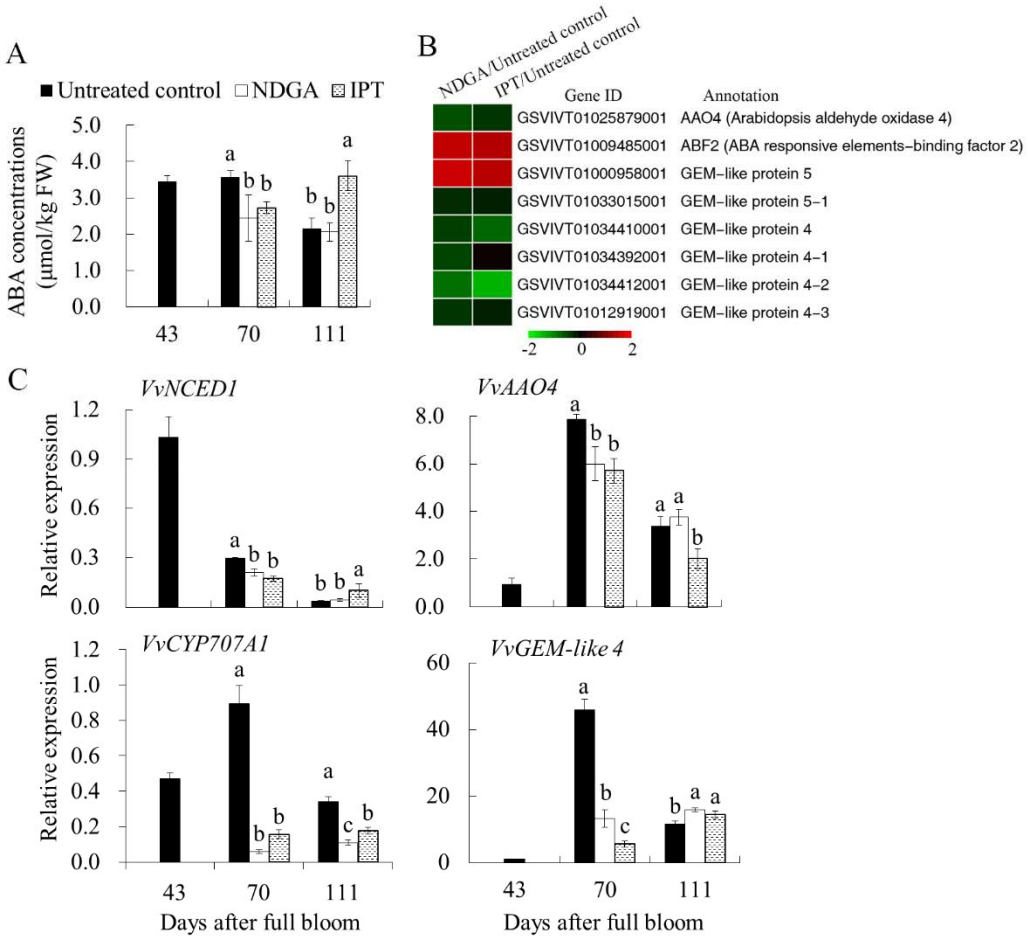

Figure 2.6 Effects of the IPT and NDGA treatments on ABA metabolism. (A) Endogenous ABA concentration. (B) Heatmap diagram of relative gene expression levels for ABA metabolism-related DEGs, using log2 fold change (|FC|) ≥ 2. The red-to-green scale was used to indicate up-regulation (red) or down-regulation (green). (C) qRT–PCR validation of selected ABA metabolism-related DEGs. Different letters indicate significant differences by Tukey-Kramer test at $p \leq 0.05$. An error bar is the standard error of the mean (n = 3).

2.3.5 Effects of IPT and NDGA treatment on sugar metabolism

Total sugar concentrations in NDGA-treated berries at 70 DAFB were lower than those in the IPT-treated or control berries. Glucose, fructose, and sucrose accumulations at 70 DAFB were inhibited by NDGA application, and IPT treatment decreased glucose production at 70 DAFB. However, at 111 DAFB the total sugar, glucose, fructose, and sucrose contents were not significantly different between IPT, NDGA, and untreated control berries (Figure 2.7A). Mapman analysis showed that both IPT and NDGA treatment down-regulated the expression levels of *sucrose synthase* (*SUS*, GSVIVT01015018001) and *acid invertase* (*AI*, GSVIVT01016869001) at 70 DAFB. In addition, three transcripts annotated as *hexose transporter* (*HT*)—namely, GSVIVT01017937001, GSVIVT01015361001 and GSVIVT01017836001—were down-regulated by IPT or NDGA treatment at 70 DAFB. However, the fructokinase gene (*fructose 6-phosphotransferase*, GSVIVT01012237001) and sucrose transport gene (GSVIVT01009254001) were up-regulated in IPT- and NDGA-treated grapes compared to the untreated control at 70 DAFB (Figure 2.7B). The expression levels of the *VvSUS*, *VvAI* and *VvHT* genes were highest in the untreated control and down-regulated upon IPT and NDGA treatment at 70 DAFB (Figure 2.7C).

2.3.6 Effects of IPT and NDGA treatment on major aromatic volatiles in grape

(*E*)-2-hexenal and hexanol concentrations increased distinctly after véraison (70 DAFB), then reached a peak at 111 DAFB. Linalool concentrations increased significantly from 70 DAFB to 111 DAFB. IPT and NDGA treatment decreased the (*E*)-2-hexenal and hexanol accumulation at 70 DAFB, but did not influence linalool accumulation (Figure 2.8A). At 70 DAFB, MapMan analysis showed that a gene

annotated as *lipoxygenase* (*LOX*, GSVIVT01000084001) and three genes annotated as *alcohol dehydrogenase* (*ADH*; GSVIVT01010644001, GSVIVT01033919001 and GSVIVT01033912001), which were involved in C_6 aroma volatile biosynthesis, were down-regulated in IPT- and NDGA-treated grapes. Furthermore, a gene annotated as *geranyl diphosphate synthase* (*GPPS*, GSVIVT01019299001) in the monoterpene biosynthesis pathway was down-regulated by IPT treatment, and a gene annotated as *terpene synthase* (TPS, GSVIVT01000401001) was down-regulated only by NDGA treatment; however, another *VvTPS* gene (GSVIVT01005220001) was up-regulated by NDGA treatment at 70 DAFB (Figure 2.8B). In addition, two genes encoding *Beta-Amyrin synthase* (GSVIVT01029508001 and GSVIVT01021474001) in the triterpene biosynthesis pathway were up-regulated by IPT or NDGA treatment. The expression levels of *VvLOX*, *VvADH*, *VvGPPS*, and *VvTPS* were higher in the untreated control berries and down-regulated by IPT and NDGA treatment at 70 DAFB.

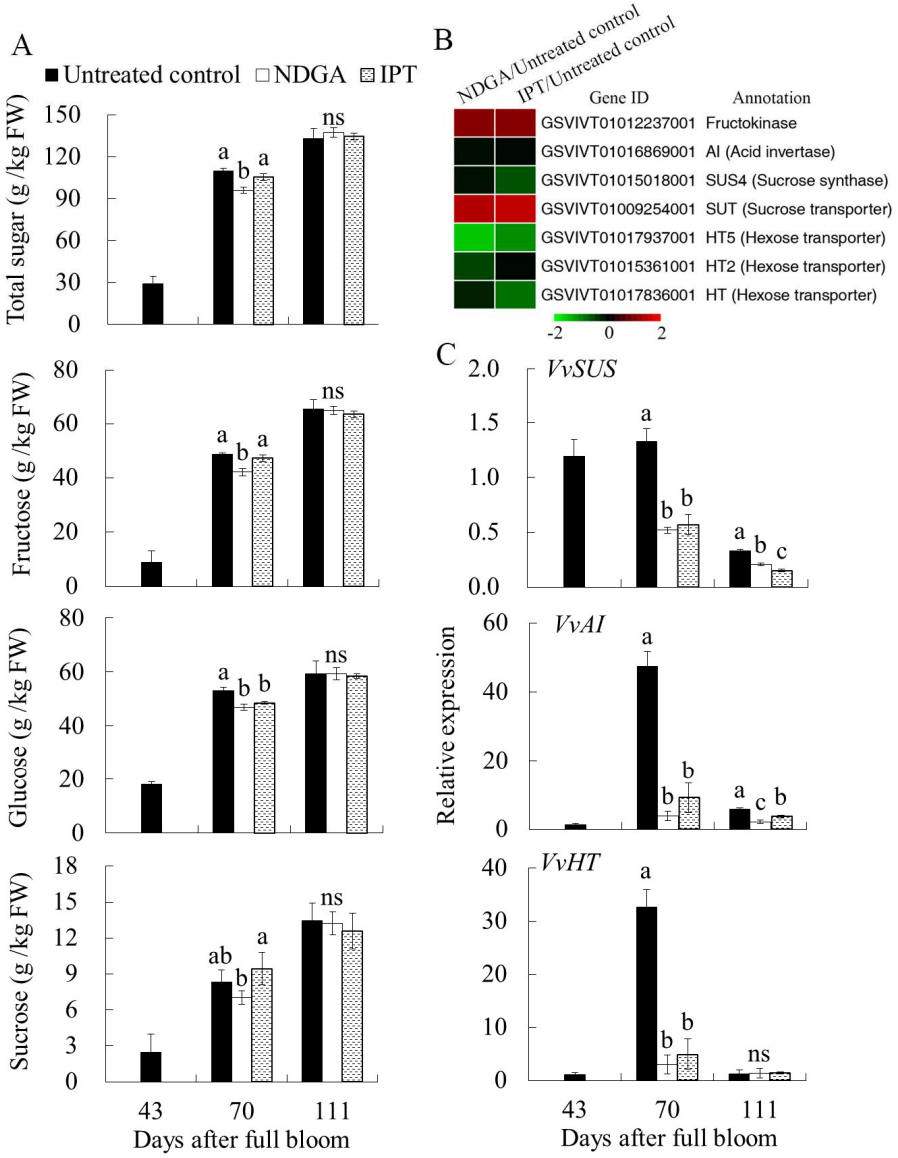

Figure 2.7 Effects of the IPT and NDGA treatments on sugar metabolism. (A) Changes in sugar contents. (B) Heatmap diagram of relative gene expression levels for DEGs in sugar metabolism, using log2 fold change (|FC|) ≥ 2. The red-to-green scale was used to indicate up-regulation (red) or down-regulation (green). (C) qRT–PCR validation of selected sugar metabolism-related DEGs. Different letters indicate significant differences by Tukey-Kramer test at $p \leq 0.05$. An error bar is the standard error of the mean (n = 3).

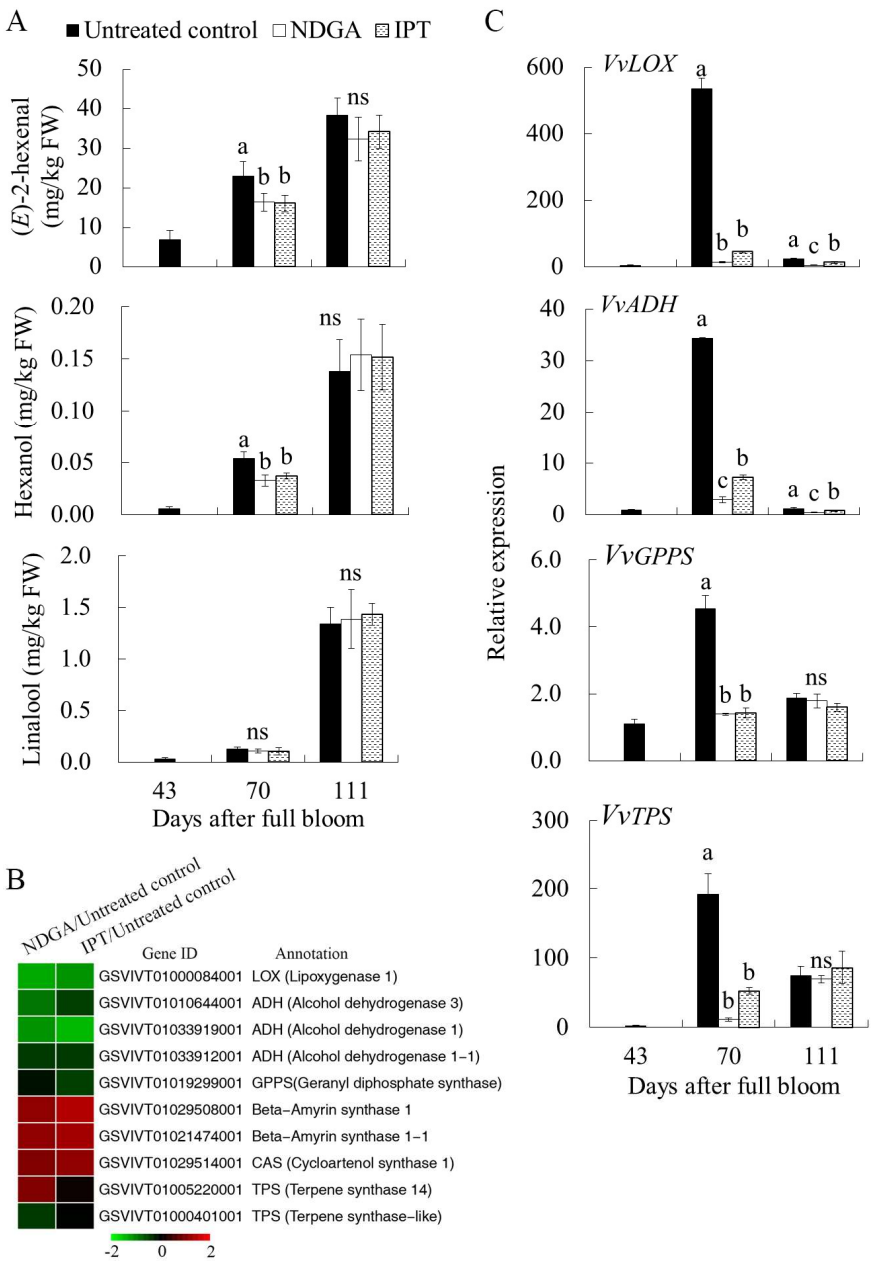

Figure 2.8 Effects of the IPT and NDGA treatments on aroma volatile metabolism. (A) Changes in aroma volatile concentrations. (B) Heatmap diagram of relative gene expression levels for DEGs in aroma volatile metabolism, using log2 fold change (|FC|) ≥ 2. The red-to-green scale was used to indicate up-regulation (red) or down-regulation (green). (C) qRT–PCR validation of selected aroma volatile metabolism-related DEGs. Different letters indicate significant differences by Tukey-Kramer test at $p \leq 0.05$. An error bar is the standard error of the mean (n = 3).

2.4 DISCUSSION

ABA increase just before véraison is one of the characteristic changes in the maturation of grape berries (Wheeler et al., 2009). Our study confirmed that NDGA treatment prior to véraison inhibited *VvNCED1* expression and ABA synthesis, which is consistent with the results of a previous report (Zhang et al., 2009). We also found that IPT similarly inhibited ABA accumulation at 70 DAFB. IPT application changed ABA, auxin and ethylene metabolism related-genes expression, and showed distinct effects on delaying grape maturation, these effects also play a role in the promotion of rice, beans and palm growth (Ohtsuka and Saka, 1988; Ohtsuka et al., 1990; Okawara et al., 2003).

It has been reported that auxin delays grape berry maturation at the transcriptional level (Ziliotto et al., 2012). In our study, IPT or NDGA application up- or down-regulated genes (e.g., genes annotated as auxin-responsive proteins) involved in auxin metabolism at 70 DAFB, respectively. Chervin et al., (2004) demonstrated that ethylene plays a potential role in grape maturation. A transcriptional analysis by Ziliotto et al. (2012) found that naphtalenacetic acid (NAA) application clearly down-regulated the expression of genes involved in ethylene metabolism, e.g., *AP2/ERF* (*APETALA2*/*Ethylene Responsive Factor*), thereby regulating grape maturation. Collectively, these results suggest that ABA may promote grape maturation through complex interactions with auxin and ethylene metabolism (Sun et al., 2010; Ziliotto et al., 2012; Kuhn et al., 2014).

To our knowledge, there is no published information on the effect of IPT on fruit development. In our study, the *AAO* gene, which encodes the enzyme that catalyzes the final step of ABA biosynthesis (Seo et al., 2000), was down-regulated in both IPT- and NDGA-treated berries. Even though the expression of *VvCYP707A1*, which is a key

enzyme in ABA catabolism (Kushiro et al., 2004), was down-regulated in IPT- and NDGA-treated berries at 70 DAFB, this might have been due to IPT and NDGA inhibiting the expression of the ABA biosynthesis genes *VvNCED1* and *VvAAO4*, and decreasing the ABA concentrations at 70 DAFB. It is noteworthy that IPT treatment up-regulated the expression of *VvABF2* at 70 DAFB in this study. A previous report showed that *VvABF2* was up-regulated by ABA during the maturation of *Vitis vinifera* 'Cabernet Sauvignon' wine grapes (Nicolas et al., 2014). These facts may suggest that the regulation of *VvABF2* in grape maturation is more complex than the regulation of other genes. On the other hand, in ABA signaling to the nucleus, key targets are the basic leucine zipper (bZIP) transcription factor ABI5 and related ABFs (Raghavendra et al., 2010). Skubacz et al. (2016) suggested that ABI5 has positive functions in core ABA signaling and influences chlorophyll breakdown. In our present study, three GEMs annotated as ABA-responsive proteins that may function downstream of ABA insensitive 5 (ABI5) in the ABA signaling pathway (Mauri et al., 2016) were down-regulated in both the IPT- and NDGA-treated grapes at 70 DAFB. These results agree with the notion that positive and negative interactions exist among bZIP proteins (Hurst, 1995), and suggest that IPT and NDGA may influence ABA metabolism at the grape maturation stage through the *VvNCED1*, *VvAAO*, and *VvGEM* genes.

Glucose and fructose concentrations increase along with the ABA concentration during grape maturation (Sun et al., 2010; Castellarin et al., 2016). In our study, NDGA treatment negatively regulated the sugar accumulation and IPT decreased the glucose concentration, which suggests that ABA may be an important factor in sugar metabolism. It is suggested that *sucrose synthase* (*SUS*) gene regulates sucrose accumulation in plant (Komatsu et al., 2002), and sucrose can be cleaved into glucose and fructose by acid

invertase (AI) in vacuoles (Desnoues et al., 2014). A previous report showed that *VvSUS* increased concomitantly with increases in endogenous glucose and ABA in 'Cabernet Sauvignon' grapes (Wang et al., 2017). It has been shown that ABA activates AI during grape development (Pan et al., 2005). Our study showing that NDGA down-regulated *VvSUS* and *VvAI* expression levels at 70 DAFB agrees with these previous findings.

In another alternative pathway, sucrose is broken down into glucose and fructose by an extracellular invertase in phloem, and glucose and fructose are subsequently transported into vacuoles by hexose transporters (HTs) (Robinson and Davies, 2000). It has been shown that the expression levels of *VvHT1* are positively regulated by both ABA and hexose (Conde et al., 2006). The results of our study showing that *VvHT* was down-regulated by NDGA in correlation with a decrease in ABA concentrations support the previous reports of Conde et al. (2006). In addition, IPT treatment also down-regulated the expression levels of *VvSUS*, *VvAI*, and *VvHT* at 70 DAFB in concert with the decrease in ABA concentrations.

Hexanol, (*E*)-2-hexenal, and linalool have been detected as major aromatic volatiles in 'Shine Muscat' grapes (Matsumoto and Ikoma, 2016; Wu et al., 2016). The C_6 aroma volatiles hexanol and (*E*)-2-hexenal are derived from the fatty acid metabolism pathways (Wang et al., 2015; Wong et al., 2016) and increase rapidly after véraison (Kalua and Boss, 2010). In agreement with the previous studies, we here found that the expression levels of *VvLOX* and *VvADH* were correlated with the production of C_6 aroma volatiles, which increased after véraison (Tesniere and Verries, 2000; Wong et al., 2016). Our results that IPT and NDGA treatment inhibited hexanol and (*E*)-2-hexenal accumulation and *VvLOX* and *VvADH* expression levels suggest that IPT and NDGA regulate C_6 aroma volatile production after véraison. Linalool as the typical aromatic compound for muscat flavor is

a monoterpene formed through the action of terpene synthase (TPS) (Martin et al., 2010) and the mid-pathway gene *VvGPPS* (Martin et al., 2012). *VvGPPS* transcripts increase significantly around véraison, whereas *VvTPS* transcripts are up-regulated after véraison (Martin et al., 2012). IPT and NDGA treatment down-regulated the expression levels of *VvGPPS* and *VvTPS* in our study, but did not influence linalool accumulation. This result suggests that linalool may be produced at a later stage (Lucker et al., 2004; Martin et al., 2012).

According to the results of the present study, we proposed a schematic graph (Figure 2.9) to explain how IPT and NDGA application regulate chlorophyll degradation, ABA metabolism, sugar accumulation, and aroma metabolism in grape berries. The application of IPT or NDGA significantly regulated ABA, auxin, and ethylene metabolism gene expression, and inhibited endogenous ABA accumulation. Chlorophyll degradation, and sugar and aroma accumulation were all negatively regulated by the respective treatments.

2.5 CONCLUSIONS

Our study indicated that IPT treatment showed similar trends in both gene expression and metabolite movements to those caused by NDGA treatment. These results indicate that IPT may function to inhibit maturation through the ABA signaling process in concert with auxin and ethylene.

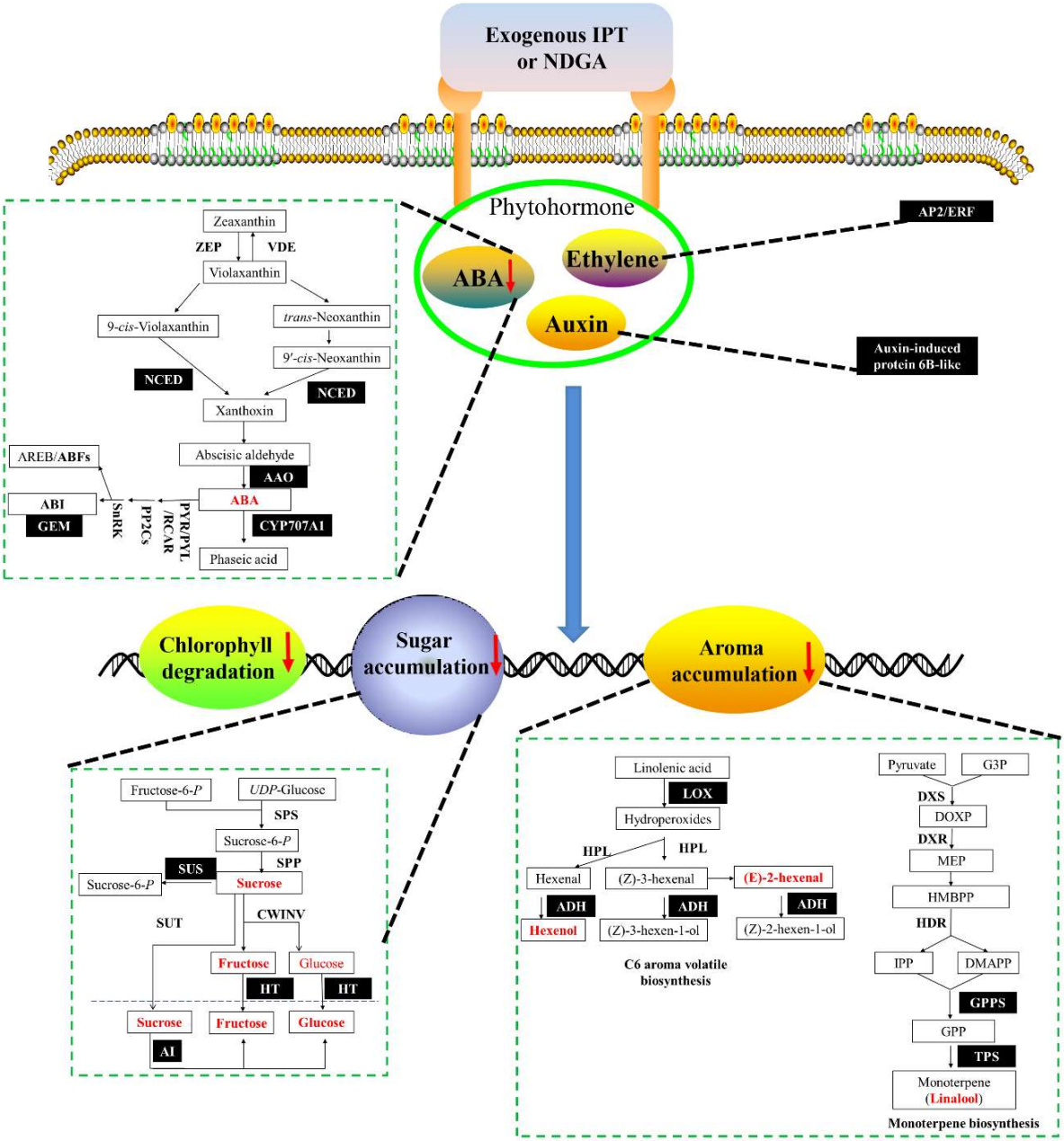

Figure 2.9 The schematic diagram of the regulation of 'Shine Muscat' grape maturation by the IPT or NDGA applications. Red arrows behind ABA, chlorophyll degradation, sugar accumulation, and aroma accumulation represent negative regulation. Genes in the black box are significantly regulated by the IPT or NDGA treatments.

CHAPTER 3

ABZ-E3M OR ETHEPHON APPLICATION AFFECTS 'SHINE MUSCAT' GRAPE BERRY MATURATION

3.1 INTRODUCTION

'Shine Muscat' grape (*Vitis labruscana Baily* × *Vitis vinifera* L.) is a popular commercial cultivar in Japan with a strong muscat flavor and high sugar concentrations (Yamada et al., 2008). As in the commercial production of any table grape, strategies for controlled, timely maturation are constantly being sought.

Abscisic acid (ABA) has been considered one of the crucial maturation control factors during fruit development (Koyama et al., 2010). ABA application has been shown to increase berry softening and skin anthocyanin concentrations (Cantín et al., 2007). However, Chervin et al. (2004) showed that grape berry maturation may be associated with ethylene responses. In addition, it is reported that ethylene application at véraison induced grape diameter increase (Chervin et al., 2008). In contrast, 1-methylcyclopropene (1-MCP), a specific inhibitor of ethylene receptors, delayed anthocyanin formation during grape development (Chervin et al., 2004). Interestingly, ABA treatment was shown to up- or down-regulate genes related to ethylene biosynthesis in grape berries (Koyama et al., 2010), and ABA plays an important role as an inducer of maturation along with ethylene in grapes and peaches (*Prunus persica* L.) (Zhang et al., 2009). Meanwhile, abscinazole-E3M (Abz-E3M), a specific inhibitor of ABA 8'-hydroxylase (CYP707A), was shown to increase endogenous ABA production (Takeuchi et al., 2016).

In this study, the effects of Abz-E3M and ethephon applications on 'Shine Muscat' berry maturation were investigated.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Fifteen 4-year-old 'Shine Muscat' grape vines grafted on rootstock 'Teleki-Kober 5BB' (*V. berlandieri* × *V. ripariahybrids*) were cultivated in 45-L pots containing well-drained soil at a Chiba University greenhouse, located at 35 °N Lat., 140 °E Long., and altitude of 37m, in 2016. Forty-five clusters (15 clusters per treatment) were randomly applied with one of three treatments at 44 days after full bloom (DAFB, 10 days before véraison): 50 μM Abz-E3M, 500 μM ethephon, or distilled water, each of which was mixed with 0.1% (v/v) of the surfactant Approach BI™. Grape berries were sampled at 44, 48, 54, 64, and 95 DAFB. The skin samples were frozen by liquid nitrogen after flesh firmness, TSS (total soluble solids) and titratable acid (TA) were measured. Berry firmness was measured after peeling the skin using a specialized penetrometer (CR-100, SUN SCIENTIFIC CO., LTD., Tokyo, Japan).

3.2.2 Determination of ethylene, ABA, chlorophyll, and soluble sugar concentrations

Ethylene production was determined using 60 berries (3 replications of 20 berries) according to a previous report (Kondo et al. 1991). Endogenous ABA and soluble sugar concentrations in the skin were analysed by the method of Kondo et al. (2014). Total chlorophyll concentrations were determined by a spectrophotometer (U-2910; HITACHI, Tokyo, Japan) according to the modified method of Hu et al. (2013).

3.2.3 RNA extraction and qRT-PCR analysis

Total RNA was isolated from the skin using the cetyltrimethylammonium bromide (CTAB) and silica column-based extraction method (Henderson and Hammond, 2013).

The cDNAs used for qRT-PCR were synthesized from 1 μg of total RNA using a cDNA Synthesis Kit (FSQ-201, Toyobo, Osaka, Japan) according to the manufacturer's instructions. For qRT-PCR validation, analysis was performed with a StepOnePlus™ system (Applied Biosystems, CA, USA). The reaction mixture was prepared using a KAPA SYBR FAST Master Mix (Kapa Biosystems, Boston, MA, USA). Primers used for real-time PCR for *VvNCED1, VvCYP707A1* and *VvActin* were constructed based on the sequences reported by Sun et al. (2010). The primers for *VvACO1, VvERF2*, *VvAI*, *VvSPS*, *VvPPH*, and *VvRCCR* were designed using Primer 5 software (http://www.premierbiosoft.com/) and are listed in Table 3.1. Expression levels were calculated as $2^{-\Delta\Delta Ct}$ and normalized to the Ct value of *VvActin*.

3.2.4 Statistical analysis

Data are presented as means ± SEs of three replicates, subjected to analysis of variance (ANOVA) procedures, and separated by the Tukey-Kramer test at $p \leq 0.05$ using the SAS statistical analysis package version 8.2 (SAS Institute, Cary, NC, USA).

Table 3.1 Primers used for qRT-PCR in Abz-E3M or ethephon application assay.

Gene name		Forward/reverse primer (5'–3')	Gene number
VvNCED1	(F)	GGTGGTGAGCCTCTGTTCCT	AY337613
	(R)	CTGTAAATTCGTGGCGTTCACT	
VvCYP707A1	(F)	GGTCACTTGGAGGGTAATTAC	XM_002282197
	(R)	TGTTGTCGGCGATTTGATCCT	
VvACO1	(F)	GCCATCATTTCACCAGCTCC	GSVIVT01015220001
	(R)	TCAAATCTGGGGCCCTTGTC	
VvERF2	(F)	GGCTAGGCACCTTCGAAACT	GSVIVT01021146001
	(R)	TTGAGCAGAGCCTTGGAACC	
VvPPH	(F)	GTCAAATGAGCAATGTGCCC	GSVIVT00028680001
	(R)	GATTCTAGGTTTCCGATCCACC	
VvRCCR	(F)	CGCCTTGACTCTTATCTACTTCC	GSVIVT00019487001
	(R)	GGAGAGGCTGGTTATGTTGAG	
VvSPS	(F)	GAACTCCTGATGTCCGAGCC	BG273723.1
	(R)	CTTGGTGGGGTGCACAAAAC	
VvAI	(F)	GCTGTGCCCAAAAATCTCTC	GSVIVT01016869001
	(R)	CCAAGCAGTCGTAGGGTCTC	
VvActin	(F)	GTGCCTGCCATGTATGTTGCC	AF369524
	(R)	GTCACGTCCAGCAAGGTCAAG	

3.3 RESULTS

3.3.1 Effects of Abz-E3M or ethephon application on firmness and TSS

Both Abz-E3M and ethephon treatments increased TSS compared to the untreated control at 48 and 94 DAFB (Figure 3.1A). The firmness values of Abz-E3M- and ethephon-treated berries were lower than that of the untreated control at 48 and 54 DAFB (Figure 3.1B). Abz-E3M and ethephon treatments decreased the TA content at 48 and 54 DAFB compared with the untreated control (Figure 3.1C). The firmness, TA, and berry weight were not significantly different at 94 DAFB except for TSS (Figure 3.1).

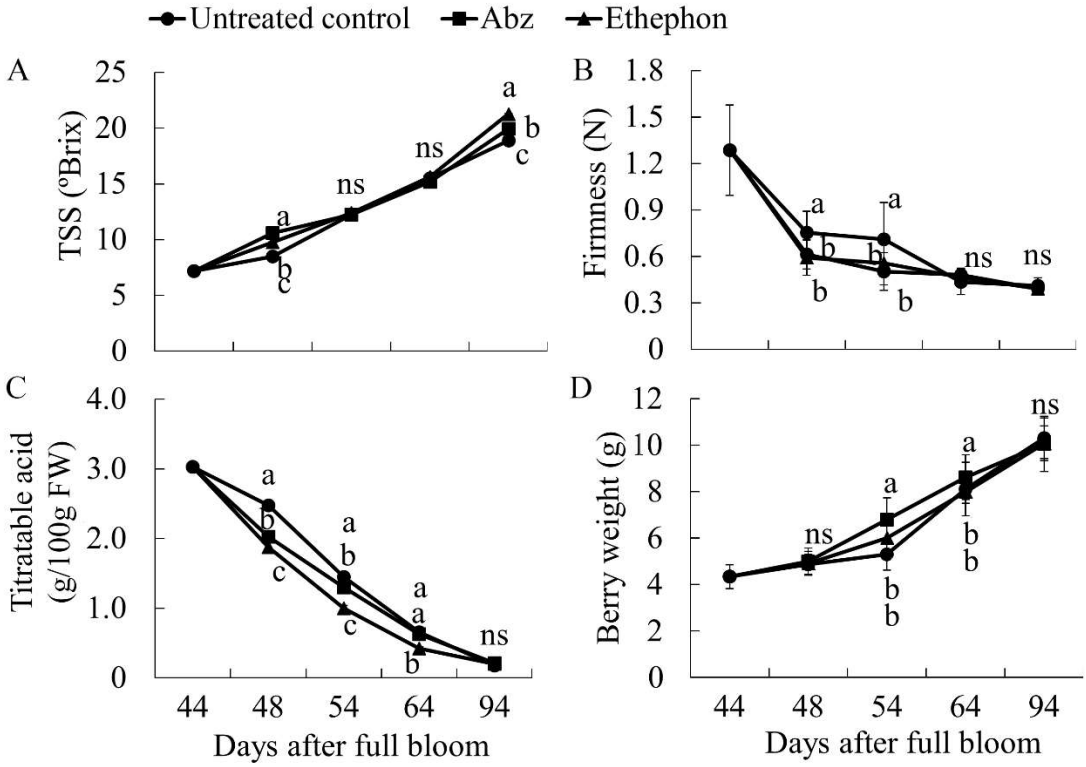

Figure 3.1 Changes of (A) TSS, (B) firmness, (C) TA, and (D) berry weight after Abz-E3M and ethephon applications in 'Shine Muscat' grape (*Vitis labruscana* Baily×*Vitis vinifera* L.). Different letters indicate significant differences by Tukey-Kramer test at $p \leq 0.05$. An error bar is the standard error of the mean (n = 3).

3.3.2 Endogenous ABA concentration, ethylene production, and related gene expressions analysis

ABA concentrations in Abz-E3M-treated berries were significantly higher than those in the untreated controls or ethephon-treated berries at 54 DAFB (Figure 3.2A). Expression levels of *VvNCED1* (9-cis-epoxycarotenoid dioxygenase) were up-regulated in the ethephon-treated group compared to those in the untreated controls at 48 and 64 DAFB. In contrast, expression levels of *VvNCED1* in Abz-E3M-treated berries were not different from those in the untreated controls at 48 or 54 DAFB. Expression levels of *VvCYP707A1* (ABA 8'-hydroxylase) were significantly down-regulated by Abz-E3M treatment at 48 DAFB and then up-regulated at 64 and 95 DAFB. Similarly, expression levels of *VvCYP707A1* were up-regulated in ethephon-treated berries at 54 DAFB (Figure 3.2B).

Ethylene peaks were observed in Abz-E3M- and ethephon-treated berries at 48 DAFB (Figure 3.3A). The expression levels of *VvACO1* (1-aminocyclopropane-1-carboxylate oxidase) and *VvERF2* (ethylene-responsive element binding factors) were significantly up-regulated in Abz-E3M- and ethephon-treated berries at 48 DAFB (Figure 3.3). The expression levels of *VvACO1* in ethephon-treated berries were higher than those in the untreated control and Abz-E3M-treated berries at 54 and 64 DAFB (Figure 3.3B).

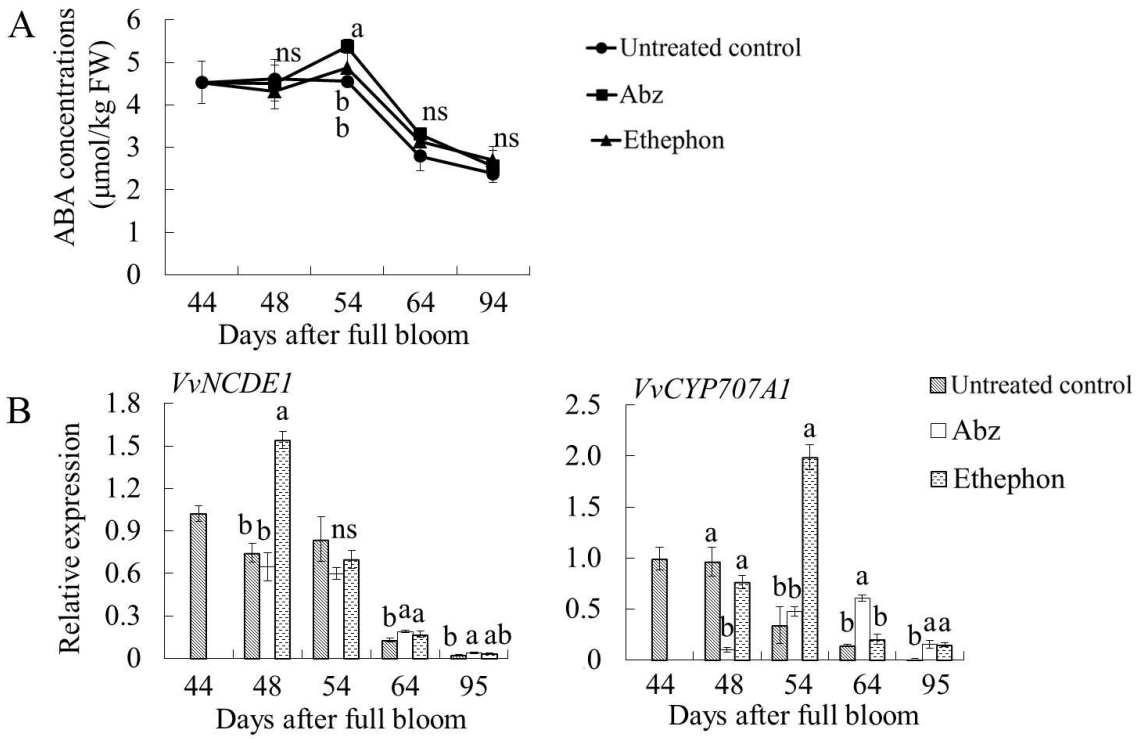

Figure 3.2 Endogenous ABA concentrations (A) and expression levels of *VvNCED1* and *VvCYP707A1* (B) in berry skin. Different letters indicate significant differences by Tukey-Kramer test at $p \leq 0.05$. An error bar is the standard error of the mean (n = 3).

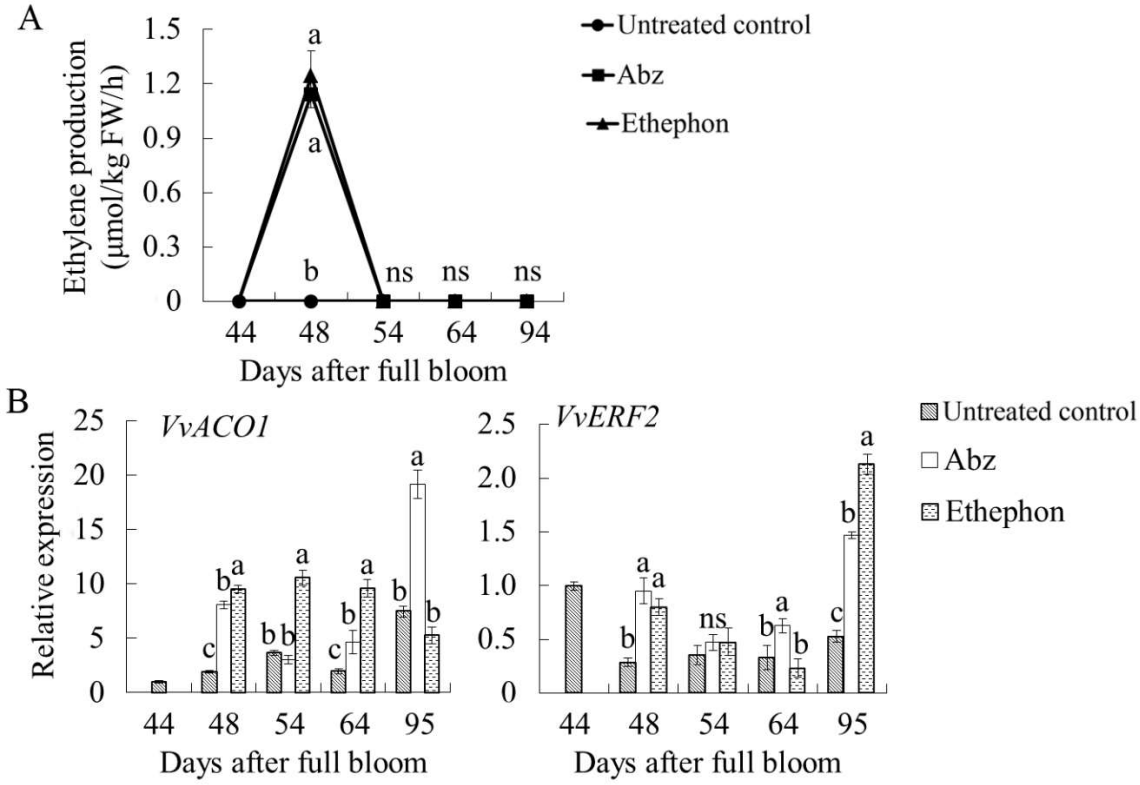

Figure 3.3 Ethylene production (A) and expression levels of *VvACO1* and *VvERF* (B) after Abz-E3M and ethephon applications in grape. Different letters indicate significant differences by Tukey-Kramer test at $p \leq 0.05$. An error bar is the standard error of the mean (n = 3).

3.3.3 Chlorophyll concentration and expressions of *VvPPH* and *VvRCCR*

Skin chlorophyll concentrations decreased from 44 to 94 DAFB across all groups, but those in Abz-E3M- and ethephon-treated groups were significantly lowerthan those in the untreated control at 48 and 54 DAFB (Figure 3.4A). Expression levels of *VvPPH* (pheophytin pheophorbide hydrolase) in Abz-E3M- and ethephon-treated berries were down-regulated at 48 DAFB, and then up-regulated from 54 to 94 DAFB. Expression levels of *VvRCCR* (red chlorophyll catabolite reductase) showed the same up-regulation from 54 to 94 DAFB as *VvPPH* (Figure 3.4B).

3.3.4 Sugar concentrations and gene expressions

Total sugar concentrations did not show significant differences between treatments at any timepoint except 54 DAFB (Figure 3.5A). Glucose and fructose showed the highest concentration in ethephon-treated berries at 54 and 64 DAFB. The Abz-E3M-treated berries showed increased fructose concentrations at 48 and 64 DAFB. Sucrose concentrations did not show a clear tendency (Figure 3.5A). The expression of *VvSPS* (sucrose phosphate synthase) and *VvAI* were down-regulated by Abz-E3M treatment at 48 DAFB, but the expression levels were up-regulated from 54 to 94 DAFB. Expression levels of *VvSPS* and *VvAI* were up-regulated by ethephon at 48 and 54 DAFB (Figure 3.5B).

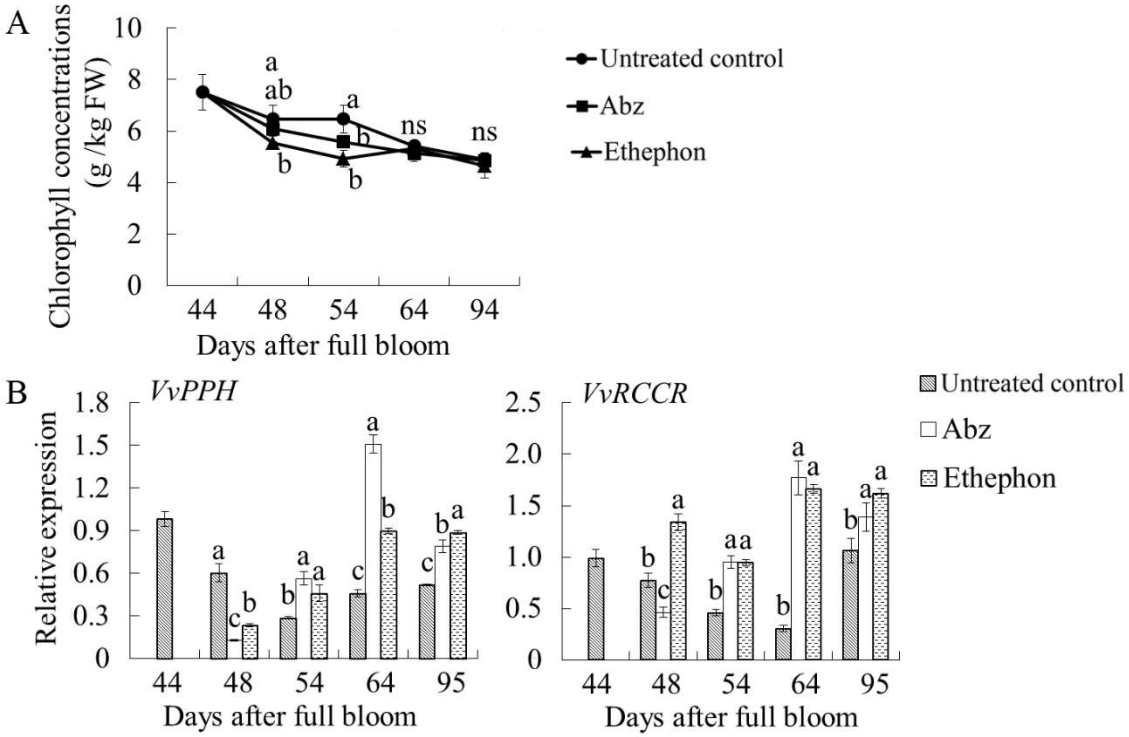

Figure 3.4 Changes of skin chlorophyll concentrations (A) and expression levels of *VvPPH* and *VvRCCR* (B) in berry skin. Different letters indicate significant differences by Tukey-Kramer test at $p \leq 0.05$. An error bar is the standard error of the mean (n = 3).

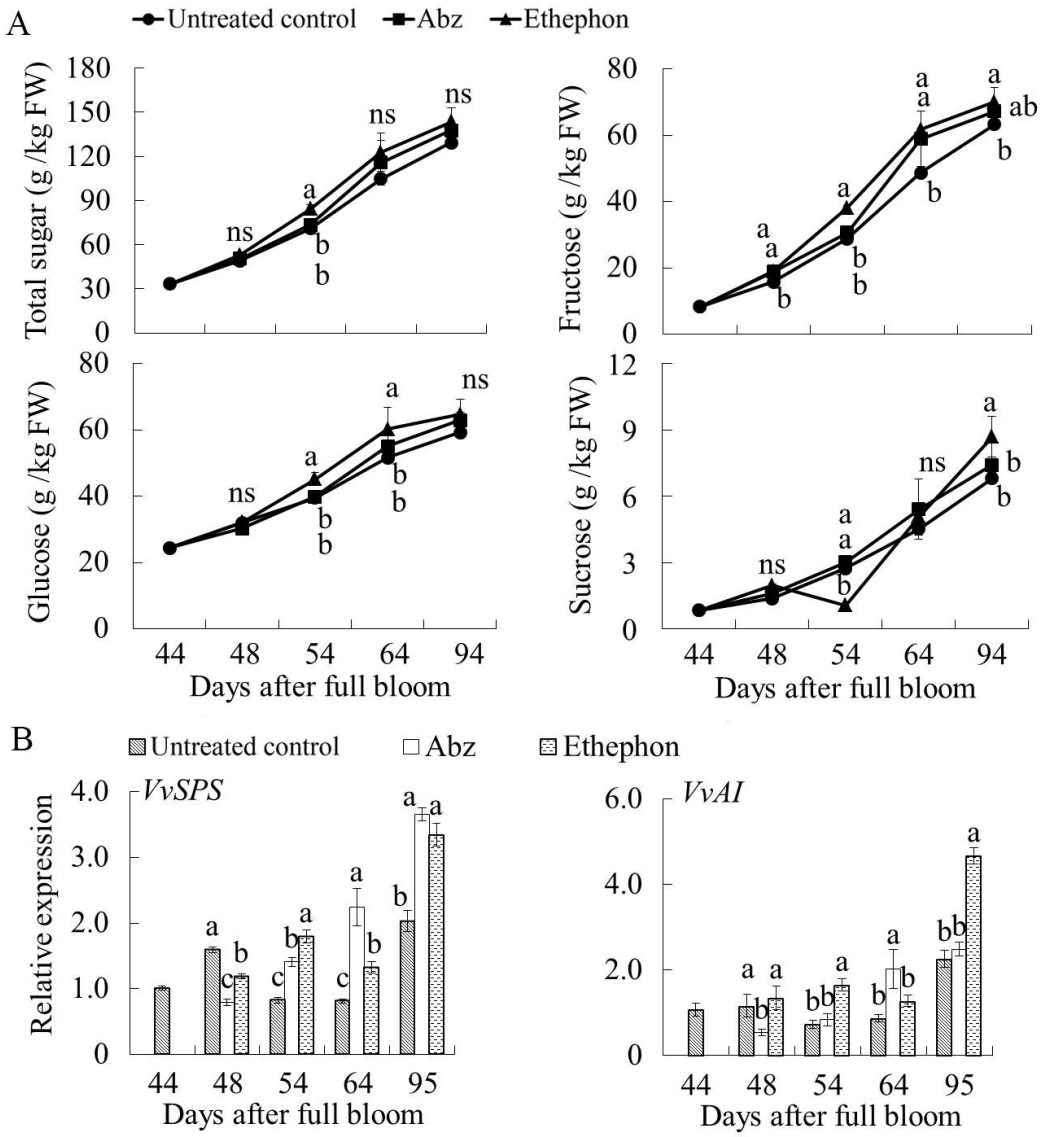

Figure 3.5 Effects of Abz-E3M and ethephon on sugar concentrations (A) and expression levels of *VvSPS* and *VvAI* (B) in berry skin. Different letters indicate significant differences by Tukey-Kramer test at $p \leq 0.05$. An error bar is the standard error of the mean (n = 3).

3.4 DISCUSSION

ABA application has been shown to accelerate physiological and biochemical changes that are related to maturation in grapes (Zoccatelli et al., 2013). Likewise, the expression level of the key ABA biosynthesis gene *VvNCED1* has been shown to be up-regulated at véraison (Pilati et al., 2017). In our study, Abz-E3M inhibited the expression levels of *VvCYP707A1* and increased endogenous ABA concentration. The results suggest that Abz-E3M treatment before véraison can stimulate grape maturation by increasing endogenous ABA. The results do not deny the relationship between *VvNCED1* and endogenous ABA biosynthesis because the inhibition of *CYP707A1* could increase endogenous ABA concentration (Okazaki et al., 2011).

Previous studies have shown that ethylene seems to regulate grape maturation (Chervin et al., 2004; Sun et al., 2010). New evidence obtained from transcriptome analysis suggested that ethylene synthesis genes (*ACO*) or transcription factors (*ERF/AP2*) are expressed during grape maturation (Koyama et al., 2010). Our study showed Abz-E3M and ethephon treatment significantly up-regulated expression levels of *VvACO1* and *VvERF2*. This up-regulation coincided with the ethylene peak in Abz-E3M- or ethephon-treated berries, suggesting that ethephon treatment can promote grape maturation similarly to Abz-E3M, through *VvACO1* and *VvERF2*. It has been suggested that ethylene may trigger transcription of ABA-related genes during grape development (Ziliotto et al., 2012), and that ABA may play an important role as an inducer of maturation along with ethylene in grapes (Zhang et al., 2009). In our study, ethephon treatment up-regulated *VvNCED1* gene expression at 48 DAFB, but did not result in increased ABA concentrations. This result suggests that the concomitant up-regulation of *VvCYP707A1* may have also impacted ABA production.

GENERAL DISCUSSION

Given the commercial importance of grapevines, it is great significance to explore the mechanism of grape development and maturation. Previous studies found that various phytohormones play important role in grape maturation, such as ABA, auxin, ethylene, gibberellin, and brassinosteroids (Davies et al., 1997; Chervin et al., 2004; Symons et al., 2006; Wheeler et al., 2009; Murcia et al., 2017).

Grapevine (*Vitis vinifera* L.) is considered as a non-climacteric fruit species due to there is no obvious ethylene production during the berries development (Coombe and Hale, 1973). ABA has been considered as one of the main maturation control factors in grape, as ABA concentration is very low in the young berries but increased and peaked at around véraison (Davies et al., 1997; Deluc et al., 2009; Wheeler et al., 2009; Giribaldi et al., 2010). Exogenous ABA application could stimulate grape maturation (Cantín et al., 2007; Peppi et al., 2008; Zoccatelli et al., 2013). In this study, the ABA inhibitor NDGA application delayed grape maturation and down-regulated ABA metabolism and signaling genes expression including *VvNCED1*, *VvAAO*, and *VvGEM*. These results agree with that NDGA application inhibit grape maturation (Zhang et al., 2009; Jia et al., 2018), and suggest that IPT and NDGA may influence ABA metabolism at the grape maturation stage through the *VvNCED1*, *VvAAO*, and *VvGEM* genes. In contract, the Abz-E3M (ABA 8'-hydroxylase inhibitor) increased the endogenous ABA contents and the gene expression levels of *VvCYP707A1*, which resulted in grape maturation advanced. The results do not deny the relationship between *VvNCED1* and endogenous ABA biosynthesis because the inhibition of *CYP707A1* could increase endogenous ABA concentration (Okazaki et al., 2011).

Traditionally, IPT has been widely used to control the rice blast fungus *Magnaporthe*

oryzae. However, numerous studies demonstrated that IPT have a potential function for plant growth regulation (Ohtsuka and Saka 1988; Ohtsuka et al., 1990; Okawara et al., 2003). This novel plant growth regulator application can enhance or modify auxin activity, cytokinin activity, and ethylene synthesis (Ohtsuka and Saka 1988). In this study, IPT application significantly decreased the ABA contents at 70 DAFB and changed *VvNCED1*, *VvAAO*, and *VvGEM* genes expression, which showed similar negative effects with NDGA. These results suggested that IPT can be used for grape maturation regulation.

Although ethylene is considered as main ripening regulator of climacteric fruit, Previous studies have shown that ethylene seems to regulate grape maturation (Chervin et al., 2004; Sun et al., 2010). Transcriptional analysis found that NAA application down-regulated the expression of genes involved in ethylene metabolism, such as *AP2/ERF* (Ziliotto et al., 2012). Koyama et al. (2010) found that ethylene synthesis genes (*ACO*) or transcription factors (*ERF/AP2*) are expressed during grape maturation. In this study, Six DEGs involved in the ethylene biosynthesis and signal transduction pathway were down-regulated in NDGA-treated grapes at 70 DAFB, and three such DEGs were down-regulated in IPT-treated grapes. In addition, Abz-E3M and ethephon treatment significantly up-regulated expression levels of *VvACO1* and *VvERF2*. This up-regulation coincided with the ethylene peak in Abz-E3M- or ethephon-treated berries, suggesting that ethephon treatment can promote grape maturation similarly to Abz-E3M, through *VvACO1* and *VvERF2*. It has been suggested that ethylene may trigger transcription of ABA-related genes during grape development (Ziliotto et al., 2012), and that ABA may play an important role as an inducer of maturation along with ethylene in grapes (Zhang et al., 2009). Together, these results suggest that ABA may promote grape maturation through complex interactions with auxin and ethylene metabolism (Sun et al., 2010; Ziliotto et al., 2012; Kuhn et al., 2014).

In summary, IPT or NDGA application on grape could delay maturation and negatively relate maturation-related genes expression. Abz-E3M or ethephon application to grape could promote maturation and increase the expression levels of the maturation-related genes. ABA and ethylene may interplay to effect on grape maturation through regulating the expression levels of *VvNCED1, VvCYP707A1, VvACO1*, and *VvERF2*.

SUMMARY

The effects of IPT, NDGA, Abz-E3M or ethephon on the maturation of 'Shine Muscat' grape berries were investigated using transcriptome analysis and qRT-PCR verification. IPT or NDGA applications retarded chlorophyll degradation in the berry skin. Transcriptional profiling revealed that 2,218 genes in the grapes treated with IPT and 2,270 genes in those treated with NDGA were differentially expressed compared to the untreated control grapes at 70 DAFB. IPT or NDGA applications regulated the expression of genes involved in ABA, auxin, and ethylene metabolism. Mapman analysis and qRT-PCR validation showed that the expression levels of *VvNCED1*, *VvCYP707A1*, *VvAAO4*, and *VvGEM-like* genes in the ABA metabolic pathway were negatively regulated by IPT and NDGA treatments at 70 DAFB. Moreover, the IPT and NDGA treatments inhibited glucose accumulation by down-regulation the expressions of *VvSUS*, *VvAI*, and *VvHT*, which are involved in sugar biosynthesis and transportation. In addition, the IPT and NDGA treatments delayed (*E*)-2-hexenal and hexanol productions, and down-regulated aroma metabolic pathway genes such as *VvLOX*, *VvADH*, *VvGPPS*, and *VvTPS*.

Abz or ethephon treatment decreased the firmness and titratable acid concentration. Abz treatment inhibited *VvCYP707A1* expression levels at 48 DAFB and increased endogenous ABA accumulation at 54 DAFB. Ethephon treatment significantly up-regulated *VvNCED1* expression levels at 48 DAFB and *VvCYP707A1* at 54 DAFB, but had no effect on ABA concentration. Ethylene and gene expression levels of *VvACO1* and *VvERF2* in Abz- and ethephon-treated berries at 48 DAFB were up-regulated. Abz or ethephon treatment also accelerated chlorophyll breakdown in berry skin with the up-regulation of *VvPPH* and *VvRCCR* expression levels. The total sugar concentrations slightly increased in both Abz- and ethephon-treated berries. These results suggest that

the Abz treatment before véraison can stimulate grape maturation by increasing endogenous ABA, and thus the ethephon treatment can promote grape maturation, similarly to Abz, through *VvACO1* and *VvERF2*. ABA and ethylene may interplay to effect on grape maturation through regulating the expression levels of *VvNCED1, VvCYP707A1, VvACO1* and *VvERF2*.

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